Estrategias dirigidas al enriquecimiento y desarrollo de formulaciones de compuestos fenólicos con actividad antioxidante y antiinflamatoria para mejorar su biodisponibilidad

Strategies for enrichment and formulation development of phenolic compounds with antioxidant and anti-inflammatory activities to improve their bioavailability

Marisol Villalva Abarca

Tesis Doctoral - Madrid 2020

UAM
Universidad Autónoma de Madrid

FACULTAD DE CIENCIAS
UNIVERSIDAD AUTÓNOMA DE MADRID

CIAL
INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN

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INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CIAL)
Departamento de Producción y Caracterización de Nuevos Alimentos

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Memoria presentada por:
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Para optar al grado de:
DOCTOR EN CIENCIAS DE LA ALIMENTACIÓN
Mención Internacional

Trabajo realizado bajo la dirección de:
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DÑA. SUSANA SANTOYO DIEZ, PROFESORA TITULAR DE LA UNIVERSIDAD AUTÓNOMA DE MADRID Y DÑA. LAURA JAIME DE PLABLO, PROFESORA TITULAR DE LA UNIVERSIDAD AUTÓNOMA DE MADRID.

CERTIFICAN,

Que el presente trabajo titulado "**Estrategias dirigidas al enriquecimiento y desarrollo de formulaciones de compuestos fenólicos con actividad antioxidante y antiinflamatoria para mejorar su biodisponibilidad** ***/ Strategies for enrichment and formulation development of phenolic compounds with antioxidant and anti-inflammatory activities to improve their bioavailability"*** y que constituye la memoria que presenta DÑA. MARISOL VILLALVA ABARCA para optar al grado de Doctor en Ciencias de la Alimentación, ha sido realizado bajo su dirección en el Instituto de Investigación en Ciencias de la Alimentación (CIAL) y la Universidad Autónoma de Madrid.

Y para que así conste firman el presente informe en Madrid a 10 de octubre de 2019.

Fdo. Dña. Susana Santoyo Diez

Fdo. Dña. Laura Jaime de Pablo

A mis padres.

A Rafa.

Consider again that blue dot.

That's here.

That's home.

That's us.

- Carl Sagan –
"Pale Blue Dot, 1994"

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ABREVIATURAS

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CAL	*Calendula officinalis* L. / caléndula
CF	Compuestos fenólicos
CGA	Ácido clorógenico
COX-2	Ciclooxigenasa 2
DCQA	Ácido dicafeoilquínico
EFSA	Autoridad Europea de Seguridad Alimentaria
GAE	Equivalentes de ácido gálico
GDL	Glucono-delta-lactona
GRAS	Generalmente reconocido como seguro
IL	Interleuquina
iNOS	Oxido nítrico sintetasa
LDL	Lipoproteínas de baja densidad
LPH	Lactasa-floricina hidrolasa
LPS	Lipopolisacárido bacteriano
MAJ	*Origanum majorana* L. / mejorana
MEL	*Melissa officinalis* L. / melisa
MG	Geles lácteos
MIL	*Achillea millefolium* L. / milenrama
NaCas	Caseinato de sodio
NE	Nanoemulsiones
NF-κB	Factor de transcripción nuclear kappa beta
NO	Oxido Nítrico
ORAC	Capacidad de absorción de radicales de oxígeno
PLE	Extracción con líquidos presurizados
RA	Acido Rosmarínico
ROS	Especies reactivas del oxígeno
RNS	Especies reactivas del nitrógeno
SAS	Tecnología supercrítica antisolvente
SC-CO_2	Dióxido de carbono supercrítico
SFE	Extracción con fluidos supercríticos
SPI	Aislado de proteína de soja
TEAC	Capacidad antioxidante en equivalentes de Trolox
TNF-α	Factor de necrosis tumoral alfa
TPC	Contenido en compuestos fenólicos totales
UAE	Extracción asistida con ultrasonidos

RESUMEN/SUMMARY

RESUMEN

El objetivo de esta tesis doctoral ha sido desarrollar estrategias de obtención y enriquecimiento de compuestos fenólicos con actividad antioxidante y antiinflamatoria, así como de diferentes formulaciones con el fin de mejorar la biodisponibilidad de dichos compuestos.

La extracción asistida con ultrasonidos (UAE) y con líquidos presurizados (PLE) con mezclas de etanol:agua o etanol puro resultaron técnicas sostenibles adecuadas para la obtención de extractos con una alta actividad antioxidante y/o antiinflamatoria, respectivamente. De entre las plantas estudiadas, la mejorana fue seleccionada por su destacada actividad antioxidante, relacionada con su alto contenido en compuestos fenólicos, en particular en ácido rosmarínico; mientras que la milenrama destacó por su actividad antiinflamatoria, debido a la presencia de determinados compuestos fenólicos como el ácido 3,5-dicafeoilquínico (DCQA) y agliconas de flavonoides, junto con compuestos del aceite esencial, como el borneol y el alcanfor.

El extracto de mejorana obtenido por PLE con un 70% de etanol, que presentó las mejores características bioactivas, se encapsuló en distintas matrices proteicas, de cara a la incorporación de los compuestos de interés en productos alimenticios. El uso de un aislado de proteína de soja resultó la matriz más adecuada, sin alterar, además, el efecto inmunomodulador del extracto.

Por otra parte, el empleo de la resina de adsorción XAD-7HP dio lugar al fraccionamiento y consiguiente incremento en compuestos fenólicos del extracto PLE-70% original de mejorana, dando lugar a un aumento en 1,7 veces del contenido en ácido rosmarínico (RA). Tras la digestión gastrointestinal *in vitro*, los extractos de mejorana registraron pérdidas importantes en la cantidad de RA; no obstante, gracias a su mayor abundancia en el extracto enriquecido, este compuesto fue detectado en mayor cantidad tras el proceso de absorción empleando células Caco-2, mejorando así la actividad antioxidante y antiinflamatoria de la fracción absorbida del extracto enriquecido respecto al extracto original.

De manera similar, el uso de la resina XAD-7HP en el fraccionamiento de un extracto UAE 100% etanol de milenrama, permitió la obtención de una fracción

dotada de mayor actividad antioxidante y antiinflamatoria que el extracto de partida. Esta mejora en ambas actividades estaría relacionada tanto con el incremento producido en el contenido en compuestos fenólicos, como con el aislamiento de compuestos con alto potencial antiinflamatorio. Una vez efectuada la digestión gastrointestinal *in vitro* de los extractos de milenrama, se observaron degradaciones de algunos compuestos fenólicos e isomerizaciones entre los diferentes DCQAs durante la etapa intestinal; sin embargo, tras la absorción en células Caco-2, la fracción biodisponible presentó cierta actividad antiinflamatoria posiblemente relacionada con la presencia de las agliconas de flavonoides metoxilados detectadas en dicha fracción.

Asimismo, la incorporación del extracto UAE-100% de milenrama en geles lácteos acidificados, evitaron la degradación de su composición fenólica durante el proceso de digestión gastrointestinal *in vitro*, preservando, de esta manera, su función antioxidante. En consecuencia, resulta una estrategia adecuada para la mejora de la bioaccesibilidad de los compuestos bioactivos de cara a la formulación de productos alimenticios.

Por otro lado, la aplicación de la tecnología de fraccionamiento supercrítica antisolvente al extracto de milenrama, condujo a la obtención de dos fracciones de composición muy distinta. De esta forma, la mayoría de los compuestos fenólicos precipitaron en la celda de extracción, mientras que el uso de CO_2 supercrítico produjo la disolución y consiguiente arrastre de la fracción volátil del extracto, lo que dio lugar a una mejora de la actividad antioxidante y antiinflamatoria, respectivamente, respecto al extracto original.

Con el desarrollo de esta tesis doctoral ha quedado de manifiesto que, tanto la mejorana como la milenrama representan fuentes valiosas de compuestos fenólicos bioactivos y que tanto su extracción, como su enriquecimiento y encapsulación mediante el uso de técnicas sostenibles ayudarían a mejorar su biodisponibilidad, incrementando así su potencial uso como ingredientes funcionales de cara a su formulación en alimentos y/o suplementos alimenticios.

SUMMARY

The aim of this PhD thesis has been to develop strategies for extraction and enrichment of phenolic compounds with antioxidant and anti-inflammatory activities, as well as the development of different formulations aimed to enhance the bioavailability of those compounds.

Ultrasound-assisted extraction (UAE) and pressurized liquids (PLE) with mixtures of etanol:water or pure ethanol resulted in sustainable techniques suitable for obtaining extracts with high antioxidant and/or anti-inflammatory activity, respectively. Among the plants studied, marjoram was selected for its outstanding antioxidant activity, related to its high content of phenolic compounds, particularly rosmarinic acid; while yarrow stood out for its anti-inflammatory activity, due to the presence of certain phenolic compounds such as 3,5-dicaffeoylquinic acid (DCQA) and flavonoid aglycones, along with essential oil compounds, such as borneol and camphor.

The marjoram extract obtained by PLE with 70% ethanol, which presented the best bioactive characteristics, was encapsulated in different protein matrices, in order to incorportate the compounds of interest into food products. The use of a soy protein isolate was the most appropriate matrix, without altering the immunomodulatory effect of the extract.

On the other hand, the use of the XAD-7HP adsorption resin resulted in the fractionation and consequent increase in phenolic compounds of the original PLE-70% marjoram extract, resulting in a 1.7-fold increase in the rosmarinic acid (RA) content. After *in vitro* gastrointestinal digestion, marjoram extracts registered significant losses in the amount of RA; however, thanks to its greater abundance in the enriched extract, this compound was detected in greater quantity after the absorption process using Caco-2 cells, therefore an improvement of the antioxidant and anti-inflammatory activity of the absorbed fraction of the enriched extract was allowed, with respect to the original extract.

Similarly, the use of the XAD-7HP resin in the fractionation of a UAE 100% ethanol yarrow extract, allowed to obtain a fraction endowed with greater antioxidant and anti-inflammatory activity than the original extract. This

improvement in both activities would be related both to the increase in the content of phenolic compounds, and to the isolation of compounds with high anti-inflammatory potential. Once the *in vitro* gastrointestinal digestion of yarrow extracts was carried out, degradations of some phenolic compounds and isomerization between the different DCQAs were observed during the intestinal stage; however, after absorption in Caco-2 cells, the bioavailable fraction exhibited certain anti-inflammatory activity possibly related to the presence of methoxylated flavonoid aglycones detected in that fraction.

In addition, the incorporation of the UAE-100% yarrow extract in acidified milk gels, prevented the degradation of its phenolic composition during the *in vitro* gastrointestinal digestion process, and preserving its antioxidant activity. Therefore, this strategy is appropriate to improve the bioaccessibility of bioactive compounds for the formulation of food products.

Moreover, the application of the anti-solvent supercritical fractionation technology to the yarrow extract, led to the obtention of two fractions with very different composition. In this way, the majority of the phenolic compounds precipitated in the extraction cell, while the use of supercritical CO2 caused the dissolution and consequent drag of the volatile fraction of the extract, which resulted in an improvement of the antioxidant and anti-inflammatory activity, respectively, with regards to the original extract.

With the development of this PhD thesis it has become evident that both marjoram and yarrow represent valuable sources of bioactive phenolic compounds. Their extraction, enrichment and encapsulation, through the use of sustainable techniques, would help to improve their bioavailability, increasing its potential use as functional ingredients for its formulation in foods and/or nutritional supplements.

1. INTRODUCCIÓN GENERAL

1. Introducción General

En las últimas décadas, se han llevado a cabo un gran número de investigaciones en el campo de la alimentación y la nutrición humana referente a la preservación o mejora de la salud mediante la dieta. De tal manera que, el avance científico en ese campo, nos ha permitido descubrir y comprobar la actividad biológica de algunos componentes presentes en los alimentos de consumo habitual en nuestra dieta (frutas, verduras, semillas y sus derivados) y en otras fuentes naturales como especies marinas, hongos y plantas.

A su vez, el cambio en los hábitos alimenticios del consumidor actual ha favorecido el desarrollo y crecimiento del sector de los denominados alimentos funcionales, cuyo valor global para el 2020 se estima en más de $300 billones (USD) (Santeramo y col., 2018). Estos alimentos funcionales tienen la característica de que, además de cumplir su función energética y nutritiva, son capaces de aportar un beneficio fisiológico funcional. Ese beneficio adicional para la salud, por lo general, se consigue gracias a la presencia, o adición, de uno o varios *compuestos bioactivos,* integrados en el alimento en una cantidad suficiente para ejercer su bioactividad. Asimismo, es cada más fuerte la tendencia de diseñar alimentos libres de aditivos artificiales, que en ciertas ocasiones se han relacionado con efectos nocivos para la salud y que a su vez responde también a la demanda de un etiquetado limpio o la denominada *clean label*. Por lo tanto, estas nuevas exigencias han llevado a la búsqueda de ingredientes de origen natural, y por consiguiente han dado lugar a la exploración de nuevas vías para su obtención y aislamiento desde sus matrices de origen. De entre la gran diversidad de compuestos bioactivos con un uso potencial como ingredientes naturales, podemos destacar particularmente al grupo de los compuestos fenólicos, que ha sido ampliamente estudiado en las últimas décadas por demostrar una gran serie de actividades biológicas con posibles repercusiones fisiológicas.

1.1. Compuestos fenólicos bioactivos.

Los compuestos fenólicos (CF) son metabolitos secundarios que están presentes en los alimentos que consumimos habitualmente, como las frutas, los cereales, semillas, leguminosas, etc., aunque también se ha descrito su presencia en algunas especies de macro y microalgas, así como en algunos hongos y setas (Heleno y col., 2015; Matos y col., 2017). Además de las fuentes ya mencionadas, las plantas superiores representan una de las más importantes, e interesantes, fuentes de compuestos fenólicos debido a su fácil disponibilidad y amplia diversidad.

Los compuestos fenólicos constituyen un grupo muy amplio, donde se han llegado a identificar más de 8.000 compuestos con estructuras muy variadas (Cory y col., 2018). De manera general, este grupo de compuestos proceden de dos rutas de síntesis, la ruta del ácido shiquímico y/o del ácido malónico y se caracterizan por poseer en su estructura uno o varios anillos aromáticos, unidos a uno o más grupos hidroxilo. Además, generalmente en la naturaleza, se encuentran de forma conjugada, de manera que pueden estar vinculados a una o más unidades funcionales como ésteres, metil ésteres, glicósidos, entre otros.

En función de su esqueleto carbonado, los compuestos fenólicos pueden clasificarse en dos grandes grupos, los flavonoideos y los no flavonoideos. Dentro del grupo de los no flavonoideos se encuentran aquellos compuestos que poseen un único grupo fenol, dando lugar a diferentes estructuras según los sustituyentes carbonados que presente dicho núcleo (Fig. 1A). Este grupo a su vez se divide en varios subgrupos en función de las cadenas hidrocarbonadas laterales, destacando principalmente dos tipos: los compuestos hidroxibenzoicos y los compuestos hidroxicinámicos. Los hidroxibenzoicos, que se corresponden con los derivados del ácido benzoico (Fig. 1B), poseen una cadena lateral de un solo carbono, como el ácido gálico, ácido vainíllico y el ácido protocatéquico. Por su parte, los hidroxicinámicos, que derivan del ácido cinámico (Fig. 1C), tienen una cadena lateral de tres átomos de carbono, como el ácido cumárico y el ácido cafeico.

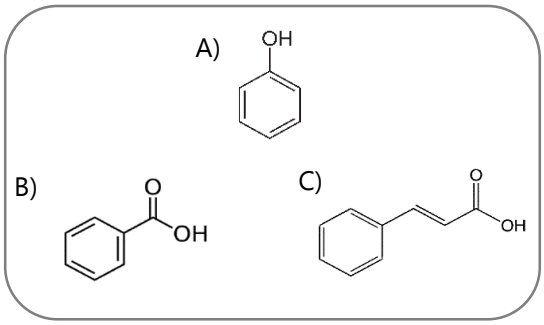

Figura 1. Estructura química del grupo fenol (A), del ácido benzoico (B) y del ácido cinámico (C).

Además, estos compuestos se pueden encontrar de forma polimerizada, como los lignanos y los galotaninos (taninos hidrolizables). Igualmente, en este grupo de los no flavonoideos se incluyen a los estilbenos, de entre los que destaca el *trans*-resveratol.

A su vez, los compuestos fenólicos flavonoideos o flavonoides comparten una estructura química común (C6-C3-C6) formada por dos ciclos bencénicos (A y B) unidos por una cadena de tres átomos de carbono, donde, normalmente, estos tres átomos de carbono dan lugar a un heterociclo de oxígeno (C), tal y como se muestra en la Fig. 2. Según el grado de oxidación que alcance el anillo heterociclo, mediante la incorporación de grupos funcionales y la posición relativa de estos, los flavonoides se pueden clasificar en 7 subgrupos: flavonas, flavonoles, flavanoles, flavanonas, isoflavonas y antocianos (Pan y col., 2010).

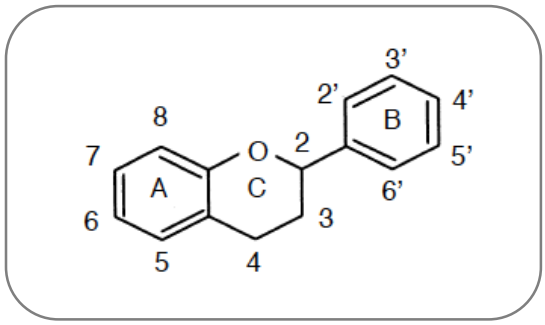

Figura 2. Estructura básica de los flavonoides.

Dentro del grupo de los flavonoides también se encuentran las auronas, chalconas y las dihidrochalconas. Estos compuestos comparten la estructura general C6-C3-C6, pero se diferencian de los anteriores en que no contienen, en general, el heterociclo de oxígeno y por tanto el anillo C se presenta de forma abierta.

1.1.1. Actividades biológicas asociadas a los CF.

La mayor parte de los compuestos fenólicos tienen una función estructural y son responsables, en cierta medida, del color, sabor y aroma de ciertas especies de plantas y frutos. Asimismo, los compuestos fenólicos, funcionan como un mecanismo de autodefensa ante patógenos o protección contra la radiación/oxidación (Ferreira y col., 2017).

En ese sentido, dado que estos compuestos desempeñan una importante función fisiológica, se ha generado un gran interés hacia el estudio de sus propiedades biológicas. Tanto los estudios *in vitro*, como en animales, humanos y epidemiológicos muestran que varios compuestos fenólicos tienen propiedades antioxidantes y antiinflamatorias que podrían tener efectos preventivos y/o terapéuticos para enfermedades cardiovasculares, trastornos neurodegenerativos, cáncer y obesidad (Pérez-Jiménez y col., 2010).

Asimismo, otras tantas y muy diversas propiedades bioactivas, han sido atribuidas a los compuestos fenólicos, incluyendo su actividad antimicrobiana, antiviral, hepatoprotectora, ansiolítica y analgésica (Ferreira y col., 2017).

- ***Actividad Antioxidante***

Los compuestos fenólicos se han propuesto como compuestos con una destacada actividad antioxidante gracias a su capacidad de captación de radicales libres y otras especies reactivas, como las especies reactivas de oxígeno (ROS) o nitrógeno (RNS), a la donación de átomos de hidrógeno o electrones y a su capacidad de atrapar metales pro-oxidantes o inhibir enzimas, protegiendo de este modo a las células frente al daño oxidativo (Trivellini y col., 2016). De manera general, la capacidad antioxidante de los compuestos fenólicos depende del número y la posición de los sustituyentes hidroxilo en relación con el grupo funcional carboxílico,

como es el caso de los ácidos fenólicos. En lo que respecta a los flavonoides, además de las características estructurales, la naturaleza de las sustituciones en los anillos B y C, determinan su actividad antioxidante (Balasundram y col., 2006). Asimismo, se he observado que el desarrollo de varias enfermedades como el cáncer, la ateroesclerosis, la diabetes y las enfermedades neurodegenerativas tienen una base oxidativa derivada de la sobreproducción de radicales libres. Esta alteración del equilibrio oxidante y antioxidante fisiológico, puede provocar graves repercusiones a nivel celular incluyendo daños oxidativos en el ADN, en los lípidos o proteínas, y a su vez inducir procesos como el envejecimiento celular, mutagénesis o carcinogénesis (Gupta y col., 2014).

En este sentido, desde hace unas décadas, la evaluación de la actividad antioxidante de los compuestos fenólicos y su relación con el desarrollo de otras enfermedades ha dado lugar a un gran número estudios. Así, el ácido rosmarínico ha sido indicado como un ácido fenólico con una prominente actividad antioxidante, por ejemplo, se ha asociado a la estabilización y protección de membranas contra el daño oxidativo (Bulgakov y col., 2012), protección contra el efecto dañino de radicales libres en distintos tejidos en ratas diabéticas (Sotnikova y col., 2015). Por su parte, la naringenina redujo la formación de ROS en las células de neuroblastoma humano SH-SY5Y, además de incrementar la activación de un factor de transcripción que coordina la respuesta antioxidante neuroprotectora en ratones (Lou y col., 2014). Asimismo, la luteolina mostró una alta capacidad de eliminación de ROS intracelular, asociado al proceso de apoptosis en células de cáncer de colon HT-29 (Kang y col., 2017).

- ***Actividad Antiinflamatoria***

Otra de las actividades biológicas relacionadas con los compuestos fenólicos, e influenciada en cierta medida por su capacidad antioxidante, es la actividad antiinflamatoria. La inflamación es una respuesta biológica, ante una infección microbiana o lesión (isquémica, tóxica o autoinmune) en un tejido. El sistema inmune desencadena esta respuesta con el objetivo de eliminar la causa del daño producido en el tejido, en un periodo de tiempo corto. Sin embargo, si la inflamación ocurre por un tiempo prolongado, se origina una fase de inflamación crónica. Durante el proceso de inflamación, algunas células, principalmente los macrófagos, promueven

la producción de mediadores proinflamatorios. Estos mediadores incluyen interleuquinas (IL), como las IL-1β, IL-6 e IL-8, el factor de necrosis tumoral (TNF-α), especies reactivas de oxígeno, óxido nítrico (NO) y prostaglandinas (PGs). La sobreproducción de estos mediadores en la inflamación crónica, ha sido relacionado con la aparición de enfermedades degenerativas crónicas como artritis, aterosclerosis, diabetes, cáncer o Alzheimer, entre otras (Medzhitov, 2008). Debido a la gran relevancia a nivel mundial que tienen este tipo de patologías, el estudio de la actividad antiinflamatoria de los compuestos fenólicos provenientes de fuentes vegetales se ha propuesto como un tema de suficiente interés.

En ese sentido, el resveratrol, las antocianinas y la curcumina, pueden reducir la inflamación vía inhibición de la producción de prostaglandina, inhibición de la activación del factor de transcripción nuclear-κB (NF-κB) y supresión de citoquinas proinflamatorias (Zhang y col., 2015). Así también, las flavonas apigenina, luteolina y diosmetina han demostrado ejercer su efecto antiinflamatorio al inhibir la expresión de las enzimas inflamatorias ciclooxigenasa 2 COX-2 y la óxido nítrico sintetasa (iNOS), así como la reducción en ciertas citoquinas proinflamatorias (Jungbauer y Medjakovic, 2012). Recientemente, la presencia del ácido 1,5-dicafeoilquínico permitió la reducción de los niveles de NO, IL-6 y TNF-α en células RAW264.7 activadas con lipopolisacárido bacteriano (LPS) (da Silva y col., 2017). Del mismo modo, el ácido rosmarínico, disminuyó la secreción de IL-8 e IL-6 en células de adenocarcinoma humano Caco-2 (de Magalhães, y col., 2012). Asimismo, ciertos flavanoles demostraron una dosis-respuesta en la disminución de la concentración de citoquinas IL-6 en el plasma sanguíneo de adultos con obesidad (Stote y col., 2012).

- ***Actividad anticancerígena y antimicrobiana***

Debido a que se ha informado que el daño oxidativo, así como la liberación no controlada de citoquinas en la inflamación crónica, están relacionados con el inicio y desarrollo de cáncer, muchos compuestos antioxidantes y antiinflamatorios han sido investigados como potenciales agentes anticancerígenos. De este modo, se ha indicado que los compuestos fenólicos modulan los niveles de especies reactivas de oxígeno en las células, regulando así la proliferación celular, la supervivencia y la

apoptosis. Además de mostrar un efecto inhibidor en la señal de cascadas oncogénicas o promover proteínas supresoras de tumores (Anantharaju y col., 2016).

En este contexto, los ácidos carnósico y carnosol han demostrado ejercer un efecto antiproliferativo de manera sinérgica en células de cáncer de colon y páncreas (González-Vallinas y col., 2015). De manera similar, se ha observado el efecto antitumor del carvacrol y el ácido rosmarínico, sobre células cancerígenas de colon HT-29 y de cáncer de próstata PC-3, relacionándose en parte con la inhibición de la IL-8 intracelular (Kaliora y col., 2014). En otro estudio, Kurata y col., (2007) pudieron identificar que el ácido cafeico, el ácido clorogénico, y los isómeros 3,4-, 3,5- y 4,5-dicafeoilquínico, inhibieron el crecimiento de células de cáncer de estómago (Kato III) y de leucemia (HL-60).

Por su parte, la función antimicrobiana de los compuestos fenólicos ha sido evaluada, encontrándose que los aceites esenciales provenientes de plantas y especias suelen considerarse potentes agentes antifúngicos y antibacteriales. Así, los componentes terpénicos timol, carvacrol o eugenol de algunas especias, han sido señalados con una importante actividad antimicrobiana (Devi y col., 2010; Trivellini y col., 2016). Así también, la presencia de ácido cafeico, luteolina-7-*O*-glucósido y apigenina-7-*O*-glucósido, se relacionó con la actividad antimicrobiana ante varias cepas de bacterias Gram-positivas (Kenny y col., 2014). Por otra parte, se ha indicado que las proantocianidinas de tipo A, y con mayor influencia sus metabolitos catecol, ácido benzoico, ácido vainíllico, ácido fenilacético y el ácido 3,4-dihidroxifenilacético, son los responsables de la anti adherencia de *E. coli* en relación con las infecciones en el tracto urinario (González de Llano y col., 2015).

Como ya se mencionaba anteriormente, ciertamente gracias a las actividades biológicas exhibidas por los compuestos fenólicos, el aprovechamiento y recuperación de estos se ha convertido en un objetivo primordial de la industria alimentaria con el fin de utilizarlos como ingredientes funcionales.

En la Unión Europea, es la Autoridad Europea de Seguridad Alimentaria (EFSA) la encargada de autorizar las declaraciones de salud en alimentos, y a día de hoy solo se han encontrado evidencias científicas con una relación causa-efecto para los compuestos fenólicos del aceite de oliva (hidroxitirosol, oleuropeína y tirosol) y los flavanoles de cacao. De este modo, se destaca la capacidad de los flavanoles del

cacao para mantener la elasticidad de los vasos sanguíneos, lo cual contribuye a mantener un flujo sanguíneo normal, siempre y cuando se consuman 200 mg diarios de extracto de cacao con un alto contenido en flavanoles, ya sea en forma de cápsulas, tabletas o adicionados en alimentos/bebidas. En el caso de los fenólicos del aceite de oliva, se refiere a la capacidad de estos para proteger a las lipoproteínas de baja densidad (LDL) del daño oxidativo, proponiéndose un consumo diario mínimo de 5 mg de hidroxitirosol y sus derivados por cada 20 mg de aceite de oliva.

1.1.2. Las plantas como fuente de compuestos fenólicos bioactivos.

Entre las fuentes naturales de compuestos bioactivos, las plantas es una de las más ampliamente estudiadas, debido principalmente a su inmediata disponibilidad y diversidad. En ese sentido, varias especies de plantas han sido tradicionalmente señaladas por demostrar un efecto beneficioso en la salud, incluidos los desórdenes respiratorios, problemas gastrointestinales, padecimientos y heridas musculares, problemas cardiovasculares, enfermedades asociadas al síndrome metabólico, cáncer y enfermedades neurodegenerativas (Roby y col., 2013). La acción mitigante sobre estos y otros padecimientos a través del empleo de las plantas y sus derivados, se ha relacionado principalmente con su contenido en compuestos fenólicos. Dentro de las especies más estudiadas se mencionan a aquellas pertenecientes a las familias Lamiaceae (romero, salvia, orégano), Apiaceae (comino, hinojo), Zingiberaceae (cúrcuma, jengibre), Ginkgoaceae (ginkgo), Asteraceae (manzanilla, caléndula, artemisia, milenrama) y Myrtaceae (eucalipto), entre otras.

1.1.2.1. Familia Lamiaceae

La familia Lamiaceae (Labiatae) se trata de una de las familias botánicas más extendidas a nivel global, y está conformada por unas 6900-7200 especies. Muchas de las especies de esta familia tienen un arraigado uso culinario debido a sus propiedades aromáticas y de sabor, y también son reconocidas por su amplio uso terapéutico (Roby y col., 2013; Tzima y col., 2018). Dentro de las especies más estudiadas podemos mencionar al romero, salvia, tomillo, mejorana, melisa y al

orégano. En particular los extractos de estas especies, obtenidos con distintos disolventes, se han estudiado ampliamente para explorar su funcionalidad, y se les relacionan principalmente con actividades como antioxidante, antimicrobiana, antiinflamatoria y antiproliferativa (Bessada y col., 2015; Tzima y col., 2018).

- ***Melissa officinalis* L.**

Melissa officinalis L., o melisa como se le conoce comúnmente, nativa del centro y del mediterráneo de Europa, constituye una planta con un alto valor culinario y terapéutico (Fig. 3). Esta planta es utilizada como agente sedante en el tratamiento de trastornos nerviosos y la falta de sueño, y también para tratar problemas gastrointestinales, por sus propiedades antiespamódicas. Estas propiedades se han relacionado principalmente a su alto contenido en ácidos hidroxicinámicos, como el ácido cafeico, el ácido rosmarínico y los derivados de este. Además, aunque en menor abundancia, también se menciona la presencia de flavonoides, como la luteolina, herperidina y la naringenina (Shakeri y col., 2016).

Figura 3. Detalle de la planta de melisa.

En ese sentido, los extractos acuosos y etanólicos de melisa, con importantes cantidades de ácido cafeico, rosmarínico e isómeros del ácido salvianólico, han demostrado su actividad neuroprotectora en ratas (Ozarowski y col., 2016). Además, las decocciones hechas con melisa se han señalado como bebidas con acción antimicrobiana y antitumoral, donde nuevamente se distingue el ácido rosmarínico, además del ácido litospérmico como los principales componentes (Carocho y col., 2015). La variación respecto a la abundancia en los constituyentes de *M. officinalis*

depende de las condiciones climáticas, estacionarias y geográficas, el tiempo de cosecha y también el procedimiento de obtención de sus extractos.

- ***Origanum majorana* L.**

Origanum majorana L., o mejorana, también representa una de las hierbas más utilizadas como ingrediente culinario y con más usos terapéuticos dentro de la familia Lamiaceae (Fig. 4). Esta planta es nativa de la zona del mediterráneo, aunque es extensamente cultivada en varios países de Asia, el norte de África y Europa, incluidos España, Portugal y Alemania. Ha sido utilizada tradicionalmente gracias a su eficacia para aliviar diversas patologías, incluyéndose los problemas gastrointestinales, respiratorios, reumáticos y neurológicos (Bina y col., 2017; Tzima y col., 2018).

Figura 4. Detalle de la planta de mejorana.

La mejorana presenta una gran diversidad de compuestos fenólicos en su composición, entre ellos varios ácidos hidroxibenzoicos, ácidos hidroxicinámicos, flavonoides y terpenos fenólicos, los cuales se relacionan con las propiedades beneficiosas de esta planta. De este modo, la presencia de los ácidos rosmarínico y cafeico, se relaciona principalmente con la actividad antioxidante observada en mejorana (Vallverdú-Queralt y col., 2015). Además, el aceite esencial de mejorana, donde se pueden encontrar mono y di-terpenos, como el *p*-cimeno, carvacrol, timol, ácido carnósico y carnosol, ha sido asociado con las propiedades antimicrobiana y antiinflamatoria de la mejorana (Bina y col., 2017). Así también, las infusiones de esta planta, con un contenido mayoritario en ácido rosmarínico y carvacrol, han

demostrado su actividad antiproliferativa ante células de cáncer de próstata (Kaliora y col., 2014).

1.1.2.2. Familia Asteraceae

La familia botánica Asteraceae (Compositae) es muy extensa, incluyendo más de 1600 géneros y 23.000 especies de plantas, que se distribuyen ampliamente por todo el mundo, aunque prevalecen en la zona del mediterráneo. Numerosas especies de la familia Asteraceae se corresponden con importantes flores de uso ornamental, arbustos y árboles. Algunas especies además han sido utilizadas desde hace muchos años como plantas medicinales, muchas de las cuales producen aceites esenciales utilizados con fines terapéuticos (Bessada y col., 2015). Así, entre las especies de la familia Asteraceae con propiedades beneficiosas, que han sido frecuentemente estudiadas, se pueden mencionar a la manzanilla, artemisa, centaura, caléndula y milenrama. Estas especies se han relacionado principalmente con actividades como la antimicrobiana, antiinflamatoria, antiespasmódica y antiproliferativa (De Magalhães y col., 2012; Bessada y col., 2015).

- ***Achillea millefolium* L.**

Achillea millefolium L., comúnmente conocida como milenrama, es una especie originaria de Europa y el oeste de Asia, pero su cultivo se ha extendido por diversas zonas templadas del hemisferio norte, incluyendo el norte de América. *A. millefolium* es una planta arbustiva con flores (Fig. 5), que ha sido utilizada ampliamente en la medicina tradicional para tratar diversas enfermedades tales como fiebre, afecciones respiratorias, problemas hepáticos, sanación de heridas, hemorroides y trastornos gastrointestinales (Ali y col., 2017).

Una buena parte de la funcionalidad de milenrama se debe a la presencia de sus compuestos fenólicos, los cuales presentan una amplia variedad entre ácidos fenólicos y flavonoides, destacando, por su abundancia, los derivados del ácido clorogénico, como el ácido 3,5-dicafeoilquínico (DCQA).

Por su parte, los derivados conjugados de luteolina, apigenina y quercetina también se han encontrado dentro de la composición fitoquímica de milenrama. En este sentido, extractos acuosos de milenrama con un contenido principalmente de ácido clorogénico y el ácido 3,5-DCQA, demostraron un alto poder antioxidante. A su vez, extractos metanólicos de milenrama mostraron un potente efecto antitumoral ante células de cáncer de pulmón, colon y cervical. Estos extractos, contenían además del ácido clorogénico y el 3,5-DCQA, cantidades relevantes del ácido 4,5-dicafeoilquíno y derivados acetilhexósidos de apigenina (Dias y col., 2013). Además, los flavonoides aislados de *A. millefolium*, luteolina, apigenina-7-*O*-*β*-glucósido, luteolina-7-*O*-*β*-glucósido y 6-hidroxiluteolina-7-*O*-*β*-glucósido, demostraron su capacidad inhibitoria de las enzimas acetilcolinesterasa y butirilcolinesterasa, relacionadas con el Alzheimer (Sevindik y col., 2015).

Figura 5. Detalle de la planta de milenrama.

- ***Calendula officinalis* L.**

Calendula officinalis L., o caléndula, es una flor arbustiva de la familia de las Asteraceae con un color amarillo-anaranjado que le caracteriza (Fig. 6). Esta planta es originaria de Egipto y de la zona del mediterráneo, pero su cultivo se ha extendido por toda Europa, Asia y algunos lugares de América. La caléndula también es considerada como una planta comestible y ornamental, pero sobre todo tiene un alto valor medicinal.

Figura 6. Detalle de la planta de caléndula.

Tradicionalmente los extractos de caléndula se han utilizado para tratar úlceras gástricas, conjuntivitis, heridas y quemaduras de la piel, dolores reumáticos y malestares ocasionados por las venas varicosas (Khalid y col., 2012). La presencia de compuestos fenólicos, principalmente los flavonoles glicosilados derivados de la quercetina y la isoramnetina, se han relacionado, en gran parte, con las propiedades beneficiosas de *C. officinalis*. Además, se han descrito algunos ácidos fenólicos como el ácido cafeico y los ácidos mono y dicafeoilquínicos, aunque en una menor abundancia. Así también en el aceite esencial de caléndula se han reportado terpenos fenólicos como el carvacrol y *p*-cimeno (Olenikov y col., 2014; Miguel y col., 2016). De este modo, extractos hidroetanólicos de caléndula, con rutina y quercetina-3-*O*-glucósido como componentes mayoritarios, estimularon la expresión de factores de crecimiento del tejido conectivo, favoreciendo la sanación de heridas en ratones (Dinda y col., 2016), Asimismo, infusiones de caléndula, con un alto contenido en isoramnetina-3-*O*-ramnosilrutinosido e isoramnetina-3-*O*-rutinosido, demostraron un potencial efecto antitumoral frente a células de cáncer (Miguel y col., 2016).

1.1.3. Biodisponibilidad de los compuestos fenólicos.

El estudio de la biodisponibilidad de los compuestos fenólicos se ha convertido en un tema de interés en los últimos años dado que, para que los compuestos fenólicos sean capaces de ejercer un beneficio fisiológico, es necesario que estos compuestos se encuentren biodisponibles tras su ingesta. El término biodisponibilidad, se refiere a la fracción de un determinado compuesto/nutriente ingerido que es capaz de alcanzar la circulación sistémica y que está disponible para ser utilizado en distintas funciones fisiológicas (Carbonell-Capela y col., 2014). Como se resume en la Fig. 7, la biodisponibilidad, incluye a su vez a la bioaccesibilidad. Esto implica que, una vez que los compuestos fenólicos son liberados desde la matriz alimentaria, se verán expuestos a sufrir modificaciones durante su paso por el tracto gastrointestinal, y solo cierta proporción/fracción quedará disponible para su absorción intestinal (Alegría y col., 2015).

Figura 7. Representación esquemática del proceso de biodisponibilidad de los compuestos bioactivos (incluidos los compuestos fenólicos) tras su ingesta.

Además, hay que tener en cuenta que el grado de biodisponibilidad variará ampliamente en función del tipo de compuesto fenólico y la matriz en la que se encuentre. Por lo tanto, el efecto de la matriz alimentaria y las interacciones entre los compuestos fenólicos y otros componentes, como proteínas o polisacáridos,

puede intervenir en su liberación, solubilidad y digestibilidad. Así también, su estabilidad ante la digestión y posterior absorción dependerá de una variedad de factores tales como: su estructura química, tamaño molecular, solubilidad y esterificación con otros compuestos.

A lo largo de las últimas dos décadas se han desarrollado varias metodologías para evaluar la biodisponibilidad de los compuestos fenólicos, que incluyen modelos *in vitro*, *ex vivo*, *in situ* e *in vivo* (Carbonell-Capela y col., 2014). Entre estos modelos propuestos, los modelos *in vitro* son los más utilizados, principalmente por su fácil implementación y bajo coste. Estos modelos, se centran en simular las condiciones fisiológicas del proceso de digestión gastrointestinal, aunado a la evaluación de la absorción a nivel intestinal. Respecto a la aproximación de las condiciones del proceso de digestión gastrointestinal los modelos estáticos siguen siendo utilizados con mayor frecuencia, de manera que existen diversas metodologías publicadas consistentes en el uso de enzimas digestivas, agitación y las condiciones de pH específicas dentro de las etapas oral, gástrica e intestinal. Recientemente también se ha publicado un protocolo en el que se establecen las condiciones estándar para la simulación de la digestión *in vitro* (Minekus y col., 2014), en el ámbito de la Acción COST Infogest, el cual ha sido actualizado a su última versión 2.0 muy recientemente (Brodkorb y col., 2019).

A continuación, se describen las etapas involucradas en un modelo de aproximación in vitro, considerando la digestión gastrointestinal y la absorción intestinal.

1.1.3.1. Digestión gastrointestinal

Los compuestos fenólicos provenientes de la dieta se enfrentarán, en primer lugar, al proceso de digestión, comenzando por su estancia en la cavidad oral, donde se produce la masticación (en el caso de las matrices sólidas) y la mezcla con saliva y enzimas provenientes de la microbiota oral. Dado que la liberación parcial de algunos compuestos fenólicos puede ocurrir, se ha observado que algunos flavonoides-*O*-glicosilados son susceptibles de hidrólisis por la acción de enzimas glicosídicas, y ciertos flavonoles y proantocianidinas pueden interaccionar con algunas enzimas de la saliva ricas en prolina, llegando incluso a formar precipitados (Lewandoska y col., 2013; Cueva y col., 2017). Sin embargo, las transformaciones en

esta etapa suelen ser mínimas debido a que el tiempo de estancia de los compuestos fenólicos en la boca es muy limitado (de segundos a un par de minutos).

En la etapa gástrica, se produce la liberación/extracción de compuestos fenólicos, como resultado de los movimientos peristálticos y la acción de los ácidos estomacales (pH 1-2) y enzimas gástricas (pepsina y lipasa gástricas). En general, los compuestos fenólicos se mantienen estables a pH bajos, sin embargo, se ha reportado la degradación de proantocianidinas a monómeros en condiciones ácidas. Asimismo, aunque de manera limitada, se ha reportado que algunos compuestos fenólicos pueden absorberse a nivel gástrico como la malvidina-3-glucósido, o los ácidos clorogénico, gálico, cafeico y *p*-cumárico, los cuales se han detectado en plasma de ratas, a tiempos muy cortos tras su ingesta oral (Lewandoska y col., 2013; Tajik y col., 2017).

Durante la etapa de digestión intestinal los compuestos fenólicos se enfrentarán a condiciones alcalinas de pH (7-7.5) y a la presencia de los jugos biliares y enzimas pancreáticas, por lo que algunos de ellos se verán afectados frente a estas condiciones. Las antocianinas han resultado particularmente sensibles a la digestión intestinal, mostrando grandes pérdidas sobre todo por las condiciones de alcalinidad (Tagliazzuchi y col., 2010). Por otro lado, ciertos derivados glicosilados de apigenina también se mostraron fuertemente afectados al transcurrir la digestión intestinal (Czubinski y col., 2019). Mientras tanto, en el caso del ácido clorogénico, se ha observado tanto la aparición de ácido cafeico, proveniente de su hidrólisis parcial, pero sobre todo la formación de otras formas isoméricas durante la etapa intestinal (Bouayed y col., 2012).

1.1.3.2. Absorción intestinal

Es en la zona del duodeno y yeyuno, donde se va producir la *absorción* intestinal de los compuestos fenólicos, y las células de la capa intestinal (enterocitos) propiciarán el metabolismo de los mismos. Uno de los modelos *in vitro* de absorción intestinal más estudiado, y estandarizado, es el modelo con monocapas celulares crecidas sobre soportes semipermeables (membranas Transwell®). Entre las líneas celulares más utilizadas podemos mencionar a las células de adenocarcinoma colorrectal humano Caco-2 (Artursson y Borchardt, 1997). Este modelo es elegido ya

que las células Caco-2 son capaces de diferenciarse espontáneamente y crecer formando una monocapa muy parecida en su fenotipo a la membrana celular de los enterocitos intestinales en el humano. Las células Caco-2 poseen una serie de mecanismos de transporte activo y pasivo que normalmente se encuentran en los enterocitos del intestino delgado, lo que facilita la aproximación de la absorción de metabolitos. Este modelo celular permite también evaluar el metabolismo intracelular gracias a las enzimas expresadas por las células Caco-2, las cuales, en su mayoría, son iguales a las que se encuentran en los enterocitos intestinales.

La permeabilidad de los compuestos fenólicos a través de las células intestinales tiene lugar mediante dos vías principales: la vía paracelular, cuando los compuestos son capaces de atravesar los espacios acuosos intercelulares (ruta mínima), y la vía transcelular, a través de la propia membrana celular (ruta mayoritaria), ambas representadas en la Fig. 8. Los mecanismos más importantes en la absorción se clasifican de acuerdo al consumo de energía durante su transporte. Así, podemos distinguir una difusión **pasiva** a favor del gradiente de concentración, o mediada por ciertos transportadores, generalmente proteínas de la membrana celular. Por otro lado, el mecanismo de transporte **activo** es el que se realiza en contra del gradiente de concentración y, por lo tanto, requiere un gasto de energía. En este tipo de transporte activo, según el sentido en que se produzca el transporte, podemos distinguir entre el transporte activo de absorción o *influx*, y el transporte de secreción o *efflux*, también representados en la Fig. 8.

En este punto, la naturaleza química de los compuestos fenólicos será de gran influencia para su absorción, incluyendo el grado de polimerización, tipo y número de sustituyentes unidos y su hidrofobicidad. Las formas agliconas, por su naturaleza lipofílica, se dice que atraviesan la membrana celular por difusión pasiva. Por su parte, los compuestos fenólicos glicosilados, que son más hidrófilos, no pueden atravesar fácilmente la membrana celular de los enterocitos. De esta manera se han sugerido dos mecanismos para la absorción de los compuestos fenólicos glicosilados. Por una parte, los compuestos fenólicos glicosilados son hidrolizados por la enzima lactasa-floricina hidrolasa (LPH) presente en el borde cepillo de las células epiteliales. Así, derivado de esa liberación, la parte aglicona puede absorberse vía transcelular.

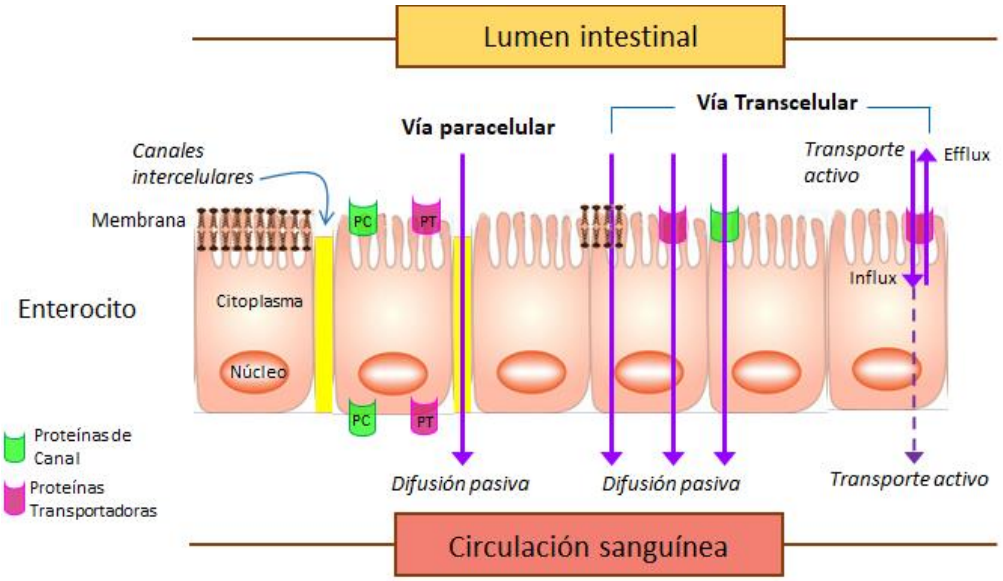

Figura 8. Tipos de transporte paracelular y transcelular en las células del epitelio intestinal.

Y, por otro lado, se ha propuesto que algunos compuestos fenólicos glicosilados pueden ser transportados a través de la membrana celular de los enterocitos por la interacción con transportadores selectivos, como el caso del transportador activo dependiente de glucosa-1 (SGLT1). Una vez dentro de los enterocitos, la enzima β-glucosidasa presente en el citosol de la célula, hará su función hidrolítica para remover el sustituyente glicosilado (Velderrain-Rodríguez y col., 2014). De manera general, las isoflavonas y el ácido gálico son los que mejor se absorben en el intestino humano, seguidos de catequina, glucósidos de quercetina, flavanonas y flavonas. La absorción de proantocianidinas es menos eficiente, ya que requerirían degradarse previamente en monómeros o dímeros (Carbonell-Capella y col., 2014).

Una vez dentro del enterocito, los compuestos fenólicos pueden ser dirigidos a la circulación sanguínea de manera intacta o en forma de metabolitos conjugados, principalmente sulfatados, glucosilados y metilados, como resultado de las reacciones enzimáticas de la fase II del metabolismo. También se ha observado que algunos compuestos fenólicos pueden ser devueltos al lumen intestinal por la acción

de transportadores activos como la glucoproteína-P 1 (P-gp) o las proteínas asociadas a la resistencia a múltiples fármacos (*multi-drug resistence associated proteins*, MRP). Finalmente, los compuestos fenólicos seguirán una ruta metabólica en el cuerpo humano como cualquier otro xenobiótico. De este modo, después de su paso por el tracto gastrointestinal y durante su absorción intestinal, serán sometidos a varios procesos metabólicos en las células del enterocito, para posteriormente seguir su metabolismo en el hígado y los riñones con el fin último de ser eliminados del organismo a través de la bilis y la orina. Además, aquellos que no se logren absorber en el intestino, seguirán su tránsito hacia el colon, donde después de estar en contacto con las enzimas colónicas y ser metabolizados, serán eliminados a través de las heces.

1.2. Técnicas de extracción y fraccionamiento de compuestos fenólicos.

1.2.1. Extracción de compuestos fenólicos.

En las últimas décadas los esfuerzos para proponer y desarrollar nuevas tecnologías de extracción enfocadas a la sostenibilidad se han hecho evidentes. El enfoque hacia la obtención de compuestos fenólicos con el uso de tecnologías que, además de ofrecer mejoras en la metodología de extracción (rendimientos, selectividad de compuestos, automatización, etc.), supongan un ahorro de recursos (tiempo y disolventes de extracción) y un menor consumo de energía, es cada vez más constante. De tal manera, han surgido las tecnologías enmarcadas en el contexto de la 'química verde'. Este último término, formulado por primera vez en la década de 1990, consiste en el diseño y aplicación de procesos que reducen o eliminan el uso y generación de sustancias peligrosas, incluyendo consideraciones físicas (por ejemplo, flamabilidad), toxicológicas (por ejemplo, mutagenicidad), y medioambientales (Anastas y Kirchhoff, 2002). Así, el empleo de disolventes ecológicos, no tóxicos, y el uso de procesos menos contaminantes y más eficientes,

es un aspecto importante a tener en cuenta a la hora de buscar la obtención de compuestos fenólicos a partir de plantas o matrices vegetales.

En este contexto, las llamadas técnicas convencionales de extracción (maceración, hidrodestilación y Soxhlet) se han visto sustituidas o complementadas por otras técnicas más avanzadas, principalmente con el fin de disminuir los tiempos de extracción y las grandes cantidades de disolvente que suponen el empleo de las técnicas convencionales. Estas técnicas alternativas de extracción, también son conocidas como técnicas no-convencionales o *técnicas limpias,* al resultar más respetuosas con el medio ambiente. Entre las técnicas no-convencionales más frecuentes para la extracción de compuestos fenólicos, a partir de matrices vegetales, se incluyen la extracción asistida por ultrasonidos (UAE), extracción con fluidos supercríticos (SFE) y extracción con líquidos presurizados (PLE) (Chemat y col., 2017; Gallego y col., 2019), además de la extracción asistida por microondas (MAE) y la extracción asistida con enzimas (EAE), entre otras (Azmir y col., 2013).

- ***Extracción con fluidos supercríticos (SFE).***

La técnica de SFE está basada en el uso de disolventes a temperaturas y presiones por encima de su punto crítico. En esas condiciones, los disolventes supercríticos tienen la densidad típica de un líquido, una viscosidad similar a la de un gas y una difusividad intermedia (entre un líquido y un gas). Debido a su baja viscosidad y alta difusividad, los fluidos supercríticos tienen una mayor penetración en la matriz de la muestra y una transferencia de masa favorable (Azmir y col., 2013). En la Figura 9 se muestra una representación de lo que es un sistema de extracción con fluidos supercríticos a partir de una matriz sólida. La muestra se coloca dentro de la celda de extracción (3), a la temperatura seleccionada. El CO_2 en estado supercrítico (SC-CO_2) es bombeado (1) hasta la celda de extracción y fluye hacia la salida de la celda junto con los compuestos fenólicos solubilizados en el disolvente. Después, por la bajada de presión (4) los compuestos extraídos se separan del disolvente gaseoso y se recolectan en el separador (5).

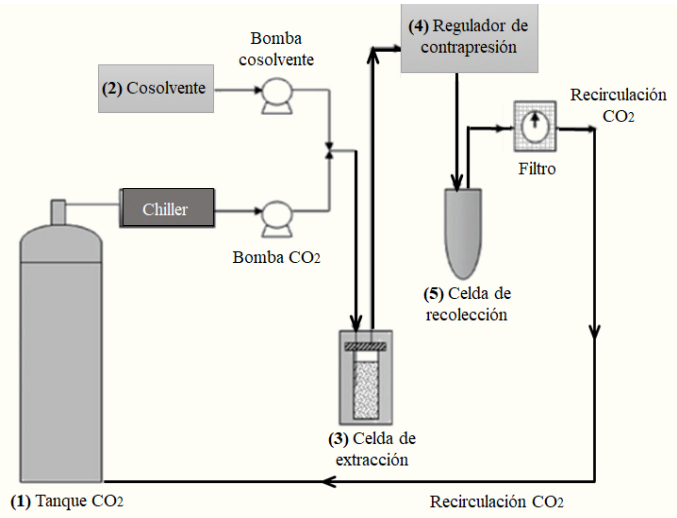

Figura 9. Esquema de un sistema de extracción con fluidos supercríticos (SFE).

Esta técnica es considerada de nulo o muy bajo consumo de disolventes, ya que el SC-CO_2, se recircula en el sistema para su reutilización. Además, otra ventaja es que el CO_2 al tratarse de un gas puro, no genera residuos tóxicos y, seguido de su despresurización, los extractos obtenidos están libres de disolvente (Jaime y col., 2014). Por otro lado, el CO_2 tiene una baja polaridad, lo que puede limitar la recuperación de compuestos hidrofílicos o de polaridad intermedia, como es el caso de muchos compuestos fenólicos provenientes de plantas. Para aliviar esta deficiencia, se puede hacer uso de ciertos solventes orgánicos, llamados cosolventes (2), que ayuden a modificar la polaridad del CO_2, siendo el etanol uno de los más utilizados para este propósito.

A pesar de que su implementación requiere de una inversión alta, en comparación a otras técnicas no-convencionales, y de personal capacitado para su ejecución, la técnica SFE es fácilmente escalable y se usa actualmente en varios procesos industriales (Chemat y col., 2017). Esta técnica ha sido ampliamente empleada para la extracción de compuestos fenólicos, incluidos los terpenos fenólicos (componentes de los aceites esenciales), a partir de varias especies de plantas (de Melo y col., 2014; da Silva y col., 2016). En ese sentido, podemos destacar el empleo de hojas de romero (*R. officinalis*) con el fin de recuperar ácido carnósico,

carnosol, ácido rosmarínico y rosmanol (Carvalho y col., 2005; del Pilar Sánchez-Camargo y col., 2014). También destaca el empleo de varias especies de tomillo (*T. vulgaris* y *T. zygis*) para la obtención de timol, como el componente mayoritario del aceite esencial (Fornari y col., 2012). Por otro lado, se logró la producción de un extracto abundante en flavonoides, entre ellos, rutina, miricetina, luteolina, apigenina y naringenina, a partir de hierbabuena (*M. spicata)* (Bimakr y col., 2012). Asimismo, los ácidos gálico, protocatéquico, *p*-hidroxibenzoico, vaínillico y siríngico, se extrajeron de melisa (*M. officinalis*) empleando la tecnología SFE (Karasová y col., 2006).

- ***Extracción con líquidos presurizados (PLE).***

La técnica de extracción con líquidos presurizados permite la obtención de extractos a partir de matrices sólidas, empleando disolventes líquidos a altas presiones y altas temperaturas. Estas condiciones, permiten mantener a los disolventes en estado líquido, a pesar de trabajar por encima de su punto de ebullición a presión atmosférica. Esta técnica ha recibido distintos nombres, y se le conoce también como extracción con fluidos presurizados (PFE) o extracción acelerada con disolventes (ASE). Asimismo, si el agua es empleada como disolvente de extracción, se le puede denominar como extracción con agua caliente presurizada (PHWE) o extracción con agua subcrítica (SWE) (Wianowska y Gil, 2019). El principio es el mismo en todos los casos, en el que un disolvente en estado líquido (1) es bombeado a hasta el compartimento del horno (4), para ingresar a la celda de extracción (5), la cual en el momento de extracción se encuentra presurizada y calentada. La muestra en estado sólido se encuentra dentro de la celda de extracción, la cual generalmente está pre-tratada (secada y molida). Además, es común el uso de un material inerte para rellenar la celda de extracción y evitar atascos dentro de la celda. Una vez finalizado el tiempo de extracción, conocido como tiempo estático, el disolvente es bombeado de dentro de la celda hacia el vial de recolección (7) donde se depositará junto con el extracto obtenido. Finalmente, una corriente de un gas inerte (8), comúnmente nitrógeno, es inyectada dentro de la celda para garantizar la completa expulsión del disolvente y purgar el sistema (Fig. 10).

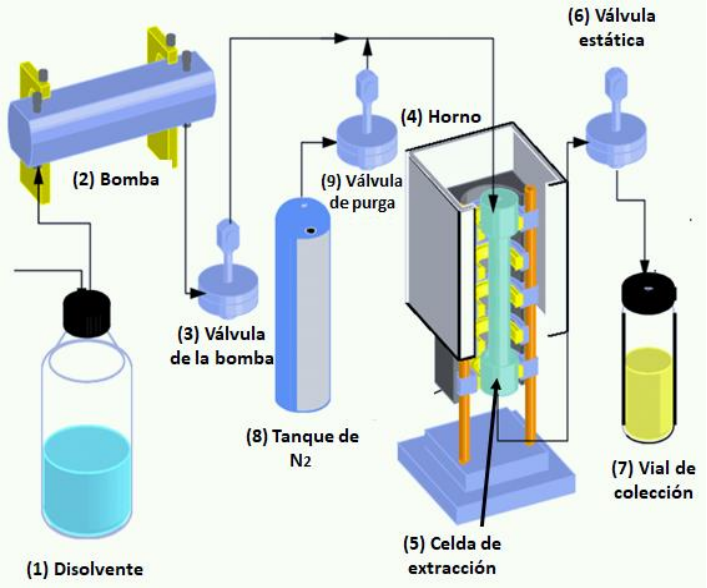

Figura 10. Esquema de un equipo de extracción con líquidos presurizados. Adaptada de Thermo Scientific website (ASE Dionex).

En las últimas décadas la técnica PLE ha tomado mayor importancia para la extracción de compuestos fenólicos, debido a que resulta en una mayor eficiencia de extracción respecto a otras técnicas. El uso de altas temperaturas reduce la viscosidad del disolvente líquido, a la vez que permite la ruptura de las interacciones matriz-analito, por lo que aumenta la penetración del disolvente en la matriz. El uso de altas presiones ayuda a su vez a aumentar el área de contacto entre la matriz y el disolvente, por lo que su difusividad se ve favorecida (Wianowska y Gil, 2019). La elección de un disolvente clasificado como seguro o GRAS (Generally Recognised As Safe), como el agua y el etanol, y sus mezclas, resulta lo más apropiado en línea con la sostenibilidad. Además, con el empleo de SWE se produce un cambio en la constante dieléctrica del agua, por lo que ésta se comporta como un disolvente orgánico, y la extracción de compuestos hidrofóbicos se ve favorecida (Morales y col., 2019). El uso de la técnica PLE ocurre sobre todo a una escala laboratorio, donde las capacidades de las celdas de extracción son aún limitadas, aunque recientemente ya se han diseñado algunos modelos, con este tipo de tecnología, en una escala piloto (Meng y Lozano, 2014).

La extracción con líquidos presurizados se ha utilizado ampliamente desde hace ya un par de décadas representando las matrices vegetales una de las principales áreas de aplicación (Gallego y col., 2019; Wianowska y Gil., 2019). De esta manera, respecto a los compuestos fenólicos se puede mencionar la obtención de miricetina, kaempferol y quercetina a partir de hojas de té negro utilizando agua subcrítica, a una temperatura óptima de 170ºC y 200ºC (Cheigh y col., 2015). Asimismo, distintas temperaturas de agua subcrítica fueron aplicadas para obtener extractos de manzanilla (*M. chamomilla*) con un alto contenido de ácidos fenólicos y flavonoides, entre ellos apigenina, luteolina-7-*O*-glucósido, ácido *p*-hidroxibenzoico y el ácido 5-*O*-cafeoilquínico, como los componentes más abundantes en los extractos (Cvetanović y col., 2019). Por otra parte, la extracción optimizada de oleuropeína proveniente de hojas del olivo (*O. europaea*) se consiguió mediante el uso de una mezcla H_2O:Etanol (44:56, v/v) a 190ºC (Xynos y col., 2014). De la misma manera, la técnica de PLE se utilizó para la obtención de extractos de melisa (*M. officinalis*) con un alto contenido en ácido rosmarínico, al utilizarse etanol puro como disolvente de extracción a 150ºC (Miron y col., 2013).

- ***Extracción asistida por ultrasonidos (UAE).***

La extracción asistida mediante ultrasonidos se fundamenta en la cavitación, un fenómeno basado en la formación, crecimiento y colapso de microburbujas generadas en una fase líquida sometida a ondas de ultrasonidos. La implosión de estas microburbujas genera condiciones puntuales de elevada presión y temperatura, dando lugar a erosiones y rupturas de la matriz, lo que mejora la transferencia de masa y acelera la difusión (Chemat y col., 2017). Así, la efectividad de esta técnica depende de la frecuencia acústica, la temperatura y presión aplicada. Uno de los sistemas de cavitación más utilizados se representa en la Fig. 11, y se corresponde con un equipo de ultrasonidos tipo sonda. Estos sistemas suelen utilizarse a una frecuencia mayor a 20 kHz y menor a 100 kHz, que es fácilmente controlable y establecida con precisión desde el controlador (1). La sonda de ultrasonidos (4) se sumerge dentro del reactor (5) que contiene la muestra sólida, generalmente seca y con un tamaño de partícula disminuido, resuspendida en un volumen adecuado de disolvente. La intensidad de los ultrasonidos transmitidos al medio líquido suele derivar en un incremento de temperatura, por lo que existe la

posibilidad de reducir/detener los pulsos generados una vez que se alcanza la temperatura límite detectada dentro del reactor (2). Al mismo tiempo, un revestimiento de enfriamiento en la cámara de reacción suele usarse para evitar el sobrecalentamiento de la muestra. Muchas de las metodologías de extracción con ultrasonidos también contemplan una agitación constante de la disolución (6) durante el tiempo que transcurra la extracción. Una vez finalizado el proceso, el extracto líquido se puede recuperar, tras la filtración/eliminación de los restos sólidos.

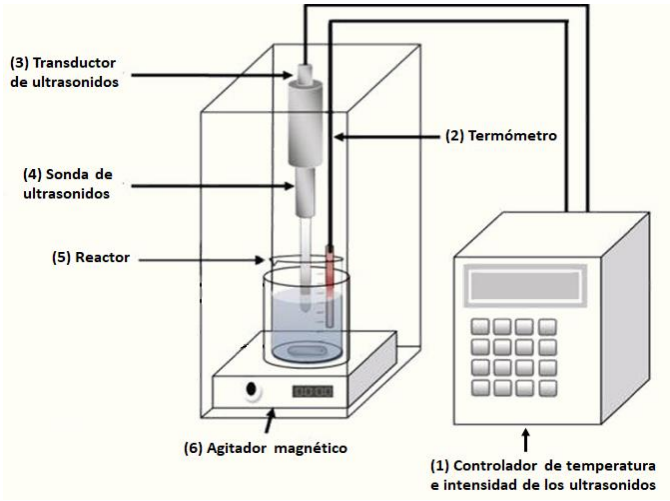

Figura 11. Esquema representativo de un equipo UAE tipo sonda. Adaptado de Gasmalla y col., 2015.

Además de seleccionar los parámetros de operación para la generación de ultrasonidos y el control de la temperatura, la optimización de la técnica de UAE va a depender de la selección del disolvente, el tiempo de extracción y la relación soluto/disolvente. En cuanto al disolvente de extracción, como en cualquier otra técnica sólido/líquido, la elección se hará conforme a la solubilidad de los compuestos que se deseen recuperar.

Igualmente, debido a la preocupación de no generar más gasto de disolventes orgánicos contaminantes, y buscando una baja toxicidad en los extractos obtenidos, la elección de etanol y agua, y las mezclas de estos, son opciones bastante apropiadas.

La técnica de extracción asistida por ultrasonidos es una técnica que requiere, generalmente, de la implementación de tecnología de bajo costo y su ejecución resulta un proceso sencillo y fácil de reproducir. Gracias a estas ventajas, la UAE es una de las técnicas de extracción no-convencionales que ha visto crecer su capacidad, tanto a nivel piloto como a escala industrial. En este sentido, empresas como Hielscher (Alemania), REUS (Francia), y algunas empresas ubicadas en China, se han encargado de desarrollar sistemas basados en la extracción con ultrasonidos de tipo sonda, y de superficie radiante con agitación constante, para la fabricación de aditivos alimentarios y cosméticos, entre otros usos (Meng y Lozano, 2014).

De esta manera, el empleo de la extracción asistida por ultrasonidos ha generado un gran número de estudios referente a la obtención de compuestos fenólicos a partir de plantas (Roselló-Soto y col., 2015; Ameer y col., 2017). Así podemos destacar el uso de UAE para lograr la optimización de compuestos fenólicos totales extraídos de hojas del olivo (*Olea europaea)* con el uso de 50% etanol y 25ºC (Şahin y col., 2013). Igualmente, el valor más alto de compuestos flavonoides totales se logró con el uso de 40% etanol y 72,3 ºC, extraídos de hojas de *Camellia fascicularis* (Liu y col., 2018). Adicionalmente, a partir de hojas de majorana (*O. majorana*) bajo condiciones de extracción de 80% etanol y 35ºC, se consiguieron las cantidades más altas de ácido rosmarínico, luteolina-7-*O*-glucósido, apigenin-7-*O*-glucósido, ácido cafeico, ácido carnósico y carnosol (Hossain y col., 2012). De manera similar, de la hoja de laurel (*L. nobilis)* se consiguió extraer una cantidad óptima de compuestos fenólicos totales con la aplicación de 35% etanol (Muñiz-Márquez y col., 2013).

1.2.2. Técnicas de fraccionamiento.

La mezcla de compuestos solubles que se obtiene a partir de matrices vegetales puede contener, además de los compuestos fenólicos, algunas sustancias adicionales como azúcares, ácidos orgánicos y grasas. Por tal motivo, pueden requerirse pasos adicionales para eliminar los componentes no deseados y purificar/enriquecer los extractos en aquellos compuestos que sean de mayor interés. La mayoría de las veces, durante estos procesos se genera la obtención de diferentes fracciones enriquecidas en uno o varios compuestos específicos, en función de la afinidad que presenten las moléculas respecto al disolvente o agente de separación.

Para lograr un enriquecimiento de compuestos fenólicos, se han empleado varias estrategias, entre ellas una técnica tradicionalmente usada es la extracción líquido-líquido secuencial con solventes orgánicos. Sin embargo, este método presenta un alto gasto de solvente, bajas eficiencias y es difícil de llevarlo a escala industrial. Otra de las técnicas usadas con mayor frecuencia es la extracción en fase sólida (SPE), donde los compuestos de interés se encuentran solubilizados y se ponen en contacto con un material adsorbente, permitiendo la separación y concentración de los compuestos de interés dependiendo del disolvente de elución que sea utilizado. Esta técnica ha ganado fama por su rapidez y alta sensibilidad, y por la amplia gama de materiales de relleno que se encuentran disponibles de manera comercial. El aislamiento de compuestos fenólicos con cartuchos C-18 de fase inversa es una técnica muy utilizada, sobre todo a nivel laboratorio. Evidentemente este sistema está limitado por la corta capacidad de los cartuchos, y el poco volumen/peso de extracto que se puede recuperar.

De esta manera, apuntando a una mayor escala, el uso de resinas macrospóricas de adsorción resulta ser una metodología más adecuada. En esa misma línea, se pueden mencionar el uso de membranas de ultra y nano-filtración como una técnica que permite el tratamiento de extractos vegetales con volúmenes más altos. Igualmente, el fraccionamiento de compuestos fenólicos utilizando la tecnología supercrítica antisolvente (SAS) ha cobrado fuerza en la última década, como una alternativa a los métodos comunes de purificación de extractos, permitiendo a su vez, la obtención de micropartículas.

1.2.2.1. Uso de resinas macrospóricas de adsorción.

La adsorción es una tecnología relativamente simple y flexible de cara al enriquecimiento de compuestos fenólicos. Se compone de un sistema de adsorción-desorción, que se ve influenciado por la naturaleza del soluto (carga iónica, polaridad, solubilidad y tamaño molecular), las condiciones de la solución en la que se encuentra disuelto el soluto (polaridad, pH, temperatura y concentración) y la naturaleza del disolvente de elución (polaridad, concentración y pH). Habitualmente, el uso de metanol con diferentes proporciones de agua como disolvente de elución, ha reportado altos rendimientos en la recuperación de compuestos fenólicos en estas resinas, sin embargo, la elección de etanol con diferentes mezclas de agua, es preferible para reducir la toxicidad de los extractos secos.

La adsorción con resinas presenta una serie de ventajas sobre otras técnicas, incluyendo su simplicidad de operación, baja toxicidad, larga vida útil y alta capacidad de adsorción para una gran diversidad de compuestos, como lo son los compuestos fenólicos. Además, gracias a su alta capacidad de regeneración, los costos de operación resultan relativamente bajos por lo que se favorece su uso a escala industrial (Pérez-Larrán y col., 2018). Siendo así que en los últimos años se ha extendido el empleo de resinas de adsorción en la industria de los alimentos para remover compuestos fenólicos que aporten color, sabor o aromas indeseados en ciertas formulaciones, como en el caso de la producción zumos de frutas, y también en la recuperación de compuestos fenólicos a partir de subproductos industriales (Soto y col., 2011; Kammerer y col., 2019). Asimismo, un sistema semicontinuo de columnas con resinas, a escala piloto, se ha propuesto para la recuperación de compuestos fenólicos a partir de alpechín de la oliva (Frascari y col., 2019).

Existe una gran variedad de resinas que resultan adecuadas para la adsorción selectiva de compuestos fenólicos, sin embargo, las resinas no-iónicas han sido utilizadas tanto para la adsorción de compuestos fenólicos puros, como de extractos de matrices vegetales (Soto y col., 2011; Pérez-Larrán y col, 2018; Kammerer y col., 2019). De tal modo, se muestran algunos ejemplos relevantes del uso de resinas de adsorción para el fraccionamiento/enriquecimiento de compuestos fenólicos a partir de matrices vegetales (Tabla 1).

Así las amberlitas XAD-7HP y XAD-16 han sido más frecuentemente utilizadas para la recuperación de compuestos fenólicos como el ácido rosmarínico a partir de hojas de *Rabdosia serra* (Lin y col., 2012) y de compuestos poliméricos como proantocianidinas (Pinto y col., 2017). Además, tras el uso de amberlitas XAD-7HP se logró la recuperación del 60% de catequinas a partir del té negro (Monsanto y col., 2014).

Tabla 1. Ejemplos de compuestos fenólicos purificados con resinas de adsorción a partir de matrices vegetales.

Técnica	Matriz / Disolvente	Compuestos de interés	Condiciones	Referencias
RESINAS DE ADSORCIÓN	*Rabdosia serra* (hojas)/ agua	Ácido rosmarínico	XAD-7HP y HP-20 / 10, 30, 50, 70, 90% etanol	Lin y col., 2012
	Eucalipto (corteza)/ 52% etanol	Polifenoles totales / proantocianidinas	XAD-4 y XAD-16 / 95% etanol	Pinto y col., 2017
	Té negro (hojas)/ agua	Catequinas / teaflavinas	XAD-7HP y FPX66 / 50% etanol	Monsanto y col., 2014
	Granada (pericarpio)/ 60% etanol	Polifenoles totales	D101, AB-8, HPD-100, HPD-450 y HPD-600 / 60% etanol	Jiang y col., 2016
	Bayas (aronia, grosella, mora, arándano, sauco)/ agua acidificada (1% ácido cítrico)	Antocianinas	XAD-7 / 96% etanol	Denev y col., 2010
	Regaliz (hojas) / 80% etanol	Flavonoides	XAD-16 y SP825 / 90% etanol	Dong y col., 2015
	Helianthus tuberosus (hojas) / 60% etanol	Ácido 3-*O*-cafeoilquínico	ADS-21 / 80% etanol	Sun y col., 2015

1.1.1.1. Tecnología Supercrítica Antisolvente (SAS)

El proceso SAS es utilizado cuando un soluto tiene baja solubilidad en el fluido supercrítico, generalmente CO_2 supercrítico. En este caso, la condición para llevar a cabo el proceso SAS es seleccionar un disolvente orgánico apropiado que disuelva el soluto de interés (o el extracto) y que, a su vez, sea miscible en el SC-CO_2. De esta manera, el extracto en forma de disolución (1) se hará pasar por una boquilla (3) hacia la celda de extracción (4). A la par, el SC-CO_2 (2), que actúa como antisolvente, será bombeado hacia la celda de extracción. Al entrar en contacto el antisolvente y la disolución del extracto, se forma un medio homogéneo en el que el disolvente orgánico pierde poder de solvatación debido a la expansión volumétrica. Debido a las diferencias en polaridad, hidrofobicidad del CO_2 y peso molecular, algunos de los compuestos fenólicos en la mezcla ya no serán solubles en el nuevo medio y precipitarán (5). Por el contrario, otros compuestos interactúan fuertemente con el disolvente orgánico y aún serán solubles a pesar de la presencia del antisolvente. Estos compuestos son arrastrados con el dióxido de carbono (7), donde se pueden recuperar una vez que se despresuriza el sistema (Fig. 12) (Padrela y col., 2018).

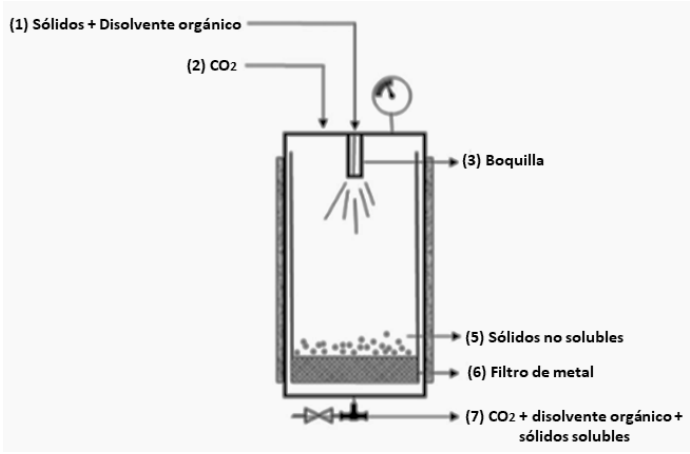

Figura 12. Esquema representativo la celda de precipitación SAS de un sistema supercrítico antisolvente. Adaptada de: Villanueva-Bermejo y col., 2017.

En general, el proceso SAS puede llevarse a cabo de manera discontinua o semicontinua. En el primer caso, el disolvente orgánico, junto con el soluto, se encuentra en el interior de la celda de precipitación y se introduce el fluido supercrítico durante un periodo lo suficientemente largo como para lograr la disolución del disolvente orgánico en la fase supercrítica y la precipitación de las partículas. En cambio, en el proceso semicontinuo, tanto el fluido supercrítico, como la disolución con el soluto de interés, se introducen al interior de la celda de precipitación de forma continua y simultánea mediante una bomba, hasta que se finaliza el proceso y se produce la despresurización del sistema y la recogida de las partículas precipitadas en la celda de precipitación. En el caso del proceso semicontinuo, la disolución se atomiza al hacerla pasar a través de una boquilla a la entrada de la celda de precipitación, donde se pondrá en contacto con el fluido supercrítico, produciéndose el efecto antisolvente (Cocero y Martín, 2008). Muchos de los trabajos con la tecnología SAS están enfocados a la purificación de compuestos fenólicos a partir de matrices vegetales (Baldino y Reverchon., 2018; Gil-Ramírez y Rodríguez-Meizoso, 2019).

En la Tabla 2 se muestran algunos ejemplos del uso de la técnica SAS, indicando las condiciones de presión y temperatura necesarias para la precipitación de compuestos fenólicos a partir de matrices vegetales. Podemos observar que los rangos de presión van desde los 8 MPa hasta los 30 MPa para estos compuestos específicos y que las temperaturas no exceden de los 70ºC. Particularmente llama la atención el caso del romero, donde en dos estudios individuales se utilizó como extracto de partida en la precipitación antisolvente. Por un lado, el uso de una disolución acuosa-etanólica (50:50, v/v), para precipitar el ácido rosmarínico (Sánchez-Camargo y col., 2016), y, por otro lado, una disolución en etanol puro, con el objetivo de recuperar al ácido carnósico en la fracción soluble (Visentín y col., 2011).

También se puede destacar el uso de tinturas como el propolis para el fraccionamiento de flavonoides (Catchopole y col., 2004) y el uso de lactato de etilo y acetona, como disolventes para solubilizar extractos de hojas de té verde y conseguir la precipitación de catequinas (Soto y col., 2011; Villanueva y col., 2015). Así se pone de manifiesto la acción antisolvente del CO_2 en las disoluciones líquidas que se desean fraccionar.

Tabla 2. Fraccionamiento de diferentes compuestos fenólicos a partir de matrices vegetales mediante la técnica SAS empleando resinas de adsorción.

Técnica	Matriz / Disolvente	Compuestos de interés	Condiciones	Referencias
SAS	Té verde (hojas)/ lactato de etilo	Catequinas	15 - 30 MPa / 50 y 70 ºC	Villanueva y col., 2015
	Té verde (hojas)/ acetona + polímero	Catequinas + polímero	8 - 12 MPa / 11 - 34 ºC	Sosa y col., 2011
	Romero (hojas)/ 50% etanol	Ácido rosmarínico	10 - 30 MPa / 40 ºC	Sánchez-Camargo y col., 2016
	Romero (hojas)/ etanol	Ácido carnósico	30 MPa / 50 ºC	Visentín y col., 2011
	Semilla de uvas (residuos)/ etanol	Polifenoles totales	8 - 15 MPa / 35 y 50 ºC	Marqués y col., 2013
	Olivo (hojas)/ etanol	Oleuropeína y tirosol (derivados)	15 MPa / 50 ºC	Chinnarasu y col., 2015
	Moras (residuos)/ etanol	Antocianinas y otros compuestos fenólicos	12,5 MPa / 30 ºC	Machado y col., 2018
	Propóleo (tintura) / etanol	Flavonoides	27,5 - 30 MPa / 60 ºC	Catchpole y col., 2004

1.3. Encapsulación de compuestos fenólicos.

Los compuestos fenólicos son compuestos naturalmente inestables debido a su conformación estructural, que los hace susceptibles a ciertas condiciones físicas y químicas, como la luz, pH, las altas temperaturas y el oxígeno. Además, su funcionalidad en el organismo puede verse limitada por su baja liberación desde la matriz alimentaria, su inestabilidad en el tracto gastrointestinal, su solubilidad y baja absorción en el intestino. De esta manera, la encapsulación se presenta como una estrategia para preservar la estabilidad de los compuestos fenólicos y mejorar su biodisponibilidad (Li y col., 2015; Esfanjani y col., 2018). Además, desde un punto de vista tecnológico, la encapsulación ayuda a la incorporación de los agentes bioactivos en nuevas matrices alimentarias, mejorando su solubilidad o enmascarando esos sabores amargos y astringentes propios de los compuestos fenólicos. Igualmente, la encapsulación ayudará a la conservación de los compuestos fenólicos durante su almacenamiento y procesado (Ray y col., 2016).

- **Agentes encapsulantes**

La encapsulación implica la incorporación de moléculas químicamente sensibles, como los compuestos fenólicos, dentro de un material secundario para formar cápsulas o matrices más resistentes. Ese material secundario es el *agente encapsulante*, al que también se le denomina material de recubrimiento, revestimiento, material portador o material de pared (Esfanjani y col., 2018). Existe una gran variedad de materiales para la encapsulación de compuestos fenólicos, y en el caso de destinarse para su consumo humano, es necesario emplear materiales clasificados de grado alimentario o reconocidos como GRAS (Jia y col., 2016). En la Tabla 3 se mencionan los materiales utilizados comúnmente, de los que podemos destacar los polímeros de carbohidratos obtenidos de plantas, como pectinas, goma arábiga, maltodextrinas y ciclodextrinas. También podemos incluir a los polisacáridos procedentes de algas, como los carragenanos y alginatos, o los dextranos, que son sintetizados por bacterias. De la misma forma, las proteínas, tanto de origen vegetal como animal, resultan materiales idóneos para su uso como agentes encapsulantes. Así podemos mencionar a las caseínas de la leche, las proteínas del suero lácteo y los aislados proteicos de soja.

Los lípidos conforman otro grupo importante de materiales de encapsulación, como los fosfolípidos de soja o de huevo, y los mono- y di-glicéridos. Igualmente, para la estabilización de las formulaciones suelen emplearse sustancias emulsionantes, destacando el caseinato de sodio y la lecitina de soja.

Tabla 3. Materiales comúnmente utilizados como encapsulantes aptos para su uso en alimentos.

Origen	**Carbohidratos**	**Proteínas**	**Lípidos**
Plantas	-Pectinas -Goma arábiga -Goma guar -Almidón -Maltodextrinas -Ciclodextrinas	-Proteína aislada de leguminosas (soja, guisantes, etc.) -Proteína aislada de cereales (trigo, maíz, etc.).	-Aceites vegetales -Lecitinas -Fosfolípidos (fosfatidílcolina de soja) -Glicéridos
Especies marinas	-Carragenanos -Alginatos		-Fosfolípidos
Microorganismos/ animales	-Dextranos -Quitosano -Goma xantana	-Caseínas -Caseinato de sodio -Proteínas de suero lácteo -Gelatina -Albúmina	-Fosfolípidos (fosfatidílcolina de huevo) -Glicéridos

La elección del material de encapsulación dependerá tanto de las propiedades físico-químicas (polaridad, solubilidad) de los agentes bioactivos que se desean encapsular, como de las mismas propiedades del agente encapsulante. Además, se tendrá en cuenta si el compuesto se encuentra en estado puro o forma parte de un extracto, lo cual podría complicar la elección del material.

En ese sentido, no existe un material de encapsulación único para cada compuesto que se desee encapsular, sino que el material ideal será aquel que, además de brindar protección/estabilidad al agente bioactivo, reúna ciertas características (Arranz y col., 2016; Ray y col., 2016):

i) El material debe ser compatible y no reaccionar con la matriz alimentaria que lo rodea.
ii) El material deberá mantenerse inalterado durante el procesamiento y almacenamiento.
iii) El material no deberá afectar negativamente la bioactividad del compuesto encapsulado.
iv) El material deberá permitir la liberación del compuesto encapsulado en el sitio apropiado.

Muchas veces resulta complicado que un solo material cumpla con todas las necesidades, por eso comúnmente suele usarse una mezcla de dos o más agentes en un mismo sistema de encapsulación.

- **Técnicas de encapsulación**

Existe una extensa variedad de técnicas que se han utilizado para la encapsulación de compuestos fenólicos, de las cuales las más comunes son: el secado por pulverización, la liofilización, emulsiones, formación de liposomas, coacervación, inclusión molecular, gelificación y la precipitación antisolvente (Dordević y col., 2015; Jia y col., 2016).

Las técnicas de encapsulación se pueden clasificar, según la tecnología aplicada, en mecánicas y químicas. Entre las técnicas mecánicas, tenemos a la liofilización, pulverización, formación de emulsiones, liposomas y precipitación antisolvente. La liofilización y el secado por pulverización, son dos de las técnicas más extendidas debido a la alta disposición de los equipos de secado. Además, para la gran mayoría de las aplicaciones en la industria de los alimentos, el fin último es conseguir partículas en polvo a partir de una mezcla líquida, para facilitar su integración en las formulaciones y el almacenamiento de los ingredientes pulverizados. Referente a las emulsiones, estas representan un sistema coloidal estable conformado por dos fases líquida inmiscibles, donde un líquido se dispersa dentro del otro (aceite-en-agua o agua-en-aceite). Para estabilizar la emulsión, la adición de un agente emulgente de naturaleza anfifílica es necesaria (caseinato de sodio, lecitinas). Las nanoemulsiones se denominan de tal manera por contener partículas con un radio entre 10-100 nm (McClements y Rao, 2011) y para su elaboración requieren de una mayor cantidad de estabilizador y de la aplicación de alta energía, conseguida con

homogeneizadores de alta presión. Las nanoemulsiones pueden utilizarse directamente en su estado líquido o en polvo, tras su secado generalmente por liofilización o pulverización.

Recientemente el uso de la técnica de precipitación antisolvente va en crecimiento debido a la posibilidad de su escalado. Generalmente, se está utilizando la tecnología de una planta de líquidos supercríticos para llevar a cabo la precipitación de partículas. En ese sentido, la mayoría de los estudios se están realizando con el uso de CO_2 en estado supercrítico, como antisolvente. La metodología sigue el mismo principio que la anteriormente mencionada técnica de fraccionamiento antisolvente o SAS (Padrela y col., 2018), con la adición de un polímero o material de recubrimiento para ayudar a una mejor formación de partículas.

Por otra parte, dentro de las técnicas de encapsulación por métodos químicos, podemos mencionar la gelificación y la inclusión molecular. La gelificación como una técnica de encapsulación es una de las propuestas más estudiadas para aprovechar las propiedades de agregación que presentan las proteínas (caseínas, albúmina, β-lactoglobulina) y algunos polisacáridos presentes en los alimentos, cuando son sometidos a temperatura elevada, condiciones de acidificación y presencia de iones o enzimas. En el caso de las proteínas, los residuos hidrofóbicos de los aminoácidos son capaces de atrapar a los compuestos fenólicos y formar una estructura tridimensional. Las estructuras gelificadas resultantes presentan una textura semisolida o como un sólido suave (Norton y col., 2015).

La inclusión molecular generalmente conlleva el uso de ciclodextrinas como agentes encapsulantes. Estos oligosacáridos cíclicos derivados del almidón, presentan en su molécula un carácter hidrofílico en su parte externa, mientras que internamente son hidrofóbicas. Por eso, son ampliamente utilizadas para incrementar la solubilidad en agua de aquellas partículas lipofílicas. Además de las ciclodextrinas, otros biopolímeros se han utilizado para la inclusión molecular de compuestos fenólicos, como los almidones y sus derivados (Lorentz y col., 2012). En ese sentido, algunos de los ejemplos de diferentes técnicas de encapsulación para extractos ricos en compuestos fenólicos y compuestos puros se citan en la Tabla 4.

Tabla 4. Tecnologías para la encapsulación de compuestos fenólicos.

Técnica de encapsulación	Agente encapsulante	Extracto de partida	Compuesto fenólico encapsulado	Referencia
Técnicas mecánicas				
Secado por pulverización	Proteína aislada de suero	Extracto de arándano	Antocianinas	Flores y col., 2014
	Proteína aislada de suero y maltodextrina	Extracto de ortosifón	Ácido rosmarínico, sinensetina, eupatorina	Pang y col., 2014
	Maltodextrina	Extracto de yerba mate	Ácido gálico, ácido 3,4-dihidroxibenzoico, ácido *p*-cumárico, ácido cafeico, ácido clorogénico y ácido 3,5-DCQA	Nunes y col., 2015
	Maltodextrina y pectina	Extracto de melisa	Compuestos fenólicos totales	Sansone y col., 2011
	Maltodextrina y goma arábiga	Extracto de propóleo	Ácidos fenólicos (11) y flavonoides (14)	Andrade y col., 2018
Emulsiones	Aceite de canola + lactoferrina y/o proteína aislada de suero	Extracto de romero	Ácido carnósico y carnosol	Arranz y col., 2017
	Aceite de maíz + glicerol (emulsión 1) y NaCl + Tween 20, Tween 80 o lecitina	Extracto de piel de mango	Ácido gálico, mangiferina, catequina y quercetina	Velderrain-Rodríguez y col., 2019
	Aceite de soja + caseinato de sodio y/o pectina	Compuesto puro	Galato de epigalocatequina (EGCG)	Sabouri y col., 2018
	Aceite de cacahuete + lecitina de soja + Tween 20	Compuesto puro	Resveratrol	Sessa y col., 2014
	Miglyol 812 + β-lactoglobulina	Compuesto puro	Curcumina	Ahmend y col., 2012

Tabla 4. Tecnologías para la encapsulación de compuestos fenólicos (Continuación).

Técnica de encapsulación	Agente encapsulante	Extracto de partida	Compuesto fenólico encapsulado	Referencia
		Técnicas mecánicas		
Precipitación antisolvente (CO_2 supercrítico)	Polímero biodegradable	Extracto de té verde	Compuestos fenólicos totales	Sosa y col., 2011
	-	Piel y semilla del mango	Mangiferina, isomangiferina; quercetina 3-*O*-galactósido; quercetina 3-*O*-glucósido; quercetina 3-*O*-xilósido; quercetina 3-*O*-arabinósido; quercetina y kaempferol	Meneses y col., 2015
	Polímero biodegradable	Compuesto puro	Resveratrol	Dal Magro y col., 2017
	-	Compuesto puro	Rutina	Montes y col., 2016
	Etilcelulosa Lecitina de soja	Compuesto puro	Quercetina	Mezzomo y col., 2016. Lévai y col., 2017
Liposomas	Fofolípidos (leche)	Extracto comercial de té	EGCG	Gülseren y col., 2012
	Fosfatidilcolina (huevo)	Compuesto puro	Quercetina	Tong-Un y col., 2010
	Lecitina de soja + colesterol	Compuesto puro	Ácido clorogénico	Feng y col., 2016
	Lecitina + colesterol	Compuesto puro	Luteolina	Wu y col., 2018
	Lecitina de colza	Compuesto puro	Apigenina	Paini y col., 2015

Tabla 4. Tecnologías para la encapsulación de compuestos fenólicos (*Continuación*).

Técnica de encapsulación	Agente encapsulante	Extracto de partida	Compuesto fenólico encapsulado	Referencia
Técnicas químicas				
Gelificación	Alginato de calcio y almidón (solución de $CaCl_2$)	Extracto de yerba mate	Compuestos fenólicos totales	López-Córdoba y col., 2014
	Leche acidificada (caseínas y suero)	Extracto de cacao	Flavanoles y procianidinas	Vega y Grover., 2011
	Micelas de caseína	Compuesto puro	EGCG	Haratifar y Corredig, 2014
	Quitosano + ácido linoleico y β-lactoglobulina	Compuesto puro	Quercetina	Ha y col., 2013
	Quitosano	Compuesto puro	Ácido clorogénico	Nallamuthu y col., 2015
Inclusión molecular	β-ciclodextrinas	Extracto de hierba de San Juan	Epicatequina, catequina y quercetina	Kalogeropoulos y col., 2010
	β-ciclodextrina	Compuesto puro	Ácido cafeico, ácido ferúlico y ácido *p*-cumárico	Liu y col., 2016
	Hidroxipropil-β-ciclodextrina (HP- β-CD)	Compuesto puro	Carvacrol	Kamimura y col., 2014
	β-ciclodextrinas	Compuesto puro	Ácido rosmarínico	Çelik y col., 2011
	Amilosa	Compuesto puro	Ácido clorogénico	Lorentz y col., 2012

Las partículas formadas tras el proceso de encapsulación, son pequeñas vesículas que llevan unido al compuesto de interés. Dependiendo de la técnica empleada, las

partículas obtenidas pueden obtenerse de diferentes tamaños y formas. De acuerdo al tamaño, se pueden clasificar como micro (1.0-5000 µm) y nano (< 1.0 µm) (Esfanjani y col., 2018). Referente a la forma, existen formas esféricas, tubulares o matrices no definidas, representadas en la Fig. 12 (Fang y Bhandari, 2010; Dordević y col., 2015; Ruiz Canizales y col., 2018).

La forma más básica es una partícula de núcleo esférico, que además puede estar compuesto de una sola capa (a) o multicapa (b), como el caso de las emulsiones. También hay algunas en las que el compuesto encapsulado queda disperso en el núcleo y rodeado por el material de pared, dando como resultado formas homogéneas y más definidas, como lo que ocurre en la técnica de secado por pulverización o la precipitación antisolvente (c), o formas no definidas, como en el secado por liofilización o la coacervación (d). Otras de las morfologías se refieren a vesículas esféricas compuestas por bicapas lipídicas, como los liposomas (e), las partículas tubulares resultantes de la inclusión con ciclodextrinas (f), o un complejo tipo micela, como lo que ocurre en la gelificación (g).

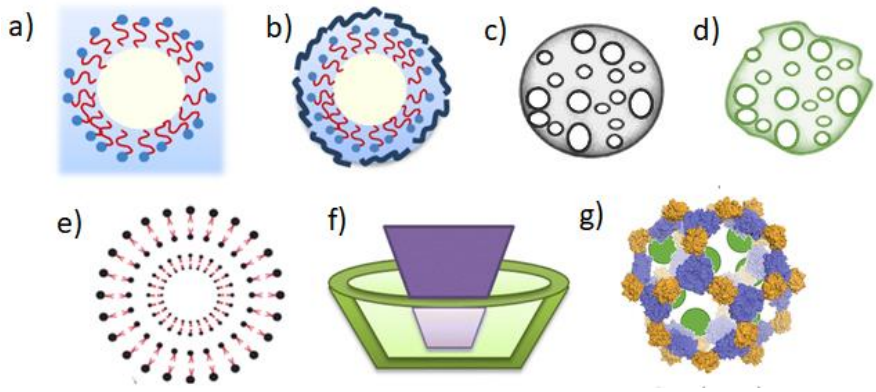

Figura 12. Ilustración esquemática de las distintas morfologías de las partículas obtenidas tras el proceso de encapsulación. Adaptada de: Ruiz Canizales y col., 2018; Dordević y col., 2015; Fang y col., 2010.

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2. JUSTIFICACIÓN Y OBJETIVOS

2. Justificación y Objetivos

Debido a la amplia variedad de actividades biológicas atribuibles a los compuestos fenólicos, estos compuestos se presentan como ingredientes potenciales para el diseño de alimentos con propiedades saludables. Desde esta perspectiva, diferentes aspectos relacionados con los compuestos fenólicos tendrán que ser evaluados previamente a su incorporación, como son: i) su obtención, ii) su caracterización química, iii) la evaluación de su actividad biológica y, iv) la evaluación y mejora de su biodisponibilidad.

En este sentido, el objetivo general de esta tesis es **desarrollar diferentes estrategias para el enriquecimiento y formulación de compuestos fenólicos, con actividad antioxidante y antiinflamatoria, con la finalidad de aumentar su biodisponibilidad.** Así, con el fin de lograr el cumplimento del objetivo general, se plantearon los siguientes *objetivos parciales*:

1. Identificar las plantas pertenecientes a las familias Lamiaceae y Asteraceae que presentan un mejor potencial como fuente de compuestos con actividad antioxidante y/o antiinflamatoria mediante el uso de técnicas de extracción sostenibles. Optimizar el proceso de extracción: técnica y solvente empleado.

2. Investigar la relación existente entre la composición de los extractos obtenidos y su actividad antioxidante y/o antiinflamatoria.

3. Aplicar el uso de técnicas de fraccionamiento sostenibles altamente escalables, como las resinas de adsorción y la tecnología supercrítica de precipitación antisolvente, para la obtención de fracciones enriquecidas en compuestos con actividad antioxidante y/o antiinflamatoria.

4. Estudiar la biodisponibilidad de los compuestos fenólicos presentes en los extractos seleccionados mediante un modelo de digestión gastrointestinal *in vitro* y su posterior absorción intestinal en células Caco-2.

5. Mejorar la biodisponibiliad y/o favorecer la incorporación en matrices alimentarias de los extractos seleccionados mediante el desarrollo de distintas formulaciones.

3. PLAN DE TRABAJO

3. Plan de Trabajo

El plan de trabajo seguido se muestra en la Fig. 13. La primera etapa consistió en la realización de un estudio comparativo del potencial antioxidante y antiinflamatorio de diversas plantas pertenecientes a las familias *Lamiaceae (Origanum majorana* y *Melissa officinalis)* y *Astereaceae (Calendula officinalis* y *Achillea millefolium)*. Para ello se llevaron a cabo diversas extracciones mediante tecnología de fluidos supercríticos (SFE) y extracción asistida con ultrasonidos (UAE), analizándose posteriormente su actividad antioxidante y antiinflamatoria mediante ensayos *in vitro*, así como su composición química.

Una vez seleccionadas las especies que presentaban un mayor potencial antioxidante y/o antiinflamatorio (mejorana y milenrama), se realizó un nuevo estudio comparativo entre técnicas de extracción avanzadas: la extracción con líquidos presurizados (PLE) y la UAE, realizando a su vez, el análisis químico y funcional de los extractos.

Posteriormente, se procedió al enriquecimiento de los compuestos con actividad antioxidante y/o antiinflamatoria presentes en los extractos seleccionados de mejorana y milenrama mediante el uso de resinas de adsorción y tecnología supercrítica de precipitación antisolvente. A continuación, se determinó la biodisponibilidad de los extractos empleando los modelos *in vitro* de digestión gastrointestinal y absorción intestinal en células Caco-2. Se analizó la actividad antioxidante y antiinflamatoria de la fracción biodisponible, junto con el análisis de su composición fenólica.

De manera paralela, se ha estudiado la formulación de los extractos óptimos con el fin de mejorar su bioaccesibilidad, lo que daría lugar a una posible mejora de la concentración de compuestos de interés biodisponibles. Igualmente, estas formulaciones de compuestos fenólicos se han encaminado hacia su adecuación para su posible uso como ingredientes funcionales.

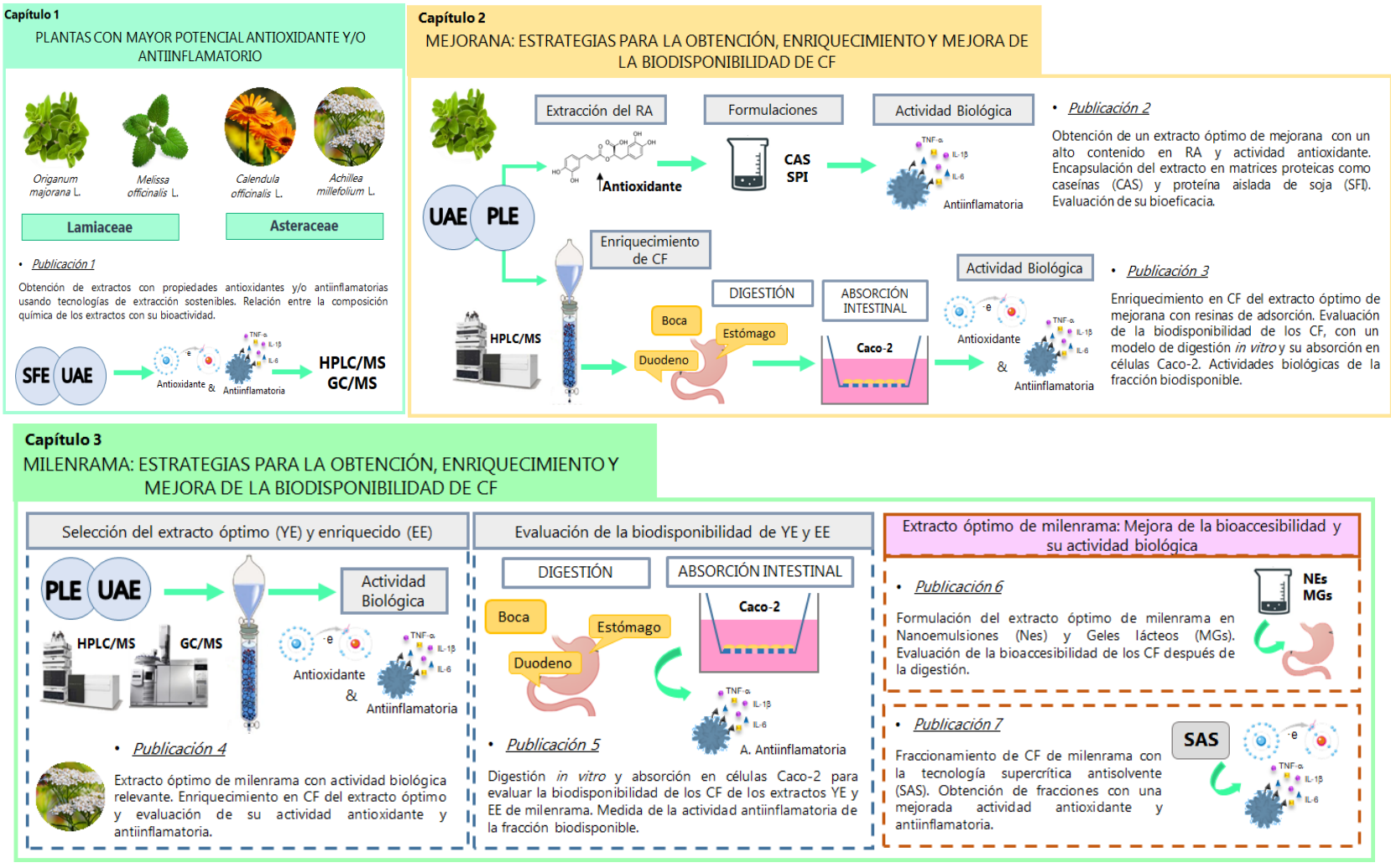

Figura 13. Esquema general del plan de trabajo.

4.RESULTADOS

CAPÍTULO 1

Identificación de las plantas con un mayor potencial antioxidante y/o antiinflamatorio.

Prefacio

Las plantas de las familias Lamiaceae y Asteraceae representan una buena fuente de compuestos fenólicos. Dentro de estas, algunas especies han sido tradicionalmente utilizadas por tener un efecto beneficioso en la salud, como *Melissa officinalis* y *Origanum majorana,* (Lamiaceae) y *Achillea millefolium* y *Calendula officinalis* (Asteraceae).

En lo que respecta a la obtención de compuestos fenólicos, es importante considerar que las técnicas y procesos de extracción sean los más apropiados y eficientes, y a su vez respetuosos con el medio ambiente. En ese sentido, la técnica de extracción con fluidos supercríticos (SFE) y la técnica de extracción asistida por ultrasonidos (UAE), permiten la recuperación de compuestos fenólicos con un menor volumen de disolventes, y además son técnicas fácilmente escalables. Teniendo en cuenta la amplia variabilidad de compuestos fenólicos que se encuentran en las plantas, el uso de disolventes con diferente polaridad es necesario cuando se pretende realizar un estudio dirigido a la obtención de extractos ricos en compuestos fenólicos.

En este primer capítulo se abordó el empleo de técnicas de extracción sostenibles (SFE y UAE), dirigidas a la obtención de extractos ricos en compuestos fenólicos con propiedades antioxidante y/o antiinflamatoria, a partir de *Melissa officinalis, Origanum majorana, Achillea millefolium* y *Calendula officinalis*.

Los resultados se muestran en la publicación *1*, titulada: **Sustainable extraction techniques for obtaining antioxidant and anti-inflammatory compounds from Lamiaceae and Asteraceae genera.**

De ese modo, cuando se empleó la técnica UAE se observó una clara influencia de la polaridad del disolvente de extracción en la recuperación de compuestos fenólicos. Así, el uso de mezclas etanólicas y acuosas (50:50, v/v), permitió la obtención de extractos con una alta actividad antioxidante, mientras que el uso de un disolvente menos polar que el agua, como es el etanol puro, propició la obtención de extractos con una destacada actividad antiinflamatoria. En el caso del uso de CO_2 supercríto con SFE, se observó que los extractos tenían un menor contenido en compuestos fenólicos totales (TPC) y una baja actividad antioxidante, así como un efecto antiinflamatorio limitado.

Respecto a las plantas estudiadas, los extractos de mejorana (*Origanum majorana*) destacaron por su alta actividad antioxidante, mientras que los de milenrama (*Achillea millefolium*) lo hicieron por su actividad antinflamatoria. Asimismo, el análisis de la composición química de los extractos, utilizando técnicas cromatográficas avanzadas (HPLC-PAD-MS/MS y GC-MS), permitió no solo la caracterización de los extractos obtenidos, sino también la determinación de los compuestos que serían los principales responsables de las actividades observadas.

Este primer estudio permitió determinar que plantas presentaban un mayor potencial antioxidante y/o antiinflamatorio, correspondiéndose en este caso con la mejorana y la milenrama, descartándose el uso de melisa y caléndula para posteriores capítulos de la presente memoria.

Publicación 1

Sustainable extraction techniques for obtaining antioxidant and anti-inflammatory compounds from Lamiaceae and Asteraceae genera.

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ABSTRACT

Within Lamiaceae and Asteraceae genera, *Melissa officinalis* L., *Origanum majorana* L., *Calendula officinalis* L. and *Achillea millefolium* L., have been considered as a good source of bioactive ingredients with a widespread therapeutic effects. In this study, supercritical fluid extraction technique using pure CO_2 (SFE) and ultrasound assisted extraction (UAE) with pure ethanol (UAE-100) or ethanol:water 50:50 (v/v) (UAE-50) have been proposed as green extraction techiniques to obtain plant-extracts with potential antioxidant and anti-inflammatory activities. Higher extraction yield, total phenolics content (TPC) and antioxidant activity were achieved in UAE-50 extracts, besides the plant used. In contrast, UAE-100 extracts showed the greater anti-inflammatory activity for the four studied plants. After the HPLC-PAD-QTOF-MS/MS analysis an important number of phenolic compounds, even unreported constituents, were identified in the four plants. *O. majorana* ethanol:water extract presented the maximum content of phenolics and the highest antioxidant activity; among its composition, both rosmarinic acid and luteolin glucoside derivatives were the most abundant compounds. The pure ethanol extract of *A. millefolium* resulted with an important content of caffeoylquinic acid derivatives and some aglycone of flavonoids, which could be related with its remarkable inhibition of TNF-α, IL-1β and IL-6 cytokines secretion. Besides, the volatile fraction of *A. millefolium*, in particular borneol and camphor, may contributed with this latter activity. Thus, *O. majorana* along with *A. millefolium* are considered as the most promising source of bioactive ingredients with potential use in health promotion.

1. INTRODUCTION

In the last decades, an increasing number of studies have been focused on the quest of new sources of food ingredients to deploy applications in pharmaceutic, cosmetics, food industry and of course in nutrition. Several compounds, such as carotenoids, saponins, phenolic compounds, terpenes, peptides, etc., have been proposed to possess interesting benefits for human being. The first step, regarding development of food ingredients, involves to find out the most suitable plant matrix. In that regard, Lamiaceae and Asteraceae genera represent a great source of valued plant species, often used as flavouring ingredients in many culinary preparations, but also considered as medicinal herbs, where these beneficial effects have been related mainly to phenolic compounds (Bessada et al., 2015; Costa et al., 2015).

Phenolic compounds, secondary metabolites of plants, have been extensively related to several biological properties, including antibacterial, antitumor, antioxidant and anti-inflammatory activity. Because of their structural characteristics, phenolic compounds are considered natural antioxidant and anti-inflammatory chemicals, able to avoid cellular damage related with the oxidative stress, and capable to reduce the occurrence of many chronic pathologies and diseases, such as diabetes, cardiovascular diseases or cancer (Premkumar et al., 2018).

Melissa officinalis L., commonly known as lemon balm, is a Lamiaceae specie typically used for prevention and treatment of nervous disturbances and gastrointestinal disorders. Different extracts of *M. officinalis* have exhibited antioxidant, antimicrobial and anti-inflammatory activities (Carocho et al., 2015; Ozarowski et al., 2016), in which, hydroxycinnamic acids, like rosmarinic acid, and luteolin glycosylated derivatives, were found as the main phenolic constituents (Miron et al., 2013). *Origanum majorana* L., also known as marjoram, is a Lamiaceae specie that has been appreciated because of its therapeutic properties against gastrointestinal, respiratory and neurological disorders (Vladimir-Knežević et al., 2014; Costa et al., 2015). Essential oil and other extracts from *O. majorana* have been indicated to possess antimicrobial, antioxidant, anti-proliferative and anti-inflammatory activities (Kaliora et al., 2014; Bina et al., 2017). Caffeic acid derivatives and flavonoids, along with ursolic acid, carnosic acid and carnosol, have been found as part of marjoram composition (Hossain et al., 2014; Vallverdú-Queralt et al., 2015).

The Asteraceae *specie Achillea millefolium* L., usually named as yarrow, is worldwide consumed for the treatment of gastrointestinal disorders and hepatobiliary complaints as well as for wound/ulcer healing and skin inflammation (Ali et al., 2017). Essential oil of *A. millefolium* is usually related to antimicrobial and anti-inflammatory activities (Chou et al., 2013; Abdossi and Kazemi, 2016). Moreover, alcoholic and water extracts, have shown antioxidant and antitumor properties (Dias et al., 2013; Pereira et al., 2018). Caffeic acid derivatives, mainly caffeoylquinic acids, and flavones has been reported as part of the yarrow's composition (Ali, et al., 2017). *Calendula officinalis* L., commonly known as marigold, has been frequently used for healing skin diseases, wounds and duodenal ulcer (Dinda et al., 2016; Miguel et al., 2016). Extracts of *this Asteraceae* specie has been reported for its anti-inflammatory, antibacterial, antioxidant and antitumor activity (Lim, 2014a). Quercetin derivatives, mainly their glycosylated and methylated forms, have been described within the phenolic composition in marigold (Hernández-Saavedra et al., 2016).

Nowadays, according to the sustainable development goals launched by United Nations (UN, 2019) the use sustainable extraction techniques in order to obtain plant-bioactives is essential. For that purpose, extraction techniques run by the Green Chemistry principles should be used (Chemat et al., 2017). Supercritical Fluid Extraction (SFE) has been widely used for the extraction of natural compounds in a more efficient and green environment (Ameer et al., 2017). SFE mostly uses CO_2 as carrier, a green and GRAS solvent (Generally Recognised as Safe), which allows the recovery of high-quality extracts. Besides, SFE represents a minimizing solvent-consuming process due to CO_2 recirculation. Ultrasound Assisted Extraction (UAE) applies ultrasonic vibrations generated at high frequencies to enhance the plant-bioactives extraction (Rodríguez-Pérez et al., 2015). UAE have been considered a more efficient technique than conventional ones, because implies solvent-reduction consumption and shorter-extraction times. As a consequence, a minor waste of solvent and energy is achieved (Chemat et al., 2017). Methanol and/or its water mixtures had been frequently used; nevertheless, this toxic solvent should be replaced with other GRAS status solvents such as ethanol in order to obtain high quality food ingredients.

Therefore, the main objective of this study was to perform a screening of the activities of Lamiaceae and Asteraceae extracts by using sustainable extraction methodologies. Thus, the antioxidant and anti-inflammatory activities of *Melissa officinalis L.* (MEL), *Origanum majorana L.* (MAJ), *Achillea millefolium L.* (MIL) and *Calendula officinalis L.* (CAL) were assessed. Furthermore, an extensive chemical analysis of the extracts was carried out in an attempt to correlate composition and bioactivities.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

HPLC grade acetonitrile was obtained from Macron Fine Chemicals (Madrid, Spain) and formic acid (99%) from Acros Organics (Madrid, Spain). Pure ethanol (99.5%) was purchased from Panreac (Barcelona, Spain). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%) were purchased from Sigma-Aldrich (Madrid, Spain). Phenolic acids' authentic standards (HPLC purity ≥ 95%) were purchased from Sigma-Aldrich, Phytolab (Madrid, Spain), Cymit Química SL (Madrid, Spain) and Extrasynthese S.A. (Genay, France) as detailed in supplementary material (Table S1).

2.2 Plant material and extraction

Origanum majorana L. leaves, *Melissa officinalis* L. leaves, *Achillea millefolium* L. inflorescences and leaves, and *Calendula officinalis* L. flowers were purchased as dry raw material from a local herbal company (Plantafarm S.A., León, Spain). Previous to the extraction process, the samples were ground (Premill 250, Leal S.A., Granollers, Spain) to diminish the particle size, preserved under vacuum and stored at room temperature until their use.

2.2.1 Ultrasound assisted extraction (UAE)

UAE plant extracts were obtained by using a Branson 250 digital device (Branson Ultrasonics, Danbury, CT, USA) with an electric power of 200 W and frequencies of 60 Hz. A corresponding solvent volume of ethanol (UAE-100) or ethanol:water (50:50, v/v) (UAE-50) was added to 35 g of ground plant material, in a ratio of 1:10 (plant/solvent, w/v). The mixture was submitted to ultrasounds for 30 min and 70% amplitude (40ºC), using a probe of ½' diameter. Next, samples were filtered and ethanol was removed under vacuum (IKA RV 10, Madrid, Spain). Samples were freeze-dried when required. Dried extracts were maintained at -20ºC protected from light until use. Extractions were carried out by duplicate.

2.2.2 Supercritical fluid extraction (SFE)

SFE assays were carried out in a pilot-plant supercritical fluid extractor (Model SF2000, Thar Technology, Pittsburgh, PA, USA) according to García-Risco et al., (2017). In order to obtain SFE extracts from the four plants, CO_2 flow was established at 70 g/min, and pressure and temperature were set at 140 bar and 40 ºC, respectively. After the extraction process (180 min), a precipitated oleoresin-type extract was recovered from the extraction vessel with ethanol. Then, ethanol was removed under vacuum to obtain a final solid residue, which was kept at -20ºC until use. Extractions were carried out by duplicate.

2.3 Total Phenolic Content (TPC) and Antioxidant activity

The total phenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method, as described by Singleton et al., (1999). A calibration curve of gallic acid was used, and results were expressed as mg of gallic acid equivalents (GAE) per gram of extract.

To determine the antioxidant activity of the extracts, two methodologies were assessed. The $ABTS^{\bullet+}$ radical scavenging capacity was performed as described by Re et al. (1999) procedure.

The radical scavenging assay was developed at four different concentrations of each extract. Results were expressed in mmol of Trolox equivalents /g of extract. The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out according to Huang y col. (2002). The reaction took place in a 96 black round-bottom plate

containing 150 µL of fluorescein stock solution ($8x10^{-8}$, PBS 0.075M), 25 µL of plant extract, PBS (blank samples) or trolox solution (standard), and 25 µL of AAPH radical fresh solution (165.94 mM). Excitation and emission wavelength were set at 485 nm and 520 nm, respectively (Infinite M200, Tecan, Barcelona, Spain) and the fluorescence intensity was recorded every 1 min at 37ºC, until the value was <5% of the initial reading. Results were expressed as mmol of Trolox /g of extract. Analyses were carried out by triplicate.

2.4 Cell culture and anti-inflammatory activity

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were maintained and cultured as described by Villalva et al. (2018). THP-1 cells were seeded in 24-well plates ($5x10^5$ cells/mL) and differentiated to macrophages with 100ng/mL of phorbol 12-myristate 13-acetate (PMA) for 48h.

The cytotoxicity of the extracts was tested (10, 20 and 50 using µg/mL) on differentiated macrophages using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide (MTT) assay (Mossmann, 1983). For anti-inflammatory assays, macrophages were incubated with or in absence of 0.05 µg/mL of bacterial lipopolysaccharide (LPS from *E. coli* O55:B5, Sigma-Aldrich, Madrid, Spain) in the presence of a non-cytotoxic extract concentration for 24h. The secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, was measured in the cells supernatants using ELISA assay kits (BD Biosciences, Aalst, Belgium), according to the manufacturer's instructions. Results were expressed as mean of three determinations ± standard deviation.

2.5 HPLC-PAD-ESI-QTOF-MS/MS analysis

Mass spectrometric detection was performed using an HPLC system (1100 series, Agilent Technologies, Santa Clara, CA, USA) connected to an ultra-high-resolution QTOF instrument (MAXIS II, Bruker, Bremen, Germany). Electrospray ionization source was used in negative mode for all the analyses and the parameters were adjusted as follows: capillary voltage 3400 V, end plate offset 500 V, in-source Collision Induced Dissociation energy (isCID) 30 eV for MS/MS spectra. Nitrogen was used as nebulizer gas (pressure of 4 Bar) and drying gas (heated to 250°C, flow 8 L/min). For accurate high resolution mass spectrometry (HRMS) internal calibration was performed after each chromatographic run by means of a mixture of

phosphazenes. The accurate masses obtained were processed using the elemental composition calculator incorporated in the data analysis software (Bruker, Bremen, Germany). Prior to mass detection, dry extracts were dissolved at 2.5 mg/mL in ethanol:water (60:40, v/v), and filtered by 0.45 μm polyvinylidene fluoride (PVDF) filter.

Separation was achieved in a reversed-phase ACE Excell 3 Super C18 column (150 mm x 4.6 mm, 3 μm, ACT, Aberdeen, Scotland) protected by a pre-column (ACE 3 C18-AR, 10 mm x 3 mm) as was described by Villalva et al., (2018) with slight modifications. Briefly, the chromatographic separation was performed using solvent A (water with 0.1% formic acid, v/v) and solvent B (pure acetonitrile), following the next gradient elution: 0-1 min, 5% B; 6 min, 15% B; 21 min, 25% B; 26 min, 35% B; 36-41 min, 50% B; 43-48 min, 100% B; 50 min, 5% B.

Identification of phenolic compounds was performed by contrasting the accurate mass and MS/MS fragmentation pattern with the literature, and by comparison of its retention time (Rt) and UV-Vis max absorption pattern with available analytical standards. Phenolic compounds quantification was carried out in a HPLC-PAD system (1260 Infinity series, Agilent Technologies, Santa Clara, CA, USA) using a five-level calibration curve of authentic phenolics standards. Those compounds which standard was not available, were quantified with the calibration curve of that compound with greater structural affinity, *e.g.* apigenin-7-*O*-glucoside was used for apigenin glycosylated derivative, and chlorogenic acid was used for caffeoylquinic acid derivative. Analyses were carried out by triplicate and the results were expressed as mg phenolic compound /g of extract.

2.6 GC-MS identification

The volatile fraction of the extracts was determined by GC-MS in an Agilent Technologies 7890A system (Agilent Technologies, Santa Clara, CA, USA) coupled to a 5975C triple-axis mass spectrometer detector. The column used was a HP-5MS (5% Phenyl methyl siloxane, Agilent 19091S-433) and the chromatographic conditions for separation were followed as described by García-Risco et al., (2017). The components were identified based on their relative retention time and mass spectrum compared to the library data of the GC-MS system (NIST MS 2.0). Analyses were done by triplicate.

2.7 Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Fisher's least significant differences (LSD) test to discriminate among means ($p < 0.05$). To determinate the correlation between the different experimental data, a Pearson test were carried out ($p < 0.05$). Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA) software was used for this purpose.

3. RESULTS AND DISCUSSION

3.1 Evaluation of TPC and Antioxidant Activity of the plant extracts

Two different green extraction techniques, SFE and UAE, were used in this study to obtain bioactive compounds from Lamiacea and Asteracea plants, following the extraction conditions previously studied in our research group (García-Risco, et al., 2017). As shown in Table 1, extraction yield was influenced by solvent polarity, regardless of plant used. Overall, the SFE extracts demonstrated a lower mass recovery in comparison with UAE. Moreover, within UAE extracts, those obtained with pure ethanol achieved significantly inferior yield values than the extracts obtained with 50% ethanol.

Two complementary assays, ABTS radical scavenging and ORAC, were used in order to evaluated the antioxidant activity of SFE and UAE extracts (Table 1). Overall, the solvent polarity seemed to exert a clear influence on antioxidant activity, despite the plant matrix. Thus, an increment in the solvent polarity allowed to obtain extracts with higher antioxidant activity. In that context, the greatest TEAC and ORAC values were achieved with the use of 50% ethanol (UAE-50 extracts), followed by UAE-100 extracts. In contrast, SFE extracts, carried out with CO_2, presented a limited antioxidant activity. Moreover, for UAE-50 extracts, both Lamiaceae species, MAJ and MEL, presented a higher antioxidant activity than the Asteraceae ones, highlighting *O. majorana* as the most effective and *C. officinalis* with the lowest values.

A similar behaviour was found regarding the TPC as can be observed in Table 1. For all the plants studied, the extracts obtained by UAE presented higher TPC values than those obtained by SFE.

Moreover, the UAE-50 extracts exhibited a significantly higher recovery of phenolic compounds (mg GAE/g extract) compared to 100% ethanol as reported before by Rodríguez-Pérez et al., (2015) regarding UAE extracts from plants. Therefore, it was confirmed the close correlation ($p < 0.05$) between the total content of phenolic compounds and the antioxidant activity (TEAC or ORAC value) of the plant extracts (Supplementary material, Fig. S1). These results also suggest that most of the phenolic compounds present in these plants have a high or mid-polarity. Moreover, despite of the extraction technique and the solvent used, the greatest TPC value was corresponded to MAJ, while the lowest was found in CAL.

Table 1. Extraction yield, Total phenolic content (TPC) and Antioxidant activity of plant extract, obtained by supercritical fluids extraction (SFE) and ultrasound assisted extraction (UAE) techniques.

		MEL	***MAJ***	***CAL***	***MIL***
Yield [1]	SFE	$0.6 \pm 0.0^{C}{}_{d}$	$1.5 \pm 0.2^{C}{}_{b}$	$2.8 \pm 0.2^{C}{}_{a}$	$0.8 \pm 0.0^{C}{}_{c}$
	UAE-100	$4.2 \pm 0.2^{B}{}_{c}$	$5.9 \pm 0.4^{B}{}_{a}$	$6.7 \pm 0.7^{B}{}_{a}$	$5.1 \pm 0.1^{B}{}_{b}$
	UAE-50	$22.7 \pm 0.6^{A}{}_{b}$	$15.6 \pm 0.1^{A}{}_{c}$	$27.9 \pm 0.6^{A}{}_{a}$	$14.8 \pm 0.1^{A}{}_{d}$
TPC [2]	SFE	$16 \pm 1^{C}{}_{b}$	$56 \pm 1^{C}{}_{a}$	$13 \pm 1^{C}{}_{c}$	$14 \pm 2^{B}{}_{bc}$
	UAE-100	$70 \pm 1^{B}{}_{c}$	$148 \pm 3^{B}{}_{a}$	$36 \pm 1^{B}{}_{d}$	$111 \pm 2^{A}{}_{b}$
	UAE-50	$112 \pm 2^{A}{}_{b}$	$247 \pm 5^{A}{}_{a}$	$82 \pm 2^{A}{}_{d}$	$106 \pm 3^{A}{}_{c}$
TEAC [3]	SFE	$0.03 \pm 0.00^{C}{}_{c}$	$0.24 \pm 0.02^{C}{}_{a}$	$0.05 \pm 0.02^{B}{}_{c}$	$0.08 \pm 0.01^{C}{}_{b}$
	UAE-100	$0.24 \pm 0.01^{B}{}_{c}$	$0.71 \pm 0.01^{B}{}_{a}$	$0.06 \pm 0.01^{B}{}_{d}$	$0.29 \pm 0.03^{B}{}_{b}$
	UAE-50	$0.71 \pm 0.02^{A}{}_{b}$	$1.46 \pm 0.03^{A}{}_{a}$	$0.33 \pm 0.02^{A}{}_{d}$	$0.52 \pm 0.04^{A}{}_{c}$
ORAC [4]	SFE	$0.76 \pm 0.01^{C}{}_{b}$	$1.59 \pm 0.04^{C}{}_{a}$	$0.38 \pm 0.02^{C}{}_{c}$	$0.73 \pm 0.02^{C}{}_{b}$
	UAE-100	$0.94 \pm 0.03^{B}{}_{c}$	$2.57 \pm 0.14^{B}{}_{a}$	$0.48 \pm 0.01^{B}{}_{d}$	$1.86 \pm 0.11^{B}{}_{b}$
	UAE-50	$2.71 \pm 0.12^{A}{}_{b}$	$5.17 \pm 0.09^{A}{}_{a}$	$1.32 \pm 0.10^{A}{}_{d}$	$2.16 \pm 0.02^{A}{}_{c}$

[1] Yield expressed in % of dried mass extract/dried mass plant sample. [2] TPC as mg GAE/g extract. [3] TEAC as mmol Trolox/g extract. [4] ORAC as mmol Trolox/g extract. $^{A-D}$ Different superscript letters denote significant differences within a column ($p < 0.05$). $^{a-c}$ Different subscript letters denote significant differences within a line ($p < 0.05$). MEL, *Melissa officinalis*. MAJ, *Origanum majorana*. MIL, *Achillea millefolium*. CAL, *Calendula officinalis*.

Overall, within the studied extracts, Lamiacea species exhibited a greater performance regarding the TPC and antioxidant activity properties. Several studies have shown the antioxidant properties of extracts from *O. majorana* and *M. officinalis*. Moreover, this radical scavenging capacity has been related to its high phenolic content (Miron et al., 2103; Hossain et al., 2014).

3.2 Anti-inflammatory activity of plant extracts

To evaluate the anti-inflammatory activity of Lamiaceae and Asteraceae species, LPS-activated THP-1 macrophages were exposed to 10 μg/mL of SFE and UAE extracts, that represented a non-cytotoxic concentration (data not shown). A significant release of the pro-inflammatory cytokines studied were observed in the stimulated THP-1 macrophages (positive control) in contrast to non-stimulated cells (negative control) (Fig. 1). The addition of plant extracts from both, SFE or UAE, techniques resulted in very different results, regarding the release inhibition of the three cytokines studied. Thus, the most relevant inhibition of TNF-α was observed in presence of the UAE-100 extracts, for all plant species (Fig 1A). Moreover, the highest inhibition was exhibited by MIL UAE-100 followed by CAL UAE-100, demonstrating a reduction approx. 60% and 50%, respectively, compared with the positive control. However, none UAE-50 extract inhibited this cytokine secretion. Regarding SFE extracts, only CAL and MIL presented a secretion reduction of approx. 30%.

The obtained results for IL-1β release in presence of plant extracts (Fig. 1B) were similar to those obtained for TNF-α, since UAE-100 extracts were the most active. In particular, MIL UAE-100 produced an important decrease in the IL-1β secretion (approx. 55%) compared with the positive control. Finally, UAE-100 extracts also exhibited an important inhibition of IL-6 (Fig. 1C), especially MIL UAE-100 demonstrated approx. an 80% inhibition. For this cytokine, all SFE extarcts presented a moderate inhibition.

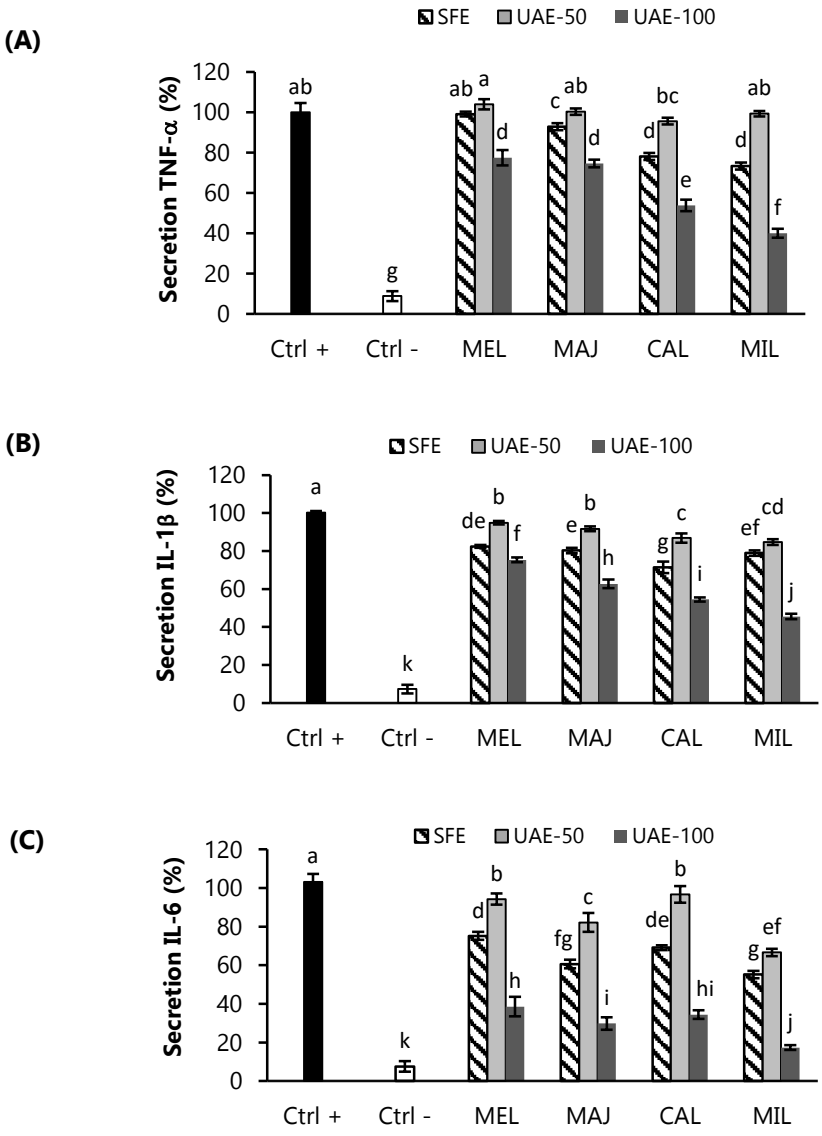

Figure 1: Levels of (A) TNF-α, (B) IL-1β and (C) IL-6 secreted by THP-1 macrophages, activated with LPS, in presence of 10 μg/mL of SFE (stripped bars), UAE-50 (light grey bars) and UAE-100 (dark grey bars) plant extracts. Ctrl+, positive control (cells stimulated with LPS without extract). Ctrl-, negative control (cells non-stimulated). MEL, *Melissa officinalis* L., MAJ, O*riganum majorana* L., CAL, *Calendula officinalis* L, MIL, A*chillea millefolium* L. Each bar is the mean of three determinations ± SD. $^{a-k}$ Different letters indicate statistical differences among LSD procedure ($p < 0.05$).

Overall, these results bring to light the higher anti-inflammatory potential of the studied Asteraceae species compared to the Lamiaceae ones, mainly UAE-100 extracts. According to the above results, a trend about pro-inflammatory cytokines inhibition could be delineated, *i.e.* UAE-100 > SFE > UAE 50 extracts. Thus, opposite to the antioxidant activity, the anti- inflammatory activity of these extracts was not totally related to their TPC. Furthermore, the observed anti-inflammatory properties of the extracts, could be linked to the presence of specific phenolic compounds (Benedek et al., 2007; Dinda et al., 2016), but the contribution of other type of compounds can not be discarded. In that sense, several studies have shown the contribution of terpenes on the anti-inflammatory effect of the essential oil of these plants (Lim, 2014a; Abdossi and Kazemi, 2016).

3.3 HPLC-PAD-ESI-QTOF-MS/MS identification and quantification of selected UAE extracts

As aforementioned, a close relationship between TPC and antioxidant activity has been established. Nevertheless, in an attempt to better understand which specific compounds are behind this activity, a comprehensive analysis of such extracts with a remarkable activity, *i.e.* UAE-50 extracts, was carried out. Although several literature regarding the phenolic composition of the four studied plants have already published, in this study an extensive exploration on their individual components were done. Thus, 146 compounds were tentatively identified, where 26 compounds have not been described before in these plant-matrices, butalthough they have been reported in related species. Accurate mass (*m/z*) and MS/MS fragmentation patterns of all identified compounds are shown at supplementary material (Table S2). Organic acids, phenolic acids (hydroxybenzoic and hydroxycinnamic acids and their derivatives), other polar compounds, flavonoids (flavones, flavonols, flavanones and their derivatives), and saponins were properly characterised.

Different studies have already published the composition of *M. officinalis* (Barros et al., 2013; Miron et al., 2013; Carocho et al., 2015; Ozarowski et al., 2016) and *O. majorana* (Hossain et al., 2014; Kaliora et al., 2014; Taamalli et al., 2015; Vallverdú-Queralt et al., 2015), mainly their phenolic one. However, an extended characterization of ethanol:water extracts is presented in the present article, even with unreported compounds for these matrices (Table S2).

Regarding phenolic acids constituents, some yunnaneic acid isomers were described for *M. officinalis* (Carocho et al., 2015; Ozarowski et al., 2016); nevertheless, to the best of our knowledge, yunnaneic acid D (peak 64, *m/z* 539) is still unreported in this specie. Similarly, sagecoumarins were identified in Lamiaceae family, mainly in *S. officinalis* and *M. officinalis* (Martins et al., 2015b; Ozarowski et al., 2016), but not in *O. majorana*. Thus, peak 120 (*m/z* 535) was tentative identified as a sagecoumarin isomer in MAJ-50, according to its fragmentation pattern (Ozarowski et al., 2016).

This study also contributes to describe an extended flavonoid composition of *O. majorana*. To the best of our knowledge, 11 compounds were not yet referenced in marjoram, despite the fact they were found in other Lamiaceae spp. For instance, the presence of luteolin diglucuronide (peak 44, *m/z* 637), eriodictyol hexoside (peak 69, *m/z* 449) and isorhamnetin hexoside (peak 84, *m/z* 477) were described in *T. vulgaris* (Martins et al., 2015a;); eriocitrin (peak 59, *m/z* 595) and kaemperide glucururonide (peak 99, *m/z* 475) were reported in *Origanum* spp. (Tuttolomondo et al., 2013; Martins et al., 2014); meanwhile, 6-hydroxyluteolin-7-*O*-glucoside (peak 53, *m/z* 463) and luteolin acetylglucoside (peak 93, *m/z* 489) were detected in *S. officinalis* (López-Lázaro, 2009; Martins et al, 2015b). Furthermore, in the MAJ-50 extract a group of methoxylated flavonoids were detected and tentatively identified as dihydroxyquercetin dimethyl ether (peak 132, *m/z* 331), trihydroxy dimethoxyflavone (peak 134, *m/z* 329), methoxyacacetin (peak 143, *m/z* 313) and dihydroxy trimethoxyflavone (peak 145, *m/z* 343). This type of methoxylated compounds have been also characterised by LC–MS/MS techniques in thyme (Pacifico et al., 2016).

Regarding the Asteraceae species, the composition of *C. officinalis* (CAL-50) was in agreement with published literature (Olennikov et al., 2014; Hernández-Saavedra et al., 2016; Miguel et al., 2016; Pires et al., 2018). However, no previous records were found for kaempferol-3-*O*-rutinoside (peak 80, *m/z* 593) which was also identified, by comparison with its authentic standard. Moreover, according to our results, a considerable number of phenolic compounds have still unreported in *C. officinalis* (peaks 10,14,16,19, 34, 35, 49, 52 and 68); nevertheless, it is worth to mention that they had recently recognized in other *Calendula spp* and *Asteracea spp* (Faustino et al., 2018; Olennikov et al., 2018; Pereira et al., 2018).

In CAL-50 the presence of saponins derivatives were a particular characteristic. Since calendasaponins B, A and G have been reported in other *Calendula* spp (Faustino et al., 2018), peaks 109, 119 and 138 were tentatively identified, respectively. With regards to *A. millefolium* the composition of this plant had been extensively addressed due to its wide range of bioactivities found in this plant; thus, most of the compounds derivate from our study were in accordance with different studies (Dias et al., 2013; Lim et al., 2014b; Ali et al., 2017; Pereira et al., 2018). In addition to the well-stablished phenolic profile, the presence of new caffeoylquinic acid (CQA) derivatives in the ethanol:water extract, namely feruloylquinic-*O*-caffeoylquinic acid (peak 111, *m/z* 529) and tricaffeoylquinic acid (peak 116, *m/z* 677) was defined. Besides, in this sample the presence of a new flavone derivate was also noticed, corresponding with 6-hydroxyluteolin-7-*O*-glucoside (peak 53, *m/z* 463), which had not been reported specifically in *A. millefolium*, but was characterized in Asteraceae family (López-Lázaro, 2009).

On the other hand, although some similarities for all UAE-50 extracts were found, such as the presence of organic acids and caffeic acid, many differences were noticed (Table S2). Within Lamiaceae species, forty-nine compounds could be identified in *M. officinalis* (MEL-50), mostly rosmarinic acid (RA) and its derivatives, either dimers, trimers or tetramers; meanwhile seventy compounds were identified in *O. majorana* (MAJ-50) showing a multivariate composition of caffeic acid derivatives and flavonoids, mainly glycosylated flavones. Besides, flavanones were only detected in this plant. With regards to Asteraceae species, forty-one compounds were identified in *C. officinalis* (CAL-50) where glycosylated and methylated derivatives of flavonols, along with caffeic acid derivatives, were particularly exhibited in this extract. Finally, within the fifty-nine compounds identified in *A. millefolium* (MIL-50), the presence of several mono- and di-CQA derivatives represented a special characteristic in this plant, along with a great variety of methoxylated and glycosylated flavones.

From all the tentative identified compounds, 107 phenolic compounds were quantified in the aqueous ethanolic extracts, as can be observed in Table 2. Within Lamiaceae species, rosmarinic acid (RA) was particularly abundant in MEL-50, while for MAJ-50 extract, RA and luteolin-7-*O*-glucoside were found as the main phenolics.

Table 2. Phenolic composition of selected UAE extracts. MEL, *Melissa officinalis* L., MAJ, O*riganum majorana* L., CAL, *Calendula officinalis* L., MIL, A*chillea millefolium* L.

Rt (min)	*Compound*	*MEL-50*	*MAJ-50*	*CAL-50*	*MIL-50*	*MIL-100*
11.00	Protocatechuic acid pentoside	nd	nd	0.14 ± 0.03	nd	nd
12.12	Hydroxyferulic acid hexoside isomer I	nd	nd	0.16 ± 0.05	nd	nd
12.17	3,4-dihydroxyphenil lactic acid	0.61 ± 0.04	1.29 ± 0.06	nd	nd	nd
12.51	Hydroxyferulic acid hexoside isomer II	nd	nd	0.20 ± 0.08	nd	nd
12.90	Neochlorogenic acid *	nd	0.27 ± 0.05	0.60 ± 0.10	0.43 ± 0.10[1]	0.26 ± 0.05
13.24	Hydroxyferulic acid hexoside isomer III	nd	nd	0.24 ± 0.00	nd	nd
13.35	Protocatechuic acid *	0.19 ± 0.07	0.22 ± 0.10	nd	0.35 ± 0.09[1]	0.11 ± 0.03
13.67	Caftaric acid isomer	nd	nd	nd	0.08 ± 0.03	0.06 ± 0.03
13.74	Caffeic acid di-hexoside	0.32 ± 0.05	nd	nd	nd	nd
13.78	Caftaric acid *	0.38 ± 0.08	nd	nd	0.16 ± 0.03[1]	0.08 ± 0.03
14.27	Caffeoylquinic acid isomer I	nd	nd	nd	0.22 ± 0.04	0.19 ± 0.04
14.30	Caffeic acid- hexoside I	nd	nd	0.20 ± 0.00	nd	nd
14.88	Chlorogenic acid *	nd	nd	7.92 ± 0.39	7.84 ± 0.57[1]	6.41 ± 0.33
15.01	Cryptochlorogenic acid *	nd	0.75 ± 0.11	0.16 ± 0.04	0.47 ± 0.09[1]	0.13 ± 0.06
15.87	Vicenin II *	nd	2.56 ± 0.17	0.11 ± 0.06	4.00 ± 0.33[1]	2.35 ± 0.13
16.08	Caffeic acid hexoside II	0.21 ± 0.08	0.29 ± 0.07	nd	nd	nd
16.81	Coumaroylquinic acid	nd	nd	0.09 ± 0.03	nd	nd
16.91	Caffeoylshikimic acid	nd	nd	0.20 ± 0.05	nd	nd
16.94	Caffeoylquinic acid isomer II	nd	nd	nd	0.64 ± 0.10[1]	0.10 ± 0.06
17.45	Apigenin-hexoside-pentoside I	nd	nd	nd	0.55 ± 0.21[1]	0.44 ± 0.12
17.82	Caffeic acid *	0.57 ± 0.09	0.78 ± 0.10	0.19 ± 0.04	0.40 ± 0.07	0.33 ± 0.04
18.01	Schaftoside isomer	nd	nd	nd	3.20 ± 0.14[1]	1.38 ± 0.10
18.23	Schaftoside *	nd	<LOQ	nd	2.73 ± 0.12[1]	2.04 ± 0.12
18.27	Luteolin-*C*-hexoside	nd	2.19 ± 0.15	nd	nd	nd
18.37	Quercetin-3-*O*-rhmanosylrutinoside	nd	nd	1.17 ± 0.73	nd	nd
18.79	Homoorientin *	nd	nd	nd	1.00 ± 0.17	2.26 ± 0.22[1]
18.82	Yunaneic acid E isomer I	1.20 ± 0.15	nd	nd	nd	nd
19.27	Yunaneic acid E isomer II	1.94 ± 0.14	nd	nd	nd	nd
19.31	Syringic acid derivative	nd	nd	0.38 ± 0.04	nd	nd
19.43	Apigenin-hexoside-pentoside II	nd	nd	nd	2.02 ± 0.18[1]	1.22 ± 0.12
19.50	Luteolin dihexoside I	nd	nd	nd	1.82 ± 0.10	3.01 ± 0.12[1]
19.82	6-hydroxyluteolin-7-*O*-glucoside	nd	35.8 ± 2.1	nd	1.99 ± 0.09	2.48 ± 0.10
20.09	Salvianolic acid I	0.77 ± 0.13	nd	nd	nd	nd
20.15	Apigenin dihexoside	nd	nd	nd	0.31 ± 0.07	0.23 ± 0.06
20.40	Quercetin 3-neohesperidoside	nd	nd	0.18 ± 0.06	nd	nd

*Identified and quantified *via* comparison with authentic standards. nd, not detected. <LOQ, below limit of quantification. [1] Indicates statistical differences between MIL-50 and MIL-100 extracts ($p < 0.05$).

Table 2. *(Continued).*

Rt (min)	*Compound*	*MEL-50*	*MAJ-50*	*CAL-50*	*MIL-50*	*MIL-100*
20.63	Isorhamnetin-3-*O*-rhamnosylrutinoside*	nd	nd	14.2 ± 1.3	nd	nd
21.11	Quercetin hexoside I	nd	nd	nd	0.72 ± 0.09	1.34 ± 0.90[1]
21.28	Quercetin-*O*-pentosylhexoside	nd	nd	0.36 ± 0.07	nd	nd
21.56	Luteolin dihexoside II	nd	nd	nd	0.27 ± 0.04	0.26 ± 0.06
21.83	Yunnaneic acid D isomer	0.37 ± 0.05	nd	nd	nd	nd
21.89	Rutin *	nd	nd	0.57 ± 0.06	0.99 ± 0.09	1.12 ± 0.11
22.43	Isovitexin	nd	nd	nd	0.50 ± 0.09	0.46 ± 0.07
22.50	Vitexin *	nd	nd	nd	0.46 ± 0.09	0.66 ± 0.11
22.76	Quercetin-malonylhexosyl-rhamnoside	nd	nd	0.65 ± 0.12	nd	nd
22.90	Apigenin deoxylhexoside	nd	nd	nd	0.26 ± 0.09	0.36 ± 0.04
23.31	Isorhamnetin-3-*O*-neohesperidoside	nd	nd	1.89 ± 0.14	nd	nd
23.37	Luteolin-*O*-glucoside	nd	17.5 ± 1.1	nd	nd	nd
23.42	Apigenin glycosylated derivative	nd	nd	nd	3.21 ± 0.13[1]	2.42 ± 0.09
23.58	Luteolin-7-*O*-*β*-glucoside *	0.21 ± 0.03	14.6 ± 1.0	nd	4.96 ± 0.35	8.23 ± 0.72[1]
23.62	Quercetin- hexoside II	nd	nd	0.19 ± 0.06	nd	nd
23.79	Rosmarinic acid hexoside	7.19 ± 0.84	nd	nd	nd	nd
23.89	Luteolin-7-*O*-glucuronide *	nd	4.09 ± 0.11	nd	0.69 ± 0.07	0.82 ± 0.07
24.64	Quercetin hexuronide	nd	nd	nd	0.45 ± 0.04[1]	0.21 ± 0.03
24.93	Kaempferol-3-*O*-rutinoside*	nd	nd	0.19 ± 0.07	nd	nd
25.01	Chicoric acid	0.75 ± 0.06	nd	nd	nd	nd
25.39	3,4-Dicaffeoylquinic acid *	nd	nd	nd	1.42 ± 0.09	1.43 ± 0.07
25.47	Ishoramnetin-3-*O*-rutinoside *	nd	nd	7.23 ± 0.62	nd	nd
25.92	Isorhamnetin hexoside I	nd	nd	nd	0.48 ± 0.07	1.18 ± 0.10[1]
26.17	Quercetin -3-*O*-acetyl-glucoside	nd	nd	0.64 ± 0.04	nd	nd
26.44	Sagerinic acid	2.22 ± 0.18	nd	nd	nd	nd
26.61	1,5- Dicaffeoylquinic acid *	nd	nd	0.31 ± 0.09	2.57 ± 0.10[1]	1.73 ± 0.09
26.77	3,5- Dicaffeoylquinic acid *	nd	nd	5.37 ± 0.96	15.3 ± 1.0	21.9 ± 1.2[1]
26.81	Salvianolic acid E	0.43 ± 0.05	nd	nd	nd	nd
26.99	Diosmin *	nd	3.77 ± 0.19	nd	nd	nd
27.33	Isorhamnetin-3-*O*-glucoside*	nd	nd	0.67 ± 0.09	nd	nd
27.53	Apigenin-7-*O*-glucoside *	nd	2.17 ± 0.09	nd	1.05 ± 0.21	2.28 ± 0.32[1]
27.80	Luteolin-*O*-malonylglucoside	nd	nd	nd	0.26 ± 0.07	0.55 ± 0.09[1]
28.16	Isorosmarinic acid	0.46 ± 0.05	nd	nd	nd	nd
28.17	Apigenin-*7*-*O*-glucuronide *	nd	1.70 ± 0.09	nd	nd	nd
28.20	4,5- Dicaffeoylquinic acid *	nd	nd	2.61 ± 0.15	5.70 ± 0.20[1]	4.41 ± 0.13
28.84	Rosmarinic acid *	19.2 ± 1.0	37.6 ± 1.9	nd	nd	nd

*Identified and quantified *via* comparison with authentic standards. nd, not detected. <LOQ, below limit of quantification. [1] Indicates statistical differences between MIL-50 and MIL-100 extracts ($p < 0.05$).

Table 2. *(Continued).*

Rt (min)	*Compound*	*MEL-50*	*MAJ-50*	*CAL-50*	*MIL-50*	*MIL-100*
29.19	Luteolin-*O*-hexuronide	1.64 ± 0.11	nd	nd	nd	nd
29.25	Ishoramnetin-3-*O*-acetylglucoside isomer I	nd	nd	1.12 ± 0.09	nd	nd
29.71	Lithospermic acid *	nd	10.5 ± 0.7	nd	nd	nd
30.34	Isorhamnetin hexoside II	nd	nd	nd	0.17 ± 0.04	0.53 ± 0.07[1]
30.38	Lithospermic acid isomer I	1.84 ± 0.09	21.3 ± 1.0	nd	nd	nd
30.61	Salvianolic acid B *	0.49 ± 0.02	2.06 ± 0.08	nd	nd	nd
31.07	Dicaffeoylquinic acid isomer	nd	nd	0.08 ± 0.03	0.18 ± 0.06[1]	0.08 ± 0.02
31.79	Lithospermic acid isomer II	8.65 ± 0.52	3.56 ± 0.23	nd	nd	nd
32.11	Feruloyl-*O*-caffeoylquinic acid	nd	nd	nd	0.13 ± 0.03	0.12 ± 0.02
32.21	Salvianolic acid L isomer I	1.75 ± 0.08	4.32 ± 0.15	nd	nd	nd
32.39	Sagecoumarin caftaride	0.64 ± 0.06	nd	nd	nd	nd
32.69	Tricaffeoylquinic acid	nd	nd	0.13 ± 0.02	0.30 ± 0.06	0.39 ± 0.07
33.24	Sagecoumarin isomer	0.40 ± 0.05	1.33 ± 0.09	nd	nd	nd
33.30	Eriodictyol	nd	0.67 ± 0.05	nd	nd	nd
33.66	Luteolin *	nd	1.10 ± 0.08	nd	1.70 ± 0.07	1.89 ± 0.09[1]
33.71	Salvianolic acid C derivative	2.19 ± 0.09	nd	nd	nd	nd
33.93	Quercetin *	nd	0.36 ± 0.03	nd	0.33 ± 0.04	0.64 ± 0.05[1]
34.45	Salvianolic acid L isomer II	0.95 ± 0.06	0.98 ± 0.07	nd	nd	nd
35.06	Methoxyquercetin isomer	nd	nd	nd	0.58 ± 0.08	0.82 ± 0.11[1]
35.20	Quercetin dimethyl ether	nd	0.81 ± 0.10	nd	nd	nd
36.26	Rosmarinic acid derivative I	nd	0.27 ± 0.07	nd	nd	nd
36.50	Jaceidin isomer	nd	0.62 ± 0.08	nd	nd	nd
36.95	Naringenin *	nd	0.94 ± 0.10	nd	nd	nd
37.12	Apigenin *	nd	0.09 ± 0.02	nd	0.42 ± 0.04	0.59 ± 0.05[1]
37.33	Dihydroxyquercetin dimethyl ether	nd	0.61 ± 0.07	nd	nd	nd
37.67	Diosmetin *	nd	nd	nd	0.34 ± 0.03	0.45 ± 0.05[1]
37.83	Trihydroxy dimethoxyflavone I	nd	0.69 ± 0.07	nd	nd	nd
37.89	Rosmarinic acid derivative II	0.30 ± 0.03	nd	nd	nd	nd
38.48	Trihydroxy dimethoxyflavone II	nd	nd	nd	<LOQ	0.24 ± 0.06
39.95	Centaureidin	nd	nd	nd	0.27 ± 0.06	1.90 ± 0.10[1]
39.99	Salvianolic acid F isomer	1.16 ± 0.09	nd	nd	nd	nd
40.21	Rosmarinic acid derivative III	0.46 ± 0.03	0.35 ± 0.04	nd	nd	Nd
42.18	Methoxyacacetin	nd	0.04 ± 0.02	nd	<LOQ	0.22 ± 0.05
42.70	Salvianolic acid C caffeoylhydroxycaffeide	3.32 ± 0.10	nd	nd	nd	nd
42.98	Dihydroxyvtrimetoxyflavone	nd	nd	nd	0.13 ± 0.03	0.31 ± 0.04[1]
45.35	Casticin *	nd	nd	nd	0.35 ± 0.03	2.53 ± 0.11[1]

*Identified and quantified *via* comparison with authentic standards. nd, not detected. <LOQ, below limit of quantification. [1] Indicates statistical differences between MIL-50 and MIL-100 extracts ($p < 0.05$).

With respect to Asteraceae, isorhamnetin-3-*O*-rhamnosylrutinoside was the most representative constituent for CAL-50, whereas 3,5-dicaffeoylquinic acid (3,5-DCQA) was for MIL-50. Nevertheless, in an attempt to better understanding the quantitative differences within the studied samples, the cumulative concentration of phenolic compounds in the four extracts was plot as shown in Fig. 2.

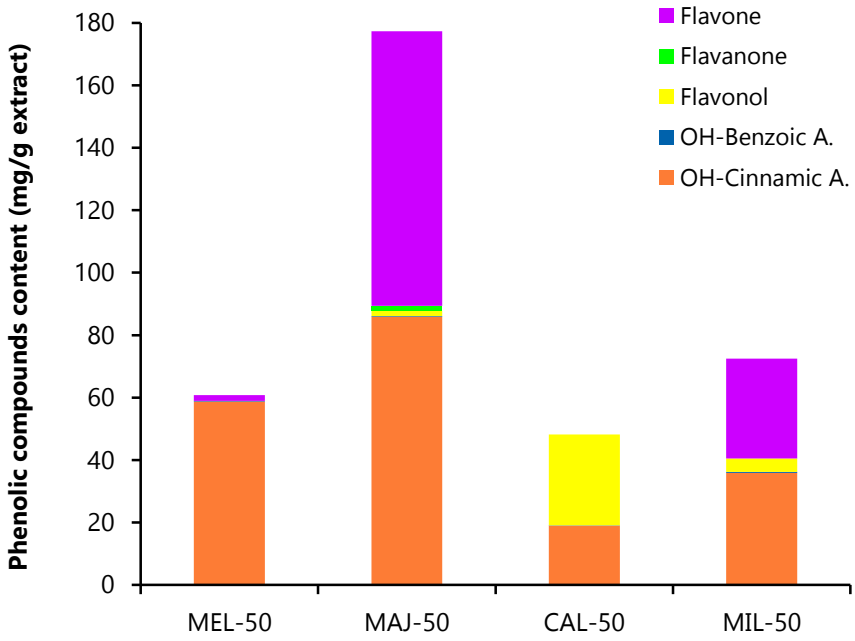

Figure 2: Phenolic compounds presented by groups in the studied UAE-50 extracts, expressed as mg/g extract. MEL, *Melissa officinalis* L., MAJ, O*riganum majorana* L., CAL, *Calendula officinalis* L., MIL, A*chillea millefolium* L.

Considering these results, the antioxidant activity of the aqueous ethanolic extract could be attributable to the sum of phenolic compounds found in the different extracts, but also to the presence of specific phenolic compounds. Thus, the greatest antioxidant activity of MAJ-50 was clearly associated to its maximum content of phenolic compounds, mainly hydroxycinnamic acids and flavones, compared to the other extracts (Fig. 2), but problably influenced by RA and its dimers (*i.e.* lithosphermic acid isomer) and also by the luteolin glucosylated derivatives (*i.e* 6-hydroxyluteolin-7-*O*-glucoside, luteolin-*O*-glucoside and

luteolin-7-*O*-β-glucoside) as the most representative compounds in this sample. All these compounds have been recognized for their antioxidant activity, especially (López-Lázaro, 2009; Miron et al., 2013).

Although, MEL-50 and MIL-50 showed a similar total accumulation of phenolics (Fig. 2), *M. officinalis* resulted more effective than *A. millefolium* in terms of antioxidant activity. In that regard, the main difference was the great concentration of hydroxycinnamic acids in MEL-50, and as a consequence its better antioxidant effect could be related. Similar to MAJ-50, the abundance of well-known antioxidants, such as RA and its derivatives, in MEL-50 was noticed. Therefore, the presence of high contents of RA in MEL-50 could be considered the principal factor to contribuite with its antioxidant activity.

Regarding MIL-50 and CAL-50, although both plants belong to the same family, their individual phenolic composition resulted quite different (Fig. 2). MIL-50 was distinguished by the presence of a similar content of both hydroxycinnamic acids and flavones, whereas for CAL-50 the abundance of flavonols was relevant. A greatest content of 3,5-DCQA and chlorogenic acid found in A. millefolium ethanolic:water extract could be mainly related to its antioxidant activity (Liang and Kitts, 2016). In contrast, although this type of antioxidant components, *i.e.* CGA and DCQAs, were found in CAL-50 too, its lack of antioxidant activity could be attributed to its low content of phenolic compounds.

Additionally, the phenolic composition of MIL-100 extract, that exhibited the highest anti-inflammatory activity, displayed a quite similar TPC compared to the aqueous ethanolic extract of yarrow (Table 2). Nevertheless, a significant increase of certain compounds such as 3,5-DCQA, luteolin-7-*O*-glucoside, casticin and centaureidin, was found. It was previously reported that apigenin, quercetin, luteolin and diosmetin, aglycones that were enriched in MIL-100 extract, reduced IL-6 and TNF-α secretion in LPS-stimulated RAW macrophages (Mueller et al., 2010). At the same time, centaureidin and casticin, also enriched in MIL-100 extract, had been described to perform anti-inflammatory activity (Jachak et al. 2011; Chan et al., 2018). Besides, Benedek et al. (2007) have reported the anti-inflammatory activity of an enriched fraction in DCQAs and flavonoids from *A. millefolium*. Thus, the release inhibition of cytokins shown by MIL-100 extract could be attributed, at least partially to these phenolic compounds.

On the other hand, as pure ethanol was used as solvent, volatile compounds with powerful anti-inflammatory propierties could have been also extracted (Chou et al., 2013; Abdossi and Kazemi, 2016). Therefore, a subsequent GC-MS analysis of MIL-100 extract was conducted.

3.4 GC-MS composition of ethanolic extracts

The composition of the volatile fraction of MIL UAE-100 extract demonstrated the presence of a wide range of monoterpenes and sesquiterpenes, found in a greater or lesser extent (Table 3).

Table 3. Chemical composition as peak area contribution of *Achillea millefolium* L. ethanolic extract (UAE-100 extract) of volatile fraction identified by GC-MS.

Rt (min)	RI	Compound	% Area
4.6	997	Yomogi alcohol	6.1
5.1	1028	Eucalyptol	5.0
5.5	1058	Artemisia ketone	4.3
5.8	1079	Artemisia alcohol	5.6
6.2	1101	Thujone	2.9
6.9	1138	Camphor	10.4
7.2	1160	Borneol	23.8
7.6	1174	Terpinene-4-ol	1.9
8.7	1262	5,9-Dimethyl-5,8-decadien-2-one	2.9
9.3	1299	Carvacrol	2.9
12.1	1478	α-curcumene	0.9
13.5	1569	Spathulenol	2.7
13.6	1578	Caryophillene oxide	2.6
13.8	1589	Viridiflorol	5.1
14.3	1630	δ-Cadinol	4.6
14.5	1640	β-Eudesmol	8.6
15.6	1718	Chamazulene	5.9
16.8	1890	Corymbolone	3.8
		∑ AUC	**4.10×10^6**

Rt: retention time. RI: retention index. AUC: area under curve.

This composition was in accordance with previous reports of *A. millefolium* extracts obtained in our research group (García-Risco et al., 2107; Villanueva et al., 2017). As can be observed, borneol (23.8%) was the most abundant compound so far, although camphor (10.4%) and β-eudesmol (8.6%) were also representative in the extract. As mentioned by other authors, borneol significantly inhibited nitric oxide production, and cytokines secretion (TNF-α, IL-1β and IL-6) in LPS-activated macrophages (Arranz et al., 2014; Abdossi and Kazemi, 2016).

In addition, champor and β-eudesmol have also been described as anti-inflammatory agents (Arranz et al., 2014; Kim et al., 2018).

In that context, regarding the anti-inflammatory properties of *A. millefolium* ethanol extract, an exclusive influence of one group of compounds cannot be totally established, but some accumulative factors like, i) a greater abundance of some specific phenolics, ii) the presence of terpenoid compounds of the volatile fraction and ii) the accumulative or synergistic effect of minor compounds, could contribute to this activity.

4. CONCLUSIONS

Ultrasound assisted extraction resulted a useful technique to obtain extracts with an important antioxidant and anti-inflammatory activity, from a sustainable approach. From the four studied plants, the aqueous ethanolic extract of *Origanum majorana* (Lamiaceae) has been displayed as the most effective in terms of its antioxidant activity, whereas *Achillea millefolium* (Asteraceae) extract, with pure ethanol, demonstrated a remarkable anti-inflammatory activity on LPS-stimulated macrophages. Moreover, a further analysis of their constituents allowed the tentative identification of the compounds responsible of these bioactivities. Thus, the antioxidant activity of *O. majorana* extracts, could be related to its high content of total phenolic compounds overall, but mainly related to the presence of rosmarinic acid and luteolin glucoside derivatves. On the other hand, the anti-inflammatory activity of *A. millefolium*, was partially explained by its richness in specific phenolic acids and flavonoids, but also by the presence of volatile compounds in its composition. Thus, both, *O. majorana* and *A. millefolium* should be considered as a good source of potent bioactive ingredients.

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ABBREVIATIONS

SFE, superctrical fluids extraction; UAE, ultrasound assisted extraction; TPC, total phenolic content; GRAS, Generally recognised as safe; MEL, *Melissa officinalis* L.*;* MAJ; *Origanum* majorana L.; *MIL, Achillea millefolium* L; CAL, *Calendula officinalis* L.; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffer solution; LPS, bacterial lipopolysaccharide; LSD, least significant difference; TNF-α, tumour necrosis factor-α; IL-1β, interleukin 1β; IL-6, interleukin 6; CQA, caffeoylquinic acid; DCQA, dicaffeoylquinic acid.

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Supplementary material

Table S1. Authentic commercial standards (HPLC purity ≥ 95%)

Supplier	Authentic standard
Cymit Química SL. Madrid, Spain	Ishoramnetin-3-*O*-rutinoside Isorhamnetin-3-*O*-glucoside Isorhamnetin-3-*O*-rhamnosylrutinoside
Extrasynthese S.A. Genay, France	Apigenin-7-*O*-glucuronide Apigenin-7-*O*-glucoside Caffeic acid Homoorientin Isoquercitrin Isorhamnetin Luteolin-7-*O*-*b*-glucoside Luteolin-7-*O*-glucuronide Quercetin Rutin
Sigma-Aldrich. Madrid, Spain	Arbutin Chlorogenic acid Diosmetin Gallic acid Rosmarinic acid Vitexin

Supplier	Authentic standard
Phytolab. Madrid, Spain	1,5- Dicaffeoylquinic acid 3,4- Dicaffeoylquinic acid 3,5- Dicaffeoylquinic acid 4,5- Dicaffeoylquinic acid Apigenin Caftaric acid Casticin Cryptochlorogenic acid Diosmin Kaempferol-3-*O*-rutinoside Lithospermic acid Luteolin Naringenin Neochlorogenic acid Orientin Protocatechuic acid Salvianolic acid B Schaftoside Vicenin II

Table S2. Composition of selected UAE 50% ethanol extracts. M, *Melissa officinalis*; O, *Origanum majorana;* C, *Calendula officinalis*; A, *Achillea millefolium*.

Peak no.	Rt (min)	Compound	Accurate mass (*m/z*)	MS/MS ions (*m/z*)	Extract
1	3.50	Gluconic acid	195.0511	129 (100)	M, O, C, A
2	3.70	Quinic acid	191.0564	127 (100), 111 (30)	M, O, C, A
3	3.90	Tartaric acid	149.0093	72 (100)	M, O
4	4.20	Malic acid	133.0144	115 (100)	M, O, C, A
5	4.50	Citric acid	191.0197	111 (100), 87 (40)	O, C, A
6	5.10	Isocitric acid	191.0197	111 (100), 87 (40)	O
7	6.20	Succinic acid	117.0950	73 (100)	M
8	10.11	Arbutin *	271.0819	108 (100)	O
9	10.45	Gallic acid *	169.0144	125 (100)	M, O
10	11.00	Protocatechuic acid pentoside	285.0622	153 (100), 109 (80)	C
11	11.58	3,4-dihydroxyphenillactic acid – hexoside	359.0987	197 (100), 179 (60)	M, O
12	11.75	Vainillic acid hexoside	329.0833	167(100), 152 (20)	O
13	11.83	Dihydroxybenzoic acid hexoside	315.0721	153 (98), 109 (80)	M, O, A
14	12.12	Hydroxyferulic acid hexoside isomer I	371.0628	209 (100), 191 (55)	C
15	12.17	3,4-dihydroxyphenil-lactic acid	197.0455	179 (80), 135 (100)	M, O
16	12.51	Hydroxyferulic acid hexoside isomer II	371.0628	209 (100), 191 (40)	C
17	12.63	Hydroxybenzoic acid hexoside	299.0774	137 (100)	M, O, C
18	12.90	Neochlorogenic acid *	353.0877	191 (100), 179 (76), 135 (40)	O, C , A
19	13.24	Hydroxyferulic acid hexoside isomer III	371.0628	209 (100), 191 (25)	C
20	13.35	Protocatechuic acid *	153.0197	109 (100)	M, O, A
21	13.67	Caftaric acid isomer	311.0410	179 (14), 149 (100)	A
22	13.74	Caffeic acid dihexoside	503.1410	179 (14), 149 (100)	M
23	13.78	Caftaric acid *	311.0410	179 (14), 149 (100)	M, A
24	14.21	Luteolin 6,8-*C*-dihexoside	609.1453	489 (100), 325 (40)	O, A
25	14.27	Caffeoylquinic acid isomer I	353.0877	191 (100), 179 (20)	A
26	14.30	Caffeic acid hexoside I	341.0882	179 (100) 135 (60)	M, C
27	14.71	Coumaric acid hexoside	325.0934	163 (100), 119 (20)	M, O
28	14.88	Chlorogenic acid *	353.0877	191 (100), 161 (10)	C, A
29	15.01	Cryptochlorogenic acid *	353.0877	191 (100), 161 (11)	O, C, A
30	15.54	Caffeic acid hexuronide	355.0678	191 (100)	C
31	15.87	Vicenin 2 *	593.1504	473 (100)	O, A
32	16.08	Caffeic acid hexoside II	341.0882	179 (100), 135 (65)	M, O
33	16.67	Coumaric acid pentoside	295.0464	163 (80), 119 (63)	M
34	16.81	Coumaroylquinic acid	337.0936	191 (100)	C
35	16.91	Caffeoylshikimic acid	335.078	179 (100), 135 (77)	C
36	16.94	Caffeoylquinic acid isomer II	353.0879	191 (100), 161 (10)	C, A
37	17.40	Feruloyl tartaric acid	325.057	193, 161, 134	M
38	17.45	Apigenin-hexoside-pentoside I	563.1401	473 (10), 443 (20)	A
39	17.82	Caffeic acid *	179.0353	135 (100)	M, O, C, A
40	18.01	Schaftoside isomer	563.1401	473 (10), 443 (20)	A
41	18.23	Schaftoside *	563.1401	473 (10), 443 (20)	O, A
42	18.27	Luteolin-*C*-hexoside	447.0925	357 (38), 327 (100)	O
43	18.37	Quercetin-3-*O*-rhmanosylrutinoside	755.2052	301 (100), 271 (23)	C

* Comparison with authentic standards.

Table S2. *(Continued).*

Peak no.	Rt (min)	Compound	Accurate mass (*m/z*)	MS/MS ions (*m/z*)	Extract
44	18.64	Luteolin diglucuronide	637.1042	285(100)	O, A
45	18.79	Homoorientin *	447.0930	429 (30), 357 (100), 327 (80)	A
46	18.82	Yunaneic acid E isomer I	571.1099	329 (31), 197 (100), 179 (24), 135 (39)	M
47	19.14	Luteolin-hexoside-hexuronide	623.1246	447 (90), 285 (100), 112 (60)	A
48	19.27	Yunaneic acid E isomer II	571.1099	329 (31), 197 (100), 179 (24), 135 (39)	M
49	19.31	Syringic acid derivative	313.0572	197 (100), 121 (25)	O, C
50	19.43	Apigenin-hexoside-pentoside II	563.1401	473 (10), 443 (20)	A
51	19.50	Luteolin dihexoside I	609.1451	447 (100), 357 (26), 327 (70), 285 (10)	A
52	19.74	Feruloylquinic acid	367.1042	191 (100), 173 (40)	C
53	19.82	6-Hydroxyluteolin-7-*O*-glucoside	463.0880	301 (100)	O, A
54	20.09	Salvianolic acid I	537.1043	493 (25) , 339(100), 295(22), 197(60)	M, O
55	20.15	Apigenin dihexoside	593.1502	269 (100)	A
56	20.29	Luteolin hexoside	447.0873	285 (100)	O
57	20.40	Quercetin-3-neohesperidoside	609.1468	301 (100)	C
58	20.63	Isorhamnetin-3-*O*-rhamnosylrutinoside*	769.2240	315 (100), 300 (20)	C
59	20.98	Eriocitrin	595.1591	287(100)	O
60	21.11	Quercetin hexoside I	463.0877	301 (100)	A
61	21.28	Quercetin-*O*-pentosylhexoside	595.1310	301 (100)	C
62	21.56	Luteolin dihexoside II	609.1750	447 (20), 285(10)	O, A
63	21.79	Luteolin rutinoside	593.1430	285 (100)	O
64	21.83	Yunnaneic acid D isomer	539.1198	197 (80), 135 (60)	M
65	21.89	Rutin *	609.1093	301 (100)	C, A
66	22.43	Isovitexin	431.0981	311 (100)	A
67	22.50	Vitexin *	431.0981	311 (100)	A
68	22.76	Quercetin-malonylhexosyl-rhamnoside	695.1472	651 (100), 301 (23)	C
69	22.85	Eriodyctiol hexoside	449.1028	287.0524 (100)	O
70	22.90	Apigenin deoxylhexoside	577.1566	269 (100)	A
71	23.31	Isorhamnetin-3-*O*-neohesperoside	623.1621	315 (100), 300 (10)	C
72	23.37	Luteolin-*O*-glucoside	447.0932	285 (100), 151 (20)	O
73	23.42	Apigenin glycosylated derivative	445.1135	269 (100)	A
74	23.58	Luteolin-7-*O*-*β*-glucoside *	447.0928	285 (100)	M, O, A
75	23.62	Quercetin hexoside II	463.0889	301 (100)	C
76	23.79	Rosmarinic acid hexoside	521.1306	359 (21), 197 (100), 179 (12), 161 (29)	M, O
77	23.89	Luteolin-7-*O*-glucuronide *	461.0722	285 (100)	O, A
78	24.01	Apigenin hexoside	431.0928	269 (100)	A
79	24.64	Quercetin hexuronide	477.0671	301 (100)	A
80	24.90	Kaempferol-3-*O*-rutinoside *	593.1521	285 (100)	C
81	25.01	Chicoric acid	473.0730	311 (50), 179 (80), 149 (100), 135 (10)	M

* Comparison with authentic standards.

Table S2. *(Continued).*

Peak no.	Rt (min)	Compound	Accurate mass (*m/z*)	MS/MS ions (*m/z*)	Extract
82	25.39	3,4-dicaffeoylquinic acid *	515.1189	353 (100), 335 (30), 179 (69), 173 (80)	A
83	25.47	Ishoramnetin-3-*O*-rutinoside *	623.1627	315 (100)	C
84	25.92	Isorhamnetin hexoside I	477.1035	315 (100)	O, A
85	26.17	Quercetin-3-*O*-acetyl-glucoside	505.0996	463 (30), 301 (100)	C
86	26.21	Quercetin pentoside	433.0718	301 (100)	O
87	26.44	Sagerinic acid	719.1619	539 (20), 521 (7), 359 (100), 197 (20), 179 (18)	M
88	26.61	1,5-dicaffeoylquinic acid *	515.1190	353 (100), 191 (40)	C, A
89	26.77	3,5-dicaffeoylquinic acid *	515.1190	353 (100), 191 (55), 179 (35), 135 (21)	C, A
90	26.81	Salvianolic acid E isomer	717.1463	537 (28), 519 (100), 339 (88), 295 (22)	M, O
91	26.99	Diosmin *	607.1668	607 (10), 299 (100), 284 (10)	O
92	27.33	Isorhamnetin-3-*O*-glucoside *	477.1045	315 (100)	C
93	27.39	Luteolin acetylglucoside	489.0973	447 (30), 285 (100)	O
94	27.53	Apigenin-7-*O*-glucoside *	431.0980	269 (100)	O, A
95	27.80	Luteolin-*O*-malonylglucoside	533.0931	489 (100), 285 (15)	A
96	28.16	Isorosmarinic acid	359.0771	197 (35), 179 (30), 161 (100), 135 (20)	M
97	28.17	Apigenin-7-*O*-glucuronide *	445.0767	269 (100)	O
98	28.20	4,5-dicaffeoylquinic acid *	515.1190	353 (100), 191 (10), 179 (30), 173 (40)	A
99	28.68	Kaempferide glucuronide	475.0821	299.0522 (100)	O
100	28.83	Apigenin-*O*-hexuronide	445.0775	269 (100)	A
101	28.84	Rosmarinic acid *	359.0771	197 (80), 179 (50), 161 (100), 135 (30)	M, O
102	29.19	Luteolin-*O*-hexuronide	461.0727	285 (100)	M
103	29.25	Ishoramnetin-3-*O*-acetylglucoside	519.1150	315 (100), 300(15)	C
104	29.71	Lithospermic acid *	537.1038	493 (44), 359 (43), 295 (93), 161 (100)	O
105	30.34	Isorhamnetin hexoside II	477.1034	315 (100)	O
106	30.38	Lithospermic acid isomer I	537.1041	493 (24), 295 (80), 197 (18), 161 (90)	M, O
107	30.61	Salvianolic acid B *	717.1461	519 (30), 359 (100), 295 (10), 179 (10)	M, O
108	31.07	Dicaffeoylquinic acid isomer	515.1191	353 (100), 191 (15),179 (35), 173 (30)	C, A
109	31.36	Calendasaponin B	971.4855	971 (100), 809 (40)	C
110	31.79	Lithospermic acid isomer II	537.1024	493 (32), 359 (30), 295 (100)	M, O
111	32.11	Feruloyl-*O*-caffeoylquinic acid	529.1350	367 (100), 193 (55), 191 (22)	A
112	32.21	Salvianolic acid L isomer I	717.1464	519 (100), 359 (30), 339 (15), 149 (18)	M, O
113	32.39	Sagecoumarin caftaride	829.1260	667 (88), 535 (80), 311 (50), 135 (47)	M
114	32.44	Salvianolic acid L hydroxycaffeide	895.1730	519 (77), 369 (73), 161 (100)	M
115	32.57	Salvianolic acid A isomer	493.1145	359 (100), 295 (10), 197 (20), 161 (39)	M, O
116	32.69	Tricaffeoylquinic acid	677.1519	515 (100), 353 (68)	C, A
117	32.81	Methylrosmarinic acid	373.0932	179 (100), 161 (20), 135 (82)	M

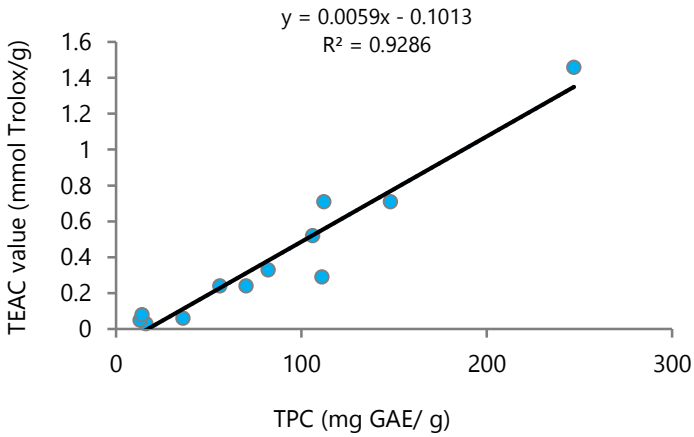
* Comparison with authentic standards.

Table S2. *(Continued).*

Peak no.	Rt (min)	Compound	Accurate mass (*m/z*)	MS/MS ions (*m/z*)	Extract
118	33.13	Luteolin dimer	569.0718	285 (100), 112 (80)	A
119	33.19	Calendasaponin A	1117.5437	1117 (100), 955 (10)	C
120	33.24	Sagecoumarin isomer	535.0886	359 (8), 197 (10), 177 (100), 161 (19)	M, O
121	33.30	Eriodictyol	287.0555	151 (100), 135 (85)	O
122	33.66	Luteolin *	285.0400	175 (80), 151 (100), 107 (51)	M, O, A
123	33.71	Salvianolic acid C derivative	715.1304	535 (100), 491 (11), 311 (9), 135 (7)	M
124	33.93	Quercetin *	301.0352	151 (60)	O, A
125	34.45	Salvianolic acid L isomer II	717.1462	519 (100), 339 (13)	M, O
126	35.06	Methoxyquercetin isomer	315.0508	301 (100)	O, A
127	35.2	Quercetin dimethyl ether	329.0665	314 (100), 299 (70)	O
128	36.26	Rosmarinic acid derivative I	565.1341	359 (60), 197 (28), 179 (20), 161 (100)	M, O
129	36.50	Jaceidin isomer	359.0767	344 (57), 329 (100)	O
130	36.95	Naringenin *	271.0607	151 (100)	O
131	37.12	Apigenin *	269.0454	112 (100)	O, A
132	37.33	Dihydroxyquercetin dimethyl ether	331.0817	299 (100)	O
133	37.67	Diosmetin *	299.0554	112 (100)	A
134	37.83	Trihydroxy dimethoxyflavone I	329.0665	314 (20), 299 (100)	O
135	37.89	Rosmarinic acid derivative II	565.1358	359 (100), 197 (30), 179 (13), 135 (36)	M
136	38.31	Isorhamnetin *	315.0511	301 (100), 209 (15)	C
137	38.48	Trihydroxy dimethoxyflavone II	329.0665	299 (100)	A
138	38.57	Calenduloside G	793.4376	631 (100), 613 (30)	CA
139	39.74	Salvianolic acid C isomer	491.0986	267 (16), 179 (100), 161 (21)	M, O
140	39.95	Centaureidin	359.0770	344 (59), 229 (100)	A
141	40.00	Salvianolic acid F isomer	313.0721	161 (100)	M
142	40.21	Rosmarinic acid derivative III	565.1358	359 (94), 197 (24), 179 (20), 161 (100)	M, O
143	42.18	Methoxyacacetin	313.0716	283 (100), 112 (60)	O,
144	42.70	Salvianolic acid C caffeoylhydroxycaffeide	849.1674	359 (100), 179 (6), 161 (20), 135 (40)	M
145	42.98	Dihydroxy trimetoxyflavone	343.0820	328 (100), 313 (20)	O, A
146	45.35	Casticin *	373.0923	358 (43), 343 (90)	A

* Comparison with authentic standards.

(A)



(B)

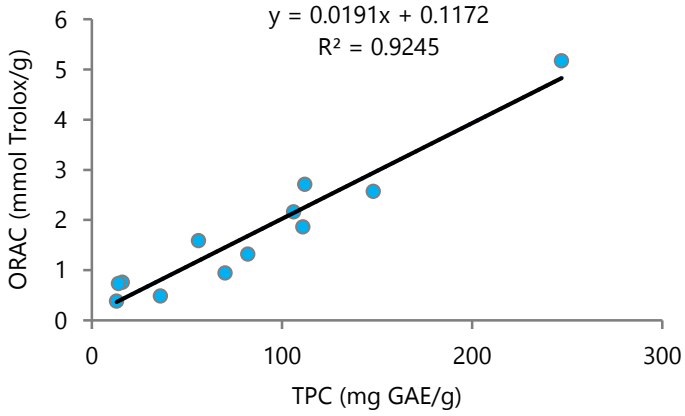


Figure S1. Antioxidant activity of Asteraceae and Lamiaceae plant extracts as a function of TPC. (A) TEAC value (mmol trolox/ g); (B) ORAC (mmol trolox / g).

CAPÍTULO 2

Estratregias dirigidas al enriquecimiento y desarrollo de formulaciones para mejorar la biodisponibilidad de los compuestos fenólicos de mejorana.

Prefacio

La mejorana (*Origanum majorana* L.) es una planta aromática, perteneciente a la familia Lamiaceae, cuyos extractos presentan diversas actividades biológicas, las cuales se han asociado principalmente a su contenido en compuestos fenólicos, en particular al ácido rosmarínico (RA).

A partir de los resultados obtenidos en el *Capítulo 1* y con el objetivo de obtener extractos de mejorana con un alto poder antioxidante, así como un alto contenido en AR, se han utilizado como técnicas de extracción, además de la UAE, la extracción con líquidos presurizados (PLE). Empleándose como disolventes mezclas etanólicas y acuosas, en diferentes proporciones (50:50; 70:30 y 100:0, v/v).

Los mejores resultados en términos del contenido en compuestos fenólicos totales, actividad antioxidante y contenido en ácido rosmarínico se consiguieron al emplear etanol al 70%, en ambas técnicas de extracción. Además, la técnica PLE dio lugar a un mayor rendimiento de extracción y extractos con una mayor actividad antioxidante. Así, los estudios posteriores relativos al enriquecimiento en ácido rosmarínico, y compuestos fenólicos en general, utilizándose resinas macrospóricas de adsorción XAD-7HP, se llevaron a cabo con el extracto de mejorana PLE-70% etanol.

La biodisponibilidad del RA y demás compuestos fenólicos presentes en los extractos enriquecidos de mejorana se evaluó mediante una digestión gastrointestinal *in vitro, o*bservándose una reducción generalizada en los compuestos fenólicos, particularmente para el RA. Tras la digestión, para los ensayos de absorción intestinal se emplearon monocapas de células Caco-2. Finalmente, la

evaluación de la bioactividad de la fracción absorbida se llevó a cabo, en términos de su actividad antioxidante y antinflamatoria, relacionándose la presencia del RA y otros compuestos fenólicos con dichas actividades.

Paralelamente, se llevó a cabo una encapsulación del extracto de mejorana PLE-70% etanol, con el objeto de estudiar su posible incorporación a formulaciones alimenticias. Para ello, se emplearon caseínas y proteínas aisladas de soja, con el fin de encontrar la matriz proteica más eficiente capaz de conseguir encapsular la mayor cantidad de ácido rosmarínico sin disminuir la bioactividad del extracto de mejorana, medida en este caso como actividad antinflamatoria del extracto.

De esta manera, el presente Capítulo 2, se compone de dos estudios simultáneos utilizando como materia prima a la mejorana, y los resultados se ven reflejados en las publicaciones *2* y *3*, tituladas:

2. **Protein matrices ensure safe and functional delivery of rosmarinic acid from marjoram (*Origanum majorana*) extracts**.
3. **Anti-inflammatory and antioxidant activities from the basolateral fraction of Caco-2 cells exposed to a rosmarinic acid enriched extract.**

Publicación 2

Protein matrices ensure safe and functional delivery of rosmarinic acid from marjoram (*Origanum majorana*) extracts.

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ABSTRACT

BACKGROUND: To understand the interactions between carriers and functional ingredients is crucial when designing delivery systems, to maximize bioefficacy and functionality. In this study, two different protein matrices were evaluated as means to protect the extract isolated from marjoram leaves (*Origanum majorana*), casein micelles from fresh skim milk and soy protein isolate (SPI).
RESULTS: Marjoram extract was obtained from pressurization of ethanol and water solvent. Protein dispersions of casein and SPI (5 g L^{-1} each) with or without marjoram extract (0.1-3 mg mL^{-1}) were prepared and homogenized. The physicochemical characterization of charge and entrapment efficiency were conducted. The results demonstrated that entrapment efficiency was highly dependent on the carrier itself where SPI formulations showed 20% higher affinity when compared to casein micelles. To investigate the physiological behaviour of the marjoram–protein dispersions, human macrophages were employed. A non-specific inflammatory response of macrophages stimulated with bacterial lipopolysaccharide was measured for TNF-α, IL-1β and IL-6 cytokine secretion.
CONCLUSION: Casein and SPI protein formulations warranted high bioefficacy of marjoram extract, showing their potential as safe carriers.

1. INTRODUCTION

Health promoting benefits of aromatic plants and spices have been extensively described. Among them marjoram (*Origanum majorana* L.) is recognized for being used as food additive with flavouring properties in addition to promoting digestive system well-being. Marjoram extract (ME) is known for its antioxidant and antimicrobial properties[1,2] and for its anti-inflammatory activity.[3] This activity is contributed to the composition of the ME mainly phenolic acids like rosmarinic, caffeic, carnosic and gallic, as well as other phenolic compounds as luteolin, apigenin or carnosol.[4] Rosmarinic acid has been reported as the main compound detected in hydroalcoholic MEs.[5]

Environmental clean technologies for extraction and concentration of compounds from plant origin are well established and offer reduced toxicity usually associated with traditional solid-liquid extractions where methanol, hexane or acetone is used as a solvent. More advanced techniques have become available aiming to reduce losses on the bioactive quantities extracted from plants and increase their purity and maximize their functionality *per se*. Consequently, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are techniques that offer high reproducibility in short time, simple manipulation and low energy input.[6] Potential industrial application of UAE to plant materials has been previously described providing higher extraction yield than classic methods (e.g. solid-liquid extraction).[7] Pressurized liquid extraction (PLE) is also employed in food technology and is considered a safe and clean technique. Extraction process using PLE occurs at solvent temperature between 50-200 °C and pressure around 6-12 MPa.[8] Optimization of extraction process has been achieved using higher temperatures to increase solubility and extraction rate, for example, water can behave as an organic solvent at certain temperature that affects its dielectric constant.[9]

Despite the advantages of applying these techniques in ingredient development, they are mainly and widely used in pharmaceutical applications. Nevertheless, their incorporation in food products represent a challenge due to low solubility of final products in water or complex matrices and the level of solvent traceability in the final product. Therefore, protein based nanostructures are ideal alternative carriers of bioactive compounds including carotenoids or phenolic compounds. Compounds, especially poor water-soluble molecules enclosed in milk or plant based proteins

have shown improved solubility and stability during processing or transit through the gastro intestinal tract.[10,11] Milk components, including milk proteins, have been described as delivery carriers for biologically active molecules. Caseins represent more than 80% of total milk proteins where about 95% is present as colloidal aggregates, so called casein micelles, due to the presence of calcium and phosphate linked to serine-phosphate residues in native milk. It is described that the hydrophobic environment of the core of casein micelles offers the possibility to entrap non-polar molecules.[12] Examples of successful delivery of low water-soluble molecules in casein micelles are curcumin or vitamin D.[13]

Noteworthy, plant based proteins are an abundant low-cost source of bioactive peptides, such as soy proteins. Soy proteins exposure to alkali solubilization and acid precipitation process outcomes protein denaturation, and as result aggregation in isolates with an average size of nanometers and low water solubility, highly dependent of preparation conditions is achieved.[14] Soy protein isolate (SPI) is the most commercially available soy protein and commonly recognized for good ability to adsorb and stabilize the interphase of oil-in-water emulsions, owing to amphiphilic properties of isolates. Moreover, SPI has been described as carrier of lipophilic compounds (e.g. β-carotene or curcumin).[15,16]

The objective of this study was to evaluate the bioefficacy and functionality of ME entrapped in protein matrices, respectively casein micelles and SPI. Physicochemical characterization of the formulations was conducted and entrapment efficiency was determined by means of high-performance liquid chromatography (HPLC). To determine the potential bioefficacy of the encapsulated ME an *in vitro* model of inflammation was employed. Immuno-modulatory response was conducted in human macrophages to confirm the hypothesis that ME entrapment in protein matrices may ensure safe delivery and therefore functionality.

2. MATERIALS AND METHODS

2.1 Marjoram samples preparation

Dried marjoram leaves (*Origanum majorana* L.) were obtained from Herboristeria Murciana company (Murcia, Spain), certified ISO 9001:2008. The sample was ground in a knife mill (Grindomix GM 200, Restch. Spain) and the particle size was determined by sieving the ground plant material to the appropriate size (< 500 μm).

2.2 Pressurized Liquid Extraction

Extraction of marjoram was performed in a Dionex ASE 350 (Dionex Corporation. Sunnyvale, CA, USA) system equipped with a solvent controller unit. Three different ratios of ethanol/water (v/v) solutions were applied (50:50, 70:30, 100:0) as extraction solvent. Powdered marjoram sample (1.0 g) was mixed with sea sand (4.0 g) and placed into an 11 mL Dionex (ASE 350) stainless-steel cell. The extraction was performed at 100 ºC for 10 min at 10.34 MPa, in duplicates. Prior freeze-drying (Labconco Corporation, Missouri, USA) the extracts, the solvent was evaporated in a rotary evaporator IKA RV 10 (VWR International, Barcelona, Spain). All the lyophilized samples were stored at -20 ºC until use.

2.3 Ultrasound Assisted Extraction

Ground marjoram (40 g) with the corresponding concentration of ethanol/water solution (50:50, 70:30, 100:0) in a ratio 1:10 (bark/solvent) were submitted to ultrasound extraction for 30 minutes using a ½ diameter disruptor horn probe at 70% amplitude (maximum power output of 400W at 60 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury, USA) maintaining the temperature at 35 ºC with an ice bath and assisted with a stir plate. After sonication, the samples were filtered, evaporated and freeze-dried. All samples were stored under -20 ºC prior to analysis.

2.4 Rosmarinic acid quantification, total phenolic content and antioxidant activity determination

HPLC-photodiode array detection (PAD) analysis of rosmarinic acid in marjoram extracts was performed as previously described.[17]

Total phenolic content (TPC) was determined using the Folin–Ciocalteu's colorimetric method developed by Singleton et al.[18] A standard curve was calculated using gallic acid, and results were expressed as gallic acid equivalents (GAE) (mg of gallic acid per gram of dried extract). The antioxidant activity of the MEs was determined by the $ABTS^{\bullet+}$ assay. This method was applied according to Re *et al.*[19] protocol. The results were expressed as TEAC values (millimoles of Trolox equivalents per gram of dried extract).

2.5 Entrapment of marjoram pressurized liquid extract in protein aggregates

Two different protein matrices, caseins and SPI were employed as carriers of marjoram pressurized liquid extract. Preliminary studies were performed to choose a SPI concentration with minimum insoluble fraction (less than 10%). A range of SPI concentrations were prepared in 50 mM sodium phosphate buffer pH 7.4, stirred for 1 h at 40 ºC and stored overnight at 4 ºC for complete hydration. Conventional homogenization was then performed using the protein solutions at 450 KPa for four passes followed by low speed centrifugation (100x*g* for 5 min) (Eppendorf, Brinkmann Instruments, Westbury, NY, USA). Supernatant aliquots were collected and protein content was determined by Lowry assay (DC Protein Assay, BioRad Laboratories, Mississauga, ON, Canada), using BSA as standard.

Caseins were isolated from skim milk by centrifugation at 62 000x*g* for 30 min and 20 ºC (OptimaTM LE-80K, with a Ti-45 rotor, Beckman–Coulter, Mississauga, ON, Canada). Protein analysis in the pellets was measured by Dumas combustion method nitrogen analyzer (FP-528, Leco Inc. Lakeview Avenue, St. Joseph, MI, USA). Casein pellets were dissolved at 5 g L^{-1} (based on protein) in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride to ensure the isotonic environment using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Scientific, Mississagua, ON, Canada). Marjoram extract stock solutions were dissolved in ethanol: imidazole buffer (1:3), final volume 1 mL, and added dropwise to achieve 0.1, 0.25, 0.5, 1, 2 and 3 mg mL^{-1} in the casein solution. The mixtures were further kept for 1 h on a magnetic stirrer at 37 ºC. Casein formulations were then submitted to high-pressure homogenization at 475 KPa for four passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA).

Protein solutions containing 5 g L^{-1} SPI were chosen to incorporate ME. Stock extract solutions were dissolved in ethanol/sodium phosphate buffer (1:3), final volume 1 mL, to achieve 0.1, 0.25, 0.5, 1, 2 and 3 mg mL^{-1} in the SPI formulations. Protein solutions were prepared as described above and after overnight storage at 4 ºC, extract solutions were added dropwise. The mixtures were further kept for 1 h on a magnetic stirrer at 37 ºC. High-pressure homogenization was then performed at 475 KPa for four passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA).

Zeta (ζ)-Potential of the fresh casein and SPI formulations was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK). Casein formulations were diluted in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride (1:1000) while SPI formulations were diluted in 50 mM sodium phosphate buffer pH 7.4 (1:100).

2.6 Rosmarinic acid entrapment efficiency

Entrapment efficiency of rosmarinic acid in casein and SPI formulations was measured after homogenization. Samples were priory filtered (0.45 µm PVDF filters, Fisher Sci, Mississauga, ON, Canada) and aliquots of 500 µL were centrifuged in concentrator microcentrifuge tubes (Spin-x UF 500 10K MWCO PES 500 µl, Corning, NY, USA), for 15 min at 3000x*g* (benchtop Eppendorf centrifuge 5415D, Brinkmann Instruments, Westbury, NY, USA). Collected permeate was further analyzed for rosmarinic acid quantification by means of HPLC-PAD as previously described.[17]

2.7 In vitro immunomodulatory activity of marjoram formulations

Human THP-1 monocytes (American Type Culture Collection, ATCC, CEDARLANE Corporation, Burlington, ON, Canada) were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mM L-glutamine and 0.05 mM β-mercaptoethanol (Sigma-Aldrich Co, Oakville, ON, Canada) at 37 ºC in 95% humidified air containing 5% CO_2. Cells were plated at a density of 5 x 10^5 cells mL^{-1} in 24 well plates. Differentiation to macrophages was induced by incubating the cells with 100 ng ml^{-1} PMA (Sigma-Aldrich Co) for 48 h.

The toxic effect of the marjoram formulations (50, 100 and 200 µL) on differentiated macrophages was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich Co) following Mosmann's method.[20]

For immunomodulatory assay, cells were washed with phosphate-buffered saline (PBS) solution and incubated with 0.05 µg mL^{-1} lipopolysaccharide (LPS) (Sigma-Aldrich Co) in presence of either 100 µL of casein or SPI formulations containing respectively, 0, 0.5 and 1 mg mL^{-1} of ME for 24 h. Formulations were tested along with a control of ME 100 and 50 µg mL^{-1}. Then, the supernatants were kept frozen at -80 ºC. The release of cytokines IL-1β, IL-6 and TNF-α was measured in the

supernatants of macrophages cells using ELISA kits (R&D Systems, Bio-Techne Corporation, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.8 Statistical analysis

For each sample, duplicate extractions were performed and the analysis of phenolic compounds was carried out in triplicate expressed as mean values and standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by LSD test with a $P \leq 0.05$ using Statgraphics Centurion XVI (Statpoint Inc., Washington, USA) software.

3. RESULTS AND DISCUSSION

3.1 Comparison of marjoram extracts and rosmarinic acid determination

Two extraction techniques (PLE and UAE) were used to obtain a ME with a high quantity of rosmarinic acid and a significant antioxidant activity using three different concentrations of ethanol/water like solvent extraction. Both ethanol and methanol had been widely used to extract phenolic compounds from plant material due to their polarity and good solubility, although, for industrial purposes ethanol is preferable since it is considered GRAS.[9,21] The effect of ethanol on yield extraction, TPC and TEAC value of marjoram extract for both techniques are presented in Table 1. The higher extraction yield occurred when a mixture of ethanol/water was used as solvent extraction, instead of absolute ethanol. Moreover, the values were significantly higher when the extraction was carried out by PLE technique, in comparison with UAE, up to 23% of yield when 50% and 70% ethanol was used. For the TPC determination, the values ranged from 143.4 to 265.9 mg GAE g^{-1} of dry extract for both techniques PLE and UAE. However, at absolute ethanol as extraction phase, PLE yields the highest activity of ME (201.2 ± 4.9 versus 143.4 ± 1.7 mg GAE g^{-1} of dry extract). The highest TPC value was achieved for a mixture of ethanol/water (70:30) (v/v).

Table 1. Evaluation of extraction yield (% dry weight ± standard deviation (SD)), total phenolic content (TPC (mg GAE g^{-1} dry extract ± SD)), antioxidant activity (TEAC (mmol TE g^{-1} dry extract ± SD)) and quantification of rosmarinic acid (RA) (mg RA g^{-1} of dried extract ± SD) for marjoram extracts obtained by ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) using different percentages of ethanol (% v/v) during the extraction process[A,B]

Extraction technique	Ethanol/Water (% v/v)	Extraction Yield (%)	TPC (mg GAE g^{-1} dry extract)	TEAC (mmol TE g^{-1} dry extract)	mg RA g^{-1} extract
UAE	50:50	11.56 ± 0.7 $^{b\,2}$	233.2 ± 2.1 $^{b\,1}$	1.44 ± 0.02 $^{b\,2}$	33.62 ± 1.18 $^{b\,1}$
	70:30	15.6 ± 0.3 $^{a\,2}$	256.6 ± 3.4 $^{a\,1}$	1.52 ± 0.04 $^{a\,2}$	35.87 ± 1.89 $^{a\,1}$
	100:0	5.86 ± 0.4 $^{c\,2}$	143.4 ± 1.7 $^{c\,2}$	0.54 ± 0.02 $^{c\,2}$	23.36 ± 0.82 $^{c\,2}$
PLE	50:50	22.9 ± 0.1 $^{a\,1}$	237.5 ± 2.2 $^{b\,1}$	1.49 ± 0.02 $^{b\,1}$	31.48 ± 0.26 $^{c\,1}$
	70:30	23.3 ± 0.1 $^{a\,1}$	265.9 ± 4.8 $^{a\,1}$	1.81 ± 0.04 $^{a\,1}$	33.94 ± 0.75 $^{a\,1}$
	100:0	11.1 ± 0.34 $^{b\,1}$	201.2 ± 4.9 $^{c\,1}$	0.81 ± 0.02 $^{c\,1}$	32.36 ± 0.39 $^{b\,1}$

[A] Within an extraction technique, different superscript letters indicate statistical differences between ethanol/water composition at $P < 0.05$.
[B] Within the same ethanol/water composition, different subscript letters indicate statistical differences between extraction technique at $P < 0.05$.

Meanwhile, the antioxidant activity of MEs, expressed as Trolox equivalent (TE) per gram of dry matter, ranged from 0.54 to 1.52 mmol TE g^{-1} for those obtained with the UAE technique and from 0.81 to 1.81 mmol TE g^{-1} for the PLE technique. Curiously, the effect of ethanol in the TEAC values in both techniques, was higher for those extractions performed with an ethanol/water of 70:30 composition rather than 100% ethanol. Particularly in this condition, the use of PLE allowed a slightly elevated value than UAE. In addition, in this study, a strong correlation between the TPC content and the antioxidant activity is exhibited, as the higher TPC values corresponding to the higher TEAC values. Other researchers have reported a positive correlation between the TPC and antioxidant activity of herbs.[22]

Rosmarinic acid quantification and its potential antioxidant activity in MEs is shown in Table 1. It can be observed that the values were similar in ME with 50% and 70% of ethanol, unlike for the absolute ethanol condition where PLE extract showed a better result.

Thereby, PLE and UAE seem to represent an appropriate approach to obtain marjoram extracts with optimum quantity of rosmarinic acid, although a better extraction yield was obtained by PLE.

Based on the above results we selected PLE technique as extraction technique using 70% ethanol, due to the advantages that PLE presents, like a remarkable higher extraction yield and a lightly higher antioxidant activity when compare it with UAE in the studied conditions.

3.2 Entrapment of rosmarinic acid from marjoram pressurized extract in delivery systems

A number of delivery systems were designed to maximise entrapment of rosmarinic acid from marjoram pressurized extract. Preliminary experiments were conducted in oil-in-water emulsions. Previous studies have demonstrated that tea polyphenols are able to associate at the interface of sodium caseinate stabilized soybean oil emulsions.[23] Different concentrations of marjoram extract were studied in 10% soybean oil and 0.5% sodium caseinate formulated emulsions, however less than 10% rosmarinic acid was adsorbed at the interface. In addition, low solubility of the extract was observed in soybean oil and emulsions were not further considered as carriers of marjoram pressurized extract. Rosmarinic acid has low solubility in water and low partition coefficient, which difficulties its formulation.[24]

Since marjoram pressurized extracts showed slight solubility in water, entrapment of PLE ME was assessed in protein carriers, caseins and SPI. Complexation of low water-soluble compounds with SPI has been described to improve water dispersibility and stability to processing treatments.[15] Previous research from our group demonstrated that the commercial SPI employed in this study has lower water solubility than that reported in the literature.[25] A range of SPI solutions in water (0.1-200 g L^{-1} were prepared to determine protein insoluble fraction using Bradford protein assay.[26] Results showed that protein concentrations below 5 g L^{-1} assure an insoluble fraction lower than 10%. As for the caseins dispersions, higher solubility in water was observed. Hence, protein dispersions of caseins and SPI were employed at 5 g L^{-1} along the study.

Table 2 shows ζ-potential results of casein and SPI formulations determined by dynamic light scattering. Furthermore, ζ-potential of formulations with SPI were not affected by incorporation of ME. Similar values were obtained in the presence of the highest concentration of ME (-14.56 ± 1.01 mV) and without extract (-13.77 ± 0.61 mV). The same effect was found in casein formulations, no differences in surface charge caused by the addition of extract (-19.96 ± 2.08 mV) and (-20.55 ± 1.49) for 0 and 1 mg mL^{-1} of marjoram extract, respectively. Hence, ζ-potential results without the extract are consistent with those previously reported in the literature for SPI (-13.40 mV) and casein micelles (−21.7 mV).[10,27] Therefore, the presence of ME did not compromise physical stability of SPI and casein.

Table 2. Measurements of ζ-potential of protein suspensions (5 g L^{-1}), caseins (CAS) or soy protein (SPI), containing 0, 0.5 or 1 mg mL^{-1} of marjoram extract (ME)[A].

Sample	ζ-Potential (mV)
CAS (0 mg mL^{-1} ME)	-19.96 ± 2.08 [a]
CAS (0.5 mg mL^{-1} ME)	-20.06 ± 1.05 [a]
CAS (1 mg mL^{-1} ME)	-20.55 ± 1.49 [a]
SPI (0 mg mL^{-1} ME)	-13.77 ± 0.61 [a]
SPI (0.5 mg mL^{-1} ME)	-14.70 ± 0.30 [a]
SPI (1 mg mL^{-1} ME)	-14.56 ± 1.01 [a]

[A] Within the same protein suspension, different superscript letters indicate statistical differences between 0, 0.5 and 1 mg mL^{-1} of marjoram at $P < 0.05$.

Entrapment efficiency of rosmarinic acid in caseins and SPI solutions was determined by means of HPLC analysis. Rosmarinic acid concentration was measured in permeate samples obtained after centrifugation in concentrator tubes. Figure 1 illustrates the results obtained for entrapment efficiency in casein (Fig. 1A) and SPI (Fig. 1B) formulations. Caseins micelles entrapped 56.40 ± 4.82% of rosmarinic acid contained in 0.1 mg mL^{-1} of ME.

Similar results were found with 0.25 mg mL^{-1} of the extract (57.40 ± 10.02%), however the entrapment efficiency rapidly dropped to 20% at 1 mg mL^{-1} of extract that remained stable at 2 and 3 mg mL^{-1}. Our previous studies also demonstrated successful delivery of aromatic plant extracts in casein micelles, particularly the two main compounds, carnosic acid and carnosol, presented in rosemary supercritical extracts.[10] Encapsulation of hydrophobic pure compounds as curcumin and vitamin D in casein micelles has also been described.[13,28] Moreover, encapsulation in casein micelles provides protection from degradation of β-carotene exposed to common industrial treatments as pasteurization, sterilization or baking.[29]

From the SPI results obtained, it is interesting to point out that at the lowest concentrations of ME (0.1 mg mL^{-1}), the entrapment efficiency of rosmarinic acid in SPI reached the highest value (87.11 ± 8.51%). As the extract concentration increased, the entrapment progressively decreased and the amount detected in the aqueous phase increased to 67.54 ± 2.58% at the highest analyzed concentration of 3 mg mL^{-1}. However, at 1 mg mL^{-1} of marjoram in SPI an entrapment efficiency of 45.07 ± 6.79% rosmarinic acid was detected. Similarly, decay in encapsulation efficiency of curcumin in SPI solutions while the concentration of curcumin was increased was also described.[11] In the study by Chen et al.,[11] complexation of curcumin was assessed using 50 g L^{-1} SPI solution and the maximum encapsulation efficiency was obtained at 0.0315 mg mL^{-1} of curcumin. Teng et al.[30] described same trend of encapsulation efficiency increased with decreasing curcumin and protein ratio. A ratio of 10 g curcumin kg^{-1} protein provided an encapsulation efficiency of 97.2% while when increased to 50 g curcumin kg^{-1} protein, the encapsulation efficiency decreased to just 52.8%.

When comparing entrapment efficiency of marjoram extract using caseins and SPI, SPI noted 20 to 30% higher entrapment efficiency than caseins at the studied concentrations. SPI nanoparticles seemed to provide a more favourable environment for rosmarinic acid than casein micelles. Similar effect was previously reported in a comparison of caseins and SPI as delivery carriers for curcumin. Chen et al.[11] noted in their study higher encapsulation of curcumin in SPI nanoparticles than casein micelles by spray-drying, 96% of encapsulation efficiency compared to 83.1% reported by Pan et al.[31].

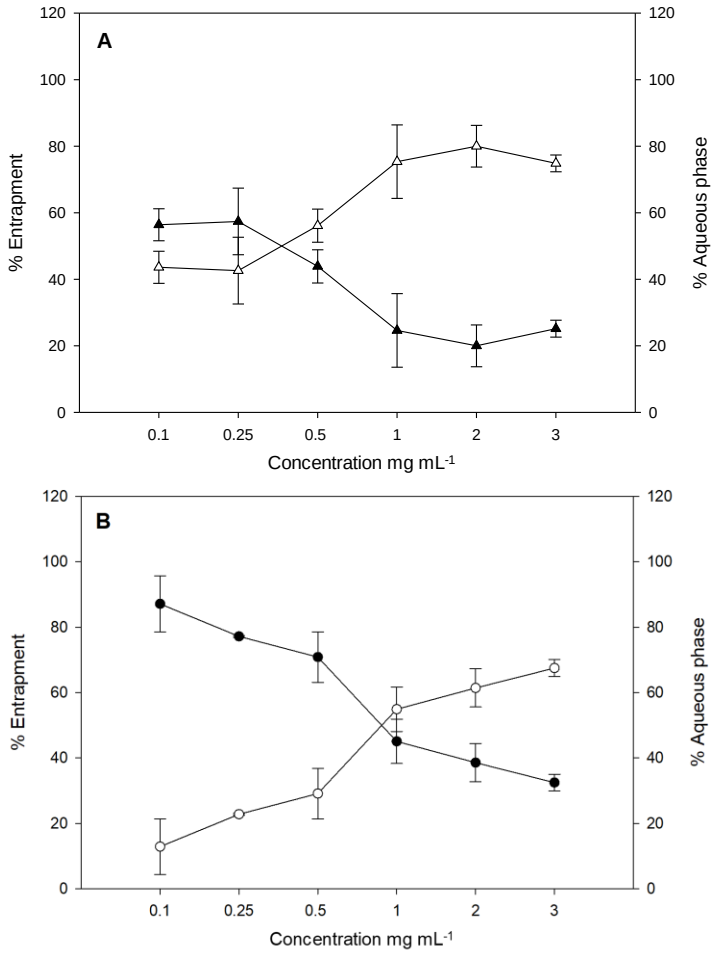

Figure 1. Percentage of rosmarinic acid entrapment (black triangles and cycles) and detected in the aqueous phase (white triangles and cycles) in caseins (A) and SPI (B) formulations at different marjoram pressurized extract concentrations. Entrapment was calculated based on the amount of rosmarinic acid detected in the aqueous phase. Results are the average of three independent experiments, with bars representing standard deviation.

3.3 Immunomodulatory activity of marjoram-protein formulations

To evaluate the bioefficacy and functionality of marjoram-protein formulations, their *in vitro* immunomodulatory activity was assessed using human macrophages differentiated from THP-1 monocytes cell line. Preliminary data was obtained to determine cytotoxicity of protein formulations containing marjoram extract (10, 50, 100 and 200 µL) and 100 µL was the maximum volume that did not induced cytotoxicity on the cells (data not shown). Figure 2 illustrates the results for TNF-α, IL-1β and IL-6 secretion. As shown, the incorporation of LPS (Control+) increased the secretion of the three measured cytokines compared with basal levels of secretion in untreated cells (Control-). Formulated marjoram protein carriers, caseins and SPI, and ME solution significantly reduced TNF-α secretion. In particular, caseins formulations showed a significant higher effect in reduction of TNF-α secretion compared to SPI formulations. Marjoram solutions (100 and 50 µg mL^{-1}) reduced TNF-α secretion to similar levels than those obtained with SPI formulations containing 1 and 0.5 mg mL^{-1} of M.

Secretion of the pro-inflammatory cytokine IL-1β was only reduced in marjoram solution treated cells up to 50% with 100 µg mL^{-1}. Neither casein alone formulations or SPI solutions with or without marjoram encapsulated showed any effect on suppressing IL-1β secretion. Both protein formulations showed elevated level of the cytokine from 130 to 180%. The presence of marjoram triggered reduction of IL-1β secretion caused by casein and SPI solutions alone. Similar to TNF-α secretion, IL-6 secreted levels were reduced in cells treated with marjoram solutions, casein and SPI formulations, compared to activated cells (Control+). However, despite SPI empty solution seemed to reduce the secretion of IL-6 (83% secretion), no statistical differences were found. When comparing casein and SPI marjoram formulations, only at the highest concentration of marjoram (1 mg ml^{-1}), casein formulation showed a higher reduction of IL-6 secretion.

Studies have shown the potential of rosmarinic acid to induce anti-inflammatory effects on different cell lines. Thus, Jiang *et al.*[32] showed evidence of rosmarinic acid down regulating the levels of TNF-α, IL-6 and high mobility box 1 protein in LPS induced RAW 264.7 cells, indicating that rosmarinic acid might inhibit activation of the nuclear factor-κB pathway by inhibiting IκB kinase activity.

Accordingly, rosmarininc acid inhibited LPS-induced up-regulation of IL-1β, IL-6, TNF-α and suppressed expression of iNOS in human gingival fibroblasts.[33] Further, Lembo *et al.*[34] indicated that rosmarinic acid produced a significant reduction in IL-1β, IL-6, IL-8 and TNF-α gene expression in HaCat cells after UVB irradiation.

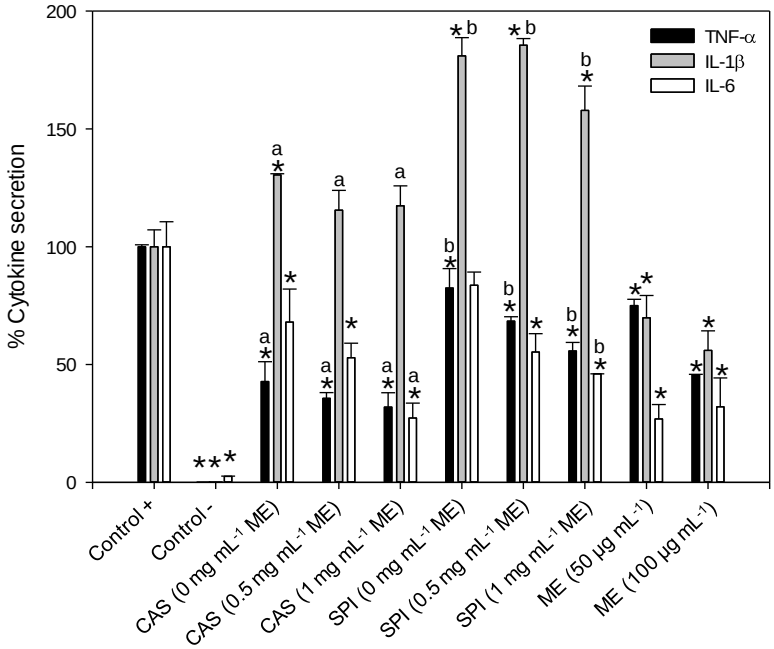

Figure 2. Effect of casein (CAS) and soy protein isolate (SPI) formulations versus marjoram extract (ME) in cytokines secretion determined by ELISA. Percentage of secretion was determined after 24 h incubation. A control lipopolysaccharide (LPS) activated macrophages (Control+) was used for comparison. TNF-α (black bars), IL-1β (grey bars) and IL-6 (white bars). Results are the average of three independent experiments, with bars representing standard deviation. Asterisk (*) denotes statistical differences between Control+ and each other sample per cytokine analyzed $P < 0.05$. Lower case letters a and b indicate statistical differences between pair comparison of casein and SPI formulation at the same concentration $P < 0.05$.

Our results showed that empty casein and SPI suspensions reduced TNF-α and IL-6 secretion. Anti-inflammatory properties of sodium caseinate has also been described in cell models.[35] TNF-α activated Caco-2 cells reduced IL-8 secretion after exposure with sodium caseinate hydrolysates for 24 h. In addition, casein derived peptides as

glycomacropeptide are described in the literature for their immunomodulatory properties.[36] Lunasin, known as a bioactive polypeptide identified in soybean with chemopreventive properties, it has also been described as anti-inflammatory in RAW 264.7 macrophages.[37,38] Similar to our study, lunasin reduces secretion of TNF-α and IL-6 in LPS activated RAW 264.7 macrophages.[39] Peptides obtained from pepsin and pancreatin hydrolysates of soy products also showed anti-inflammatory activity by means of inhibition of NO production, TNF-α and IL-1β secretion.[40]

4. CONCLUSION

The findings indicated that PLE and UAE are adequate techniques to obtain MEs with a high content of rosmarinic acid and consequently antioxidant activity. Among extracts, PLE extracted with a solvent mixture of 70:30 (*v/v*) ethanol/water presented the highest yield and antioxidant activity. Entrapment of PLE marjoram extracts in SPI provided 20 to 30% higher entrapment efficiency than caseins. The complexes of ME with caseins or SPI did not alter the immunomodulatory response of the extract itself. The results of this study would suggest that SPI and caseins could be safely used as carriers of herb extracts for applications in food product development.

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ABBREVIATIONS

SPI, soy protein isolate; ME, marjoram extract; UAE; ultrasound-assisted extraction; PLE, pressurized liquid extraction; HPLC, high-performance liquid chromatography; TPC, total phenolic content; GAE, gallic acid equivalents; TEAC, trolox equivalent antioxidant capacity; LPS, lipopolysaccharide; LSD, least significant difference, GRAS, generally recognised as safe; CAS, caseins; NO, nitric oxide.

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Publicación 3

Anti-inflammatory and antioxidant activities from the basolateral fraction of Caco-2 cells exposed to a rosmarinic acid enriched extract.

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ABSTRACT

The potential use of *Origanum majorana* L. as a source of bioavailable phenolic compounds, specifically rosmarinic acid (RA), has been evaluated. Phenolic bioavailability was tested using an *in vitro* digestion process followed by a Caco-2 cellular model of intestinal absorption. The high-performance liquid chromatography-photodiode array detector-tandem mass spectrometry (HPLC-PAD/MS/MS) analysis showed the main components in the extract were 6-hydroxyluteolin-7-*O*-glucoside and rosmarinic acid, followed by luteolin-*O*-glucoside. After digestion process, the amount of total phenolic compounds (TPC) only decreased slightly, although a remarkable reduction in RA (near 50%) was detected. Bioavailable fraction contained 7.37 ± 1.39 mg/L digested extract of RA with small quantities of lithospermic acid and diosmin and presented an important antioxidant activity (0.89 ± 0.09 mmol Trolox/L digested extract). Besides, this bioavailable fraction produced a significant inhibition in TNF-α, IL-1β and IL-6 secretion, using a human THP-1 macrophages model. Therefore, RA content in the basolateral compartment could play an important role in the antioxidant and anti-inflammatory activities found.

1. INTRODUCTION

Origanum majorana L. (marjoram) is a culinary herb often used in foods. Its essential oil and extracts have been indicated to possess antioxidant, antimicrobial, anticancer and anti-inflammatory activities.[1,2] These activities have been attributed to the presence of a high percentage of phenolic acids and flavonoids in marjoram leaves[3] particularly, to rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid naturally occurring in marjoram.[4,5] Antioxidant activity of RA has been generally admitted, since this compound may act as free radical scavenger.[6,7] Related to RA anti-inflammatory effects, Jiang et al.[8] showed that this compound down regulated the levels of TNF-α, IL-6 and high mobility box 1 protein in bacterial lipopolysaccharide (LPS) induced RAW264.7 cells, indicating that RA might inhibit activation of the nuclear factor-κB pathway by inhibiting IκB kinase activity. Accordingly, Zdarilová et al.[9] reported that RA inhibited LPS-induced up-regulation of Il-1β, IL-6, TNF-α and suppressed expression of iNOS in human gingival fibroblasts. Further, Lembo et al.[10] indicated that RA produced a significant reduction in IL-1β, IL-6, IL-8 and TNF-α gene expression in HaCat cells after UVB irradiation.

In order to extrapolate results found *in vitro* to *in vivo* situation, it is important to know the bioavailability of bioactive compounds. Therefore, the use of an *in vitro* digestion/Caco-2 cell culture model has been proposed by several authors as an economical and useful alternative to study the bioavailability of these compounds.[11,12] Thus, although the transepithelial transport of RA in intestinal Caco-2 cells monolayers has been studied,[13] it is crucial to investigate the effect that plant matrix plays on its bioavailability, since plant material matrix may alter absorption and bioavailability of phytochemicals.[14] For that matter, it would be very interesting to determine the anti-inflammatory and antioxidant effect of the bioavailable fractions of marjoram extracts enriched in RA, in order to corroborate the biological activities described for these extracts.

Solid–liquid extraction (SLE) is the most traditional technology used to extract active compounds from plant matrix. It is widely known that higher temperatures enhance the solubility of the solute in the solvent and thus improve its recovery. Nevertheless, the SLE temperature is limited by solvent boiling and, in some cases, by the loss of volatile compounds. In this regard, pressurized liquid extraction (PLE) allows the use

of solvents in a liquid state at higher temperatures. Furthermore, a compression effect is made on vegetal particles, which also contributes to improve extraction yield; moreover, lower amount of solvent is required, extraction is faster and volatiles loss is minimized.[15] Thus, several studies proposed PLE extraction as an alternative to conventional solid/liquid extraction in order to obtain phenolic compounds from herbs and spices.[16,17]

However, it is hard to obtain a highly concentrated extracts in phenolic compounds using only PLE, due to the complexity and the presence of impurities in crude extracts of herbal raw materials. Nowadays, the use of adsorption resins has been proposed as one of the most useful tools for selective enrichment of phenolic compounds from plant material, such as naringenin recovery from orange juice[18] or anthocyanins from grapes.[19] For this purpose, some of the most commonly employed resins are XAD-2, XAD-7, XAD-16 and Oasis HLB, that have been successfully used for phenolic compounds enrichment from natural extracts.[20,21] Among them, XAD-7 has been proposed for rosmarinic acid enrichment from *Lavandula vera*[22] or *Rabdosia serra.*[23]

The aim of this work was to study the anti-inflammatory and antioxidant properties of the bioavailable fraction of PLE extracts enriched in rosmarinic acid. In order to increase the quantity of RA in PLE extracts of marjoram an Amberlite XAD-7HP resin was employed. The bioavailability of both, original and enriched extracts, was determined by using an *in vitro* digestion/Caco-2 cell culture model. Thus, the anti-inflammatory and antioxidant activity of the basolateral fraction was measured.

2. MATERIALS AND METHODS

2.1 Chemicals

Ethanol of analytical grade and Folin-Ciocalteu's reagent were obtained from Panreac (Madrid, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH), gallic acid for titration (97.5%), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Madrid, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). Reference substances (analytical standard or HPLC purity ≥ 95%) for phenolic

compounds identification such as cryptochlorogenic acid, neochlorogenic acid and rosmarinic acid were purchased from Sigma Aldrich (Madrid, Spain). Apigenin, apigenin-7-*O*-glururonide, caftaric acid, diosmin, lithospermic acid, luteolin, salvianolic acid and vicenin 2 were from Phytolab (Madrid, Spain). Ethyl gallate, apigenin-7-*O*-glucoside, caffeic acid, luteolin-7-*O*-glucoside, *p*-coumaric acid and protocatechuic acid were obtained from Extrasynthese S.A. (Genay, France). Finally, luteolin-7-*O*-glucuronide was from HWI Analytic GmbH (Rülzheim, Germany). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain).

2.2 Marjoram samples and PLE extraction

Marjoram sample consisted of dried leaves obtained from an herbalist shop (Murciana herboristería, Murcia, Spain). The sample was ground in a knife mill (Grindomix GM 200, Retsch, Llanera, Spain) and the particle size was determined by sieving the ground plant material to the appropriate size (<500 μm). The whole sample was stored at -20 ºC until use.

Extractions were carried out in an ASE 350 system from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit. Each extraction cell (11 mL of capacity) was filled with a mixture of 1g of solid sample and 4 g of sea sand. Then, the cell was filled with the solvent (a mixture of ethanol: water, 70:30) up to a pressure of 1500 psi and heated to 100ºC. Static extractions were performed for 10 min. The extracts were recovered in glass vials, ethanol was eliminated by evaporation and extracts were lyophilized. The dried samples were stored at 4 ºC in the dark until analysis.

2.3 Enrichment in phenolic compounds by resin column

Enrichment experiments were carried out in a glass column (3 cm x 50 cm) packed with XAD-7HP resin (Sigma-Aldrich, St. Louis, MO, USA). In order to remove the monomers and pyrogenic agents trapped inside the pores during the synthesis process, a pre-treatment of this resin was realized following the method described by Lin et al.[23]

After pretreatment process, the resin was rehydrated overnight with ethanol at 4 ºC and packed with a bed volume (BV) of 174 mL. In order to remove the ethanol, a distillated water washing was placed (4BV) at constant flow of 4BV/h. PLE extract was dissolved in acid water (pH 3) (15 mg/mL) and 45 mL was applied onto the column.

After absorption equilibrium was reached (1h), the column was washed with 2BV of distilled water and eluted with 3BV of 80% ethanol at a constant flow of 2 BV/h. The eluted fraction was collected, evaporated to remove methanol, freeze-dried and stored at -20 ºC until evaluation.

2.4 Determination of total phenolic content (TPC) and antioxidant activity

In order to determine the TPC, Folin-Ciocalteau reagent method was applied as described by Singleton et al.[24] The results were expressed as mg of gallic acid equivalents (GAE)/g extract. Antioxidant activity was determined by DPPH method. This method was applied according to Brand-Williams et al.[25] protocol. The results were expressed as TEAC value (mmol trolox/g extract or L of digested extract). All analyses were done in triplicate.

2.5 Chemical characterization of samples

High-performance liquid chromatography array detector (HPLC-PAD) analysis of phenolic compounds was performed by using an Agilent HPLC 1260 Infinity series system with photodiode-array detector (Agilent Technologies Inc., Santa Clara, CA, USA). A reverse phase ACE Excell 3 Super C18 column (150 mm x 4.6 mm, 3 μm particle size) protected by a guard column ACE 3 C18-AR (10 mm x 3 mm) was used at 35 ºC. 20 μL of sample (extract, digested extract, apical or basolateral fractions) was injected previously filtered by 0.45 μm PVDF filter.

Chromatographic separation was achieved using solvent A (99.9/0.1 water/formic acid v/v) and solvent B (ACN) at a flow rate of 0.5 mL/min as follows: 0 min, 0% B; 1 min, 0% B; 6 min, 15% B; 21 min, 25% B; 26 min, 35% B; 36 min, 50%; 41 min, 50% B; 44 min, 100% B; 49 min, 0% B. Chromatograms were recorded at 280 nm, 320 nm and 360 nm. Peaks were tentatively identified according to its retention time and UV-Vis spectrum by comparison with analytical standards. Ethyl gallate was added as internal standard in each analyzed sample before it was filtered. Confirmation of the identified compounds was carried out by HPLC-MS analyses. HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a hybrid quadrupole-time-of-flight mass spectrometer (QTOF, QSTAR pulsar i, ABSciex) equipped with a turbo ion electrospray source was used. MS experiments in negative mode were carried out in TOF/MS and MS/MS mode. The instrumental parameters were set as follows: mass range 50-2000 Da; ion spray voltage (IS) -4500 V; ion source gas pressure (GS1): 65

psi; (GS2): 65psi; curtain gas pressure (Cur): 20 psi; declustering potencial (DP): 30 V; focusing potencial (FP): 210 V; declustering potencial 2 (DP2): 15 V; collision gas: 3 psi in MS experiments and 5 psi in MSMS experiments. In TOF/MS experiments, just before separation, an external calibration in the mass spectrometer was performed with a mixture of phosphazenes and verified after the assays. The maximum error accepted to calibrate in the whole range of mass was 5 ppm. In MSMS product ion experiments, the ion precursor was selected and the collision energy was fixed to 35 eV. Just before each MS/MS experiment, the instrument was calibrated with taurocholic acid and verified after the experiment. The accurate masses obtained were processed using the elemental composition calculator incorporated in the Analyst Software (Applied Biosystems). A margin of error up to 5 ppm for unknown compounds was allowed. Chromatographic conditions were similar as for HPLC-PAD analysis.

Quantification of identified compounds was carried out by using calibration curves of its authentic standard (Extrasynthese S.A., Genay, France) at five levels in triplicate by HPLC-PAD. Moreover, 6-hydroxyluteolin-7-*O*-glucoside, luteolin-*O*-glucoside, lithospermic acid isomer and salvianolic acid isomer were quantified by the calibration curve of luteolin-7-*O*-glucoside, lithospermic acid and salvianolic acid, respectively. Validation of the chromatographic method (LOD, LOQ, precision, repeatability, stability and recovery) was previously done (data not shown).

2.6 In vitro digestion

The digestion process was carried out following a previously published protocol[12] slightly modified. Briefly, each extract (100 mg) was dissolved in 5 mL of ethanol: water (50:50), mixed with 0.1 mL α-amylase from human saliva type XIII-A (Sigma-Aldrich, St. Louis, MO, USA) (9.3 mg in $CaCl_2$ 1 mM) and shaking for 2 min at 37 ºC (oral phase). Stomach and intestinal phases were carried out employing a titrator Titrino Plus 877 (Methrom AG, Herisau, Switzerland). Thus, oral phase was mixed with 25 mL of a gastric solution (127 mg of porcine pepsin from porcine mucosa, 536 U/mg, (Sigma-Aldrich, St. Louis, MO, USA)) at pH 2 (adjusted with 0.1 M HCL) and shaking for 1h at 37 ºC. After gastric digestion, samples were adjusted to pH 7 with 1 M NaOH prior to the pancreatic step. Next, a pancreatic-bile extract containing 9.3 mg pancreatin (Sigma-Aldrich, St. Louis, MO, USA) and 115.7 mg of bile salts in 2.8 mL of 10 mM trizma-maleate buffer was incorporated and incubated for 2h at 37 ºC.

At the end of digestion, the enzyme reaction was stopped immediately by cooling the samples in ice and samples were kept at -20ºC until analysis.

2.7 Caco-2 experiments

Caco-2 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 1% nonessential amino acids and 2 mM L-glutamine (Gibco, Paisley, UK) at 37 ºC in at humidified atmosphere containing 5% CO_2. The cytotoxic effect on Caco-2 cells of the extracts after digestion process was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay following Mosmann[26] method. Briefly, Caco-2 cells were plated in 24-well plates until cell monolayers were obtained. Then the medium was aspirated and cells were treated with different concentrations of digested extracts for 6h. After that, the medium was removed and cells were washed and incubated with MTT for 2-3h. The medium was removed and 500 μL of DMSO were added. The absorbance was measured at 570 nm.

For transport experiments, Caco-2 cells were seeded onto six-wells Transwell® plates (0.4 µm pore size, inserts of 24 mm diameter, Costar, Corning, Madrid, Spain) at a density of 3 x 10^5 cells per insert. The cells were maintained along 21 days, once the monolayer was formed, during which time culture medium was replaced every three days. The Caco-2 monolayer was used when transepithelial electrical resistance (TEER) (EVOM2, World Precision Instruments, Hitchin, UK) values were larger than 350 $\Omega \cdot cm^2$. Apical and basolateral compartments were washed once with PBS and then incubated with 1.5 mL and 2.6 mL of supplement DMEM without FBS. A volume of 150 μL of digested extracts were incorporate in the apical compartment and incubated for 6 h at 37ºC. The TEER value was measured twice before and after experiment to monitor the integrity of the Caco-2 monolayer. Then apical and basolateral samples were freeze-dried and stored at –20 ºC prior analysis.

2.8 Anti-inflammatory activity of basolateral samples from Caco-2 experiments

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 culture medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Gibco, Paisley, UK) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain) at 37 ºC in 95% humidified air containing 5% CO_2. Cells were plated at a density of 5 x 10^5 cells/mL in 24 wells plates. Differentiation of monocytes to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 48 h. The viability of THP-1/M cells in presence of basolateral medium from Caco-2 experiments was tested used the MTT assay following Mosmann[26] method. The assays were performed in triplicate.

After differentiation, cells were washed with PBS and incubated with 0.05 μg/mL of LPS from *E. coli* O55:B5 (Sigma-Aldrich, Spain) in presence of the basolateral medium from Caco-2 experiments for 24 h. Then, the supernatant was frozen at -20 ºC. Positive controls represented THP-1/M cells incubated with LPS but without basolateral medium and negative controls indicated cells nonstimulated with LPS and without basolateral medium. The release of TNF-α, IL-1β and IL-6 was measured in the supernatants of THP-1/M cells using ELISA kits (BD Biosciences, Aalst, Belgium), according to the manufacturer's instructions. The generated color was quantified by measuring the optical density at 450 nm with substrate correction at 570 nm using a multiscanner autoreader (InfiniteM200 Tecan, Barcelona, Spain). The results were expressed as the mean of three determinations ± standard deviation (SD).

2.9 Statistical analysis

Experimental results are expressed as means ± SD. One-way analysis of variance (ANOVA) followed by least significant difference test was used at $p \leq 0.05$ to look for differences between means. Statistical analyses were performed using Statgraphics v. Centurion XVI software for Windows (Statpoint Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1 Phenolic and functional characterization of PLE and enriched extracts

PLE extracts from marjoram were carried out using 70% ethanol, 100 ºC and 10 min. These extraction conditions were supported by previous studies (data not shown) developed to obtain extracts with a high content of TPC and an important antioxidant activity. The PLE obtained extract presented a TPC of 265.9 mg GAE/g extract and a TEAC value of 1.81 mmol Trolox/g extract. For the purpose to achieve extract enrichment in phenolic compounds, XAD-7HP resin was used. The effectiveness of this kind of resins for rosmarinic acid enrichment has been previously reported.[23] As Table 1 shows; the use of this resin achieved a TPC 1.5-fold superior than the original PLE extract. Thus, the amount of phenolic compounds presented in the new extract represented a 39% of total extract. Moreover, this enriched extract also presented significantly higher antioxidant activity (Table 1).

Table 1. TPC content and antioxidant activity in original and enriched extracts[A].

		TPC (mg GAE/g)	TEAC value (mmol Trolox/g)
Original extract	initial	266 ± 4.80 $^{a}{}_{2}$	1.81 ± 0.04 $^{a}{}_{2}$
	after digestion	222 ± 12.6 $^{b}{}_{2}$	1.71 ± 0.05 $^{b}{}_{2}$
Enriched extract	initial	389 ± 38.6 $^{a}{}_{1}$	2.81 ± 0.01 $^{a}{}_{1}$
	after digestion	312 ± 4.86 $^{b}{}_{1}$	2.67 ± 0.03 $^{b}{}_{1}$

[A] Data shown represent means ± S.D. (n = 3). [a,b] Different superscript letters denote significant differences within the same extract before and after digestion ($p \leq 0.05$). [1,2] Different subscript numbers denote significant differences between both extracts in the same condition (before or after digestion) ($p \leq 0.05$).

The HPLC-PAD-MS-QTOF analysis of the phenolic compounds (Table 2) resulted in the identification of 17 compounds in the extracts (original and enriched), whereas *p*-coumaric and neochlorogenic acids were also identified in the digested extracts. All the identified compounds in the extracts had been previously described in marjoram extracts. [27-29]

Moreover, according to its specific λ_{max}, accurate mass and MS/MS fragments 6-hydroxyluteolin-7-*O*-glucoside, luteolin-*O*-glucoside, lithospermic acid isomer and salvianolic acid isomer were tentatively identified in the extracts. [28,30,31]

Table 2. Phenolic compounds identified in the samples using HPLC-PAD-MS/MS.

Peak	Compound	R_t (min)	λ_{max} (nm)	Acc. mass (*m/z*)	Error (ppm)	MS^2 (*m/z*)
1	Neochlorogenic acid[a]	12.9	300, 326	353.0878	+1.3	191(100), 179(75), 135(30)
2	Protocatechuic acid[a]	13.4	260, 294	153.0193	+1.2	153(30), 109(100)
3	Caftaric acid[a]	13.7	300, 328	311.0408	+2.5	149(100)
4	Cryptochlorogenic acid[a]	15.1	300, 326	353.0878	+4.0	191(100), 179(75), 135(30)
5	Vicenin II[a]	15.9	272, 336	593.1511	-1.8	503(20), 473(100), 383(20), 353(30)
6	Caffeic acid[a]	17.8	299, 324	179.0349	+2.6	135(100)
7	6-hydroxyluteolin-7-*O*-glucoside	19.8	282, 344	463.0882	-4.1	463(20), 301(100)
8	*p*-coumaric[a]	22.9	310	163.0400	+2.1	119(100)
9	Luteolin-*O*-glucoside	23.4	282, 334	447.0932	-3.4	285(100), 151(20)
10	Luteolin 7-*O*-glucoside[a]	23.8	254, 266, 348	447.0932	-2.6	285(100), 151(20)
11	Luteolin 7-*O*-glucuronide[a]	24.0	254, 266, 348	461.0725	-4.7	285(100), 151(20)
12	Diosmin[a]	27.1	352, 266, 346	607.1668	-2.7	607(10), 299(100), 284(10)
13	Apigenin 7-*O*-glucoside[a]	27.6	266, 336	431.0983	-4.6	431(20), 269(100)
14	Apigenin 7-*O*-glucuronide[a]	28.1	266, 336	445.0776	-4.3	445(20), 269(100)
15	Rosmarinic acid[a]	28.9	288, 330	359.0772	+0.7	197(20), 161(100), 179(30), 135(20)
16	Lithospermic acid isomer	29.7	288, 310, 334	537.1038	-4.9	493(10), 359(30), 295(30), 197(20), 161(100), 135(80)
17	Salvianolic acid isomer	30.4	288, 310, 334	717.1461	-2.8	717(10), 519(30), 475(10), 359(100), 295(10), 179(10)
18	Luteolin[a]	33.7	254, 266, 348	285.0404	-3.0	285(20), 217(35), 175(100), 151(50)
19	Apigenin[a]	37.0	266, 336	269.0444	-3.8	269(20), 225(30), 151(100), 119(50)

[a] Comparison with standards.

Both extracts showed a similar qualitative composition, although important quantitative differences were found (Table 3). Therefore, enriched extract presented, in general, an increase between 1.8 and 1.5 times in the quantity of all compounds in relation to the original extract. Accordingly, the main components in both extracts were 6-hydroxyluteolin-7-*O*-glucoside and rosmarinic acid, followed by a luteolin-*O*-glucoside and an isomer of salvianolic acid. This phenolic composition was consistent with other research works where marjoram extracts had been characterized by an extended composition in phenolic acids and flavonoids. These works reported that, among phenolic acids, RA was the main compound detected. In addition, luteolin and its glucosyl derivatives have been also detected as the main flavonoids.[3,16]

Table 3. Effect of *in vitro* gastrointestinal digestion process in extracts composition.

Compound	Original extract (mg/g extract)		Enriched extract (mg/g extract)	
	Initial	After digestion	Initial	After digestion
Neochlorogenic acid	N.D. [a]	0.57 ± 0.01 [b]	N.D. [a]	0.88 ± 0.02 [b]
Protocatechuic acid	0.17 ± 0.01	0.19 ± 0.00 [b]	0.30 ± 0.01	0.18 ± 0.00 [b]
Caftaric acid	0.06 ± 0.00	0.04 ± 0.00 [b]	0.10 ± 0.01	0.09 ± 0.00
Cryptochlorogenic acid	0.86 ± 0.00	0.53 ± 0.00 [b]	1.49 ± 0.01	0.76 ± 0.00 [b]
Vicenin II	2.32 ± 0.02	2.24 ± 0.03 [b]	4.00 ± 0.01	3.73 ± 0.01 [b]
Caffeic acid	0.93 ± 0.07	0.57 ± 0.01 [b]	1.32 ± 0.01	0.88 ± 0.01 [b]
6-hydroxyluteolin-7-*O*-glucoside	33.9 ± 0.09	14.5 ± 0.18 [b]	60.9 ± 0.04	21.0 ± 0.23 [b]
p-coumaric	N.D. [a]	0.36 ± 0.00 [b]	N.D. [a]	0.52 ± 0.00 [b]
Luteolin-*O*- glucoside	25.2 ± 0.03	10.5 ± 0.12 [b]	44.7 ± 0.02	15.3 ± 0.17 [b]
Luteolin 7-*O* glucoside	15.5 ± 0.08	14.6 ± 0.02 [b]	25.5 ± 0.01	21.8 ± 0.04 [b]
Luteolin 7-*O*-glucuronide	5.69 ± 0.02	5.38 ± 0.02 [b]	9.29 ± 0.01	8.17 ± 0.04 [b]
Diosmin	6.48 ± 0.04	6.46 ± 0.03	10.3 ± 0.01	10.8 ± 0.06 [b]
Apigenin 7-*O*-glucoside	2.45 ± 0.02	3.22 ± 0.03 [b]	4.69 ± 0.01	4.90 ± 0.04 [b]
Apigenin 7-*O*-glucuronide	3.64 ± 0.49	5.74 ± 0.06 [b]	7.20 ± 0.01	8.70 ± 0.02 [b]
Rosmarinic acid	33.9 ± 0.05	19.0 ± 0.11 [b]	57.2 ± 0.05	29.0 ± 0.34 [b]
Lithospermic acid isomer	9.10 ± 0.02	6.91 ± 0.11 [b]	15.9 ± 0.05	11.0 ± 0.11 [b]
Salvianolic acid isomer	17.2 ± 0.35	2.60 ± 0.14 [b]	24.9 ± 0.16	4.27 ± 0.09 [b]
Luteolin	1.39 ± 0.08	0.55 ± 0.03 [b]	2.07 ± 0.01	0.73 ± 0.02 [b]
Apigenin	0.45 ± 0.01	0.22 ± 0.00 [b]	0.75 ± 0.01	0.31 ± 0.04 [b]

[a] N.D. = non detected. [b] Denotes significant difference when compares initial and after digestion concentration within same extract ($p \leq 0.05$). Data represent means ± S.D. (n = 4).

3.2 Phenolic composition and antioxidant activity of the extracts during *in vitro* digestion

Digestion effect in TPC and antioxidant activity for both extracts is shown in Table 1. After the digestion process, the amount of TPC only decreased slightly for both extracts, as well as antioxidant activity. Notwithstanding, digestion step produced a remarkable reduction of RA (approximately 50%) in both original and enriched extracts (Table 3). This decrease in RA content during gastrointestinal digestion was in agreement with other studies that reported RA degradation or transformation into other compounds during *in vitro* gastrointestinal digestion.[32,33] Moreover, Zoric et al.[34] showed that gastrointestinal stability of RA was highly influenced by the plant matrix, indicating that the presence of some flavonoids such as luteolin or apigenin enhances the stability rate of RA during digestion process.

Moreover, the quantitative analysis of phenolic composition after the digestion process (Table 3) showed that most compounds, presented in both extracts, were affected by digestion step to a lesser or greater extent. For that matter, although RA was reduced after digestion, it represented the main compound in both digested extracts, followed by luteolin-*7*-*O*-glucoside, 6-hydroxyluteolin-7-*O*-glucoside and luteolin-*O*-glucoside. Among these compounds, 6-hydroxyluteolin-7-*O*-glucoside and luteolin-*O*-glucoside were most affected by the digestion step, meanwhile, luteolin-7-*O*-glucoside and glucuronide, were much less affected, even more apigenin-7-*O*-glucoside and glucuronide increased after digestion.

It should be also noted the appearance of neochlorogenic and *p*-coumaric acids in both digested extracts, compounds not detected in the nondigested extracts and whose presence could be attributed to isomerization and degradation processes of initial phenolic acids. Accordingly, Xie et al.[35] observed isomeric transformations of chlorogenic acids, where cryptochlorogenic acid was mainly turned into chlorogenic acid at pH 7 and 37 ºC, in agreement with intestinal conditions. Moreover, some studies have shown the presence of coumaric acid as a related metabolite of rosmarinic acid in human and animal plasma.[36,37]

3.3 Caco-2 cell transport experiments

Transport experiments of digested extracts were carried out using an *in vitro* model of the intestinal barrier: Caco-2 cells differentiated to enterocytes[38]. Prior to transport experiments, the cytotoxicity of the digested extracts was evaluated at 6h. The results showed that 150 μL of the digested extracts was the maximum concentration that did not significantly affected cell viability (data not shown). In addition, the integrity of the Caco-2 monolayer during exposure experiments was evaluated as TEER-value.

Total phenolic compounds content recovered in apical and basolateral fractions for both digested extracts was measured to determine the quantity of these compounds unabsorbed and bioavailable, respectively (Table 4). For both extracts, only a small amount of the phenolic compounds presented in the digested extracts was detected in the bioavailable fraction. Thereby, the bioavailable fraction from enriched extract presented a quantity of 84.8 mg GAE/L digested extract, meanwhile for original extract only 68.5 mg GAE/L digested extract was detected in basolateral fraction.

Table 4. TPC content and antioxidant activity in original and enriched extracts after caco-2 absorption experiments[A].

		TPC (mg GAE/L digested extract)	TEAC value (mmol Trolox/ L digested extract)
Original extract	After digestion	502 ± 18.1$^{a}{}_{2}$	3.41 ± 0.16 $^{a}{}_{2}$
	Apical fraction	358 ± 26.7 $^{b}{}_{2}$	1.77 ± 0.21$^{b}{}_{2}$
	Basolateral fraction	68.5 ± 6.40 $^{c}{}_{2}$	0.73 ±0.01 $^{c}{}_{2}$
Enriched extract	After digestion	684 ± 31.1$^{a}{}_{1}$	4.78 ± 0.58 $^{a}{}_{1}$
	Apical fraction	556 ± 40.5 $^{b}{}_{1}$	3.74 ± 0.36 $^{b}{}_{1}$
	Basolateral fraction	84.8 ± 7.86 $^{c}{}_{1}$	0.89 ±0.09 $^{c}{}_{1}$

[A] Data shown represent means ± S.D. (n = 3). [a,b,c] Different superscript letters denote significant differences within the different fractions of the same extract ($p \leq 0.05$). [1,2] Different subscript numbers denote significant differences among the same fractions of both extracts ($p \leq 0.05$).

The phenolic compounds recovered in apical and basolateral fractions for both digested extracts were also analysed by HPLC in order to determine the bioavailability of individual components (Table 5). The results indicated that, for both extracts, the main component detected in basolateral fraction was RA, although enriched extract presented a quantity 1.6 times superior. Besides RA, lithospermic acid isomer and diosmin were the major components of basolateral fraction in both extracts, meanwhile 6-hydroxyluteolin-7-*O*-glucoside and luteolin-7-*O*-glucoside, compounds with an important presence after digestion process, only were presented in less than 5% in that fraction.

The permeability of RA across Caco-2 cells monolayer has been studied by several authors. Accordingly, Konishi and Kobayashi[13] reported that RA transport throughout Caco-2 cells was mainly *via* paracellular diffusion and its intestinal absorption efficiency was low. These authors supported this idea with the fact that RA transport increased linearly with the concentration and did not reach a plateau even at 30 nM. However, more recently, Qiang et al.[39] suggested that RA was absorbed across Caco-2 cells *via* both paracellular and transcellular diffusion. Moreover, Falé et al.[40] reported that the transport of RA across these cells was increased in presence of a mixture of luteolin and apigenin, since these flavonoids may interfere in several mechanisms involved in the permeation of RA, such as uptake and efflux mechanisms, indicating that the flux of RA may be mediated by transport systems. Data obtained in this work for RA were consistent with the idea that RA transport throughout Caco-2 cells was mainly *via* paracellular diffusion, since in this case the transport increased linearly with the concentration. Thus, enriched extract (after digestion) with a quantity of rosmarinic acid 1.6 times higher than original extract, also presented in basolateral fraction 1.6-fold of RA than original extract. However, it could not be discarded the influence of the flavonoid content in the absorption efficiency of RA obtained in this work (approximately 14%).

Table 5. Extracts phenolic composition after caco-2 absorption experiments[a].

Compound	Original extract (mg/L digested extract)			Enriched extract (mg/L digested extract)		
	Initial	Apical	Basolateral	Initial	Apical	Basolateral
Neochlorogenic acid	1.45 ± 0.41	0.94 ± 0.04	0.31 ± 0.03	2.09 ± 0.03	1.77 ± 0.09	0.41 ± 0.07
Protocatechuic acid	0.45 ± 0.03	0.37 ± 0.00	N.D. [b]	0.43 ± 0.03	0.39 ± 0.04	N.D. [b]
Caftaric acid	0.06 ± 0.00	N.D. [b]	N.D. [b]	0.15 ± 0.03	0.05 ± 0.00	N.D. [b]
Cryptochlorogenic acid	1.27 ± 0.00	0.81 ± 0.08	0.13 ± 0.03	1.75 ± 0.02	1.39 ± 0.09	0.21 ± 0.07
Vicenin II	4.81 ± 0.54	4.07 ± 0.52	0.63 ± 0.09	8.35 ± 0.14	7.74 ± 0.42	1.00 ± 0.40
Caffeic acid	1.51 ± 0.17	1.08 ± 0.10	0.37 ± 0.03	2.39 ± 0.01	1.86 ± 0.07	0.54 ± 0.09
6-hydroxyluteolin-7-*O*-glucoside	17.1 ± 1.04	2.66 ± 0.35	0.47 ± 0.29	20.5 ± 0.43	6.69 ± 1.09	1.01 ± 0.62
p-coumaric	0.80 ± 0.15	0.50 ± 0.03	0.30 ± 0.01	1.38 ± 0.05	0.81 ± 0.01	0.45 ± 0.06
Luteolin-*O*-glucoside	12.8 ± 0.84	2.81 ± 0.10	0.42 ± 0.21	15.9 ± 0.41	6.61 ± 0.89	1.02 ± 0.58
Luteolin 7-*O* glucoside	17.2 ± 1.35	2.69 ± 0.41	0.54 ± 0.32	23.84 ± 0.58	8.34 ± 1.22	1.03 ± 0.05
Luteolin 7-*O*-glucuronide	7.03 ± 0.99	2.26 ± 0.04	0.74 ± 0.06	8.36 ± 0.23	4.71 ± 0.28	1.26 ± 0.03
Diosmin	10.5 ± 0.19	8.56 ± 0.02	1.06 ± 0.21	15.7 ± 0.17	18.6 ± 1.36	1.96 ± 0.62
Apigenin 7-*O*-glucoside	4.25 ± 0.10	1.66 ± 0.02	0.17 ± 0.06	6.20 ± 0.08	3.72 ± 0.14	0.46 ± 0.21
Apigenin 7-*O*-glucuronide	9.03 ± 0.36	3.23 ± 0.38	0.75 ± 0.16	13.4 ± 0.24	7.33 ± 0.19	1.40 ± 0.47
Rosmarinic acid	32.2 ± 2.30	23.1 ± 4.05	4.45 ± 0.54	51.6 ± 2.61	42.4 ± 5.63	7.37 ± 1.39
Lithospermic acid isomer	11.4 ± 1.02	8.53 ± 1.37	1.45 ± 0.29	18.3 ± 0.76	14.4 ± 0.08	2.13 ± 0.42
Salvianolic acid isomer	4.27 ± 0.41	3.20 ± 0.33	0.61 ± 0.08	6.44 ± 0.10	4.82 ± 0.10	0.87 ± 0.10
Luteolin	0.23 ± 0.02	1.92 ± 0.75	0.15 ± 0.01	0.45 ± 0.00	3.82 ± 0.69	0.61 ± 0.47
Apigenin	0.04 ± 0.00	0.70 ± 0.39	N.D. [b]	0.07 ± 0.00	1.48 ± 0.37	0.17 ± 0.00

[a] Data represent means ± S.D. (n= 4). [b] N.D. = non detected.

In this work, the bioavailability of luteolin and apigenin derivatives was, in general, lower than for RA, although, in this case the transport also increased linearly with the concentration. However, in this study is noteworthy that, after 6h of experiment, the amount of luteolin and apigenin aglycone in the apical compartment substantially increased in relation to the amount of these components at t = 0 h. According to those results, Yasuda et al.[41] suggested that luteolin glucoside is partially hydrolyzed by LPH (lactase-phlorizin hydrolase), and moreover, only a fraction of the released aglycone is absorbed inside the cells, where it is converted to its glucuronide conjugate, and subsequently secreted to basolateral compartment. In this study, glucoside and glucuronide forms of luteolin and apigenin were detected in basolateral solution after 6 h, inferring that a portion of glycoside derivatives are also transported in an unchanged way across Caco-2 cells.[42,43] Nevertheless, for a better comprehensive absorption path for flavones, further analysis should be submitted.

In addition, antioxidant activity for both digested extracts was also measured in apical and basolateral compartments and data are shown in Table 4. As can be observed, the antioxidant activity detected in basolateral fraction from enriched extract was a 13% significantly higher than that measured in this fraction when original extract was used. This result was in agreement with the greater amount of phenolic compounds detected in the basolateral chamber for this extract. Thus, mainly RA, lithospermic acid and diosmin presented in the bioavailable fraction could be responsible of its antioxidant activity.

3.4 Anti-inflammatory activity of basolateral samples from Caco-2 experiments

The activation of THP-1/M was carried out by LPS incorporation into the medium. After 24h of incubation, LPS treated cells shown an important increase in all pro-inflammatory cytokines measured (TNF-α, IL-1β and IL-6), compared to nonactivated controls (Figure 1). These activated cells were considered as positive controls for all the cytokines tested. The cytotoxicity assays (data not shown) indicated that 20 μL of the basolateral fraction was the maximum concentration that did not affected THP-1 viability. Thus, when the activation of THP-1/M was carried out in presence of 20 μL of basolateral medium from Caco-2 experiments, an important decrease in TNF-α secreted level was observed compared with levels obtained in absence of extracts (positive control). Moreover, basolateral samples from enriched extract after digestion achieved an 80% of inhibition in TNF-α secretion, higher to that obtained

with the original extract (40%). IL-1β and IL-6 secretion were also reduced (60%) in presence of 20 μL basolateral fractions of the original digested extracts (with respect to positive control). For both interleukins, the enriched extract decreases their release in a greater extent than the original extract, 70% for IL-1β and an 85% for IL-6. Besides, the basolateral fraction from control digestion did not reduced significantly the secretion of any of the pro-inflammatory cytokines studied, compared to the positive control.

Considering these results, basolateral fractions of both digested extracts presented an important anti-inflammatory activity, although enriched extract showed a higher inhibition in the release of all pro-inflammatory cytokines studied. In this regard, several studies have reported that RA, either as pure standard or incorporated into a vegetable matrix, inhibited LPS-induced up-regulation of IL-1β, TNF-α and IL-6 in different cells lines.[11,12] Besides, luteolin and its derivatives have also been reported to be able to inhibit the production of pro-inflammatory cytokines, such as TNF-α and IL-1β.[44] However, none of these studies measured the inhibition in the production of these cytokines by extracts or pure compounds after a digestion and absorption processes.

In conclusion, this study showed the potential use of marjoram extracts as a source of bioavailable compounds with an important antioxidant and anti-inflammatory activities. Thus, the results indicated that PLE extracts from *Origanum majorana* L. represented a rich source of bioavailable RA, especially when using the enriched extract. Besides RA, luteolin derivatives, lithospermic acid isomer and diosmin were also detected in the basolateral fraction in both extracts. Moreover, the bioavailable fractions of both extracts showed a remarkable antioxidant and anti-inflammatory activities, being more prominent when using enriched extract. Thus, RA could have an important role in these activities, although other phenolic compounds detected in the basolateral fractions could also interact synergistically.

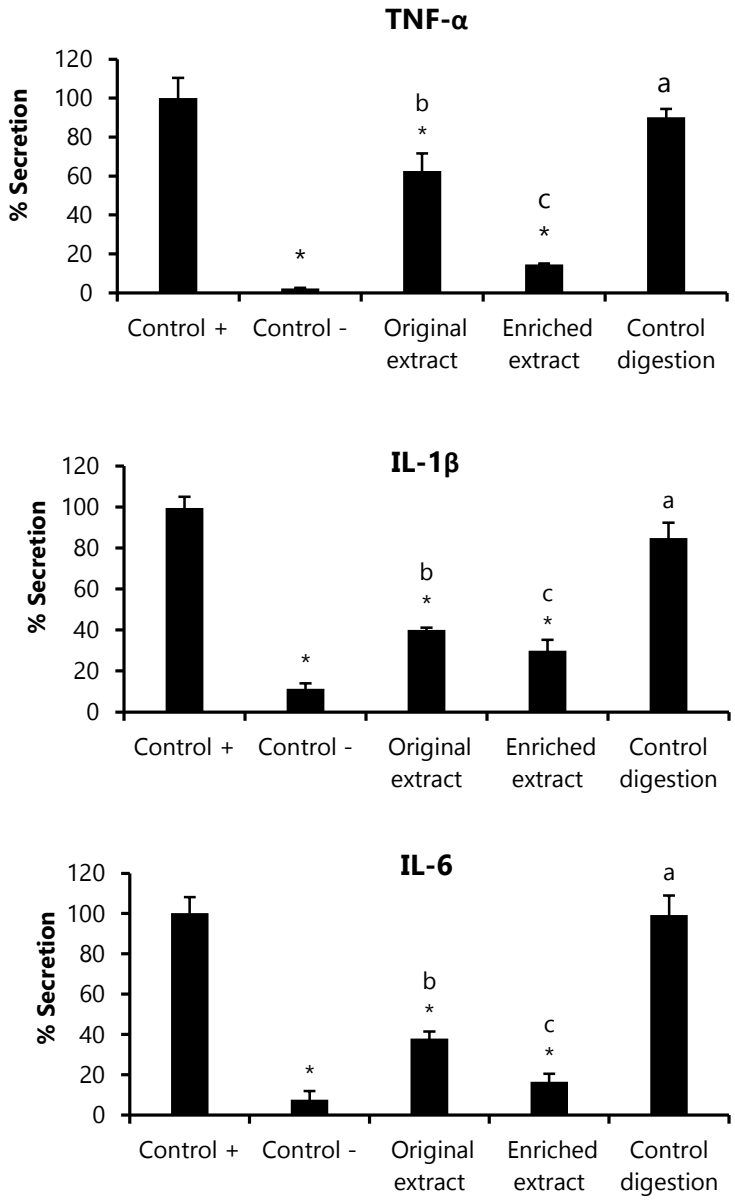

Figure 1: Levels of TNF-α, IL-1β and IL-6 secreted by THP-1/M, activated with LPS, in presence of bioavailable fractions from extracts. Positive control (cells stimulated with LPS but in absence of extract), negative control (cells in contact just with RPMI media), control digestion (bioavailable fraction from a digestion without extract, only digestion juices). Each bar is the mean of three determinations ± S.D. *Denotes statistical differences when compares with positive control. [a,b,c] Different letters indicate statistical differences among original extract, enriched extract and digestion control. Significance level at $p \leq 0.05$ with LSD procedure.

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Notes

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Abbreviations

RA, rosmarinic acid; TPC, total phenolic content; LPS, bacterial lipopolysaccharide; SLE, solid-liquid extraction; PLE, pressurized liquid extraction, BV; bed volume; GAE, gallic acid equivalents; TEAC, trolox equivalent antioxidant capacity; ACN, acetonitrile; LOD, limit of detection; LOQ, limit of quantification; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; TEER, transepithelial electrical resistance; THP-1/M, differentiated macrophages.

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CAPÍTULO 3

Estrategias dirigidas al enriquecimiento y desarrollo de formulaciones para mejorar la biodisponibilidad de los compuestos fenólicos de milenrama.

Prefacio

La milenrama (*Achillea millefolium* L.) es una planta que ha sido ampliamente utilizada por sus propiedades curativas, señalándose a los compuestos fenólicos, donde destacan los ácidos fenólicos derivados del cafeico y los derivados de la luteolina y la apigenina, como los principales responsables de los beneficios a la salud.

Teniendo en cuenta los resultados del *Capítulo 1*, los extractos UAE de milenrama presentaron una elevada actividad antiinflamatoria. Así, en este capítulo también se ha utilizado la PLE para la obtención de extractos de esta planta. El uso de mezclas etanólicas y acuosas (etanol 50% y etanol 70%), dieron lugar a extractos con una mayor actividad antioxidante, mientras que aquellos obtenidos con etanol puro presentaron una mejor respuesta antiinflamatoria, en ambas técnicas de extracción. No obstante, la técnica UAE ofrece la ventaja de poder procesar cantidades mayores de material, en tiempos reducidos, además de ser una tecnología fácilmente escalable.

Dada la relevancia de su actividad antiinflamatoria, se seleccionó el extracto UAE-100% y se enriqueció en compuestos fenólicos mediante el uso de resinas macrospóricas de adsorción XAD-7HP y distintas mezclas de etanol/agua como eluyentes. Se caracterizaron 3 fracciones distintas, destacando la fracción del 80% etanol por presentar una actividad antioxidante, y antinflamatoria superior a la del extracto. Los resultados de la obtención y enriquecimiento de los extractos de milenrama, se recogen en la *publicación 4* titulada: **Antioxidant and anti-inflammatory activities of yarrow extracts obtained by sustainable extraction and isolation procedures.**

Posteriormente, tanto el extracto original de milenrama como el enriquecido en compuestos fenólicos se sometieron a un proceso de digestión gastrointestinal *in vitro*. Así, durante la fase intestinal de la digestión se registraron algunas pérdidas en la cantidad de fenólicos totales y una reducción de la actividad antioxidante, pero sobre todo se produjeron procesos de isomerización para ciertos compuestos fenólicos. Seguidamente, se realizaron los estudios de absorción usando células Caco-2, detectándose solo una pequeña cantidad de compuestos fenólicos en la fracción biodisponible, principalmente agliconas de flavonoides metoxilados. Dicha fracción exhibió una cierta actividad antiinflamatoria. Este estudio dio lugar a la *publicación 5* con el título: **Bioavailability assessment of yarrow phenolic compounds using an *in vitro* digestion/Caco-2 cell model: anti-inflammatory activity of the basolateral fraction.**

Debido a los cambios registrados por los compuestos fenólicos del extracto de milenrama durante la etapa de la digestión intestinal, se diseñaron dos formulaciones para minimizar dichos cambios. Así, se formularon nanoemulsiones con caseinato de sodio, y geles lácteos acidificados donde se incorporó el extracto de milenrama. Además de evaluarse la eficiencia de encapsulación, las formulaciones fueron digeridas para determinar el grado de protección de los compuestos fenólicos durante la digestión. Los hallazgos de este estudio se encuentran recogidos en la *publicación 6* titulada: **Improved *in vitro* bioaccessibility and antioxidant activity of yarrow phenolic compounds formulated in emulsions and acidified milk gels.**

Finalmente, se realizó un fraccionamiento del extracto UAE-100% etanol de milenrama con la tecnología antisolvente y el uso de CO_2 supercrítico. Así, fue posible obtener por un lado fracciones con una elevada actividad antioxidante, y por otro, fracciones con una importante actividad antiinflamatoria. Además, gracias los análisis por HPLC-MS y GC-MS se pudo establecer una cierta relación entre la composición de las fracciones y sus actividades biológicas. Los resultados derivados de este estudio se encuentran reunidos en la *publicación 7* que lleva por título: **Supercritical anti-solvent fractionation for improving antioxidant and anti-inflammatory activities of an *Achillea millefolium* L. extract.**

Publicación 4

Antioxidant and anti-inflammatory activities of yarrow extracts obtained by sustainable extraction and isolation procedures.

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ABSTRACT

This study was aimed to the development of a sustainable procedure for obtaining extracts with antioxidant and anti-inflammatory activities from *A. millefolium* (yarrow). To that purpose, advance extraction techniques, Pressurized Liquid Extraction (PLE) and Ultrasound Assisted Extraction (UAE), were compared. Three different ethanol:water mixtures were used as green solvents. No statistical differences were found in terms of the total phenolic content (TPC) and antioxidant activity within both techniques, while higher extraction yields were recovered by using PLE, and slightly higher anti-inflammatory activity were achieved in UAE extracts. In contrast, aqueous-ethanol solvent influence was a determinant factor. Extracts with 70% ethanol achieved higher antioxidant activity, whereas pure ethanol extracts exhibited the greatest anti-inflammatory activity. A further fractionation, using macroporous XAD-7HP resins and ethanol:water mixtures as eluents, was carried out to goal the isolation of phenolic compounds and study their implication in both bioactivities. From the collected fractions, the one obtained with 80% ethanol proved to be effective increasing the phenolic compounds content (more than 2-fold), accomplished an extract with higher antioxidant activity (3-fold superior), and a higher inhibition of TNF-α, IL-β and IL-6 cytokines than initial yarrow extract. These results highlight the role that phenolic compounds may play in antioxidant and anti-inflammatory activities, both because of their total content and for the increased presence of specific compounds.

1. INTRODUCTION

Achillea millefolium L., commonly known as yarrow, is a flowering plant native to the Northern hemisphere. It represents one of the most widely used plants from the Asteraceae family in folk medicine, due to its antimicrobial, choleretic, hepatoprotective, antiproliferative, antioxidant and anti-inflammatory activities. As several studies have been focus on the antioxidant properties of *A. millefolium* and its related species less attention has been paid to their anti-inflammatory activity (García-Risco et al., 2017; Mohammadhosseini et al., 2017; Hussein et al., 2019).

Inflammation represents a self defence mechanism in the human body. This response is mediated by the action of immune cells, such as natural killer and macrophages, upon activation of many types of mediators, including cytokines, chemokines and eicosanoids (Aziz et al., 2018). However, a prolonged inflammation state could lead to chronic inflammatory conditions, being related to many diseases, including rheumatoid arthritis, atherosclerosis, obesity and diabetes (Aziz et al; 2018). To control this anomaly, an anti-inflammatory response is required. In this regard, consumption of anti-inflammatory compounds derived from plants could reinforce the endogenous anti-inflammatory system. Yarrow essential oil, along with its organic and aqueous extracts has been shown to possess anti-inflammatory activities (Burk et al., 2010; Hussein et al., 2019). In particular, borneol and camphor have been proposed as potent anti-inflammatory essential oil constituents (Abdossi and Kazemi, 2016; Villalva et al., 2019), whereas dicaffeoylquinic acids and flavonoids, mainly luteolin and apigenin aglycones, were the main anti-inflammatory agents associated to yarrow extracts (Benedek et al., 2007; Tadić et al., 2017).

The extraction of bioactive compounds from yarrow has been generally performed by conventional techniques, *e.g.* maceration, hydrodistillation and steam distillation, which are time-consuming and require large volume of solvents (Chemat et al., 2017). In contrast, advance technologies, such as Ultrasound Assisted Extraction (UAE), Pressurised Liquid Extraction (PLE) or Supercritical Fluid Extraction (SFE), could be used as an efficient alternative to these classical extraction methodologies. These techniques have been proposed as being more efficient, allowing solvent use reduction, shorter-time processing as well as obtaining higher extraction yields (Pan et al., 2012; Cai et al., 2016). In addition, the use of green solvents, such as ethanol

or water, is completely essential for the sustainable production of bioactive food ingredients according to United Nations sustainable development goals 2030 (UN, 2019).

Moreover, after the extraction of bioactive compounds from a specific matrix, a fractionation or purification step is used to be needed in order to concentrate targeted compounds, eliminating the non-desirable ones. To this purpose, several approaches have been proposed, including membrane separation (Conidi et al., 2017), high pressure techniques (Fernández-Ponce et al., 2015), antisolvent-supercritical fluid precipitation (Villanueva-Bermejo et al., 2017) or adsorption resins (Soto et al., 2011). Membrane separation represents a low operating and maintenance cost, as well as an easy scale-up technology, applied for phenolic compounds separation (Zagklis et al., 2015). Nevertheless, this kind of methodology sometimes allowed only a partial separation since the membrane filtration is mainly related to a size exclusion process (Conidi y col., 2017). On the other hand, the application of high pressure and precipitation with antisolvent-supercritical fluids allow an efficient separation of compounds (Fernández-Ponce et al., 2015; Villanueva-Bermejo et al., 2017) however, the use of specialised equipment with a high cost of installation is implied.

The use of macroporous adsorption resins represents a sustainable option used for phenolic compounds fractionation (Pérez-Larrán et al., 2018; Kammerer et al., 2019). It represents a non-toxic, easy control, and scalable methodology. In addition, these resins can be easily regenerated without thermal procedures, and it can be used for several cycles, which results in a low-cost application that reduces waste generation. Adsorption fractionation comprehends the separation of targeted compounds through its polarity characteristics. To this purpose, different type of resins has been applied for the selective recovery of phenolic compounds from natural sources, such as XAD-16 for flavanones, flavanols and chlorogenic acid or SP-70 for polymethoxylated flavones (Pérez-Larrán et al., 2018; Soto et al., 2011), or XAD-7HP for enrichment of rosmarinic acid and flavones derivatives (Lin et al., 2012; Villalva y col., 2018).

In this context, the main objective of this study was the development of a sustainable procedure for obtaining antioxidant and anti-inflammatory enriched fractions from

yarrow. Green extraction technologies, such as UAE and PLE, were used, whereas a selective enrichment with XAD-7HP resins, as a sustainable fractionation process was carried out. In addition, a thorough analysis of the phenolic composition and the volatile fraction was performed in order to elucidate the compounds potentially related to the antioxidant and anti-inflammatory activities of yarrow.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%) were purchased from Sigma-Aldrich (Madrid, Spain). HPLC grade acetonitrile was obtained from Macron Fine Chemicals (Madrid, Spain) and formic acid (99%) from Acros Organics (Madrid, Spain). Authentic standards HPLC purity (≥ 95%) were purchased from Sigma-Aldrich, Phytolab (Madrid, Spain), Cymit Química SL (Madrid, Spain) and Extrasynthese S.A. (Genay, France) accordingly to the information provided as supplementary material (Table S1). Pure ethanol (99.5%) from Panreac (Barcelona, Spain) and ultrapure milliQ water (Merck Millipore, Madrid, Spain) were used in this study.

2.2. Sample preparation and extraction procedure

Yarrow plant, including inflorescences and upper dried leaves (water content <50.0 g/kg of leaves), was obtained from a local herbalist supplier (Plantafarm S.A., León, Spain). The sample was ground (Premill 250, Leal S.A., Granollers, Spain) and sieved to reduced its particle size (< 500 µm).

PLE was performed in an ASE 350 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a solvent controller unit. The ground yarrow (1.0 g) was mixed with 4.0 g of sea sand (Sigma Aldrich, Madrid, Spain) in a ceramic mortar, and placed into a 11 mL stainless-steel extraction cell. The cell was loaded into the oven, heated up to 100 ºC and filled with the corresponding ethanol:water solution (50:50, 70:30, 100:0, v/v). Static extraction were performed for 10 min at 1500 psi. The resulted extract was collected in a glass vial and the ethanol was evaporated at 35ºC in a vacuum rotary evaporator (IKA RV 10, VWR, Madrid, Spain) and subsequently freeze-

dried when required. Samples were maintained at -20ºC until use. All experiments were done by triplicate.

UAE was carried out using a high power (400 W, 60 kHz) ultrasonic probe system (1/2" diameter) at 50% amplitude for sonication output (Branson 450, Branson Ultrasonics, Danbury, CT, USA). Ground yarrow (40 g) was soaked with ethanol:water solution (50:50, 70:30, 100:0, v/v) in a ratio of 1:10 (yarrow/solvent) and submitted to extraction for 30 min with a cooling coating to control the temperature (40 ºC) and continuous stirring. After that process, the samples were filtered and were processed as aforementioned for PLE, to obtain a dry extract. All experiments were done by triplicate.

2.3. Fractionation of yarrow extract

Yarrow extract fractionation was performed on a XAD-7HP resin column (Sigma-Aldrich, St. Louis, MO, USA). Prior fractionation experiments, the resin pre-treatment and activation were accomplished following the method described by Villalva et al. (2018). The column was packed with a bed volume (BV) of 225 mL and washed with distilled water (4BV) at a constant flow (4BV/h). UAE yarrow extract was dissolved in ethanol:water (pH 3, 60:40 v/v) to obtain a concentration of 15 mg/mL. Following, 70 mL extract were placed onto the column, standing for 1 h to achieve the absorption equilibrium. First, the column was washed with 2BV of distilled water (discarded washing fraction), and then, phenolic compounds were isolated by using 3BV of different ethanol:water mixtures (v/v), *i.e.* 20% ethanol (F-20), 80% ethanol (F-80) and 100% ethanol (F-100) at a constant flow of 2BV/h. A final washing step was completed with acetone (2BV). The three eluted fractions (F-20, F-80 and F-100) were processed as above-mentioned for yarrow extracts in order to obtain a dry sample. The whole fractionation process was done by duplicate.

2.4. Cell culture and anti-inflammatory activity

Human THP-1 cells (ATCC, Manassas, VA, USA) were growth and maintained according to the methodology described by Villalva et al. (2018). To perform anti-inflammatory experiments, cells were seeded in 24-well plates ($5x10^5$ cells/mL) with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) and allowed to differentiate into macrophages (48h). Extracts cytotoxicity over macrophages was evaluated using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) according to

Mosmann (1983) method. Afterwards, the macrophages were washed with PBS and treated with serum-free medium containing a non-cytotoxic quantity of yarrow extracts, followed by the addition of 0.05 μg/mL LPS (*E. coli* O55:B5, Sigma-Aldrich, Spain). After 24h incubation, supernatants were collected and the release of TNF-α, IL-1β and IL-6 interleukins were measured at 450 nm with substrate correction at 570 nm (Infinite M200 multiscanner, Tecan, Barcelona, Spain) after the ELISA immunoassay (BD Biosciences, Aalst, Belgium). Results were expressed as mean of three determinations ± standard deviation.

2.5. Total phenolic content (TPC) and antioxidant activity determination

TPC was determined according to the Folin–Ciocalteu's colorimetric method proposed by Singleton et al. (1999). Results were expressed as gallic acid equivalents (GAE) (mg of gallic acid/ g dry extract). For antioxidant activity determination the $ABTS^{\bullet+}$ radical-scavenging assay was followed (Re et al., 1999). Trolox was used as reference standard, and results were expressed as TEAC values (mmol Trolox Equivalents (TE) / g dry extract). Triplicate measurements were carried out.

2.6. Analysis of phenolic compounds

Phenolic analysis was carried out in a HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with a Photodiode Array Detector (PAD). A reverse phase ACE Excel 3 Super C18 column (3 μm, 150 mm x 4.6 mm) (ACT, Aberdeen, Scotland) protected by a pre-column of the same material, was used to phenolic compounds separation, according to the chromatographic conditions described by Villalva et al. (2018). Assignation of phenolic compounds was accomplished by comparison of their retention time and UV-Vis max absorption (280, 320, 340 and 360 nm) using their respective authentic standard. Quantification was carried out using a calibration curve established with five dilutions of each pure standard. For those phenolics which authentic standard was not available, a calibration curve from other compound of the same phenolic group was used as reference as described at supplementary material information (Table S2). The results were expressed as mg / g dry extract. All analyses were done by triplicate.

2.7. GC-MS analysis

The GC-MS analysis was carried out in an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA) comprising a split/splitless injector, a FID detector, a 5975C triple-axis mass spectrometer detector and GC/MS Solution software. The analysis was performed as previously described by Villalva et al. (2019). The compounds identification was performed by contrasting of the obtained mass spectral fragmentation patterns with the Wiley 229 mass spectral library and comparing its corresponding retention index with that published in the literature.

2.8. Statistical analysis

Experimental results were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Fisher's least significant differences (LSD) test at $p < 0.05$ was used to discern differences between means. Statistical analyses were performed using Statgraphics v. Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1. Extraction yield, TPC and Antioxidant activity of yarrow extracts

In order to obtain phenolic compounds from yarrow, PLE and UAE techniques using three different solvent compositions, *i.e.* pure ethanol (E-100), and its aqueous mixtures (v/v) ethanol 70% (E-70) and ethanol 50% (E-50), were studied. It is known that methanolic:water solutions are used as efficient solvents for phenolic compounds extraction (Mustafa and Turner, 2011); however, in order to develop a sustainable extraction procedure, green solvents were proposed in the present study. Moderate temperatures were used in both PLE (100 ºC) and UAE (40 ºC) in order to avoid compounds degradations (García-Risco et al., 2017; Wianowska and Gil, 2019).

As shown in Table 1, the solvent composition significantly influenced the extraction yield, TPC and antioxidant activity of the yarrow extracts albeit in a different way. Higher extraction yields were fulfilled by increasing the percentage of water in the extraction solvent, regardless of the extraction technique used. It should be considered the higher water affinity displayed by other organic and inorganic compounds, apart from phenolics (*e.g.* carbohydrates or minerals), which might

contribute to the mass recovery from vegetal matrices (Machado et al., 2017). Concerning the total phenolic content, E-70 extract shown the highest TPC value, followed by E-100 and E-50 extracts, despite of the extraction technique. This behaviour was in agreement with previous reports in terms of the total amount of phenolics found in *A. millefolium* aerial parts' extracts (Benetis et al., 2008).

Table 1. Extraction yield, total phenolic content and antioxidant activity of PLE and UAE yarrow extracts.

		Extraction Yield [1]	TPC [2]	TEAC Value [3]
PLE				
	E-50	20.8 ± 0.1 [a]	103 ± 2 [c]	0.56 ± 0.03 [b]
	E-70	17.9 ± 0.2 [b]	130 ± 2 [a]	0.68 ± 0.01 [a]
	E-100	7.6 ± 0.1 [e]	113 ± 3 [b]	0.33 ± 0.01 [c]
UAE				
	E-50	13.6 ± 0.8 [c]	101 ± 3 [c]	0.55 ± 0.04 [b]
	E-70	11.4 ± 0.4 [d]	129 ± 2 [a]	0.66 ± 0.05 [a]
	E-100	5.7 ± 0.9 [f]	111 ± 2 [b]	0.31 ± 0.01 [c]

[1] Yield expressed as the percentage of mass of dried extract / mass of initial plant material.
[2] TPC=Total phenolic content, expressed as mg gallic acid equivalent (GAE) / g extract.
[3] TEAC value expressed in mmol Trolox equivalent / g extract.
[a,b,c] Different letters denote significant differences in the same column ($p < 0.05$).

With respect to the antioxidant activity, the highest TEAC value was also achieved at ethanol 70%; however, in this case, aqueous ethanol mixtures seemed to enhance the antioxidant activity of the extracts in comparison to those obtained with pure ethanol, since E-70 and E-50 extracts presented a significantly higher TEAC value than E-100. In the light of these results, it seems that the antioxidant activity of yarrow extracts could be partially attributable to the total phenolic content, but also to the presence of specific phenolic compounds with high and mid-polarity (Dias et al., 2013).

Regarding the extraction technique, PLE allowed to increase the extraction yield despite the solvent used. On the other hand, no statistical differences were found in

terms of TPC and antioxidant activity between both techniques (Table 1). These results could be attributed to the use of a higher temperature (100 ºC) along with high pressure conditions, inherent to the pressurised liquid extraction technique. It is known that higher temperatures in PLE decrease the solvent viscosity, which increases its ability to wet the matrix and to solubilize the target analytes. In addition, the introduction of a solvent under pressure to the extraction vessel will allow a maximum penetration into the pore improving the contact with the analyte, and as a result, the extraction efficiency would be enhanced (Wianowska and Gil, 2019).

3.2. Anti-inflammatory activity of PLE and UAE yarrow extracts

The anti-inflammatory activity of yarrow extracts was determined using THP-1 macrophages activated with LPS and incubated with 20 µg/mL of each extract, which resulted in a non-toxic concentration in previous studies (cell viability ≥ 95%). As can be seen in Fig. 1, the macrophages activated with LPS (positive control), exhibited an important release of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, after 24h incubation, compared to non-activated cells (negative control). In general, the same trend, despite of the extraction technique, was observed for all extracts in terms of the anti-inflammatory response. However, a clear influence of the solvent composition was shown. Therefore, the larger percentage of ethanol in the solvent was used, the better anti-inflammatory effect was achieved. The addition of 20 µg/mL of E-70 and E-100 yarrow extracts, from both PLE and UAE techniques, caused an important decrease of TNF-α secreted to the media compared to the positive control. However, it can be noticed that E-100 extracts exhibited the greatest inhibition of TNF-α secretion, showing at least a 70% of inhibition.

Interleukin-1β secretion (IL-1β) was effectively inhibited with the addition of 20 µg/mL of all extracts. In that regard, E-50 and E-70 samples from both extraction techniques allowed an approx. 30% reduction, whereas the most effective decrease, approx. 70%, was observed when E-100 extracts were added (Fig. 1). In the same way, the IL-6 release was highly supressed in the presence of all studied yarrow extracts, but a remarkable reduction, near to the basal levels, was achieved by the E-100 samples (Fig. 1).

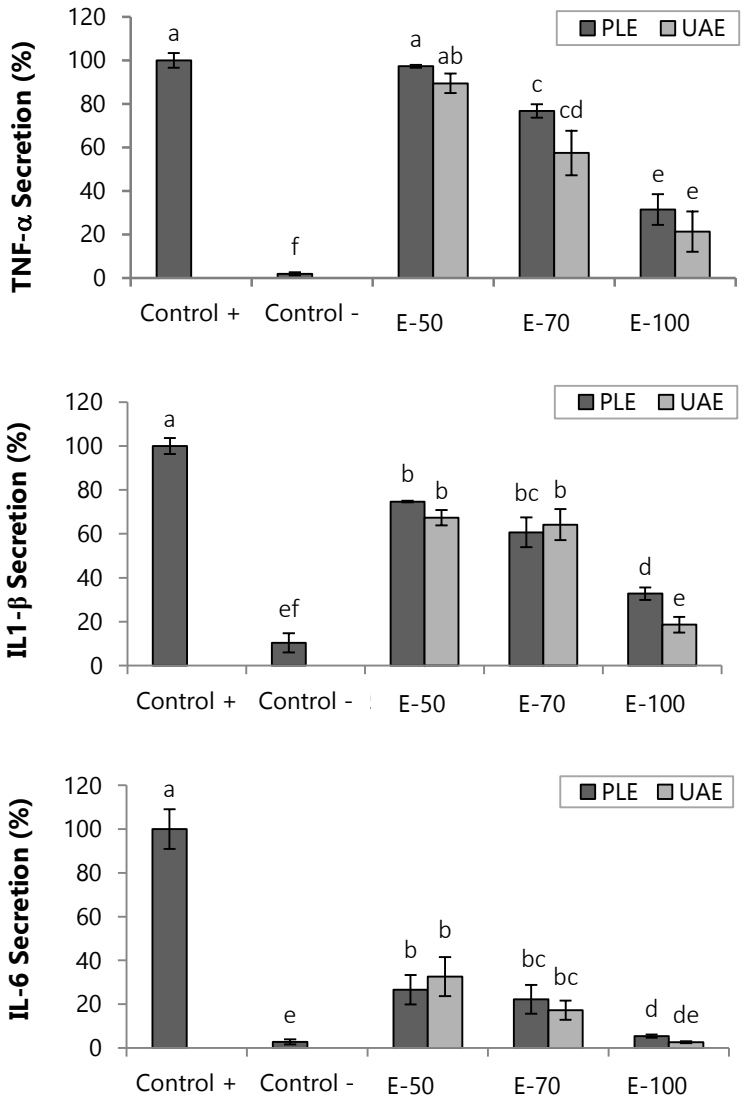

Figure 1: Levels of TNF-α, IL-1β and IL-6 secreted by THP-1 activated macrophages after 24 h incubation, in presence of 20 µg/mL of extract. E-50, extracts obtained with 50% ethanol; E-70, extracts obtained with 70% ethanol; E-100, extracts obtained with 100% ethanol. Positive control (Control +) represents the LPS activated cells without extract. Negative control (Control -) refers to the non-activated in absence of extract. Each bar is the mean of three determinations ± standard deviation. [a-f] Different letters denotes statistical differences at $p < 0.05$.

Regarding the foregoing TPC and TEAC results (Table 1), there was not a clear relationship between the anti-inflammatory activity of extracts and the total phenolic content, nor to their antioxidant activity. Therefore, these results suggest that both yarrow bioactivities could not be attributable, at least partially, to the same type of compounds. Hence, a detailed chemical analysis of the yarrow extracts' phenolic composition was conducted.

3.3. Chemical analyis of yarrow extracts

After HPLC-PAD chromatographic analysis, 49 phenolic compounds were identified as part of the UAE and PLE yarrow extracts, as can be observed in Table 2. The identified compounds were in agreement with previous HPLC-MS/MS analysis of yarrow extracts carried out in our research group (Villalva et al., 2020 *unpublished*).

It should be noticed that the phenolic composition of all yarrow extracts, despite of their extraction technique, was represented by a mixed group of flavonoids, mainly flavones, and hydroxycinnamic acids. Both apigenin and luteolin glycosylated derivatives were found in a greater or lesser extent, being the luteolin-7-*O*-glucoside the most representative flavonoid. On the other hand, for hydroxycinnamic acids, mainly caffeic acid and its quinic acid derivatives were found, highlighting the presence of the 3,5-dicaffeoylquinic acid (DCQA) as the most abundant phenolic acid. These results are in accordance with previous phenolic composition reported for *A. millefolium* (Vitalini et al., 2011; Dias et al., 2013).

Table 2. Phenolic compounds (mg/g extract) identified in yarrow extracts obtained by PLE and UAE.

	PLE			UAE		
Compounds	E-50	E-70	E-100	E-50	E-70	E-100
Neochlorogenic acid *	0.46 ± 0.01^{b}	0.53 ± 0.01^{a}	0.30 ± 0.01^{c}	0.46 ± 0.01^{b}	0.56 ± 0.01^{a}	0.30 ± 0.02^{c}
Protocatechuic acid *	0.14 ± 0.02^{b}	0.16 ± 0.01^{b}	0.12 ± 0.01^{bc}	0.25 ± 0.02^{a}	0.24 ± 0.01^{a}	0.15 ± 0.01^{b}
Caftaric acid isomer	0.23 ± 0.02^{b}	0.27 ± 0.02^{b}	0.15 ± 0.01^{c}	0.16 ± 0.01^{c}	0.32 ± 0.01^{a}	0.08 ± 0.02^{d}
Caftaric acid *	0.20 ± 0.02^{c}	0.27 ±0.01^{b}	0.15 ± 0.01^{d}	0.18 ± 0.02^{c}	0.33 ± 0.02^{a}	0.07 ± 0.03^{e}
Caffeoylquinic acid isomer I	0.27 ± 0.02^{bc}	0.31 ± 0.01^{b}	0.25 ± 0.01^{c}	0.25 ± 0.01^{c}	0.36 ± 0.02^{a}	0.19 ± 0.01^{d}
Chlorogenic acid*	8.77 ± 0.12^{c}	10.8 ± 0.2^{b}	7.03 ± 0.11^{f}	8.31 ± 0.28^{d}	11.4 ± 0.2^{a}	7.33 ± 0.09^{e}
Cryptochlorogenic acid *	0.21 ± 0.02^{b}	0.24 ± 0.01^{b}	0.15 ± 0.01^{c}	0.36 ± 0.01^{a}	0.37 ± 0.02^{a}	0.12 ± 0.02^{c}
Vicenin 2 *	4.75 ± 0.19^{c}	6.60 ± 0.16^{b}	3.93 ± 0.11^{e}	4.35 ± 0.12^{d}	7.08 ± 0.19^{a}	2.81 ± 0.07^{f}
Caffeoylquinic acid isomer II	0.51 ± 0.02^{c}	0.54 ± 0.01^{bc}	0.15 ± 0.01^{d}	0.57 ± 0.03^{ab}	0.62 ± 0.02^{a}	0.08 ± 0.01^{e}
Apigenin-hexoside-pentoside I	0.71 ± 0.03^{d}	1.07 ± 0.05^{b}	0.84 ± 0.03^{c}	0.65 ± 0.03^{e}	1.26 ± 0.06^{a}	0.54 ± 0.02^{f}
Caffeic acid *	0.49 ± 0.02^{b}	0.56 ± 0.01^{a}	0.47 ± 0.02^{b}	0.41 ± 0.02^{c}	0.55 ± 0.01^{a}	0.39 ± 0.02^{c}
Schaftoside isomer	3.20 ± 0.07^{d}	4.57 ± 0.09^{b}	3.47 ± 0.09^{c}	3.11 ± 0.13^{d}	4.86 ± 0.12^{a}	2.02 ± 0.06^{e}
Schaftoside *	3.83 ± 0.10^{b}	4.97 ± 0.09^{a}	3.45 ± 0.11^{c}	3.00 ± 0.15^{d}	5.12 ± 0.12^{a}	2.62 ± 0.05^{e}
Homoorientin *	0.56 ± 0.02^{e}	0.75 ± 0.02^{d}	1.49 ± 0.04^{b}	0.74 ± 0.09^{d}	0.92 ± 0.03^{c}	2.76 ± 0.09^{a}
Apigenin-hexoside-pentoside II	2.27 ± 0.09^{c}	3.44 ± 0.11^{a}	2.68 ± 0.10^{b}	2.12 ± 0.09^{c}	3.66 ± 0.12^{a}	1.36 ± 0.04^{d}
Luteolin dihexoside I	2.04 ± 0.08^{d}	3.37 ± 0.08^{b}	3.09 ± 0.10^{c}	1.85 ± 0.11^{d}	3.61 ± 0.11^{a}	3.53 ± 0.09^{ab}
6-hydroxyluteolin-7-*O*-glucoside	4.38 ± 0.20^{b}	5.80 ± 0.21^{a}	3.90 ± 0.17^{c}	4.13 ± 0.12^{bc}	5.94 ± 0.21^{a}	2.77 ± 0.09^{d}
Apigenin dihexoside	0.26 ± 0.03^{bc}	0.24 ± 0.01^{c}	0.28 ± 0.01^{b}	0.26 ± 0.02^{bc}	0.35 ± 0.01^{a}	0.23 ± 0.01^{b}
Quercetin hexoside	0.27 ± 0.01^{e}	0.48 ± 0.02^{c}	0.56 ± 0.03^{b}	0.63 ± 0.09^{b}	0.39 ± 0.02^{d}	1.71 ± 0.13^{a}
Luteolin dihexoside II	0.49 ± 0.04^{b}	0.50 ± 0.03^{b}	0.57 ± 0.03^{a}	0.30 ± 0.03^{c}	0.53 ± 0.04^{ab}	0.25 ± 0.02^{c}
Rutin *	1.44 ± 0.07^{b}	2.10 ± 0.08^{a}	2.22 ± 0.10^{a}	1.01 ± 0.07^{c}	2.28 ± 0.20^{a}	1.42 ± 0.09^{b}
Isovitexin	0.43 ± 0.02^{b}	0.44 ± 0.02^{b}	0.50 ± 0.05^{a}	0.47 ± 0.03^{ab}	0.41 ± 0.03^{b}	0.58 ± 0.08^{a}
Vitexin *	0.49 ± 0.06^{b}	0.71 ± 0.06^{a}	0.74 ± 0.05^{a}	0.44 ± 0.04^{b}	0.79 ± 0.07^{a}	0.83 ± 0.09^{a}
Apigenin deoxylhexoside	0.32 ± 0.02^{cd}	0.37 ± 0.03^{bc}	0.55 ± 0.06^{a}	0.29 ± 0.01^{d}	0.40 ± 0.02^{b}	0.42 ± 0.02^{b}
Apigenin glycosylated derivative	4.20 ± 0.19^{b}	5.02 ± 0.12^{a}	3.67 ± 0.14^{c}	4.12 ± 0.19^{b}	5.24 ± 0.20^{a}	3.10 ± 0.15^{d}
Luteolin-7-*O*-glucoside *	5.93 ± 0.19^{c}	7.91 ± 0.21^{b}	8.68 ± 0.28^{a}	5.13 ± 0.20^{d}	8.03 ± 0.21^{b}	8.26 ± 0.17^{ab}

[*] Identified and quantified via comparison with its authentic standard. <LOQ, below limit of quantification. [a-f] Different letters denote statistical differences at $p < 0.05$. PLE, pressurised liquid extraction. UAE, ultrasound liquid extraction.

Table 2. *(Continued).*

Compounds	PLE			UAE		
	E-50	E-70	E-100	E-50	E-70	E-100
Luteolin-7-*O*-glucuronide *	0.37 ± 0.03^{cd}	0.41 ± 0.02^{c}	0.31 ± 0.04^{d}	0.69 ± 0.05^{b}	0.38 ± 0.03^{cd}	0.83 ± 0.06^{a}
Quercetin hexuronide	0.60 ± 0.02^{c}	0.72 ± 0.03^{b}	0.79 ± 0.04^{b}	0.45 ± 0.03^{d}	0.90 ± 0.07^{a}	0.22 ± 0.01^{e}
3,4-Dicaffeoylquinic acid *	1.53 ± 0.05^{c}	1.94 ± 0.07^{a}	1.69 ± 0.06^{b}	1.42 ± 0.04^{d}	2.09 ± 0.11^{a}	1.68 ± 0.08^{b}
Isorhamnetin hexoside I	0.06 ± 0.01^{e}	0.10 ± 0.02^{d}	0.18 ± 0.03^{c}	0.42 ± 0.07^{b}	0.12 ± 0.03^{cd}	1.45 ± 0.11^{a}
1,5-Dicaffeoylquinic acid *	2.95 ± 0.10^{b}	3.22 ± 0.12^{ab}	2.20 ± 0.09^{d}	2.66 ± 0.10^{c}	3.52 ± 0.18^{a}	2.17 ± 0.16^{d}
3,5-Dicaffeoylquinic acid *	15.5 ± 0.6^{d}	18.8 ± 0.6^{c}	22.9 ± 0.9^{b}	15.9 ± 0.6^{d}	19.4 ± 0.6^{c}	25.4 ± 0.9^{a}
Apigenin-7-*O*-glucoside *	1.47 ± 0.13^{d}	2.10 ± 0.16^{c}	2.99 ± 0.24^{a}	1.16 ± 0.11^{e}	2.42 ± 0.13^{b}	2.21 ± 0.15^{bc}
Luteolin-*O*-malonylglucoside	0.56 ± 0.02^{b}	0.58 ± 0.03^{b}	0.20 ± 0.01^{d}	0.31 ± 0.02^{c}	0.95 ± 0.08^{a}	0.82 ± 0.07^{a}
4,5-Dicaffeoylquinic acid *	6.02 ± 0.27^{b}	6.87 ± 0.23^{a}	5.41 ± 0.12^{c}	5.92 ± 0.32^{b}	7.02 ± 0.39^{a}	5.15 ± 0.21^{c}
Isorhamnetin hexoside II	0.31 ± 0.03^{c}	0.34 ± 0.03^{c}	0.21 ± 0.02^{d}	0.26 ± 0.03^{d}	0.42 ± 0.04^{b}	0.66 ± 0.05^{a}
Dicaffeoylquinic acid isomer	0.26 ± 0.04^{a}	0.29 ± 0.06^{a}	0.26 ± 0.02^{a}	0.21 ± 0.02^{a}	0.25 ± 0.02^{a}	0.11 ± 0.02^{b}
Feruloyl-*O*-caffeoylquinic acid	0.17 ± 0.03^{a}	0.20 ± 0.03^{a}	0.14 ± 0.02^{b}	0.13 ± 0.01^{b}	0.22 ± 0.02^{a}	0.17 ± 0.03^{a}
Tricaffeoylquinic acid	0.24 ± 0.02^{c}	0.29 ± 0.03^{bc}	0.34 ± 0.03^{b}	0.25 ± 0.03^{c}	0.32 ± 0.03^{b}	0.46 ± 0.06^{a}
Luteolin *	1.45 ± 0.06^{d}	1.66 ± 0.07^{c}	2.01 ± 0.09^{a}	1.72 ± 0.12^{bc}	1.92 ± 0.10^{ab}	1.99 ± 0.09^{a}
Quercetin *	0.29 ± 0.02^{c}	0.34 ± 0.04^{bc}	0.66 ± 0.06^{a}	0.37 ± 0.04^{b}	0.34 ± 0.02^{bc}	0.70 ± 0.07^{a}
Methoxyquercetin	0.19 ± 0.01^{c}	0.21 ± 0.02^{c}	0.23 ± 0.03^{c}	0.60 ± 0.07^{b}	0.20 ± 0.02^{c}	0.82 ± 0.08^{a}
Apigenin *	0.34 ± 0.02^{d}	0.40 ± 0.02^{c}	0.69 ± 0.05^{a}	0.41 ± 0.05^{c}	0.51 ± 0.03^{b}	0.60 ± 0.04^{a}
Diosmetin *	0.25 ± 0.03^{c}	0.29 ± 0.03^{c}	0.57 ± 0.06^{a}	0.32 ± 0.04^{c}	0.40 ± 0.04^{b}	0.49 ± 0.03^{a}
Trihydroxy dimethoxyflavone	<LOQ	0.05 ± 0.01^{b}	0.14 ± 0.09^{a}	<LOQ	0.06 ± 0.01^{b}	0.23 ± 0.10^{a}
Centaureidin	0.28 ± 0.02^{c}	0.27 ± 0.01^{c}	1.27 ± 0.11^{b}	0.28 ± 0.01^{c}	0.26 ± 0.02^{c}	2.22 ± 0.19^{a}
Methoxyacacetin	0.17 ± 0.01^{c}	0.20 ± 0.03^{bc}	0.19 ± 0.01^{c}	0.07 ± 0.01^{d}	0.28 ± 0.02^{a}	0.25 ± 0.02^{ab}
Dihydroxy trimetoxyflavone	0.23 ± 0.02^{c}	0.36 ± 0.03^{b}	0.38 ± 0.02^{b}	0.12 ± 0.02^{d}	0.40 ± 0.02^{ab}	0.45 ± 0.04^{a}
Casticin *	0.16 ± 0.03^{d}	0.16 ± 0.01^{d}	2.12 ± 0.09^{b}	0.33 ± 0.03^{c}	0.15 ± 0.01^{d}	3.03 ± 0.10^{a}
Σ Phenolic compounds	80.3 ± 1.0^{d}	101.8 ± 1.6^{b}	95.3 ± 1.2^{c}	77.5 ± 1.1^{d}	108.2 ± 1.7^{a}	96.0 ± 1.4^{c}

* Identified and quantified via comparison with its authentic standard. <LOQ, below limit of quantification. $^{a-f}$ Different letters denote statistical differences at $p < 0.05$. PLE, pressurised liquid extraction. UAE, ultrasound liquid extraction.

Despite of the similar profile overall, the content of individual phenolic compounds was influenced by the solvent type used in the extraction methodology. In that regard, as a bigger presence of ethanol was used in the aqueous solvent mixture, a larger quantity of phenolic compounds was observed in the extracts. Thus, E-70 extracts allowed a higher recovery than E-50 ones, in particular for those polar or intermediate polar phenolic compounds (*i.e.* chlorogenic acid (CGA), vicenin 2, luteolin-7-*O*-glucoside, 3,5-DCQA and 4,5-DCQA). Regarding the E-100 extracts, polar compounds amount was decreased, but the presence of intermediate and less polar compounds was enhanced, *e.g.* 3,5-DCQA and luteolin-7-*O*-glucoside, as well as some aglycones (*e.g.* luteolin and apigenin) and methoxylated flavonoids, such as casticin and centaureidin.

Considering these results, it could be confirmed the contribution of the sum of individual phenolic compounds to the antioxidant activity of yarrow, but surely the presence of certain polar compounds, in a greater or lesser extent, might contribute to this activity. In particular, the greatest abundance of CGA, known to possess a high antioxidant activity (Liang and Kitts, 2016), was evident in E-70 extracts, as well as the great quantity of most polar glycosylated derivatives of luteolin and apigenin (Park and Song; 2013; Wang et al., 2018). With respect to the remarkable anti-inflammatory activity exhibited by the E-100 extracts, their slightly highest content of 3,5-DCQA and luteolin-7-*O*-glucoside might be related (Francisco et al., 2014; Hong et al., 2015), but also the contribution of those flavonoids' aglycones found significantly in representative amounts, *e.g.* luteolin, centaureidin and casticin, cannot be ruled out (Jachak et al., 2011; Liou et al., 2014). However, it is well known that the presence of volatile compounds in yarrow extracts may exert anti-inflammatory activity as well (Villalva et al., 2019); therefore, the study of the volatile fraction of the extracts was required.

The GC-MS analysis was only performed on the UAE yarrow extract, due to the similar composition found despite of the extraction technique. As was expected, just a barely quantity of two monoterpenes, borneol and yomogi alcohol, was detected in the E-70 extract, whereas non-detectable volatile compounds were found in the E-50 (data not shown). Opposite to that, E-100 extract showed the larger presence of volatile compounds, thanks to their higher affinity for the solvent (ethanol) used during sonication process. In that regard, 34 compounds were identified in the E-

100 extract (Table 5), being borneol the most abundant (17.9%), followed by caryophyllene oxide (9.7%) and camphor (9.6%). Also, viridiflorol and β-eudesmol were found in important amounts in this extract. The identified compounds were in accordance with previous studies of the volatile fraction from inflorescences and aerial parts of *A. millefolium* (Fierascu et al., 2015; Villanueva et al., 2017). Several studies have been pointed out the anti-inflammatory activity of borneol and camphor, the main volatile components of E-100 extract (Arranz et al., 2014; Abdossi and Kazemi, 2016). Therefore, these results suggest that the anti-inflammatory properties of yarrow could be influenced by this type of terpenes found in this extract.

3.4 UAE-ethanolic yarrow extract fractionation

The foregoing results demonstrated that either PLE or UAE techniques could be sustainable in order to obtain a yarrow extract with an important antioxidant and anti-inflammatory activities, that is it, both techniques allowed the extraction of bioactive compounds in shorter extraction times with the use of GRAS solvents. PLE allowed higher extraction yields than UAE; nevertheless, the latter has been recognized for being a more feasible and scalable technology, which has resulted in its widespread use for several food industry and pharmaceutical applications (Chemat et al., 2017). Moreover, E-100 from both extraction techniques, showed similar TEAC values, although a slightly higher anti-inflammatory properties of E-100 UAE were found. Therefore, E-100 extract obtained by UAE, was selected in order to carried out the following assays.

As it was aforementioned, phenolic compounds have been linked to the antioxidant activity of *A. millefolium* extracts. However, the contribution of total or particular phenolic compounds to this antioxidant properties, or even more, to anti-inflammatory activity remains unclear. To that purpose, a phenolic compounds separation was done. E-100 UAE extract was submitted to phenolic fractionation using XAD-7HP macroporous adsorption resins, packed in a glass column. This type of resin was chosen as it has been efficiently used for obtaining fractions enriched in certain phenolic compounds, *e.g.* rosmarinic acid from *O. majorana* extracts, improving its bioactivity (Lin et al., 2012; Villalva et al., 2018).

In the present study, different aqueous ethanolic mixtures were used as mobile phase throughout the fractionation process. After a washed procedure, an increasing aqueous ethanolic mixture was used as eluent (20%, 80% and 100% ethanol), allowing to achieve three different fractions, namely F-20, F-80 and F-100, respectively.

The recovery yield, TPC and antioxidant activity of the three collected fractions are shown in Table 3. The whole fractionation process allowed a total yield of 97 ± 2%, which included the discarded washing step with water (nearly 20%) as well as the final washing step with pure acetone (approx. 7%). However, as can be observed, the highest percentage of mass was recovered with the use of 80% ethanol, followed by the fraction with pure ethanol, whereas F-20 showed a lower recovery yield. With respect to the TPC, a 2-fold increment was achieved in F-80 fraction, compared to the initial yarrow extract (E-100, Table 1); in contrast, F-20 shown a slightly decrement, whilst F-100 resulted in a scarce TPC value. In accordance to TPC results, the TEAC value was remarkably improved in the F-80 fraction (3 times higher than initial yarrow extract) and F-20 (1.6-fold superior), whereas F-100 showed a limited antioxidant activity, being even lower than the initial yarrow extract.

Table 3. Fractionation recovery, total phenolic content and antioxidant activity in recovered F-20, F-80 and F-100 fractions from E-100 by using XAD-7HP amberlite.

Yarrow Fractions	Recovery Yield [1]	TPC [2]	TEAC value [3]
F-20	7 ± 1	101 ± 2 [b]	0.49 ± 0.01 [b]
F-80	38 ± 2	224 ± 1 [a]	1.12 ± 0.05 [a]
F-100	25 ± 2	39 ± 2 [c]	0.17 ± 0.01 [c]

[1] Recovery yield calculated as the percentage (%) of dried mass (in grams) collected in each fraction respect to the initial amount of loaded extract (in grams).
[2] TPC=Total phenolic content, expressed as mg gallic acid equivalent (GAE) / g of dried extract.
[3] TEAC value expressed in mmol Trolox equivalent / g of dried extract.
[a-c] Different letters denote significant differences within the same column ($p < 0.05$).

Regarding the anti-inflammatory activity, 10 and 20 µg/mL of the yarrow fractions, as well as the initial yarrow extract used for fractionation, were tested *in vitro*. The mentioned concentrations were indicated as non-cytotoxic doses evaluated in THP-1 macrophages (data not shown). After 24 h of incubation with LPS and all yarrow samples, the activated cells showed a significant reduction of studied cytokines (TNF-α, IL-1β and IL-6) compared to the positive control (Fig. 2). A more evident reduction on TNF-α secreted level was observed when 20 µg/mL was added. Thus, initial yarrow extract (E-100) reduced TNF-α secretion approx. 80%. Furthermore, F-100 fraction presented a decreased in TNF-α superior than F-20, but similar to that observed with the yarrow extract. Nevertheless, the maximum reduction of TNF-α secretion was mediated by F-80 fraction, allowing a nearly 85% inhibition.

Interleukin-1β was also inhibited in the presence of yarrow extract and its fractions, at both evaluated concentrations, however 20 µg/mL exhibited a better effect in the activated cells (Fig. 2). Whereas the yarrow extract showed an inhibition near to 75%, the F-80 fraction shown the greatest inhibition, closely to 85%, on IL-1β secretion level. Both F-20 and F-100 inhibition shown an important suppression on this cytokine liberated to medium, but in a lesser extent than yarrow extract. In the same way, IL-6 secretion was efficiently inhibited by both studied concentrations of all tested samples. Moreover, F-80 was the most active fraction, allowing a decrease comparable to the basal levels for IL-6 secretion.

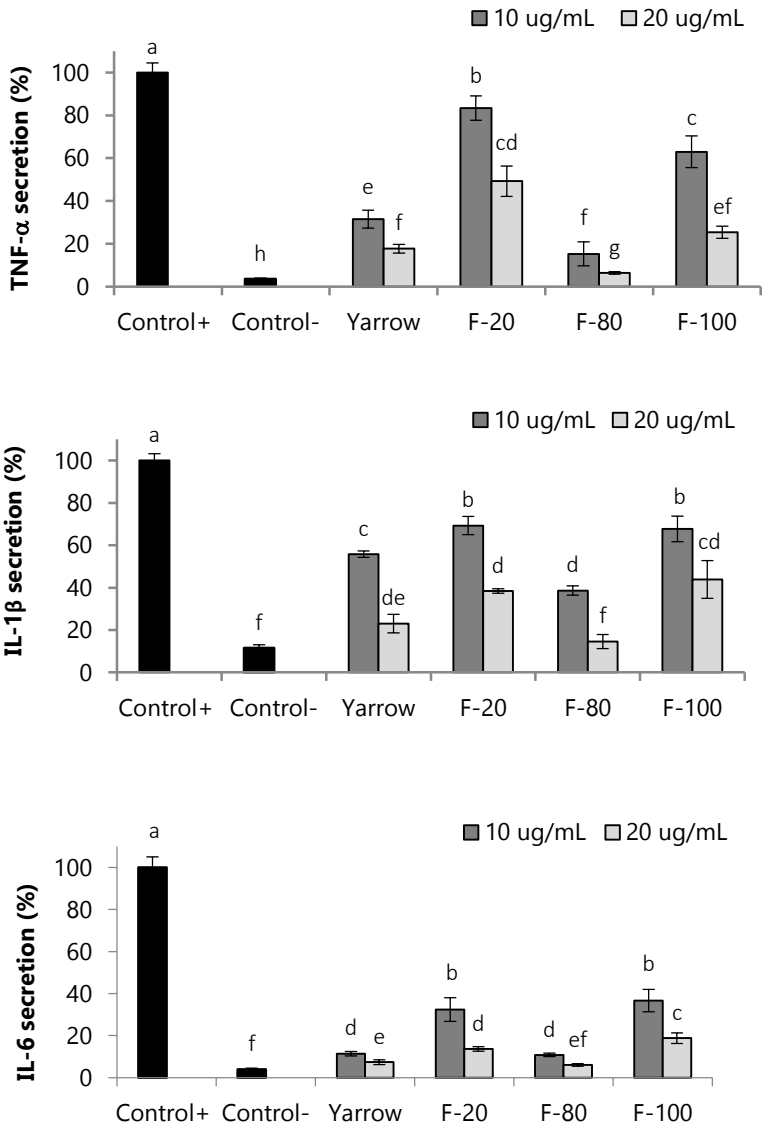

Figure 2: Levels of TNF-α, IL-1β and IL-6 secreted by THP-1 activated macrophages with LPS in presence of different yarrow fractions at 10 and 20 µg/mL (24 h of incubation). Positive control (Control +) represents the LPS activated cells without extract. Negative control (Control -) refers to the non-activated in absence of the extracts. Each bar is the mean of three determinations ± standard deviation. * Denotes statistical differences between positive control and the other samples at $p < 0.05$.

In order to elucidate the potentially related compounds to the antioxidant and anti-inflammatory activity of yarrow fractions, subsequent analyses of phenolic and volatile compounds were performed. The phenolic composition, of the initial yarrow extract and its fractions are shown in Table 4. As was expected, a selective separation and enrichment of phenolic compounds was exhibited after the fractionation process in accordance to their affinity for the solvent elution (Lin et al., 2012). The most polar compounds were collected in F-20 fraction, whereas flavonoids aglycones, the less polar compounds, were dragged in the F-100 one. Nevertheless, the greatest quantity of phenolics, either polar or less polar compounds, were preferable eluted at the F-80 fraction (more than 2-fold) (Table 4). In general, a notorious increment was observed for individual phenolics presented in F-80, around 2-3 times higher than the initial yarrow extract. In this fraction, 3,5-DCQA was the most abundant compound, followed by luteolin-7-*O*-glucoside and 4,5-DCQA, representing the 52% of all identified phenolics. Additionally, an important increment was observed on casticin, centaureidin and luteolin content. Besides, chlorogenic acid was the most predominant compound quantified in F-20 fraction, representing a 60% of the total phenolic compounds found in this sample. Regarding F-100, only a few compounds were detected, however the flavonoids aglycones resulted significantly higher than those in the initial extract, mostly casticin, centaureidin and luteolin.

Thus, the higher antioxidant and anti-inflammatory activities in F-80, could be related to the significant increment on phenolic compounds overall, compared to the initial extract, but also in particular to the most abundant compounds in this fraction, *i.e.* DCQAs (Hong et al., 2015) and luteolin-7-*O*-glucoside (Francisco et al., 2014). However, it should also notice the significant enrichment in those less polar flavonoids, mainly in its aglycone form, *e.g.* luteolin, centaureidin and casticin, which have been pointed to possess anti-inflammatory activities too (Guerra et al., 2006; Liou et al., 2014; Wang et al., 2017).

Table 4. Phenolic compounds (mg/g extract) identified in the E-100 yarrow extract and its fractions.

Compounds	Yarrow E-100	F – 20	F – 80	F – 100
Neochlorogenic acid*	0.30 ± 0.02 ^{b}	4.30 ± 0.95 ^{a}	0.15 ± 0.01 ^{c}	--
Protocatechuic acid*	0.15 ± 0.01 ^{b}	2.44 ± 0.06 ^{a}	0.13 ± 0.02 ^{b}	--
Caftaric acid isomer	0.08 ± 0.02 ^{c}	1.14 ± 0.09 ^{a}	0.15 ± 0.02 ^{b}	--
Caftaric acid*	0.07 ± 0.03 ^{c}	1.85 ± 0.04 ^{a}	0.19 ± 0.02 ^{b}	--
Caffeoylquinic acid isomer I	0.19 ± 0.01 ^{c}	0.89 ± 0.05 ^{a}	0.46 ± 0.04 ^{b}	--
Chlorogenic acid*	7.33 ± 0.09 ^{b}	37.9 ± 1.04 ^{a}	7.60 ± 0.45 ^{b}	--
Cryptochlorogenic acid*	0.12 ± 0.02 ^{b}	2.20 ± 0.01 ^{a}	0.12 ± 0.01 ^{b}	--
Vicenin II*	2.81 ± 0.07 ^{b}	3.39 ± 0.12 ^{a}	3.20 ± 0.16 ^{a}	--
Caffeoylquinic acid isomer II	0.08 ± 0.01 ^{c}	1.15 ± 0.01 ^{a}	0.22 ± 0.02 ^{b}	--
Apigenin-hexoside-pentoside I	0.54 ± 0.02 ^{b}	0.41 ± 0.01 ^{c}	0.76 ± 0.04 ^{a}	--
Caffeic acid*	0.39 ± 0.02 ^{b}	0.16 ± 0.02 ^{c}	0.90 ± 0.04 ^{a}	--
Schaftoside isomer	2.02 ± 0.06 ^{b}	1.28 ± 0.02 ^{c}	2.33 ± 0.14 ^{a}	--
Schaftoside*	2.62 ± 0.05 ^{b}	2.04 ± 0.01 ^{c}	3.74 ± 0.14 ^{a}	--
Homoorientin*	2.76 ± 0.09 ^{b}	0.32 ± 0.01 ^{c}	6.31 ± 0.31 ^{a}	--
Apigenin-hexoside-pentoside II	1.36 ± 0.04 ^{b}	0.78 ± 0.02 ^{c}	1.90 ± 0.12 ^{a}	--
Luteolin dihexoside I	3.53 ± 0.09 ^{b}	1.12 ± 0.01 ^{c}	7.68 ± 0.39 ^{a}	--
6-hydroxyluteolin-7-*O*-glucoside	2.77 ± 0.09 ^{b}	0.20 ± 0.02 ^{c}	6.46 ± 0.34 ^{a}	--
Apigenin dihexoside	0.23 ± 0.01 ^{b}	--	0.44 ± 0.25 ^{a}	--
Quercetin hexoside	1.71 ± 0.13 ^{b}	--	4.10 ± 0.25 ^{a}	--
Luteolin dihexoside II	0.25 ± 0.02 ^{b}	--	0.63 ± 0.03 ^{a}	--
Rutin*	1.42 ± 0.09 ^{b}	0.12 ± 0.01 ^{c}	2.86 ± 0.15 ^{a}	--
Isovitexin	0.58 ± 0.08 ^{b}	--	1.75 ± 0.10 ^{a}	--
Vitexin*	0.83 ± 0.09 ^{b}	--	2.51 ± 0.10 ^{a}	--
Apigenin deoxylhexoside	0.42 ± 0.02 ^{b}	--	0.85 ± 0.04 ^{a}	--
Apigenin glycosylated derivative	3.10 ± 0.15 ^{b}	0.34 ± 0.02 ^{c}	6.44 ± 0.91 ^{a}	--
Luteolin-7-*O*-glucoside*	8.26 ± 0.17 ^{b}	0.13 ± 0.02 ^{c}	24.2 ± 0.3 ^{a}	--

* Identified and quantified *via* comparison with its authentic standard. . $^{a-d}$ Different letters denote statistical differences at $p < 0.05$.

Table 4. *(Continued).*

Compounds	Yarrow E-100	F – 20	F – 80	F – 100
Luteolin-7-O-glucuronide*	0.83 ± 0.06 [b]	0.19 ± 0.03 [c]	1.57 ± 0.08 [a]	--
Quercetin hexuronide	0.22 ± 0.01 [b]	--	0.95 ± 0.06 [a]	--
3,4-dicaffeoylquinic acid*	1.68 ± 0.08 [b]	0.37 ± 0.03 [c]	3.78 ± 0.18 [a]	0.18 ± 0.01 [d]
Isorhamnetin hexoside I	1.45 ± 0.11[b]	--	3.36 ± 0.30 [a]	--
1,5-dicaffeoylquinic acid*	2.17 ± 0.16 [b]	0.23 ± 0.02 [c]	4.29 ± 0.47 [a]	0.27 ± 0.02 [c]
3,5-dicaffeoylquinic acid*	25.4 ± 0.9 [b]	0.86 ± 0.02 [c]	72.4 ± 0.9 [a]	0.79 ± 0.03 [d]
Apigenin-7-*O*-glucoside*	2.21 ± 0.15 [b]	0.09 ± 0.01 [c]	7.30 ± 0.37 [a]	0.08 ± 0.01 [c]
Luteolin-*O*-malonylglucoside	0.82 ± 0.07 [b]	--	1.08 ± 0.04 [a]	--
4,5-dicaffeoylquinic acid*	5.15 ± 0.21 [b]	0.21 ± 0.01 [c]	13.3 ± 0.8 [a]	0.19 ± 0.02 [c]
Isorhamnetin-O-hexoside	0.66 ± 0.05 [b]	--	1.57 ± 0.17 [a]	--
Dicaffeoylquinic acid isomer	0.11 ± 0.02 [b]	--	0.26 ± 0.03 [a]	--
Feruloyl-O-caffeoylquinic acid	0.17 ± 0.03 [b]	--	0.29 ± 0.07 [a]	--
Tricaffeoylquinic acid	0.46 ± 0.06 [b]	--	0.86 ± 0.05 [a]	0.40 ± 0.02 [b]
Luteolin*	1.99 ± 0.09 [b]	--	3.33 ± 0.15 [a]	3.46 ± 0.10 [a]
Quercetin*	0.70 ± 0.07 [c]	--	0.89 ± 0.04 [b]	1.51 ± 0.10 [a]
Methoxyquercetin	0.82 ± 0.08 [b]	--	0.83 ± 0.02 [b]	1.18 ± 0.03 [a]
Apigenin*	0.60 ± 0.04 [b]	--	0.39 ± 0.02 [c]	1.89 ± 0.9 [a]
Diosmetin*	0.49 ± 0.03 [b]	--	0.24 ± 0.01 [c]	0.82 ± 0.01 [a]
Trihydroxy dimethoxyflavone	0.23 ± 0.10 [b]	--	0.35 ± 0.02 [a]	--
Centaureidin	2.22 ± 0.19 [c]	--	3.34 ± 0.14 [b]	3.95 ± 0.11 [a]
Methoxyacacetin	0.25 ± 0.02 [b]	--	0.23 ± 0.01 [b]	0.79 ± 0.01 [a]
Dihydroxy-trimetoxyflavone	0.45 ± 0.04 [b]	--	0.34 ± 0.02 [c]	1.13 ± 0.02 [a]
Casticin*	3.03 ± 0.10 [c]	--	4.08 ± 0.17 [b]	6.41 ± 0.14 [a]
Σ Phenolic compounds	96.0 ± 1.4 [b]	64.1 ± 0.2 [c]	211.1 ± 1.1 [a]	23.1 ± 0.1 [d]

* Identified and quantified *via* comparison with its authentic standard. [a-d] Different letters denote statistical differences at $p < 0.05$.

On the other hand, the great richness of CGA found in F-20 suggests a strong contribution on its antioxidant and anti-inflammatory properties (Hwang et al., 2014; Liang and Kitts, 2016). Moreover, considering that the total content of phenolics was scarce in F-100, its antioxidant activity was limited either; however, the presence of luteolin and methoxylated flavonoids, such as casticin and centaureidin could be also related to the anti-inflammatory properties observed in this fraction.

As aforementioned, the presence of volatile compounds was related with the yarrow's anti-inflammatory activity, in addition to the influence that could exert some phenolic compounds. Therefore, an analysis of the volatile compounds in the three recovered fractions was carried out. The results of GC-MS analyses of F-20, F-80 and F-100 fractions are presented in Table 5. Regarding the F-20 fraction, only a few compounds were detected compared to the yarrow E-100, however, piperitone and eugenol were the most representative compounds, being even superior than in E-100.

Similarly, in F-100 a smaller abundance of volatile compounds was detected; however, chamazulene was outstanding as the principal compound in this fraction, being 3-fold superior than its respective amount found in E-100. Furthermore, in F-80 fraction, 21 of 34 compounds originally found in the yarrow E-100 were characterised, showing important amounts of sesquiterpenes, such as saussurea lactone, corymbolone, chamazulene and β-eudesmol. In that context, the presence in a greater abundance of certain compounds with recognized anti-inflammatory properties, such as chamazulene, the most abundant terpene found in F-100 and F-80, might be related, at least partially, with the observable anti-inflammatory activity in those fractions (Zargaran et al., 2014). In addition, β-eudesmol, presented in F-80, and eugenol found in F-20, have been also pointed out as anti-inflammatory compounds (Seo et al., 2011; Huang et al., 2015).

Table 5. Peak area contribution (%) of identified volatile compounds by GC-MS after fractionation process.

RI	*Compounds*	Yarrow	XAD-7HP fractions		
		E-100	F-20	F-80	F-100
997	Yomogi alcohol	2.1	--	0.3	0.5
1021	Cymene	0.3	0.2	0.8	0.7
1028	Eucalyptol	0.2	--	--	--
1037	y-Vinyl-y-valerolactone	0.2	--	0.7	--
1049	γ-Terpinene	0.3	--	0.3	1.7
1058	Artemisia ketone	2.8	--	--	--
1079	Artemesia alcohol	2.8	--	0.3	--
1097	β- Linalool	0.3	--	--	--
1101	Thujone	0.9	--	--	--
1138	Camphor	9.6	--	--	0.3
1140	cis-Verbenol	2.6	--	0.4	1.0
1160	Borneol	17.9	--	3.1	6.9
1164	Lavandulol	1.6	--	0.9	0.8
1170	Menthol	0.4	--	--	--
1174	Terpinene-4-ol	0.6	--	--	--
1179	α-Terpineol	1.7	--	--	--
1203	Verbenone	0.9	14.7	1.4	--
1225	cis-Carveol	0.3	--	--	--
1236	trans-Chrysanthenyl acetate	0.3	--	--	--
1250	Piperitone	0.3	42.5	--	--
1261	(5E)-5,9-Dimethyl-5,8-decadien-2-one	2.3	7.9	4.8	2.0
1276	2,6-Dimethyl-1,7-octadiene-3,6-diol	1.1	--	2.3	-
1353	Eugenol	0.6	34.7	1.4	--
1478	α-Curcumene	0.9	--	0.5	5.6
1548	Elemol	1.8	--	--	--
1569	Spathulenol	3.5	--	4.9	--
1578	Caryophyllene oxide	9.7	--	3.6	--
1589	Viridiflorol	8.5	--	3.6	--
1640	β-Eudesmol	6.2	--	11.7	2.3
1658	α-Bisabolol oxide B	4.3	--	8.7	--
1710	Chamazulene	2.6	--	12.8	44.8
1810	Saussurea lactone	5.0	--	16.8	--
1845	Hexahydrofarnesyl acetone	3.9	--	--	30.5
1890	Corymbolone	3.7	--	17.9	--
∑ AUC		**2.89×10^6**	**8.77×10^5**	**1.29×10^6**	**6.23×10^5**

RI= retention index. AUC= area under curve.

4. CONCLUSIONS

The present study proposes the use of UAE, together with the subsequent employ of XAD-7HP amberlite resin, using pure ethanol and ethanol:water (80%) as the most efficient solvent and eluent, respectively, for obtaining extracts enriched in phenolic compounds with antioxidant and anti-inflammatory activity from *A. millefolium*. This represents a sustainable and easily scalable technique procedure for the use of yarrow as a source of functional phenolic compounds.

In addition, the results showed in this paper highlight the possible role played by phenolic compounds, both as whole and individually, on the antioxidant and anti-inflammatory properties of yarrow extracts, along with the presence of volatile terpenes.

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ABBREVIATIONS
PLE, pressurised liquid extraction; UAE, ultrasound assisted extraction; TPC, total phenolic content; BV, bed volume; LPS, bacterial lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-1β, interleukine 1β; IL-6, interleukine 6; GAE, gallic acid equivalents; TEAC, trolox equivalent antioxidant capacity; LSD, least significant difference; E-100, extracts obtained with 100% ethanol; E-70, extracts obtained with 70% ethanol; E-50, extracts obtained with 50% ethanol; DCQA, diccafeoylquinic acid; CGA, chlorogenic acid; F-20, fraction collected with 20% ethanol; F-80, fraction collected with 80% ethanol; F-100, fraction collected with 100% ethanol.

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Supplementary material

Table S1. Authentic commercial standards (HPLC purity ≥95%)

Supplier	Authentic standard
Cymit Química SL, Madrid, Spain	Isorhamnetin-3-*O*-glucoside
Extrasynthese S.A., Genay, France	Apigenin-7-*O*-glucoside Caffeic acid Homoorientin Isoquercitrin Homoorientin Isoquercitrin Luteolin-7-*O*-*b*-glucoside Luteolin-7-*O*-glucuronide Quercetin Rutin
Sigma-Aldrich, Madrid, Spain	Chlorogenic acid Diosmetin Vitexina

Supplier	Authentic standard
Phytolab, Madrid, Spain	1,5- Dicaffeoylquinic acid 3,4- Dicaffeoylquinic acid 3,5- Dicaffeoylquinic acid 4,5- Dicaffeoylquinic acid Apigenin Caftaric acid Casticin Cryptochlorogenic acid Neochlorogenic acid Orientin Protocatechuic acid Luteolin Schaftoside Vicenin II

Table S2. Phenolic compounds references used for quantification of commercial standard unavailable.

Identified Phenolic Compound	Commercial standard
Caftaric acid isomer	Caftaric acid
Caffeoylquinic acid isomer	Chlorogenic acid
Caffeoylquinic acid isomer II	Chlorogenic acid
Apigenin-hexoside-pentoside I	Schaftoside
Schaftoside isomer II	Schaftoside
Apigenin-hexoside-pentoside II	Schaftoside
Luteolin dihexoside I	Orientin
6-hydroxyluteolin-7-*O*-glucoside	Luteolin-7-*O*-*b*-glucoside
Apigenin dihexoside	Schaftoside
Quercetin hexoside	Quercetin-3-*O*-glucoside
Luteolin dihexoside II	Orientin
Isovitexin	Vitexin
Apigenin deoxylhexoside	Apigenin-7-*O*-glucoside
Apigenin glycosylated derivative	Apigenin-7-*O*-glucoside
Quercetin hexuronide	Quercetin-3-*O*-glucoside
Isorhamnetin hexoside I	Isorhamnetin-3-*O*-glucoside
Luteolin-*O*-malonylglucoside	Luteolin-7-*O*-*b*-glucoside
Isorhamnetin hexoside II	Isorhamnetin-3-*O*-glucoside
Dicaffeoylquinic acid isomer	4,5-dicaffeoylquinic acid
Feruloyl-*O*-caffeoylquinic acid	Chlorogenic acid
Tricaffeoylquinic acid	Chlorogenic acid
Methoxyquercetin	Quercetin
Trihydroxy dimethoxyflavone	Luteolin
Centaureidin	Casticin
Methoxyacacetin	Apigenin
Dihydroxy-trimetoxyflavone	Luteolin

Publicación 5

Bioavailability assessment of yarrow phenolic compounds using an *in vitro* digestion/Caco-2 cell model: anti-inflammatory activity of the basolateral fraction.

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ABSTRACT

In this study, a combined *in vitro* digestion/Caco-2 model was performed with the aim to determine the phenolic compounds bioavailability of two yarrow extracts. HPLC-PAD characterisation indicated that the main components in both extracts were 3,5- dicaffeoylquinic acid (DCQA) and luteolin-7-*O*-glucoside. Analyses after the simulated digestion process revealed that phenolic composition was not affected during the oral phase, whereas gastric and intestinal phases represented critical steps for some individual phenolics, especially intestinal step. The transition from gastric medium to intestinal environment caused an important degradation of 3,5-DCQA (63-67% loss), whereas 3,4- and 4,5-DCQAs increased significantly, suggesting an isomeric transformation within these caffeic acid derivatives. However, an approx. 90% of luteolin-7-*O*-glucoside was recovered after intestinal step. At the end of Caco-2 absorption experiments, casticin, diosmetin and centaureidin represented the most abundant compounds in the basolateral fraction. Moreover, this fraction presented anti-inflammatory activity since was able to inhibit the secretion of IL-1β and IL-6 pro-inflammatory cytokines. Thus, the presence in the basolateral fraction of flavonoid-aglycones from yarrow, could be related with the observed anti-inflammatory activity.

1. INTRODUCTION

Achillea millefolium L. (yarrow) is a flowering plant traditionally used in the treatment of digestive and hepatobiliary disorders, inflammation and diabetes (Akram, 2013). Recent reports indicated that *Achillea* genus presented important biological activities, such as antioxidant, anti-inflammatory and antitumor activities (Mohammadhosseini et al., 2017). Most health benefits of aqueous and alcoholic yarrow extracts have been associated with its composition in phenolic compounds, mainly phenolic acids and flavones (Dias et al., 2013). Thus, Trumbeckaite et al. (2011) related the antioxidant properties of an *Achillea millefolium* hydroalcoholic extract with the presence of luteolin and chlorogenic acid in the extract, and in a lesser extent with rutin and luteolin-7-*O*-glucoside. Pereira et al., (2018) also reported that an *A. millefolium* hydroethanolic extract, containing 3,5-*O*-dicaffeoylquinic acid, 5-*O*-caffeoylquinic acid, luteolin-*O*-acetylhexoside and apigenin-*O*-acetylhexoside as main phenolic compounds, inhibited the growth of human tumour cell lines. Furthermore, both essential oils and hydroethanolic yarrow extracts have demonstrated anti-inflammatory properties, causing the inhibition of NO (nitric oxide) production and IL-8 secretion *in vitro* (Zaidi et al., 2012; Abdossi and Kazemi, 2016).

Nevertheless, after oral consumption, phenolic compounds must be bioavailable in order to perform their potential health benefits. The bioavailability is dependent upon the stability of the compound during gastrointestinal digestion, its release from the food-matrix and the efficiency of its intestinal absorption. In this context, the stability of phenolic compounds during the gastrointestinal digestion is strongly influenced by their chemical structure, since phenolics present a different sensitivity to pH variations and digestive enzymes activity (Goulas and Hadjisolomou, 2019; Lima et al., 2019). Moreover, Siracusa et al. (2011) indicated that stability of phenolic compounds under gastrointestinal conditions highly depends on the nature of the matrix in which these compounds are included. Thereby, Lingua et al., (2019) reported that phenolic acids and quercetin were the most resistant polyphenols in white grape after a simulated digestion. However, Ortega-Vidal et al., (2019) indicated that caffeoylquinic acids in herbal infusions were highly reduced by gastrointestinal digestion (approx. 10% remain). Moreover, Spínola et al., (2018) carried out extracts of *Rumex maderensis* and reported that the degradation of

different phenolic classes after digestion varied within morphological parts employed (leaves, flowers and stems). Thus, flavanols were the most stable compounds, although in flowers presented a reduction of 29.7% against 40.4% in stems. Hydroxycinnamic acids from leaves and flowers, presented a similar degradation rate (approx. 56.5%), meanwhile in stems extracts hydroxycinnamic acids were very unstable (71.8% reduction).

After gastrointestinal digestion, the intestinal absorption of phenolic compounds has also been reported to be highly influenced by phenolic compounds chemical structure. Bowles et al. (2017) studied the intestinal transport across Caco-2 monolayer of nine phenolic acids found in an aqueous extract of *Athrixia phylicoides,* concluding that *p*-coumaric acid presented the highest transport. Besides, Wu et al. (2017) reported that the absorption of caffeic acid was higher than chlorogenic acid in the Caco-2 model as well as in the rat jejunum. Therefore, the use of an *in vitro* digestion/Caco-2 cell culture model has been proposed by several authors as an economical and useful alternative to *in vivo* analysis, in order to investigate the bioavailability of phenolic compounds (Soler-Rivas et al., 2010; Lingua et al., 2019).

Concerning phenolic compounds extraction, several studies proposed the ultrasound-assisted extraction (UAE) as an adequate technique to obtain phenolic compounds from vegetal matrices (Goltz et al., 2018; Irakli et al., 2018). In this regard, UAE has been reported to reduce extraction time and solvent consumption, as well as to maximizing the recoveries of bioactive compounds (Corbin et al., 2015). However, sometimes it is difficult to obtain highly concentrated extracts using only UAE, due to complexity of vegetable raw materials. Therefore, the use of adsorption resins (XAD-2, XAD-7, XAD-16 and Oasis HLB) has been successfully employed as a tool for selective enrichment of phenolic compounds from plant material (Sandhu and Gu, 2013; Villalva et al., 2018).

The aim of this work was to study the bioavailability of yarrow phenolic compounds, by using a combined *in vitro* digestion/Caco-2 cell model. In addition, the influence of phenolics compounds concentration in the matrix on their bioavailability was also determined. Besides, the biological activity of Caco-2 basolateral fraction, in terms of anti-inflammatory activity was measured.

2. MATERIALS AND METHODS

2.1 Yarrow extract (YE) and yarrow phenolic compounds-enriched extract (EE)

Upper-dried inflorescences of yarrow were obtained from a local supplier (Plantafarm S.A., León, Spain). The sample was ground (Premill 250, Leal S.A., Granollers, Spain) and sieved to diminish its particle size (< 500µm). YE and EE were obtained as previously described by Villalva et al. (2020, *unpublished*). Briefly, to obtain YE, the ground yarrow was soaked with pure ethanol (plant/solvent 1:10, w/v) and conducted to extraction (30 min, < 40ºC) in a Branson 450 ultrasonic device (Branson Ultrasonics, Danbury, CT, USA). In order to obtain the yarrow enriched-extract (EE), a fractionation process was conducted using XAD-7HP macroporous resins and ethanol:water (80:20, v/v) as elution solvent. This EE extract was corresponded to the F-80 fraction obtained in the above mentioned manuscript. Both YE and EE dried extracts were dissolved in ethanol or ethanol:water (50:50, v/v), filtered (PVDF, 0.45 µm) and analysed by HPLC-PAD.

2.2 HPLC-PAD phenolic compounds analysis

Phenolic compounds analysis was performed using an HPLC 1260 Infinity series system with a photodiode-array detector (PAD) (Agilent Technologies Inc., Santa Clara, CA, USA) according to Villalva et al. (2020, *unpublished*). Chromatographic separation was carried out with a reverse phase ACE Excel SuperC18 column (ACT, Aberdeen, Scotland), equipped with a guard-column of the same material. Peaks were identified according to their retention time and UV-Vis maximum absorption using authentic standards (HPLC purity ≥ 95%). Quantification was performed according to the calibration curve established of each pure standard.

2.3 Determination of total phenolic content (TPC) and antioxidant activity

Total phenolic content was determined by Folin-Ciocalteau reagent as described by Singleton et al. (1999). The results were expressed as mg of gallic acid equivalents (GAE)/g extract. DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, Madrid, Spain) free radical methodology was used to evaluate the antioxidant activity according to Brand-Williams et al. (1995). The results were expressed as TEAC value (mmol Trolox/g of extract or L of digested extract). All analyses were done by triplicate.

2.4 *In vitro* gastrointestinal digestion

YE and EE were subjected to a three steps digestion process. Briefly, 5 mL of extract solution (20 mg/mL) with 0.1 mL α- amylase from human saliva (9.3 mg in Cl_2Ca 1 mM) (Type XIII-A, Sigma-Aldrich, St. Louis, MO, USA) were stirred for 2 min in a titrator Titrino Plus 877 thermostated at 37ºC (Methrom AG, Herisau, Switzerland) (oral phase). Then, 25 mL of a gastric solution (pH 2.0 ± 0.5) containing 127 mg of pepsin from porcine gastric mucosa (536 U/mg, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 1 h (gastric phase). After gastric digestion, pH was adjusted to 7.5 ± 0.5 by addition of 10 mL intestinal solution composed by 5.3% (v/v) of NaOH 0.1M, 1.4% (v/v) of NaCl 3.25 M, 0.5% of $CaCl_2$ 325 mM and 2.8% (v/v) of a pancreatic-bile extract solution (9.3 mg pancreatin (4 x USP) and 115.7 mg bile salts in 10 mM trizma-maleate buffer), allowing stirring for 2h to simulate intestinal phase. When digestion finished, the solutions were immediately cooled and filtrated (0.45 µm, PVDF) to conduct the HPLC-PAD analysis, TPC and antioxidant activity assays. Additionally, digestion steps, without yarrow sample addition, were also carried out as control digestion.

2.5 Caco-2 cell culture and transport experiments

Maintenance conditions for Caco-2 cell line (ATCC, Manassas, VA, USA), as well as the cell viability experiments, were followed as previously described by Villalva et al. (2018). To assess the transport assays, Caco-2 cells (density 3 x 10^5 cells/insert) were seeded in polyester Transwell® inserts (24 mm diameter, 0.4 µm pore size, Corning Life Science) and cultured for 21 days at 37 ºC (5% CO_2). The day of the transport experiments, the inserts were carefully washed with Phosphate Buffer Solution (PBS) and filled with 1.5 mL (apical) and 2.6 mL (basolateral) of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Basel, Switzerland) without phenol red, and a specific volume of digested yarrow extract was added in the apical compartment (extract's final dilution 1:25, v/v). At the end of 2, 4 and 6h of incubation apical and basolateral supernatants were collected, freeze-dried and stored (-20 ºC) until analysis. Cell monolayer integrity was measured before and after the transport assays using an EVOM2 epithelial volt-ohm meter (World Precision Instruments, Hitchin, UK) and only inserts with transepithelial electric resistance (TEER) values > 700 Ωcm^2 were used. In addition, lucifer yellow (Sigma-Aldrich, Madrid, Spain) permeation was determined to validate the integrity of cell barrier, according to Uchida et al., (2009).

To performed the HPLC-PAD analysis, lyophilized samples from apical and basolateral sides, were conducted to extraction with 60:40 ethanol:water (v/v) (150 µL and 175 µL, respectively) followed by centrifugation (15,000 RPM, 5 min). The supernatants were filtered (0.45 µm, PVDF) before HPLC analysis.

Cell monolayers were washed with cold PBS, followed by 500 µL pure ethanol addition. After incubation (4ºC, 30 min), cells were scraped off the membrane, sonicated (5 min) and centrifuged (4,500 RPM, 15 min) to recover the supernatant. This process was repeated three times, and finally all supernatants were evaporated until dryness with pure NO_2. The final residue was re-dissolved in 60:40 ethanol:water (v/v) (100 µL) and filtered prior HPLC injection.

The apparent permeability coefficient (P_{app}, cm s^{-1}) of each compound detected in the basolateral supernatant was determined according to D'Antuono et al. (2015) with the following equation:

$$P_{app} = \frac{(dC/dt)\,V}{C_o A}$$

Where *dC/dt* is the apparent rate of polyphenols transported to the basolateral compartment over the time (µg L^{-1} s^{-1}), *V* is the volume of the basolateral compartment (cm^3); C_o is the initial concentration in the apical compartment (µg L^{-1}) and *A* is the surface area of the membrane (cm^2).

2.6 Anti-inflammatory assays of basolateral samples from Caco-2 experiments

Differentiated macrophages from the human monocyte THP-1 cell line (ATCC, Manassas, VA, USA) was used to conduct anti-inflammatory assays according to Arranz et al. (2019). THP-1 cells were seeded in 24 well-plate (5×10^5 cells/mL) and differentiated with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) maintained for 48h (37 °C, 5% CO_2).

The cytotoxic effect of the basolateral supernatants from Caco-2 over THP-1 macrophages, was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). Afterwards, the macrophages were washed and filled with RPMI (serum-free) medium along with a non-toxic concentration of basolateral supernatants and 0.05 μg/mL LPS (*E. coli* O55:B5, Sigma-Aldrich, Madrid, Spain). After 24h incubation, the medium was collected and the release of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, was measured by an enzyme-linked immunosorbent assay (ELISA) (BD Biosciences, Aalst, Belgium) according to the manufacturer protocol. Cells with LPS but without basolateral sample, represented the positive control of the immunomodulatory assay; negative control was the non-stimulated cells in absence of basolateral sample. Results were expressed as mean of three determinations ± standard deviation.

2.7 Statistical analysis

Experimental results are expressed as means ± standard deviation (SD). Variance one-way analysis (ANOVA) followed by Fisher's least significant differences (LSD) test were used to distinguish differences between means at $p < 0.05$. Statgraphics Centurion XVI software (Statpoint Technologies Inc., Warrenton, VA, USA) was used for that purpose.

3. RESULTS AND DISCUSSION

3.1 Influence of *in vitro* gastrointestinal digestion steps on phenolic composition and antioxidant activity of the extracts

The quantitative analysis of phenolic composition of YE and EE extracts before and after the three-steps digestion process (oral, gastric and intestinal) were shown in Table 1 and 2 respectively.

Table 1. Phenolic composition (mg/g extract) of yarrow extract (YE) before and after oral, gastric and intestinal digestion steps.

Compound	Undigested YE	Oral	Gastric	Intestinal
Neochlorogenic acid	0.24 ± 0.11 ^{b}	0.21 ± 0.06 ^{b}	0.29 ± 0.09 ^{b}	0.56 ± 0.07 ^{a}
Protocatechuic acid	0.13 ± 0.10 ^{b}	0.12 ± 0.07 ^{b}	0.13 ± 0.08 ^{b}	0.47 ± 0.12 ^{a}
Caftaric acid isomer	0.08 ± 0.03 ^{a}	0.08 ± 0.04 ^{a}	0.06 ± 0.03 ^{ab}	0.04 ± 0.03 ^{b}
Caftaric acid	0.07 ± 0.03 ^{a}	0.07 ± 0.04 ^{a}	0.18 ± 0.09 ^{a}	0.13 ± 0.07
Caffeoylquinic acid isomer I	0.39 ± 0.09 ^{a}	0.39 ± 0.08 ^{a}	0.24 ± 0.08 ^{ab}	0.22 ± 0.06 ^{b}
Chlorogenic acid	5.67 ± 0.25 ^{a}	5.02 ± 0.21 ^{ab}	5.90 ± 0.30 ^{a}	4.75 ± 0.20 ^{b}
Cryptochlorogenic acid	0.13 ± 0.05 ^{b}	0.10 ± 0.03 ^{b}	0.17 ± 0.04 ^{b}	0.75 ± 0.12 ^{a}
Vicenin 2	2.11 ± 0.10 ^{bc}	2.02 ± 0.10 ^{c}	2.45 ± 0.15 ^{a}	2.24 ± 0.10 ^{ab}
Caffeoylquinic acid isomer II	0.10 ± 0.03 ^{a}	0.12 ± 0.04 ^{a}	0.10 ± 0.02 ^{a}	0.10 ± 0.03 ^{a}
Apigenin-hexoside-pentoside I	0.46 ± 0.06 ^{a}	0.48 ± 0.05 ^{a}	0.49 ± 0.06 ^{a}	0.43 ± 0.06 ^{a}
Caffeic acid	0.34 ± 0.04 ^{a}	0.36 ± 0.06 ^{a}	0.40 ± 0.05 ^{a}	0.42 ± 0.06 ^{a}
Schaftoside isomer	1.34 ± 0.10 ^{a}	1.32 ± 0.09 ^{a}	1.43 ± 0.10 ^{a}	1.43 ± 0.12 ^{a}
Schaftoside	1.77 ± 0.18 ^{ab}	1.61 ± 0.15 ^{b}	2.14 ± 0.19 ^{a}	2.01 ± 0.16 ^{a}
Homoorientin	2.10 ± 0.19 ^{a}	1.94 ± 0.12 ^{a}	2.20 ± 0.15 ^{a}	1.89 ± 0.12 ^{a}
Apigenin-hexoside-pentoside II	1.04 ± 0.11 ^{a}	0.97 ± 0.09 ^{a}	1.04 ± 0.10 ^{a}	0.98 ± 0.08 ^{a}
Luteolin dihexoside I	2.60 ± 0.18 ^{ab}	2.32 ± 0.12 ^{b}	2.77 ± 0.11 ^{a}	2.52 ± 0.11 ^{b}
6-hydroxyluteolin-7-*O*-glucoside	2.03 ± 0.12 ^{b}	1.97 ± 0.08 ^{b}	2.34 ± 0.12 ^{a}	1.74 ± 0.09 ^{c}
Apigenin dihexoside	0.15 ± 0.09 ^{a}	0.16 ± 0.06 ^{a}	0.21 ± 0.07 ^{a}	0.16 ± 0.04 ^{a}
Quercetin hexoside	1.33 ± 0.13 ^{a}	1.31 ± 0.08 ^{a}	1.10 ± 0.10 ^{a}	0.25 ± 0.07 ^{b}
Luteolin dihexoside II	0.23 ± 0.04 ^{a}	0.23 ± 0.06 ^{a}	0.27 ± 0.07 ^{a}	0.24 ± 0.04 ^{a}
Rutin	1.06 ± 0.07 ^{a}	1.08 ± 0.09 ^{a}	1.16 ± 0.07 ^{a}	1.02 ± 0.09 ^{a}
Isovitexin	0.50 ± 0.04 ^{a}	0.47 ± 0.07 ^{a}	0.57 ± 0.06 ^{a}	0.52 ± 0.06 ^{a}
Vitexin	0.67 ± 0.07 ^{a}	0.61 ± 0.09 ^{a}	0.72 ± 0.08 ^{a}	0.64 ± 0.07 ^{a}
Apigenin deoxylhexoside	0.40 ± 0.05 ^{a}	0.42 ± 0.04 ^{a}	0.25 ± 0.04 ^{b}	0.22 ± 0.03 ^{b}
Apigenin glycosylated derivative	2.52 ± 0.12 ^{b}	2.49 ± 0.09 ^{b}	2.54 ± 0.10 ^{b}	2.72 ± 0.11 ^{a}

^{a,b,c} Different letter denote statistical differences within a line according to Fisher's least significant difference (LSD) procedure ($p < 0.05$).

Table 1. *(Continued).*

Compound	Undigested YE	Oral	Gastric	Intestinal
Luteolin-7-*O*-*β*-glucoside	8.29 ± 0.28 [a]	8.12 ± 0.32 [a]	6.70 ± 0.25 [c]	7.24 ± 0.33 [b]
Luteolin-7-*O*-glucuronide	0.72 ± 0.09 [a]	0.69 ± 0.08 [ab]	0.57 ± 0.05 [b]	0.69 ± 0.07 [ab]
Quercetin hexuronide	0.15 ± 0.03 [b]	0.12 ± 0.05 [b]	0.25 ± 0.03 [a]	0.20 ± 0.04 [ab]
3,4-Dicaffeoylquinic acid	1.49 ± 0.10 [b]	1.37 ± 0.08 [b]	1.42 ± 0.08 [b]	6.26 ± 0.27 [a]
Isorhamnetin hexoside I	1.59 ± 0.12 [a]	1.49 ± 0.09 [a]	1.00 ± 0.07 [b]	1.00 ± 0.06 [b]
1,5-Dicaffeoylquinic acid	1.65 ± 0.11 [a]	1.66 ± 0.10 [a]	1.49 ± 0.07 [ab]	1.37 ± 0.08 [b]
3,5-Dicaffeoylquinic acid	23.8 ± 1.81 [a]	22.9 ± 1.13 [a]	18.8 ± 0.90 [b]	8.77 ± 0.11 [c]
Apigenin-7-*O*-glucoside	2.27 ± 0.10 [a]	2.15 ± 0.07 [ab]	2.01 ± 0.08 [b]	1.81 ± 0.09 [c]
Luteolin-*O*-malonylglucoside	0.53 ± 0.04 [a]	0.52 ± 0.03 [a]	0.50 ± 0.04 [ab]	0.44 ± 0.03 [b]
4,5-Dicaffeoylquinic acid	4.25 ± 0.20 [b]	4.05 ± 0.18 [b]	3.61 ± 0.12 [c]	11.5 ± 0.51 [a]
Isorhamnetin hexoside II	0.62 ± 0.06 [b]	0.60 ± 0.04 [b]	0.50 ± 0.04 [c]	1.35 ± 0.10 [a]
Dicaffeoylquinic acid isomer	0.06 ± 0.01 [b]	0.05 ± 0.02 [b]	0.06 ± 0.02 [b]	0.10 ± 0.01 [a]
Feruloylcaffeoylquinic acid	0.14 ± 0.03 [a]	0.12 ± 0.02 [a]	0.07 ± 0.02 [b]	0.11 ± 0.03 [ab]
Tricaffeoylquinic acid	0.36 ± 0.06 [a]	0.31 ± 0.04 [ab]	0.09 ± 0.01 [c]	0.25 ± 0.04 [b]
Luteolin	1.90 ± 0.10 [a]	1.94 ± 0.11 [a]	0.95 ± 0.08 [c]	1.32 ± 0.10 [b]
Quercetin	0.63 ± 0.05 [a]	0.60 ± 0.07 [a]	0.29 ± 0.06 [b]	0.16 ± 0.04 [c]
Methoxyquercetin	0.36 ± 0.03 [a]	0.34 ± 0.04 [ab]	0.26 ± 0.04 [b]	0.32 ± 0.04 [ab]
Apigenin	0.56 ± 0.05 [a]	0.58 ± 0.04 [a]	0.18 ± 0.02 [c]	0.38 ± 0.05 [b]
Diosmetin	0.40 ± 0.05 [a]	0.38 ± 0.04 [a]	0.22 ± 0.03 [c]	0.29 ± 0.04 [b]
Trihydroxy dimethoxyflavone	0.27 ± 0.02 [a]	0.29 ± 0.02 [a]	0.13 ± 0.01 [c]	0.20 ± 0.02 [b]
Centaureidin	2.02 ± 0.12 [a]	2.07 ± 0.09 [a]	1.22 ± 0.05 [c]	1.76 ± 0.08 [b]
Methoxyacacetin	0.25 ± 0.03 [a]	0.26 ± 0.02 [a]	0.09 ± 0.02 [c]	0.16 ± 0.02 [b]
Dihydroxy trimetoxyflavone	0.44 ± 0.05 [a]	0.46 ± 0.06 [a]	0.17 ± 0.03 [c]	0.31 ± 0.05 [b]
Casticin	2.93 ± 0.10 [a]	2.92 ± 0.11 [a]	1.45 ± 0.09 [c]	2.31 ± 0.10 [b]

[a,b,c] Different letter denote statistical differences within a line according to Fisher's least significant difference (LSD) procedure ($p < 0.05$).

Table 2. Phenolic compounds (mg/g extract) of yarrow enriched-extract (EE) before and after oral, gastric and intestinal digestion steps.

Compound	Undigested-EE	Oral	Gastric	Intestinal
Neochlorogenic acid	0.15 ± 0.03 ^{c}	0.15 ± 0.02 ^{c}	0.22 ± 0.04 ^{b}	0.86 ± 0.06 ^{a}
Protocatechuic acid	0.13 ± 0.02 ^{b}	0.13 ± 0.03 ^{b}	0.14 ± 0.03 ^{b}	0.74 ± 0.07 ^{a}
Caftaric acid isomer	0.15 ± 0.02 ^{a}	0.13 ± 0.02 ^{a}	0.05 ± 0.01 ^{b}	0.05 ± 0.02 ^{b}
Caftaric acid	0.19 ± 0.06 ^{b}	0.18 ± 0.05 ^{b}	0.30 ± 0.08 ^{a}	0.30 ± 0.06 ^{a}
Caffeoylquinic acid isomer I	0.46 ± 0.07 ^{a}	0.42 ± 0.06 ^{a}	0.48 ± 0.07 ^{a}	0.39 ± 0.06 ^{a}
Chlorogenic acid	7.60 ± 0.35 ^{a}	7.46 ± 0.21 ^{a}	7.49 ± 0.01 ^{a}	6.28 ± 0.25 ^{b}
Cryptochlorogenic acid	0.12 ± 0.01 ^{b}	0.14 ± 0.02 ^{b}	0.15 ± 0.02 ^{b}	1.11 ± 0.07 ^{a}
Vicenin 2	3.20 ± 0.12 ^{b}	3.22 ± 0.10 ^{b}	3.37 ± 0.10 ^{a}	3.49 ± 0.11 ^{a}
Caffeoylquinic acid isomer II	0.22 ± 0.02 ^{a}	0.24 ± 0.02 ^{a}	0.27 ± 0.03 ^{a}	0.28 ± 0.02 ^{a}
Apigenin-hexoside-pentoside I	0.76 ± 0.04 ^{b}	0.77 ± 0.05 ^{b}	0.96 ± 0.08 ^{a}	0.80 ± 0.07 ^{ab}
Caffeic acid	0.90 ± 0.06 ^{a}	0.91 ± 0.06 ^{a}	0.90 ± 0.04 ^{a}	0.94 ± 0.05 ^{a}
Schaftoside isomer	2.33 ± 0.14 ^{b}	2.29 ± 0.11 ^{b}	2.62 ± 0.12 ^{a}	2.77 ± 0.10 ^{a}
Schaftoside	3.64 ± 0.10 ^{b}	3.57 ± 0.11 ^{b}	4.02 ± 0.15 ^{a}	3.92 ± 0.12 ^{a}
Homoorientin	6.31 ± 0.21 ^{a}	6.03 ± 0.16 ^{a}	6.36 ± 0.18 ^{a}	5.50 ± 0.13 ^{b}
Apigenin-hexoside-pentoside II	1.90 ± 0.10 ^{a}	1.76 ± 0.09 ^{a}	1.88 ± 0.08 ^{a}	1.87 ± 0.09 ^{a}
Luteolin dihexoside I	7.68 ± 0.19 ^{a}	7.35 ± 0.12 ^{b}	7.20 ± 0.10 ^{b}	7.56 ± 0.11 ^{ab}
6-hydroxyluteolin-7-*O*-glucoside	6.46 ± 0.20 ^{a}	6.25 ± 0.16 ^{a}	6.58 ± 0.21 ^{a}	5.26 ± 0.18 ^{b}
Apigenin dihexoside	0.44 ± 0.08	0.41 ± 0.06	0.52 ± 0.06	0.42 ± 0.04
Quercetin hexoside	4.10 ± 0.20 ^{a}	4.00 ± 0.14 ^{a}	3.37 ± 0.15 ^{b}	0.96 ± 0.10 ^{c}
Luteolin dihexoside II	0.63 ± 0.05 ^{b}	0.66 ± 0.04 ^{b}	0.78 ± 0.06 ^{a}	0.69 ± 0.04 ^{ab}
Rutin	2.86 ± 0.11 ^{b}	3.19 ± 0.12 ^{a}	3.30 ± 0.10 ^{a}	3.00 ± 0.13 ^{ab}
Isovitexin	1.75 ± 0.08 ^{b}	2.06 ± 0.10 ^{a}	2.25 ± 0.11 ^{a}	2.24 ± 0.10 ^{a}
Vitexin	2.51 ± 0.10 ^{a}	2.44 ± 0.09 ^{a}	2.52 ± 0.10 ^{a}	2.42 ± 0.08 ^{a}
Apigenin deoxylhexoside	0.85 ± 0.04 ^{a}	0.89 ± 0.05 ^{a}	0.72 ± 0.04 ^{b}	0.56 ± 0.03 ^{c}
Apigenin glycosylated derivative	6.44 ± 0.21 ^{c}	6.80 ± 0.22 ^{c}	7.60 ± 0.21 ^{b}	8.23 ± 0.30 ^{a}

^{a,b,c} Different letter denote statistical differences within a line according to Fisher's least significant difference (LSD) procedure ($p < 0.05$).

Table 2. *(Continued).*

Compound	Undigested-EE	Oral	Gastric	Intestinal
Luteolin-7-*O*-*β*-glucoside	24.2 ± 1.30 [a]	23.6 ± 1.12 [a]	19.5 ± 1.06 [c]	21.8 ± 1.02 [b]
Luteolin-7-*O*-glucuronide	1.57 ± 0.08 [a]	1.45 ± 0.07 [a]	1.06 ± 0.06 [c]	1.17 ± 0.09 [b]
Quercetin hexuronide	0.95 ± 0.06 [a]	0.88 ± 0.05 [a]	0.87 ± 0.04 [a]	0.97 ± 0.02 [a]
3,4-Dicaffeoylquinic acid	3.78 ± 0.18 [b]	3.73 ± 0.12 [b]	3.80 ± 0.10 [b]	20.9 ± 1.22 [a]
Isorhamnetin hexoside I	3.36 ± 0.10 [a]	3.39 ± 0.09 [a]	3.57 ± 0.11 [a]	3.50 ± 0.12 [a]
1,5-Dicaffeoylquinic acid	4.29 ± 0.27 [ab]	4.75 ± 0.21 [a]	4.10 ± 0.12 [b]	3.62 ± 0.14 [c]
3,5-Dicaffeoylquinic acid	72.4 ± 2.92 [a]	72.5 ± 1.91 [a]	60.4 ± 2.10 [b]	24.2 ± 1.33 [c]
Apigenin-7-*O*-glucoside	7.30 ± 0.33 [a]	7.11 ± 0.21 [a]	7.00 ± 0.18 [a]	6.28 ± 0.15 [b]
Luteolin-*O*-malonylglucoside	1.08 ± 0.08 [a]	1.05 ± 0.05 [a]	1.08 ± 0.07 [a]	1.09 ± 0.08 [a]
4,5-Dicaffeoylquinic acid	13.3 ± 0.87 [b]	12.6 ± 0.63 [b]	10.5 ± 0.51 [c]	36.9 ± 1.21 [a]
Isorhamnetin hexoside II	1.57 ± 0.10 [b]	1.61 ± 0.09 [b]	1.27 ± 0.07 [c]	1.73 ± 0.09 [a]
Dicaffeoylquinic acid isomer	0.26 ± 0.04 [a]	0.28 ± 0.05 [a]	0.23 ± 0.03 [a]	0.23 ± 0.05 [a]
Feruloylcaffeoylquinic acid	0.29 ± 0.05 [a]	0.30 ± 0.06 [a]	0.15 ± 0.03 [b]	0.25 ± 0.04 [a]
Tricaffeoylquinic acid	0.86 ± 0.08 [a]	0.79 ± 0.06 [a]	0.15 ± 0.02 [c]	0.60 ± 0.06 [b]
Luteolin	3.33 ± 0.15 [a]	3.17 ± 0.11 [a]	1.78 ± 0.09 [c]	2.57 ± 0.10 [b]
Quercetin	0.89 ± 0.06 [a]	0.86 ± 0.06 [a]	0.50 ± 0.09 [b]	0.35 ± 0.05 [c]
Methoxyquercetin	0.83 ± 0.08 [a]	0.80 ± 0.07 [a]	0.61 ± 0.06 [b]	0.75 ± 0.07 [a]
Apigenin	0.39 ± 0.04 [a]	0.39 ± 0.05 [ab]	0.12 ± 0.01 [c]	0.31 ± 0.03 [b]
Diosmetin	0.24 ± 0.02 [a]	0.24 ± 0.03 [a]	0.16 ± 0.03 [b]	0.23 ± 0.03 [a]
Trihydroxy dimethoxyflavone	0.35 ± 0.03 [a]	0.35 ± 0.04 [a]	0.18 ± 0.02 [b]	0.31 ± 0.03 [a]
Centaureidin	3.34 ± 0.14 [a]	3.30 ± 0.15 [a]	2.09 ± 0.13 [c]	2.93 ± 0.17 [b]
Methoxyacacetin	0.23 ± 0.03 [a]	0.23 ± 0.02 [a]	0.06 ± 0.01 [c]	0.20 ± 0.02 [a]
Dihydroxy trimetoxyflavone	0.34 ± 0.04 [a]	0.32 ± 0.05 [a]	0.16 ± 0.02 [c]	0.20 ± 0.03 [b]
Casticin	4.18 ± 0.17 [a]	4.02 ± 0.14 [a]	2.27 ± 0.11 [c]	3.62 ± 0.12 [b]

[a,b,c] Different letter denote statistical differences within a line according to Fisher's least significant difference (LSD) procedure ($p < 0.05$).

As can be observed, both extracts presented a similar behaviour during the gastrointestinal process. In general, the phenolic composition of both yarrow extracts was not affected during the oral phase, whereas gastric and intestinal phases, especially intestinal one, resulted as critical steps for some individual phenolic compounds. Chlorogenic acid (CGA), the most abundant mono-caffeoylquinic acid in both extracts, was stable under oral and gastric conditions, but showed a loss of about 17% at the end of the intestinal step.

However, it should be highlight the higher quantity of neochlorogenic and cryptochlorogenic acids measured after intestinal step, whose increase could be attributed to isomerization of CGA. This behaviour was also found by Bouayed et al. (2012), who reported that CGA was stable to gastric conditions but degraded (between 23-41%) during intestinal digestion, with partial isomerisation to neochlorogenic and cryptochlorogenic acids. Yu et al. (2019) also reported an important bioaccessibility (68.39-91.34%) after digestion process for chlorogenic acid obtained from mulberry leaves.

With respect to dicaffeoylquinic acids (DCQAs), these compounds seem to be stable under oral conditions in both extracts. Gastric conditions mainly affected 3,5- and 4,5-DCQAs with a significant loss of approx. 20%. The transition from gastric medium to intestinal environment caused an important degradation of 3,5-DCQA (63-67% loss), whereas 3,4- and 4,5-DCQAs increased significantly their quantity after intestinal step. This increment could be related with isomerization processes among different DCQAs at intestinal pH. Moreover, at the end of the intestinal step, the sum of all DCQAs represented the 90% of these compounds in the undigested extract. D'Antuono et al. (2015), previously described that 3,5-DCQA (pure individual compound) gastrointestinal digestion produced a higher isomerization effect with the presence of 3,4- and 4,5-DCQAs.

Both extracts, YE and EE, also presented an important quantity of flavonoids, either in glycosylated or in aglycone form. Among the glycosylated forms, luteolin-7-*O*-β-glucoside, the most abundant compound within flavonoids group, was stable to oral digestion but gastric conditions produced a decrease of approx. 20%. However, this compound increased up to 87-90% at the end of intestinal step.

Gutiérrez-Grijalva et al. (2017) also found that in digestion process, the quantity of luteolin-7-*O*-glucoside decreased after gastric step, but increased at the end of intestinal step. They indicated that the loss of luteolin-7-*O*-*β*-glucoside after gastric phase could be related, in addition to pH changes, to a possible interaction between the compound and gastric enzymes that render it undetectable in chromatographic analysis.

Regarding aglycones, luteolin was also stable to oral step but hardly affected by gastric conditions (approx. 50% loss). Moreover, luteolin registered an increased when intestinal phase ended. This behaviour was observed in other aglycones such as casticin and centaureidin. According with previous results, this effect could be also related with possible interactions between digestive enzymes and phenolics, as was detected for luteolin-7-*O*-glucoside.

Digestion effect on TPC and antioxidant activity for both extracts is shown in Table 3. During digestion process, the amount of TPC only decreased slightly for both extracts (between 7-13%). With regard to the antioxidant activity, this was not significantly affected during oral phase, however, stomach and intestinal phases resulted in critical steps for this activity (26-40% decrease). This loss of antioxidant activity could be related with the losses registered in some phenolic compounds, such as luteolin and its glucosilated derivatives, since these compounds have been reported to present an important antioxidant activity (Song and Park, 2014). However, the isomerization effect occurred in some compounds (*i.e* CGA and DCQAs) could also be related. Shang et al. (2010) indicated that among DCQA isomers from a *L. fischeri* leaves ethanolic extract, 3,5-DCQA presented the highest radical scavenging activity.

Table 3. Total phenolic content (TPC) and antioxidant activity (TEAC value) of yarrow ultrasound-assisted extract (YE) and yarrow enriched-extract (EE) after oral, gastric and intestinal digestion.

		Undigested	Oral	Gastric	Intestinal
TPC [1]	YE	105 ± 3 [a]	96 ± 3 [b]	87 ± 2 [c]	91 ± 2 [b]
	EE	224 ± 3 [a]	214 ± 2 [b]	201 ± 3 [d]	208 ± 3 [c]
TEAC value [2]	YE	0.36 ± 0.01 [a]	0.35 ± 0.01 [a]	0.20 ± 0.01 [b]	0.22 ± 0.04 [b]
	EE	1.12 ± 0.06 [a]	1.06 ± 0.03 [a]	0.75 ± 0.06 [b]	0.83 ± 0.04 [b]

[1]TPC= mg GAE/g extract. [2]TEAC value= mmol Trolox/g extract. [a-d] Different letters denote statistical differences within a same line, according to Fisher's least significant difference (LSD) procedure ($p < 0.05$).

3.2 Caco-2 cell transport experiments

In order to investigate the potential bioavailability of digested yarrow phenolic compounds, their intestinal uptake was evaluated using Caco-2 cells monolayers at 2, 4 and 6h. Due to EE digested extract presented a 2-3fold superior concentration of phenolic compounds, this extract was selected to carry out the transport experiments. The cytotoxicity assays, performed by the MTT method, indicated that 40 µL/mL of digested EE was the maximum concentration that did not affect the cell viability during 6 h (data not shown). Thus, the concentration of EE phenolic compounds detected in the apical compartment, cellular monolayer and basolateral compartment after 2, 4 and 6h of transport experiments are shown in Table 4. After 2h of incubation, 11 phenolic compounds were identified in the cell monolayer, mainly flavonoid aglycones (casticin, diosmetin and centaureidin) and DCQAs isomers (3,4-DCQA and 3,5-DCQA). The concentration of those compounds in cell monolayer decreased after 4 and 6h of experiment.

Regarding the basolateral compartment, after 2h of incubation, casticin was the main compound, followed by 3,4 and 3,5-DCQAs. Data obtained after 4h showed an increase in casticin, diosmetin and centaureidin concentration in the basolateral compartment, meanwhile the amount of 3,5-DCQA remains constant and 3,4-DCQA slightly decreased.

Successively, an increment in the quantity of casticin, diosmetin and centaureidin continued until 6h of experiment, while neither 3,4 nor 3,5-DCQAs were detected at that time.

Casticin (a methoxylated flavonol) was the most abundant compound in the basolateral fraction (after 6h, a 41.7% from digested extract). The apparent permeability coefficients (P_{app}) for casticin presented a maximum value at 2h (P_{app} = 16.7 ± 0.1 × 10^{-6} cm s^{-1}) in comparison with 4h and 6h (P_{app} = 10.9 ± 0.1 × 10^{-6} and 10.2 ± 0.3 × 10^{-6} cm s^{-1}, respectively). These results suggested that casticin permeability was time-dependent and transported across the Caco-2 monolayers with a faster rate at a shorter incubation time. In spite of *in vitro* studies of casticin's permeability are still scare; recently Piazzini et al. (2017) reported a casticin's P_{app} value of 8.1 ± 0.9 × 10^{-6} cm s^{-1} across Caco-2 cells, after 4h incubation with a *Vitex agnus-castus* extract.

Diosmetin's uptake also increased with incubation time. Surprisingly, the sum of diosmetin amount in cell monolayer and basolateral fraction (at 2, 4 or 6h), was higher than the concentration of this compound initially placed in the apical side (0.84 ± 0.1 mg/L of digested extract). Thus, considering that diosmetin is the 4'-methyl derivative of luteolin, the detected increment could be originated from the metabolism of luteolin (aglycone) and/or luteolin glycosylated-derivatives presented in the digested extract. The occurrence of diosmetin as a principal methylated metabolite from luteolin was reported in rats (Chen et al., 2011).

Centaureidin represented the third most abundant compound in the basolateral fraction after 6h. This methoxylated flavonol, also showed a time-dependent absorption through Caco-2 cell monolayer, being more rapidly transported at earlier incubation time (P_{app} at 2h = 7.0 ± 0.4 × 10^{-6} cm s^{-1}). To the best of our knowledge, no previous studies had been reported for centaureidin's *in vitro* absorption.

In general, a passive transcellular diffusion through Caco-2 monolayer could be related with casticin, diosmetin and centaureidin absorption. Nevertheless, interactions of diosmetin with selected transporters such as multidrug resistance-associated protein isoform 1 (MRP1) and monocarboxylate transporter isoform 1 (MCT1), also expressed in Caco-2 cells, have been previously described (Ziberna et al., 2014).

Table 4. Phenolic compounds (mg/L of digested extract) detected in the apical compartment, Caco-2 cell monolayer and basolateral compartment at 2, 4 and 6 h incubation with digested yarrow enriched-extract (EE).

	Apical compartment			Cell monolayer			Basolateral compartment		
Compounds	2h	4h	6h	2h	4h	6h	2h	4h	6h
Apigenin glycosylated derivative	18.50 ± 0.04 ^{a}	18.30 ± 0.07 ^{b}	17.70 ± 0.10 ^{c}	0.37 ± 0.01 ^{a}	0.37 ± 0.01 ^{a}	0.36 ± 0.01 ^{a}	0.45 ± 0.01 ^{a}	0.46 ± 0.01 ^{a}	0.42 ± 0.01 ^{b}
Luteolin-7-*O*-*β*-glucoside	41.64 ± 0.10 ^{a}	40.98 ± 0.30 ^{a}	39.77 ± 0.04 ^{b}	0.39 ± 0.02 ^{a}	0.36 ± 0.01 ^{a}	0.28 ± 0.01 ^{b}	0.25 ± 0.01 ^{a}	0.24 ± 0.01 ^{a}	0.21 ± 0.01 ^{b}
3,4-Dicaffeoylquinic acid	14.85 ± 0.10 ^{a}	12.09 ± 0.10 ^{b}	9.18 ± 0.05 ^{c}	0.68 ± 0.01 ^{a}	0.66 ± 0.02 ^{a}	n.d.	0.76 ± 0.01 ^{a}	0.71 ± 0.01 ^{b}	n.d.
3,5-Dicaffeoylquinic acid	4.95 ± 0.12 ^{a}	4.26 ± 0.05 ^{b}	2.99 ± 0.07 ^{c}	0.75 ± 0.01	n.d.	n.d.	0.71 ± 0.01 ^{a}	0.70 ± 0.01 ^{a}	n.d.
Apigenin 7-*O*-glucoside	13.34 ± 0.06 ^{a}	12.06 ± 0.08 ^{b}	11.58 ± 0.15 ^{c}	0.24 ± 0.01 ^{a}	0.22 ± 0.01 ^{ab}	0.20 ± 0.01 ^{b}	0.14 ± 0.01	n.d.	n.d.
4,5-Dicaffeoylquinic acid	22.24 ± 0.14 ^{a}	20.74 ± 0.11 ^{b}	16.28 ± 0.11 ^{c}	0.08 ± 0.01	n.d.	n.d.	0.26 ± 0.01 ^{a}	0.13 ± 0.01 ^{b}	0.09 ± 0.02 ^{c}
Apigenin	0.16 ± 0.01 ^{a}	0.09 ± 0.02 ^{b}	n.d.	0.04 ± 0.01	n.d.	n.d.	0.13 ± 0.01 ^{c}	0.18 ± 0.01 ^{b}	0.27 ± 0.01 ^{a}
Diosmetin	0.95 ± 0.02 ^{a}	0.94 ± 0.03 ^{a}	0.94 ± 0.01 ^{a}	0.89 ± 0.01 ^{a}	0.66 ± 0.01 ^{b}	0.41 ± 0.01 ^{c}	0.40 ± 0.01 ^{c}	0.79 ± 0.02 ^{b}	1.53 ± 0.11 ^{a}
Centaureidin	3.27 ± 0.02 ^{a}	2.18 ± 0.07 ^{b}	0.75 ± 0.01 ^{c}	0.58 ± 0.01 ^{a}	0.41 ± 0.01 ^{b}	n.d.	0.48 ± 0.01 ^{c}	0.84 ± 0.07 ^{b}	1.01 ± 0.02 ^{a}
Methoxyacacetin	0.05 ± 0.01 ^{a}	n.d.	n.d.	0.13 ± 0.01 ^{a}	0.10 ± 0.01 ^{b}	0.10 ± 0.01 ^{b}	0.12 ± 0.01 ^{c}	0.17 ± 0.01 ^{b}	0.20 ± 0.02 ^{a}
Casticin	5.17 ± 0.11 ^{a}	4.44 ± 0.09 ^{b}	3.73 ± 0.07 ^{c}	1.87 ± 0.01 ^{a}	1.53 ± 0.01 ^{b}	1.08 ± 0.01 ^{c}	1.77 ± 0.02 ^{c}	2.31 ± 0.02 ^{b}	3.43 ± 0.06 ^{a}

^{a,b,c} Different letters denote statistical differences within a line according to Fisher's least significant difference (LSD) procedure ($p < 0.05$). n.d., not detected

The amounts of 3,4- and 3,5-DCQAs detected in the basolateral compartment after 2 and 4h of incubation were quite smaller with respect to their amounts in the apical side, showing a P_{app} calculated values (at 4h) of 1.0 ± 0.1 × 10^{-6} cm s^{-1} for 3,4-DCQA and 2.2 ± 0.1 × 10^{-6} cm s^{-1} for 3,5-DCQA. Similarly, Zhou et al. (2015) indicated that DCQAs showed, after 4h, P_{app} values of approx. 2.5 × 10^{-6} cm s^{-1}. However, at 6h, unexpectedly no DCQAs isomers were detected in basolateral fraction (table 4). In consistence with this result, D'Antuono et al., (2015), did not also detect any DCQAs isomer in the basolateral side, but coumaric and caffeic acids were found in this fraction, suggesting a cellular metabolism activity. However, in our results neither caffeic nor coumaric acids were found in the basolateral fraction at detectable amounts with the analytical technique employed. Regarding Caco-2 transport, Zhou et al. (2015) described DCQAs absorption mainly by passive diffusion *via* paracellular pathways, although some interactions of DCQAs with certain transporters were also reported *in vitro* (Farrell et al., 2013). In that context, when evaluating the absorption of a complex plant-extract, we would have to consider that certain phenolics may act like substrates or inhibitors of some transporters expressed in Caco-2 cells, thus, they could act as permeability modifiers for other compounds (Velderrain-Rodríguez et al., 2014).

3.3 Anti-inflammatory activity of the Caco-2 basolateral fraction

Basolateral fraction recovered at 6h was used to carry out the anti-inflammatory assays, using THP-1 macrophages (stimulated *via* LPS). In addition, the basolateral media from control digestion (digestion fluids without extract) was also tested. As it is shown in Fig. 1, after 24 h the stimulated macrophages (positive control) revealed a significant release of the three pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, compared to non-stimulated cells (negative control). Previous experiments to assess the cytotoxicity of the basolateral fraction indicated that 20 μL/mL did not compromise the macrophages viability (data not shown). Thus, when THP-1 macrophages were incubated with LPS in presence of 10 and 20 μL/mL of the basolateral media, TNF-α secretion was not modified, compared with the levels obtained in absence of the extracts (Fig. 1A).

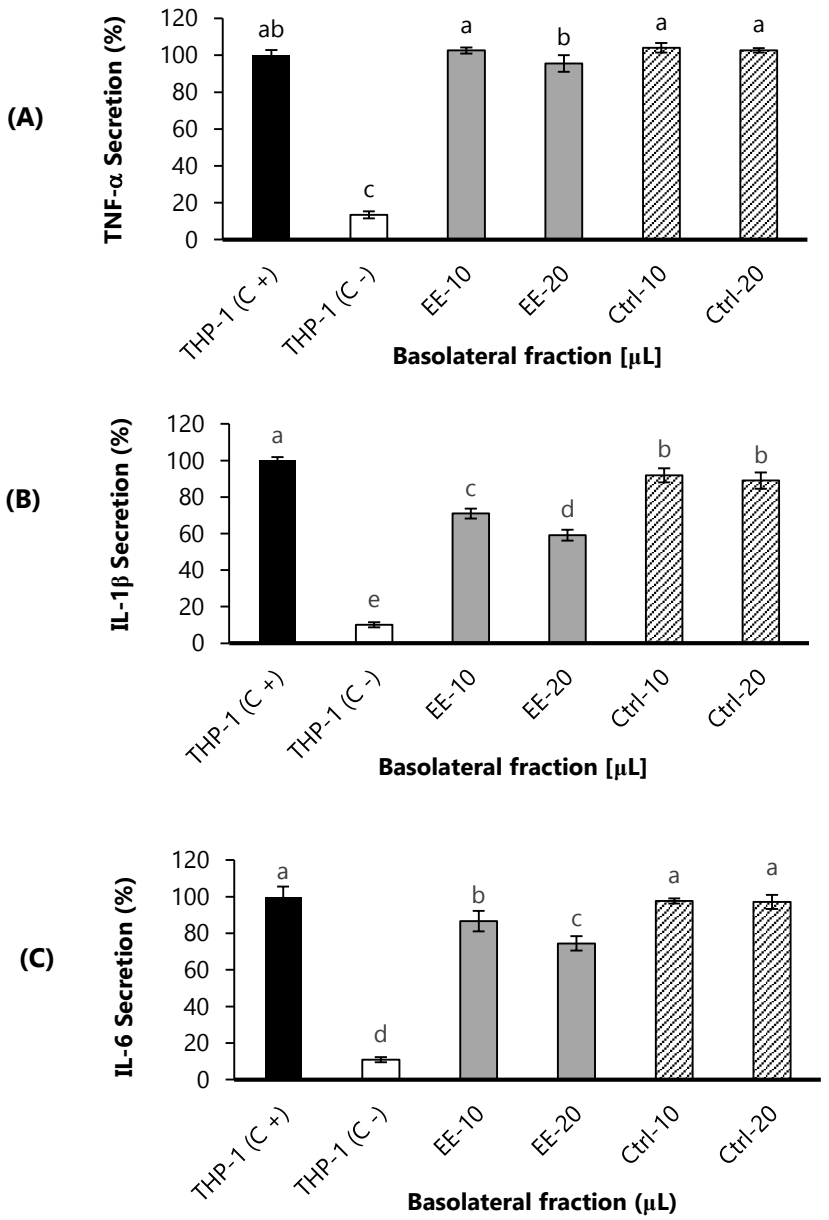

Figure 1. Levels of TNF-α, IL-1β and IL-6 secreted by THP-1 macrophages, activated with LPS, in presence of 10 and 20 µL/mL basolateral fraction from EE (yarrow enriched-extract) extract. Positive control (THP-1 C+, cells stimulated with LPS without basolateral sample), negative control (THP-1 C-, cells in contact just with RPMI medium), control digestion (Ctrl, basolateral supernatant from digested fluids without extract). Each bar is the mean of three determinations ±S.D. [a-d] Different letters indicate statistical differences among samples. Significance level at $p < 0.05$ according to Fisher's least significant difference (LSD) procedure.

In contrast, a significant reduction of IL-1β secreted was observed in presence of both concentrations of basolateral fraction, approx. 30% and 40% for 10 and 20 μL/mL (Fig. 1B). The IL-6 release was also supressed approx. 25% when applied 20 μL/mL of basolateral fraction (Fig. 1C).

Thus, the basolateral fraction from EE exhibited a moderate inhibition of IL-1β and IL-6 cytokines. Considering that this fraction was mainly composed by casticin, diosmetin and centaureidin, these flavonoids could be related, at least partilly, with the anti-inflammatory activity. Casticin was shown to decrease the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α in RAW264.7 cells treated with LPS (Liou et al., 2014). Moreover, Diosmetin also reduced pro-inflammatory IL-6 or TNF-α secretion in murine macrophages (Patel et al., 2013). Finally, Jachak et al. (2011) also reported centaureidin effectively inhibited expression of COX-1 and COX-2 enzymes related with the inflammatory response. Nevertheless, the influence of other compounds, including those found in minor concentrations or even those non-detected metabolites of phenolic compounds, cannot be ruled out.

4. CONCLUSIONS

Phenolic compounds from yarrow showed a great stability at oral step during the simulated digestion, however gastric and intestinal phases caused important modifications. Mostly CGA and DCQAs suffered an isomerization effect after intestinal step. Besides flavonoids, either in their glycosylated or aglycone form, were also reduced after intestinal phase. Casticin, diosmetin and centaureidin were the most abundant compounds found in the basolateral fraction of after Caco-2 experiments at 6h. This fraction also exhibited a certain inhibition of IL-1β and IL-6 pro-inflammatory cytokines, thus the phenolic composition of this fraction, mainly methoxylated flavonoids, could be related with the observed bioactivity. Although *in vitro* results cannot be directly extrapolated to human *in vivo* conditions, our findings exhibit a potential bioavailability of phenolic compounds present in yarrow extracts.

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ABBREVIATIONS

DCQA, dicaffeoylquinic acid; UAE, ultrasound assisted extraction; TPC, total phenolic content; YE, yarrow extract; EE, yarrow enriched-extract; GAE, gallic acid equivalent; TEAC, trolox equivalent antioxidant capacity; TEER, transepithelial electric resistance; PBS, phosphate buffer solution; LPS, bacterial lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-1β, interleukine 1β; IL-6, interleukine 6; LSD, least significant difference; CGA, chlorogenic acid; $P_{app,}$ apparent permeability coefficient; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2.

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Publicación 6

Improved *in vitro* bioaccessibility and antioxidant activity of yarrow phenolic compounds formulated in emulsions and acidified milk gels.

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ABSTRACT

The aim of this study was to improve the bioaccessibility and antioxidant activity of yarrow phenolic compounds upon an *in vitro* simulated gastrointestinal digestion. Therefore, two types of caseins-based delivery systems, sodium caseinate stabilized nanoemulsions (NEs) and glucono delta-lactone acidified milk gels (MGs), were formulated containing an ultrasound-assisted yarrow extract (YE) at two concentrations (1 and 2.5 mg/mL). Formulations with 1 mg/mL of YE were chosen based on their higher encapsulation efficiency to perform the *in vitro* digestion experiments. After digestion, YE-loaded NEs only partially protected phenolic compounds from degradation; meanwhile the phenolic composition of YE including in MGs after digestion was quite similar to undigested YE. Moreover, the antioxidant activity of MGs after digestion was higher than NEs digested samples, which confirms the higher protection of YE phenolic compound by the milk gels systems. This research demonstrated the potential use of acidified MGs as carriers to improve the bioaccessibility and antioxidant activity of yarrow phenolic compounds. Therefore, these matrices could be employed to develop new dairy products enriched with phenolic compounds.

1. INTRODUCTION

Achillea millefolium L., commonly known as yarrow, has been traditionally used to treat gastrointestinal and hepatobiliary disorders, inflammation or diabetes (Akram, 2013). The bioactivity of aqueous and alcoholic yarrow extracts has been associated with the presence of phenolic compounds, mainly phenolic acids and flavones (Dias et al., 2013). Nevertheless, after oral consumption, the potential health benefits of phenolics compounds could be limited due to instability under certain pH conditions, poor water solubility, gastrointestinal enzyme degradation, low intestinal permeability or metabolism during digestion (Lu, Kelly & Miao, 2016). Therefore, oral administration of these compounds requires strategies to preserve their chemical integrity and successful delivery in physiological targets. In recent years, the encapsulation of phenolics compounds has been extensively studied (Ghayour et al., 2019; Velderrain-Rodríguez, Acevedo-Fani, González-Aguilar & Martín-Belloso, 2019). Thus, several encapsulation techniques (*e.g.*, spray drying, emulsion, inclusion complexation…), as well as carrier materials (*e.g.*, chitosan, soy proteins, glucan…), have already been proposed for phenolic compounds (Fang & Bhandari, 2010; Lu et al., 2016; Munin & Edwards-Lévy, 2011).

Ideally, food-grade encapsulation materials, as proteins, are selected to formulate food products. Proteins have multiple applications in encapsulation matrices of phenolic compounds, as emulsifiers, gelling agents and also as entities to formulate micro and nano-particles (Jia, Dumont & Orsat, 2016). The ability of phenolic compounds to associate with milk proteins is well documented, particularly to proline-rich proteins such as α- and β-caseins through hydrophobic interactions and hydrogen bonds. Thus, this ability has been used to develop delivery carriers (Arranz et al., 2019; Ranadheera, Liyanaarachchi, Chandrapala, Dissanayake & Vasiljevic, 2016; Tavares, Croguennec, Carvalho & Bouhallab, 2014).

Nanoemulsion-based encapsulation systems for phenolic compounds have been well established, due to its high efficacy encapsulation, maintenance of chemical stability and controlled release (Artiga-Artigas, Lanjari-Pérez & Martín-Belloso, 2018; Lu et al., 2016).

In order to develop these systems, sodium caseinate (a food grade emulsifier) has been described as an emulsifier that improves delivery of phenolic compounds. Sabouri, Geng & Corredig (2015) demonstrated that sodium caseinate absorbed at the oil water interface, can be associated to high concentrations of epigallocatechin-gallate (EGCG). Moreover, these authors (Sabouri, Arranz, Guri & Corredig, 2018) also indicated that the incorporation of EGCG in sodium caseinate emulsions improved its bioaccessibility and preserved its functional properties during gastrointestinal digestion. Besides, Casanova et al., (2018) also reported a complexation of cyaniding-3-*O*-glucoside with sodium caseinate, suggesting that sodium caseinate is a potential carrier for antocyanins.

The gelation of protein matrices has also been indicated as an alternative method to encapsulate phenolic compounds (Jia et al., 2016). Caseins have excellent gelation properties and have been proposed as carriers for phenolic compounds since they are able to interact with phenolics, form complexes and entrap them through their gelation process (Ozdal, Capanoglu & Alta, 2013). Thus, Harbourne, Jacquier & O'Riordan (2011) studied the effects of adding phenolic compounds during acid gelation of milk, indicating that gels increased hydrogen bonding in the presence of phenolic compounds. Moreover, Vega & Grover (2011) also reported that in acidified skim milk gels containing cocoa flavanols, most of the flavonols were associated to the casein fraction.

The aim of this study was first to evaluate the stability of yarrow phenolic compounds after *in vitro* simulated gastrointestinal digestion. Then, two different casein-based delivery systems were developed and characterized; as a strategy to improve yarrow phenolic compounds bioaccessibility. Finally, the bioaccessibility and antioxidant activity after simulated digestion of encapsulated phenolic compounds was evaluated.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

Ethanol (EtOH) (99.5% purity) was obtained from Panreac (Barcelona, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%) and 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) were purchased from Sigma-Aldrich (Madrid, Spain). Reference standards (HPLC purity ≥95%) for phenolic compounds identification such as chlorogenic acid, diosmetin and vitexin were purchased from Sigma-Aldrich. 1,5-Diccafeoylquinic acid (DCQA), 3,4-DCQA, 3,5-DCQA, 4,5-DCQA, apigenin, luteolin, orientin, schaftoside and vicenin 2 were obtained from Phytolab (Madrid, Spain). Apigenin-7-*O*-glucoside, caffeic acid, homoorientin, luteolin-7-*O*-*β*-glucoside and rutin were from Extrasynthese S.A. (Genay, France). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain).

2.2. Yarrow samples and Ultrasound-Assisted Extraction (UAE)

Achillea millefollium L. (yarrow) was obtained from an herbalist´s local supplier (Murcia, Spain). The sample included inflorescences and upper dried leaves of the plant (water content less than 5%). The plant was ground using a Premill 250 hammer mill (Leal S.A., Granollers, Spain) and sieved to the appropriate size (<500 μm). The extraction process was performed by UAE in an ultrasonic device (Branson digital sonifier 450, Danbury, USA) at 400 W and 60 kHz. Ethanol was used as extraction solvent (1:10 plant/solvent ratio) during 30 min at 40 ºC and an output of 50% nominal amplitude. Ethanol was evaporated in a rotary evaporator (IKA®, Werke GmbH and Co., KG, Germany) and samples were stored at −20ºC until use.

2.3. Chemical characterization of phenolic compounds

Phenolic chromatographic analyses were conducted in an Agilent 1260 Infinity series HPLC-PAD (Santa Clara, CA, USA). A reverse phase ACE Excel 3 Super C18 column (150 mm x 4.6 mm, 3 · m particle size) from ATC (Aberdeen, Scotland, UK) equipped with a pre-column from the same material was employed according to the chromatographic method reported by Villalva et al. (2018). Besides, accurate mass from HPLC-PAD-QTOF-MS/MS in a negative mode analysis was used for compounds identification.

Quantification of identified compounds was performed using calibration curves of pure analytical standards. In addition, luteolin-di-hexoside and 6-hydroxyluteolin-7-*O*-glucoside were quantified by the calibration curves of orientin and luteolin-7-*O*-glucoside respectively. Correspondingly, apigenin glycosylated derivative were quantified using apigenin-7-*O*-glucoside calibration curve and schaftoside isomer and apigenin-hexoside-pentoside respect to schaftoside calibration curve.

2.4. Preparation and characterization of yarrow extract (YE)-loaded nanoemulsions (NEs).

NEs were prepared using high pressure homogenization (HPH). Oil in water emulsions, containing 7% of soybean oil (w/w) (Sigma Aldrich, Oakville, ON, Canada) and 0.0, 1.0 or 2.5% (w/w) of YE (dissolved in less than 1% of EtOH), were formulated with 2% sodium caseinate (NaCas) (New Zealand Milk Proteins, Mississauga, ON, Canada) as emulsifier. Initially, NaCas was dispersed in ultrapure water with magnetic stirrer and stored overnight at 4ºC to allow complete hydration. Emulsions were prepared by addition of YE to soybean oil and subsequent incorporation of NaCas solution. Both phases were pre-homogenized using a Polytron mixer (Brinkmann Inst. Corp., Mississauga, ON, Canada) at 30,000 RPM for 2 min, and then HPH was performed at 450 kPa for five passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA). Then NEs were characterized (particle size and Z (ζ)-potential) and stored under refrigeration at 4ºC for two weeks in order to evaluate its stability.

The particle size distribution was measured using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK), with water as dispersant. The refractive indices were 1.33 and 1.47, for water and soybean oil, respectively. Mean particle diameter was reported as the volume-weighted mean diameter ($d_{4,3}$) calculated as the average of triplicate measurements. ζ-potential was determined by dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments, Malvern, UK) in samples diluted in ultrapure grade water at 1:500 ratio. All measures were done by triplicate.

2.5. Preparation and characterization of milk gels (MGs) of yarrow extract

Pasteurised skimmed milk, acidified with 2% (w/w) of glucono delta-lactone (GDL) (Sigma Aldrich) and containing 0.0, 1.0 or 2.5% (w/w) of YE, was used to prepare MGs. Initially, a suspension of heated (85ºC for 30 min) or non-heated milk with YE was stirred for 1 hour at room temperature and protected from direct light. Then, this suspension was warmed up to 30ºC, the GDL was added, and the mixture was kept at 30ºC for 180 min. After milk gelation, the samples were stored at 4ºC until analyses.

The dynamic rheological measurements were performed by applying a constant strain of 0.05% at a frequency of 1 Hz by means of a Physica MCR301 stress-controlled rheometer (Anton-Paar, Graz, Austria) using a concentric cylinder with solvent trap cover, at 30°C. The gelation point was taken at the time of cross-over between G' (storage modulus) and G" (loss modulus) (Haratifar & Corredig, 2014). In parallel, the pH was monitored during milk gel formation using an Accumet pH meter (Fisher Scientific, Edmonton, Canada). All experiments were done in triplicate.

2.6. Encapsulation efficiency of phenolic compounds

The encapsulation efficiency (EE) of yarrow phenolic compounds in NEs and MGs was measured after centrifugation of fresh samples for 1 h (60.000 g (NEs) and 20.500 g (MGs), respectively). Supernatants (aqueous phase) were recovered and filtered using 0.22 μm polyvinylidene fluoride (PVDF) filters prior injection in a HPLC-PAD. Analysis was performed as previously described in section 2.3.

Encapsulation efficiency of individual phenolic compounds (individual EE%) was determined by Eq. 1 and the total encapsulation efficiency (total EE%) of the formulation was calculated by Eq. 2, as followed:

Eq. (1) $$\text{Individual } EE(\%) = \left[1 - \frac{Individual\ \ phenolic\ compound\ in\ aqueus\ phase}{Individual\ \ phenolic\ compound\ quantified\ in\ extract}\right] x\ 100$$

Eq. (2) $$\text{Total } EE(\%) = \left[\frac{\sum Encapsulated\ phenolic\ compounds}{Total\ phenolic\ compounds\ in\ extract}\right] x\ 100$$

2.7. *In vitro* simulated gastrointestinal digestion

A two steps *in vitro* simulated digestion, gastric and intestinal, was performed according to a standardised protocol (Minekus et al., 2014), with slight modifications. Briefly, 10 mL of each sample were mixed with 10 mL of simulated gastric fluids containing 25,000 U/mL of pepsin at a final pH 3 in amber jars. Samples were incubated in a shaking water bath (220 strokes/min) (New Brunswick Scientific Co., Inc., NJ, USA) for 30 min at 37°C. Then, 20 mL of simulated intestinal fluids were incorporated containing 5.0 mg/mL of pancreatin (800 U/mL), 160 mM bile salts, 1 mM phospholipids and 5 µL phospholipase A2 (stock solution 6.7 mg/mL), and the mixture was incubated for 2 h at 37 °C and pH 7. At the end of the digestion, the enzyme reaction was immediately stopped by keeping the samples on ice and stored at −20 °C. Samples (4 mL) were then freeze-dried for further experiments. Digestion process was performed in triplicate for every formulation (free YE, YE-NEs and YE-MGs). In addition, control digestions without YE (water, NEs and MGs) were performed.

For quantification of phenolic compounds after digestion, digested freeze-dried samples were re-suspended in 1 mL of EtOH and acidified water with 0.1% formic acid (60:40, v/v). Later, the mixture was placed into an ultrasound water-bath (5 min) and centrifuged at room temperature (4,500 RPM, 5 min). The clear supernatant was filtered using 0.45 µm PVDF filters prior analysis by HPLC-PAD as described in 2.3 section.

2.8. Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) method was applied according to Brand-Williams et al. (1995), with some modifications. Briefly, a volume of 975 µL of 6.1 x 10^{-5} M DPPH$^{\bullet}$ methanol solution was used. The reaction was started by adding 25 µL of sample or blank. The bleaching of DPPH$^{\bullet}$ was followed at 517 nm at room temperature until the reaction was completed (3 h). All the samples were prepared at 4 different concentrations, in triplicate, to obtain the inhibition curve of DPPH$^{\bullet}$. The results were calculated as mmol Trolox equivalent/g extract (TEAC value). Analysis was done in triplicate.

2.9. Statistical analysis

For each sample, experiments were performed at least in triplicate and expressed as mean values and standard deviation. A one-way analysis of variance (ANOVA) followed by LSD test (Fisher's least significant difference) post-test were used to discriminated significant differences between means at $p < 0.05$. Analysis was performed using Statgraphic v. Centurion XVI package for Windows (Statpoint Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1. Effect of *in vitro* simulated gastrointestinal digestion on YE phenolic compounds

Phenolic compounds characterization of YE, before and after *in vitro* simulated gastrointestinal digestion, was performed by HPLC-PAD analysis (Table 1). Therefore, twenty-one phenolic compounds were identified in YE, mainly glycosylated flavonoids and phenolic acids. The main compounds were 3,5-dicaffeoylquinic acid (DCQA) and luteolin-7-*O*-glucoside, followed by vicenin 2 and an apigenin glycosylated derivate. These phenols represented 49% of the total phenolic compounds quantified in YE. Other caffeoylquinic acid derivatives like chlorogenic acid and 4,5-DCQA were found in representative amounts within the phenolic acids in YE. Glycosylated flavones like 6-hydroxyluteolin-*7*-*O*-glucoside and schaftoside, besides its isomers, were also identified. These results were consistent with previously reported studies of yarrow extracts (Benedek, Gjoncaj, Saukei & Kopp, 2007; Dias et al., 2013; Pereira et al., 2018).

The quantitative analysis of phenolic fraction of YE after digestion showed that the concentration of almost all detected compounds was modified. Several authors indicated that the stability of phenolic compounds during the gastrointestinal digestion process is strongly influenced by their chemical structure, since phenolics have a different sensitivity to pH variations and digestive enzymes activity (Goulas & Hadjisolomou, 2019; Lima et al., 2019). Regarding the main components of the extract, 3,5-DCQA concentration was highly reduced after digestion (from 17.0 ± 0.47 to 5.84 ± 0.28 mg/g of extract), while 3,4-DCQA and 4,5-DCQA were increased.

Table 1. Phenolic composition of yarrow extract (YE) and digested samples: YE, NE-1.0 and MG-1.0.

Compound	Concentration (mg /g sample)			
	YE undigested	YE digested	NE-1.0 digested	MG-1.0 digested
Chlorogenic acid †	4.49 ± 0.20	1.24 ± 0.31*	3.03 ± 0.19*	3.28 ± 0.40*
Vicenin 2 †	5.33 ± 0.25	4.93 ± 0.24	5.03 ± 0.32	5.09 ± 0.60
Caffeic acid †	0.68 ± 0.22	1.09 ± 0.13*	0.63 ± 0.11	0.69 ± 0.14
Schaftoside isomer	3.50 ± 0.07	3.27 ± 0.17	3.38 ± 0.46	3.36 ± 0.45
Schaftoside †	3.49 ± 0.22	3.25 ± 0.30	3.43 ± 0.56	3.52 ± 0.38
Homoorientin †	0.46 ± 0.07	0.25 ± 0.01*	0.28 ± 0.03*	0.35 ± 0.04
Apigenin-hexoside-pentoside	2.63 ± 0.16	2.28 ± 0.19	2.54 ± 0.28	2.68 ± 0.21
Luteolin-dihexoside	2.72 ± 0.21	2.08 ± 0.10*	2.29 ± 0.14*	2.60 ± 0.24
6-Hydroxyluteolin-7-*O*-glucoside	3.67 ± 0.31	1.08 ± 0.13*	1.14 ± 0.25*	3.51 ± 0.29
Rutin †	1.37 ± 0.28	0.86 ± 0.14*	0.98 ± 0.31	1.20 ± 0.15
Vitexin †	0.71 ± 0.03	0.41 ± 0.05*	0.57 ± 0.02*	0.70 ± 0.07
Apigenin glycosylated derivative	4.79 ± 0.16	4.09 ± 0.19*	4.78 ± 0.23	4.88 ± 0.20
Luteolin-7-*O*-*β*-glucoside †	7.57 ± 0.17	5.71 ± 0.14*	6.51 ± 0.10*	7.36 ± 0.30
3,4-diccafeoylquinic acid †	1.79 ± 0.26	4.59 ± 0.34*	3.54 ± 0.19*	2.62 ± 0.25*
1,5-diccafeoylquinic acid †	1.91 ± 0.11	1.43 ± 0.18	1.73 ± 0.19	1.88 ± 0.25
3,5-diccafeoylquinic acid †	17.0 ± 0.47	5.84 ± 0.28*	10.8 ± 0.11*	15.9 ± 0.23*
Apigenin-7-*O*-glucoside †	2.20 ± 0.15	1.41 ± 0.08*	1.40 ± 0.25*	2.16 ± 0.26
4,5-diccafeoylquinic acid †	3.36 ± 0.19	7.48 ± 0.13*	3.96 ± 0.14*	3.23 ± 0.28
Luteolin †	2.15 ± 0.27	1.22 ± 0.13*	2.10 ± 0.13	2.13 ± 0.10
Apigenin †	0.61 ± 0.13	0.24 ± 0.20*	0.50 ± 0.26	0.58 ± 0.04
Diosmetin †	0.49 ± 0.07	<LOQ	0.35 ± 0.05*	0.44 ± 0.06

YE, yarrow extract. NE-1.0, YE-loaded nanoemulsions at 1 mg/mL. MG-1.0, milk gels including 1 mg/ mL of YE. <LOQ, below limit of quantification. † Phenolic compound identified and quantified *via* comparison with its authentic standard. * An asterisk indicates statistical differences of digested samples respect to the YE undigested extract (first column). Significance level at $p < 0.05$ with Fisher's Least Significant Difference (LSD) test.

In fact, 4,5-DCQA was the main compound in the digested extract. These results suggest an isomerization of 3,5-DCQA towards 3,4-DCQA and 4,5-DCQA during digestion. Similarly, D'Antuono, Garbetta, Linsalata, Minervini & Cardinali (2015), also detected the presence of 4,5- and 3,4-DCQAs after gastrointestinal digestion of 3,5-DCQA (pure individual compound). Luteolin-7-*O*-glucoside, the second most abundant compound in YE, was also reduced in approx. 25%, after digestion. Therefore, the encapsulation of YE phenolic compounds in delivery systems could be a useful strategy to improve their stability during gastrointestinal digestion.

3.2. Formulation and characterization of YE-loaded nanoemulsions (NEs)

YE-loaded NEs with two different concentrations of YE, 1mg/mL (NE-1.0) and 2.5 mg/mL (NE-2.5), were formulated in soy oil in NaCas water emulsions. NEs control, without YE, was also formulated. The particle size distribution (Fig. 1) indicated that the control emulsion had a monomodal distribution. This distribution did not change after YE incorporation, although NE-2.5 showed a wider size distribution than control and NE-1.0.

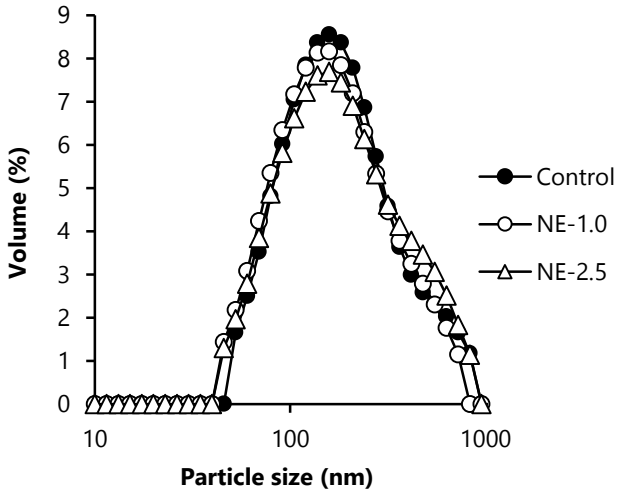

Figure 1. Particle size distribution of freshly (day 0) nanoemulsions in the presence or not of yarrow extract (YE). Control: without YE. NE-1.0: YE-loaded nanoemulsions at 1 mg/mL. NE-2.5: YE-loaded nanoemulsions at 2.5 mg/mL.

Freshly prepared control emulsion (day 0) had a mean particle diameter ($d_{4,3}$) of 248 ± 1.0 nm (Table 2). The addition of YE did not modify the emulsions particle diameter. All NEs had a negative net surface charge (ζ-potential), with no statistical differences between the control and YE-loaded NEs.

The physical stability of YE-loaded NEs was monitored during two weeks. The values of mean particle diameter and ζ-potential after 7 and 14 days of storage at 4ºC are shown in Table 2. After 14 days, no statistical differences were observed neither in the mean particle diameter nor in the ζ-potential of any formulation. These data denote the stability of YE-loaded NEs. Therefore, NEs could be suggested as carriers of yarrow phenolic compounds. Sodium caseinate stabilized nanoemulsions have also been used as carriers of several compounds such as tea polyphenols (Sabouri et al., 2015), thyme oil (Xue & Zhong, 2014) or clove oil (Sharma et al., 2017).

Table 2. Characterization of fresh nanoemulsions (0 day) and throughout storage (7 and 14 days).

	Mean particle diameter ($d_{4,3}$) (nm)			**ζ– potential (mV)**		
Sample	*0 d*	*7 d*	*14 d*	*0 d*	*7 d*	*14 d*
Control	248 ± 1.0	247 ± 1.0	247 ± 1.2	-39.1 ± 0.9	-39.2 ± 1.1	-38.7 ± 0.2
NE-1.0	250 ± 0.8	249 ± 1.2	249 ± 1.6	-38.6 ± 0.5	-38.2 ± 0.6	-37.9 ± 0.7
NE-2.5	251 ± 2.0	251 ± 1.0	250 ± 1.0	-38.2 ± 0.7	-38.0 ± 0.9	-37.8 ± 0.7

NE-1.0: YE-loaded NEs at 1 mg/mL.
NE-2.5: YE-loaded NEs at 2.5 mg/mL.
ζ–potential: zeta potential.

3.3. Formulation and characterization of acidified milk gels (MGs) including YE

Milk gels including two different concentrations of YE, 1mg/mL (MG-1.0) and 2.5 mg/mL (MG-2.5), were prepared using heated or unheated milk, acidified with GDL. Control MGs (without YE) were also formulated.

MGs formulated with heated milk showed a shorter gelation time and higher pH values at gelation time than those obtained with unheated milk (Table 3). Heated MGs also showed higher final storage modulus (G') values than those formulated with unheated milk (Table 3), indicating that heated gels presented a higher stiffness at the end of the acidification process. Besides, the final loss tangent values (tan δ) were significantly lower for heated gels. At moderate heating temperatures (70-90ºC), whey proteins unfold and associate with casein micelles. During acidification, these unfolded-associated proteins act as a cross-link between protein particles and increase the number (and strength) of bonds in the gel network, consequently higher G' values are obtained, pH increases at gelation and shorter gelation times are required (Donato, Alexander & Dalgleish, 2007; Lucey, 2002).

Despite the differences found in the rheological properties between heated and unheated formulations, the addition of different concentration of YE did not significantly modify the gelation time, the pH at gelation time, the final G' value or the final tan δ value (Table 3). Similarly, Vega & Grover (2011) formulated acidified MGs including a commercial cocoa extract, rich in flavanols, showing that final G' values and pH values at gelation time were not modified with extract addition, compared to the control gel. However, the addition of tannic and gallic acids to milk have resulted in faster gelation times and gels with G' values higher than control samples (Harbourne et al., 2011). These controversial results could be explained by interactions between milk proteins and phenolic compounds and it was described that these interactions are highly affected by the type and structure of phenolic compounds employed (Ozdal et al., 2013).

Table 3. Properties of acidified skim milk gels formulated with heated and unheated milk.

	Gelation time (min)		pH at gelation time		Final G' value (Pa)		Final tan δ	
Sample	**Heated**	**Unheated**	**Heated**	**Unheated**	**Heated**	**Unheated**	**Heated**	**Unheated**
Control	27 ± 2.1[a]	74 ± 1.6[a]	5.51 ± 0.11[a]	5.11 ± 0.09[a]	424 ± 23[a]	15.6 ± 0.0[b]	0.28 ± 0.01[a]	0.31 ± 0.01[a]
MG-1.0	27 ± 0.6[a]	74 ± 1.1[a]	5.37 ± 0.12[a]	5.08 ± 0.16[a]	397 ± 18[a]	15.5 ± 0.1[b]	0.28 ± 0.01[a]	0.31 ± 0.01[a]
MG-2.5	26 ± 2.7[a]	75 ± 0.6[a]	5.33 ± 0.09[a]	5.03 ± 0.23[a]	389 ± 13[b]	14.1 ± 1.0[a]	0.29 ± 0.01[a]	0.32 ± 0.01[a]

MG-1.0: milk gels with 1.0 mg/mL of YE. MG-2.5: milk gels with 2.5 mg/mL of YE. G': storage modulus values. tan δ: loss tangent values. [a,b] Different superscript letters mean statistical difference among different formulations for each evaluated property. Significance level at $p < 0.05$ with Fisher's Least Significant Difference (LSD) test.

3.4. Encapsulation efficiency (EE) of YE phenolic compounds in NEs and MGs.

The encapsulation efficiency of YE individual phenolic compounds in NEs and MGs is shown in Table 4.

Table 4. Encapsulation efficiency (EE) of individual yarrow phenolic compounds presented in yarrow extract (YE).

Compound	Encapsulation Efficiency (%)			
	NE-1.0	NE-2.5	MG-1.0	MG-2.5
Chlorogenic acid †	88.5 ± 6.8	6.6 ± 1.2	47.1 ± 7.0	28.3 ± 6.7
Vicenin 2 †	88.3 ± 6.7	17.5 ± 1.1	34.3 ± 8.0	8.2 ± 2.3
Caffeic acid †	89.7 ± 6.1	20.5 ± 6.9	55.0 ± 7.5	11.0 ± 5.0
Schaftoside isomer	89.4 ± 5.9	18.9 ± 5.0	56.8 ± 2.9	25.7 ± 7.3
Schaftoside †	88.1 ± 7.9	17.0 ± 4.7	40.9 ± 3.6	19.8 ± 2.1
Homoorientin †	94.6 ± 4.1	34.5 ± 7.7	65.3 ± 7.0	29.5 ± 6.5
Apigenin-hexoside-pentoside	89.9 ± 6.6	13.4 ± 2.5	33.8 ± 9.8	12.5 ± 4.8
Luteolin-dihexoside	92.3 ± 4.8	23.5 ± 7.0	56.6 ± 2.6	25.1 ± 6.0
6-Hydroxyluteolin-7-*O*-glucoside	97.0 ± 1.4	42.0 ± 5.9	74.0 ± 0.8	79.6 ± 4.5
Rutin †	93.1 ± 3.4	20.0 ± 6.6	47.4 ± 5.4	34.0 ± 7.8
Vitexin †	93.2 ± 3.6	36.6 ± 7.8	51.3 ± 0.5	31.8 ± 7.0
Apigenin glycosylated derivative	91.1 ± 5.2	34.7 ± 7.9	55.0 ± 7.4	48.4 ± 4.6
Luteolin-7-*O*-*β*-glucoside †	95.4 ± 2.8	44.5 ± 5.7	80.0 ± 3.3	73.5 ± 3.3
3,4-diccafeoylquinic acid †	91.0 ± 7.0	22.2 ± 7.6	81.4 ± 6.1	61.9 ± 6.7
1,5-diccafeoylquinic acid †	92.3 ± 4.9	43.0 ± 7.1	91.6 ± 1.6	85.0 ± 5.9
3,5-diccafeoylquinic acid †	93.2 ± 4.4	36.4 ± 6.0	98.0 ± 0.2	97.0 ± 1.0
Apigenin-7-*O*-glucoside †	94.4 ± 1.9	54.4 ± 7.8	89.4 ± 2.3	85.0 ± 2.9
4,5-diccafeoylquinic acid †	93.9 ± 3.7	43.8 ± 5.0	91.6 ± 1.6	87.5 ± 1.1
Luteolin †	97.6 ± 2.9	92.6 ± 1.1	100 ± 0.0	100 ± 0.0
Apigenin †	99.3 ± 0.3	93.4 ± 2.8	100 ± 0.0	100 ± 0.0
Diosmetin †	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Total %EE	92.3 ± 4.6 [a]	33.7 ± 0.9 [b]	72.0 ± 2.7 [A]	63.1 ± 2.6 [B]

† Phenolic compound identified and quantified *via* comparison with its authentic standard. NE-1.0: YE-loaded nanoemulsion at 1.0 mg/mL. NE-2.5: YE-loaded nanoemulsion at 2.5 mg/mL. MG-1.0.: milk gels with 1.0 mg/mL of YE. MG-2.5: milk gels with 2.5 mg/mL of YE. [a,b] Different lower case letters mean statistical difference within nanoemulsions. [A,B] Different super case letters mean statistical difference within milk gels. Significance level at $p < 0.05$ with Fisher's Least Significant Difference (LSD) test.

Regarding NEs, the encapsulation efficiency was related to the YE concentration, NE-1.0 showed higher EE for all the compounds than NE-2.5. Encapsulation efficiency of all phenolic compounds in NE-1.0 ranged from 88.1 to 100%. However, in NE-2.5 the values were under 55%, except for luteolin, apigenin and diosmetin. Hence, the total encapsulation efficiency calculated in NE-1.0 was significantly higher than in NE-2.5 (92.3 ± 4.6% vs. 33.7 ± 0.9% respectively).

Regarding MGs, the encapsulation efficiency was also affected by the concentration of YE, but in a lesser extent than in NEs. Thus, MG-1.0 had a total encapsulation efficiency of 72.0 ± 2.7%, meanwhile MG-2.5 showed a 63.1 ± 2.6%. In MGs formulations, apigenin and luteolin glycosylated forms presented a lower EE than their aglycone forms. Thus, Xiao et al. (2011) also indicated that the glycosylation of flavonoids lowered their affinity for milk proteins. These authors endorsed the decrease of affinity after glycosylation to the non-planar structure, since molecules with near-planar structure can easily enter the hydrophobic pockets in proteins. Among phenolic acids, the DCQAs showed higher encapsulation efficiency than chlorogenic and caffeic acids. DCQAs higher encapsulation could be related to the higher hydrophobicity of DCQAs (with respect to chlorogenic and caffeic acids), that induces a stronger association between proteins and polyphenols (Adrar, Madani & Adrar, 2019). Nevertheless, their different structures (size, length and flexibility) could also affect protein-phenolic compounds interactions.

3.5 Effect of *in vitro* digestion on YE phenolic compounds included NEs and MGs.

Formulations with 1 mg/mL of YE (NE-1.0 and MG-1.0) were chosen based on their higher EE to perform *in vitro* digestion experiments. The quantitative analysis of individual phenolic compounds was performed after gastrointestinal digestion (Table 1). The quantification of phenolic compounds after NE-1.0 digestion showed that the digestion process modifies their concentration. In total 10 of the 21 phenolic compounds detected in digested NE-1.0 decreased their concentration, compared to undigested YE. Regarding 3,5-DCQA, the most abundant compound in YE, a 3.4 times decreased was observed in comparison with the undigested extract. Luteolin-7-*O*-glucoside's concentration was reduced from 7.57 ± 0.17 to 6.51 ± 0.10 mg/g of sample.

These results suggested that some encapsulated compounds were partially released and exposed to digestion conditions, indicating that the formulation of YE-loaded NEs only partially protected the YE phenolic compounds during gastrointestinal transit.

The effect of gastric digestion on NaCas stabilized emulsions was previously reported. Li, Ye, Lee, & Singh (2012) described that caseinate-stabilized emulsions flocculate after digestion at acidic conditions (pH 1-3) due to pepsin hydrolysis over interfacial proteins in the droplets surface. Nevertheless, Sabouri et al. (2018) reported a higher recovery of EGCG (but not 100%) after digestion, when it was formulated with NaCas stabilized emulsions compared to water solutions. They concluded that the complexation of EGCG and NaCas at the interface of emulsions protected EGCG from digestive degradation.

After MG-1.0 digestion process, most of the phenolic compounds remained stable, comparing to undigested YE (Table 1), except chlorogenic acid, 3,4-DCQA and 3,5-DCQA. Regarding 3,5-DCQA, the concentration detected after MG-1.0 digestion was 15.9 ± 0.23 *versus* 17.0 ± 0.26 mg/g of sample from the undigested extract. Moreover, 3,4-DCQA concentration increased from 1.79 ± 0.26 to 2.62 ± 0.25 mg/g of sample after digestion. These data indicated a possible isomerization of 3,5-DCQA to 3,4-DCQA during digestion. This isomerization was also found in the digestion of the free YE (non-encapsulated), but in a greater extent. These findings are in line with the protection effect of YE phenolic compounds against degradation during digestion by MGs. Indeed, Lamothe, Azimy, Bazinet, Couillard & Britten (2014) described that the addition of a green tea extract to a yogurt matrix significantly decreased the rate and extent of proteolysis in the gastric phase. They suggested that the formation of complexes between milk proteins and green tea polyphenols increase protein stability during pepsin digestion. However, they also reported that the presence of tea extract did not influence protein hydrolysis in the intestinal phase, with the release of 100% of the polyphenols initially added. They concluded that interactions between green tea polyphenols and milk proteins help to maintain the integrity of polyphenols during digestion. Recently, Adrar et al. (2019) reported that polyphenols-proteins interactions would play a certain role in the preservation of polyphenols structure and function through the gastrointestinal tract transit.

3.6 Effect of *in vitro* digestion on free and encapsulated YE antioxidant activity

The antioxidant activity of undigested YE and digested samples: free YE, NE-1.0 and MG-1.0 was evaluated (Table 5). Empty (without YE) NEs and MGs were also digested and its antioxidant activity was determined (data not shown). The values obtained with these empty formulations were subtracted from the formulations with the extract. The digestion process reduced almost 50% of the antioxidant activity of free YE samples, which could be related with the reduction found in phenolic compounds. The gastrointestinal digestion of NE-1.0 also decreased the antioxidant activity of the samples, however, in a lesser extent than free YE solutions. These results agree with the partial protection provided by the NE to YE phenolic compounds. Moreover, the antioxidant activity of MG-1.0 after digestion was the highest compared to free YE and NE-1.0, which confirms the higher protection of YE phenolic compounds by the milk gels systems. Thus, several authors previously reported that protein-polyphenol complexes formation preserved polyphenols bioactivities, including antioxidant activity (Foegeding, Plundrich, Schneider, Campbell & Lila, 2017).

Table 5. Antioxidant activity of yarrow extract (YE) and digested samples: free YE, NE-1.0 and MG-1.0.

TEAC Value (mmol Trolox/g sample)	
YE undigested	0.349 ± 0.007[a]
Free YE digested	0.186 ± 0.002[d]
NE-1.0 digested	0.202 ± 0.003[c]
MG-1.0 digested	0.270 ± 0.005[b]

NE-1.0: YE-loaded nanoemulsions at 1 mg/mL. MG-1.0: milk gels including 1 mg/ mL of YE. [a-d] Different letters mean statistical difference between samples with Fisher's LSD test ($p < 0.05$).

4. CONCLUSIONS

The quantitative analysis of YE phenolic fraction after *in vitro* gastrointestinal digestion showed that the concentration of all phenolic compounds was modified. Two types of casein-based delivery systems, sodium caseinate stabilized NEs and GDL-acidified MGs, were proposed to improve stability of YE phenolic compounds during digestion process. Recovery of YE phenolic compounds after digestion was significantly higher in MGs than NEs. Similarly, the antioxidant activity of MGs after digestion was higher compared to NEs. Thus, the encapsulation of yarrow phenolic compounds using acidified MGs can be proposed as a strategy to improve their stability during gastrointestinal digestion and consequently, enhanced their bioaccessibility. In addition, such matrices could be employed to develop new dairy products enriched with phenolic compounds.

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ABBREVIATIONS

NEs, nanoemulsions, MGs, milk gels; YE, yarrow extract; EtOH; ethanol; UAE, Ultrasound-Assisted Extraction; DCQA, dicaffeoylquinic acid; HPH, high pressure homogenization; NaCas, sodium caseinate; DLS, dynamic light scattering; GDL; glucono delta-lactone; G', storage modulus; G"; loss modulus; tan δ, loss tangent value; EE, encapsulation efficiency; PVDF, polyvinylidene fluoride; TEAC, Trolox equivalent antioxidant capacity; LSD, least significant difference.

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Publicación 7

Supercritical anti-solvent fractionation for improving antioxidant and anti-inflammatory activities of *Achillea millefolium* L. extracts.

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ABSTRACT

Achillea millefolium L. is a plant widely used in traditional medicine. Nowadays, there is a growing concern about the study of its bioactive properties in order to develop food and nutraceutical formulations. Supercritical anti-solvent fractionation (SAF) of an *A. millefollium* extract was carried out to improve its antioxidant and anti-inflammatory activities. A selective precipitation of phenolic compounds was achieved in the precipitation vessel fractions, which presented an antioxidant activity twice than original extract, especially when fractionation was carried out at 10 MPa. The main phenolic components identified in this fraction were luteolin-7-*O*-glucoside, 3,5-dicaffeoylquinic acid, 6-hydroxyluteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside. However, separator fractions presented higher anti-inflammatory activity than precipitation vessel ones, particularly at 15 MPa. This fact could be related to separator fractions enrichment in anti-inflammatory compounds, mainly camphor, artemisia ketone and borneol. Therefore, SAF produced a concentration of antioxidant and anti-inflammatory compounds that could be used as high-added valued ingredients.

1. INTRODUCTION

Achillea millefolium L. (yarrow) is a flowering plant widely used in folk medicine in Europe. Aqueous and alcoholic extracts from dried upper parts of yarrow have been employed in the treatment of digestive problems, hepato-biliary disorders and externally, for the treatment of skin and mucous membrane inflammation (Dias et al., 2013). The study of this plant, both its composition and biological activities, has awakened a growing interest in order to develop pharmaceutical, food and nutraceutical products. Vitas, Cvetanović, MašKović, Švarc-Gajić & Malbaša (2018) produced kombucha beverages from a yarrow infusion and extracts. In addition, nowadays there are on the market several herbal tea mixtures (containing yarrow), mainly indicated for digestive problems.

Certain naturally occurring bioactive compounds present in *A. millefolium*, such as phenolic compounds, particularly chlorogenic and dicaffeoylquinic acids (DCQA) and flavonoids, as well as those belonging to the volatile oil fraction have been associated with health benefits (Mohammadhosseini, Sarker, & Akbarzadeh, 2017). Moreover, recent reports indicated that *Achillea* genus presents an important antioxidant activity, related to its flavonoids and total phenolic content (Giorgi, Mingozzi, Madeo, Speranza, & Cocucci, 2009; Mohammadhosseini et al., 2017). In addition, Trumbeckaite et al. (2011) reported that the radical-scavenging properties of a hydroalcoholic extract of *A. millefolium* were related to the presence of luteolin and chlorogenic acid in the extract, an in a lesser extent, to the presence of rutin and luteolin-7-*O*-glucoside. *A. millefolium* extracts have also been reported to present anti-inflammatory activity (Tadíc et al., 2017). Moreover, Kazemi (2015) showed that an *A. millefolium* essential oil, with high quantities of thymol and borneol, was able to inhibit nitric oxide production in macrophages stimulated with LPS (lipopolysaccharide).

Different approaches have been carried out in order to obtain fractions with high concentrations of phenolic compounds or essential oils components than original plants extracts; such as applying anion exchange resins (Kammerer, Boschet, Kammerer, & Carle, 2011), high pressure techniques (Fernández-Ponce, Casas, Mantell, & de la Ossa, 2015), membrane separation (Cissé, Vaillant, Pallet, & Dornier, 2011), supercritical fluid extraction with fractionation (Reverchon, & de Marco, 2006)

or chromatography methods (Pedan, Fischer, & Rohn, 2016; Shaheen, Lu, Geng, Shao & Wei, 2017). Recently, supercritical anti-solvent fractionation (SAF) has been proposed for the fractionation of complex plants extracts. In addition, the use of carbon dioxide as a supercritical fluid offers many advantages, such as the low critical temperature of CO_2 and the absence of oxygen during extraction, which allows minimize or avoid the degradation of solutes, as well as the possibility of recovering a free-solvent fraction (Wijngaard, Hossain, Rai, & Brunton, 2012). In the SAF process, a polar liquid solution of a plant extract, containing several families of compounds, is sprayed continuously in a co-current with supercritical CO_2 (SC-CO_2), which acts as antisolvent. This contact allows the precipitation of more polar components from the liquid solution, insoluble in SC-CO_2, whereas the remaining compounds, that are mainly less polar components, remained dissolved and are recovered by downstream pressure reduction (Meneses, Caputo, Scognamiglio, Reverchon, & Adami, 2015).

For that matter, SAF technique has recently used to fractionate phenolic compounds from plants extracts. Therefore, Natolino, Da Porto, Rodríguez-Rojo, Moreno & Cocero (2016) used this technique to obtain fractions enriched in polyphenols from a grape marc extract. Operating at 12MPa, 45 ºC and 0.99 CO_2 molar fraction, they obtained fractions with a relative enrichment of 350% of total polyphenols and a proanthocyanidins enrichment between 300-450%. Visentín, Cismondi, & Maestri (2011) also applied the SAF to improve carnosic acid (CA) recovery from an ethanolic extract of rosemary leaves, obtaining two different fractions, one insoluble with low concentration in CA (<5%) and another resinous extract with 33% of CA. Moreover, Villanueva et al. (2015) carried out the fractionation of green tea extracts obtaining decaffeinated fractions with high concentration in catechins.

Nevertheless, there are only few studies relating the enrichment in phenolic compounds or essential oil components of the fractions obtained by this technique, with their biological activities. Chinnarasu et al. (2015) obtained precipitates with high antioxidant activity from the fractionation of olive leaves extracts and Marqués, Porta, Reverchon, Renuncio and Mainar (2013) concentrated antioxidants from a defatted grape seed waste extract, achieving concentrations up to 2.7 higher than those in the starting solution. Sánchez-Camargo et al. (2016) also enhanced the

antiproliferative activity of a rosemary fraction enriched in carnosic acid and carnosol.

In this context, and based on a previous work (Villanueva-Bermejo et al., 2017), the aim of the present study was to improve the antioxidant and anti-inflammatory activities of an *A. millefollium* extract using SAF technology. Besides, the original extract and fractions obtained were analyzed in order to relate its chemical composition with the biological activities found.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Ethanol (99.5% purity) and Folin-Ciocalteu's reagent were obtained from Panreac (Barcelona, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid for titration (> 97.5%), potassium persulfate (99.9%) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Madrid, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). Reference standards for phenolic compounds, such as chlorogenic acid, cryptochlorogenic acid, diosmetin, ferulic acid, neochlorogenic acid, rosmarinic acid and vitexin (all analytical standard or HPLC purity ≥ 95%) were purchased from Sigma Aldrich (Madrid, Spain). 1,5-Dicaffeoylquinic acid (DCQA), 3,4-DCQA, 3,5-DCQA, 4,5-DCQA, apigenin, caftaric acid, casticin, luteolin, orientin, schaftoside and vicenin II were obtained from Phytolab (Madrid, Spain). Finally, amentoflavone, apigenin-7-*O*-glucoside, caffeic acid, homoorientin, luteolin-7-*O*-*β*-D-glucoside, quercetin and rutin were obtained from Extrasynthese S.A. (Genay, France) and luteolin-7-*O*-*β*-D-glucuronide from HWI Analytic GmbH (Rülzheim, Germany). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain). CO_2 (N38) was purchased from Carburos Metalicos (Madrid, Spain).

2.2. Yarrow samples and Ultrasound-Assisted Extraction (UAE)

A. millefolium from Bulgaria was supplied by a local herbalist (Murcia, Spain). According to supplier specifications, the sample included inflorescences and upper dried leaves of the plant (harvested in spring) and sun-dried (water content < than

5% wt). The plant was ground using a Premill 250 hammer mill (Leal S.A., Granollers, Spain) and sieved (particle size < 500 μm). UAE plant extraction was carried out by using an ultrasonic device (Branson Digital Sonifier 250, Danbury, USA) with a power of 200 W and frequencies of 60 kHz. Extraction conditions employed were ethanol as extraction solvent (1:10 plant/solvent ratio), 30 min time, 40 ºC temperature and an output of 70% with respect to the nominal amplitude. Finally, the extract was concentrated, until the final volume contained 17.9 mg/mL of total solid concentration (2.3% wt.) by rotary evaporation and stored at −20 ºC until its use in the SAF process.

2.3. SAF process

A detailed explanation of the device and the process design employed can be found elsewhere (Villanueva-Bermejo et al., 2017). Briefly, fractionation of UAE yarrow solution (concentration of 17.9 mg/mL) was carried out at two different pressures (10 and 15 MPa), 40 ºC and CO_2/extract flow ratio of 31.3 g/g (50 g/min for CO_2 and 1.6 g/min for UAE extract). The experiment started by pumping SC-CO_2 into the precipitation vessel until the pressure and temperature conditions were attained. Then, the UAE yarrow solution was pumped into the precipitator. After mixing, the yarrow extract components that were not soluble in SC-CO_2 + ethanol mixture precipitated in the precipitation vessel and were collected (precipitation vessel fraction). The fraction soluble in SC-CO_2 + ethanol went to separators where reduced pressure turned CO_2 into a gas and this fraction together with ethanol was also collected. The samples obtained in both separators were combined in a single fraction and ethanol removed by rotary evaporation under vacuum (separator fraction). Fractions were kept at -20 ºC under darkness until analysis.

2.4. HPLC-PAD-ESI-QTOF-MS analysis

Phenolic compounds were analyzed by HPLC following the chromatographic method developed by Villalva et al. (2018). An Agilent HPLC 1260 Infinity series system (Agilent Technologies Inc., Santa Clara, CA, USA) was used for that purpose. Chromatographic separation was carried out by using a reverse phase ACE Excell 3 Super C18 column (150 mm x 4.6 mm, 3 μm particle size) from Advanced Chrmatography Technologies (Aberdeen, Scotland) protected by an ACE 3 C18-AR (10 mm x 3 mm) guard column. Dry samples were dissolved in ethanol to reach a 5 mg/mL concentration and filtered by 0.45 μm polyvinylidene fluoride (PVDF) filter

before injection (20 µL). Retention time and UV-Vis spectrum of each chromatographic peak was compared with analytical standards for identification purpose; moreover, accurate mass from HPLC-ESI-QTOF-MS in negative mode analysis was used for compounds assignment.

Compounds quantification was carried out by using calibration curves from analytical standard, as previously described in Villalva et al. (2018). In addition, luteolin-6,8-di-*C*-glucoside, 6-hydroxyluteolin-7-*O*-glucoside and non-identified flavones were quantified by the calibration curve of orientin, luteolin-7-*O*-glucoside and luteolin respectively. Likewise, schaftoside and vicenin II calibration curves were used for schaftoside isomer and apigenin-*C*-hexoside-*C*-pentoside quantification.

2.5. GC-MS analysis of the separator fractions

The analysis of the UAE extract and SAF fractions collected from the separator was carried out in an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA). The unit comprised a split/splitless injector, a FID detector and a mass spectrometer detector (5975C triple-axis). The analysis was performed using an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm phase thickness) and the following chromatographic method: 40 ºC initial temperature, from 40 ºC to 150 ºC at 3 ºC min^{-1}, isothermal at 150 ºC for 10 min, then increased from 150 to 300 ºC at 6 ºC min^{-1} and finally isothermal at 300 ºC for 1 min. Samples were dissolved in ethanol (at 5 mg/mL), filtered by 0.45 µm filters and inyected (1 µL) in splitless mode. Helium (99.99 %) was employed as carrier gas (1 mL/min flow rate). The temperatures used were 250 ºC for the injector and 230, 280 and 150 ºC for the mass spectrometer ion source, interface and quadrupole, respectively. The mass spectrometer operated under electron impact mode (70 eV) and it was used in total ion current (TIC) mode (mass range from 40 to 500 m/z). The identification of compounds was performed by matching the mass spectral fragmentation patterns with the Wiley 229 mass spectral library, as well as comparing their corresponding retention index to those reported in the literature. Analyses were done in triplicate.

2.6. Determination of total phenolic content (TPC) and antioxidant activity

TPC determination was carried out according to Folin-Ciocalteau reagent method as described by Singleton, Orthofer & Lamuela-Reventos (1999) using gallic acid as standard. In brief, 10 µl of samples (5 mg/mL for all samples, except for P10 and P15 which working solution was 3 mg/mL) were mixed with 50 µL of Folin-Ciocalteau

reagent and 790 µL of deionized water. After 3 min, 150 µL of sodium carbonate solution (20% w/v) were added and mixed. After 2h, the absorbance at 760 nm was recorded. The results were expressed as mg of gallic acid equivalents (GAE)/g dry sample. Analyses were performed at least in triplicate.

The antioxidant activity was measured using the $ABTS^{\bullet+}$ radical scavenging assay as described in Re et al. (1999). The reaction was placed with 990 µL of the diluted $ABTS^{\bullet+}$ radical solution and 10 µL of plant extract dilutions in order to achieve a 20% to 80% of radical inhibition (sample concentration varying from 5 mg/mL to 20 mg/mL). The reaction was allowed to stand until the absorbance reached a plateau, and the absorbance was recorded at 734 nm. Results were expressed as mmol Trolox equivalent/g dry sample (TEAC value). Analyses were done at least in triplicate.

2.7. Anti-inflammatory activity

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were plated at a density of $5x10^5$ cells/mL in 24 wells plates. Culture medium consisted in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Gibco, Paisley, UK) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain) at 37 ºC in 95% humidified air containing 5% CO_2. Monocytes differentiation to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 48 h. THP-1/M cells viability, in presence of yarrow extract or fractions, was tested by MTT assay following Mosmann (1983) method. The assays were performed in triplicate.

After differentiation, THP-1/M cells were washed with PBS and incubated with 0.05 µg/mL of LPS from *E. coli* O55:B5 (Sigma-Aldrich, Spain) in presence of yarrow extract or fractions for 24 h. Then, the supernatants were collected and frozen at -20 ºC. An anti-inflammatory drug, indomethacin (5 µg/mL), was used as a reference.
ELISA kits (BD Biosciences, Aalst, Belgium), according to manufacturer's instructions, were used to measure the release of TNF-α, IL-1β and IL-6 in the supernatants of THP-1/M cells. The quantification was carried out at 450 nm with substrate correction at 570 nm using a multiscanner autoreader (InfiniteM200 Tecan, Barcelona, Spain). The results were expressed as the mean of three determinations ± standard deviation.

2.8. Statistical analysis

Statistical analysis was performed using Statgraphics v. Centurion XVI package for Windows (Statpoint Inc., Warrenton, VA, USA). Statistical differences between samples were analyzed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) procedure was applied to determine significant differences between means at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. TPC content and antioxidant activity of yarrow extract and SAF fractions.

UAE extracts from yarrow were carried out using ethanol as extraction solvent, at 40 ºC and 30 min. Previous studies carried out in the research group (data not shown) showed that those conditions were the most appropriated to obtain extracts with a high content of TPC and an important antioxidant activity. Thus, this UAE extract (called original extract) presented a yield of 5.7± 0.9% (% dry wt.), a TPC of 54.30 ±1.07 mg GAE/g extract and a TEAC value of 0.173 ± 0.004 mmol Trolox/g extract.

SAF process was carried out in order to enhance the antioxidant activity of this extract. Consequently, two different experiments, at 10 and 15 MPa of pressure, 40 ºC and CO_2/extract flow ratio of 31.3 g/g, were developed. These conditions were based in a previous work (Villanueva-Bermejo et al., 2017).

Fractions obtained at 10 and 15 MPa from the precipitation vessel (called P10 and P15) and separators (called S10 and S15) were collected and its yield value, TPC content and antioxidant activity were determined (Table 1). Results indicated that SAF process achieved an important phenolic compounds enrichment in the precipitation vessel fractions. It is worth to mention that this enrichment was significantly higher when used pressure was 10 MPa, where P10 fraction presented an antioxidant activity 2-fold superior than the original extract. Regarding separator fractions, S10 and S15 showed a similar antioxidant activity and near 5-fold lower than original extract. This result was related to the lower quantity of TPC presented in these fractions.

Table 1. Yield values (mass precipitated/mass of solid pumped), TPC content and antioxidant activity (TEAC value) in original extract and SAF fractions: P10 (precipitation vessel fraction at 10 MPa), P15 (precipitation vessel fraction at 15 MPa, S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa). Data shown represents mean ± S.D. (n=3).

Sample	Yield (%)	TPC (mg GAE/g)	TEAC value (mmol Trolox/g)
Original extract	5.7 ± 0.9	54.30 ± 1.07 ^{c}	0.173 ± 0.004 ^{c}
P-10	6.6 ± 1.1	136.44 ± 1.28 ^{a}	0.345 ± 0.002 ^{a}
S-10	52.1 ± 5.3	30.30 ± 0.25 ^{d}	0.035 ± 0.002 ^{d}
P-15	13.3 ± 2.4	119.20 ± 2.23 ^{b}	0.317 ± 0.003 ^{b}
S-15	42.6 ± 4.2	23.83 ± 0.85 ^{e}	0.033 ± 0.002 ^{d}

$^{a-e}$ Different superscript letters denote statistical differences between samples within the same column. Significance level at $p \leq 0.05$ with Fisher's Least Significant Difference (LSD) test.

Therefore, phenolic composition of the original extract and fractions were analyzed in order to establish a relationship between its composition and the antioxidant activity found.

3.2. Phenolic characterization of yarrow extract and SAF fractions.

The HPLC analysis of phenolic compounds in the original extract and SAF fractions are shown in Table 2. The main compounds identified in the original extract were flavonoids, either in glycosylated or in aglycone form, and phenolic acids.

Therefore, the compounds presented in higher concentration corresponded to the flavonoids luteolin and its glycosylated form (luteolin-7-*O*-glucoside), as well as the dicaffeoylquinic acids, where the 3,5-DCQA stood out. These results are in agreement with others previously reported, where the main flavonoids found in several yarrow extracts were luteolin-7-*O*-glucoside, luteolin, apigenin-7-*O*-glucoside and apigenin, while within the dicaffeoylquinic acids, 3,5-DCQA was the one in a greater extent (Benedek, Gjoncaj, Saukei, & Kopp, 2007; Vitalini et al., 2011).

Table 2. Phenolic composition of original extract and SAF fractions (mg compound/g dry fraction). P10 (precipitation vessel fraction at 10 MPa), P15 (precipitation vessel fraction at 15 MPa), S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa). Data shown represents mean ± S.D. (n=3).

Compound	Original	P-10	S-10	P-15	S-15
Neochlorogenic acid	0.06 ± 0.00	0.18 ± 0.00$^{*}_{a}$	-	0.14 ± 0.00$^{*}_{b}$	-
Caftaric acid	< L.Q.	0.18 ± 0.00$^{*}_{b}$	-	0.22 ± 0.00$^{*}_{a}$	-
Chlorogenic acid	0.62 ± 0.00	2.32 ± 0.0 $^{*}_{a}$	0.10 ± 0.00*	1.91 ± 0.04$^{*}_{b}$	-
Cryptochlorogenic acid	0.01 ± 0.01	0.03 ± 0.00 $_{a}$	-	0.04 ± 0.01 $_{a}$	-
Vicenin II	0.37 ± 0.01	1.61 ± 0.00$^{*}_{a}$	-	1.12 ± 0.01$^{*}_{b}$	-
Caffeic acid	0.17 ± 0.00	-	0.19 ± 0.00$^{*}_{A}$	-	0.18 ± 0.00$^{*}_{B}$
Schaftoside isomer	0.26 ± 0.00	1.49 ± 0.05$^{*}_{a}$	-	0.90 ± 0.00$^{*}_{b}$	-
Schaftoside	0.27 ± 0.00	1.36 ± 0.00$^{*}_{a}$	-	0.89 ± 0.00$^{*}_{b}$	-
Homoorientin	0.02 ± 0.01	0.32 ± 0.00$^{*}_{a}$	-	0.16 ± 0.00$^{*}_{b}$	-
Apigenin-*C*-hexoside -*C*-pentoside	0.30 ± 0.00	1.43 ± 0.01$^{*}_{a}$	-	0.85 ± 0.00$^{*}_{b}$	-
Luteolin-6,8-di-*C*-glucoside	0.47 ± 0.00	2.39 ± 0.01$^{*}_{a}$	-	1.51 ± 0.00$^{*}_{b}$	-
6-hydroxyluteolin-7-*O*-glucoside	1.45 ± 0.01	7.65 ± 0.02$^{*}_{a}$	0. 02 ± 0.00*	4.66 ± 0.00$^{*}_{b}$	-
Rutin	0.51 ± 0.01	1.44 ± 0.00$^{*}_{a}$	-	1.34 ± 0.02$^{*}_{b}$	-
Vitexin	0.13 ± 0.01	0.15 ± 0.00$^{*}_{b}$	-	0.25 ± 0.01$^{*}_{a}$	-
Luteolin-7-*O*-glucoside	7.69 ± 0.08	33.2 ± 0.07$^{*}_{a}$	0.39 ± 0.00*	23.9 ± 0.97$^{*}_{b}$	-
Luteolin-7-β-glucuronide	0.20 ± 0.01	0.56 ± 0.00$^{*}_{a}$	-	0.59 ± 0.03$^{*}_{a}$	-
Ferulic acid	0.08 ± 0.02	0.09 ± 0.00$^{*}_{a}$	-	0.04 ± 0.00$^{*}_{b}$	-
3,4-DCQA	0.38 ± 0.05	1.23 ± 0.00$^{*}_{a}$	-	0.69 ± 0.00$^{*}_{b}$	-
1,5-DCQA	0.69 ± 0.01	2.86 ± 0.01$^{*}_{a}$	-	1.80 ± 0.09$^{*}_{b}$	-
3,5-DCQA	3.62 ± 0.02	17.8 ± 0.03$^{*}_{a}$	0.28 ± 0.01$^{*}_{A}$	11.6 + 0.10$^{*}_{b}$	0.10 ± 0.00$^{*}_{B}$
Apigenin-7-*O*-glucoside	1.79 ± 0.01	6.89 ± 0.03$^{*}_{a}$	0.32 ± 0.00*	5.88 ± 0.01$^{*}_{b}$	-
4,5-DCQA	0.97 ± 0.01	4.30 ± 0.01$^{*}_{a}$	0.05 ± 0.00*	3.19 ± 0.00$^{*}_{b}$	-
Rosmarinic acid	0.18 ± 0.00	-	-	-	-
Luteolin	4.47 ± 0.01	4.58 ± 0.01$^{*}_{b}$	3.18 ± 0.00$^{*}_{A}$	13.0 ± 0.00$^{*}_{a}$	0.95 ± 0.01$^{*}_{B}$
Quercetin	0.47 ± 0.00	0.68 ± 0.00$^{*}_{b}$	0.32 ± 0.01*	1.44 ± 0.01$^{*}_{a}$	-
Flavone n.i.	1.66 ± 0.00	1.15 ± 0.00$^{*}_{b}$	1.42 ± 0.00$^{*}_{A}$	3.33 ± 0.00$^{*}_{a}$	1.16 ± 0.00$^{*}_{B}$
Apigenin	1.96 ± 0.00	1.00 ± 0.00$^{*}_{b}$	1.83 ± 0.00$^{*}_{A}$	4.74 ± 0.00$^{*}_{a}$	0.93 ± 0.00$^{*}_{B}$
Diosmetin	0.50 ± 0.00	0.31 ± 0.00$^{*}_{b}$	0.53 ± 0.00$^{*}_{A}$	0.73 ± 0.00$^{*}_{a}$	0.50 ± 0.00$^{*}_{B}$
Amentoflavone	0.42 ± 0.00	0.16 ± 0.00$^{*}_{b}$	0.52 ± 0.00$^{*}_{B}$	0.41 ± 0.00$^{*}_{a}$	0.62 ± 0.00$^{*}_{A}$
Flavone n.i.	2.14 ± 0.00	0.57 ± 0.00$^{*}_{a}$	2.68 ± 0.00$^{*}_{B}$	0.57 ± 0.00$^{*}_{a}$	3.68 ± 0.00$^{*}_{A}$
Casticin	0.29 ± 0.00	-	0.44 ± 0.01$^{*}_{B}$	-	0.62 ± 0.01$^{*}_{A}$

< L.Q.: below limit of quantification. *An asterisk indicates statistical differences between original extract and fractions. ^{a,b} Different lowercase letters denote statistical differences between P10 and P15 fractions. ^{A,B} Different capital letters denote statistical differences between S10 and S15 fractions. Significance level at $p \leq 0.05$ with Fisher's Least Significant Difference (LSD) test.

Related to precipitation vessel fractions, its principal components were luteolin-7-*O*-glucoside, 3,5-DCQA, luteolin, apigenin-7-*O*-glucoside and 6-hydroxyluteolin-7-*O*-glucoside, representing between 73% (P10) and 60% (P15) of all phenolic compounds identified. Meanwhile, separator fractions contained a reduced quantity of phenolic compounds, where flavonoids aglycones stood out.

Precipitation vessel fractions and the original sample presented a similar phenolic composition, although the compounds concentration in the fractions was, in general, much higher, highlighting luteolin-7-*O*-glucoside and 3,5-DCQA. These results could be related to the low solubility of glycosylated flavonoids and phenolic acids in the SC-CO_2 + ethanol mixture, since these compounds are poorly soluble in low-polar solvents (Chebil et al., 2007). On the other hand, the flavonoids aglycones, as less polar compounds, would be more soluble in the mixture SC-CO_2 + ethanol and therefore they would be dragged to the separator fraction.

However, it should be noted that there were some differences between fractions P10 and P15, since P10 fraction presented a greater amount of luteolin-7-*O*-glucoside and 3,5-DCQA than P15 fraction, whereas P15 contained a higher quantity of luteolin and apigenin than P10. These results could indicate that an increase in pressure during the fractionation process, would increment the presence of aglycones in the precipitation vessel fraction. In order to explain these results, it must be taken into account the complex multicomponent structure of extracts, comprising substances in a wide polarity range and with different solubility in SC-CO_2, which could exert a strong effect regarding the partial solubility of compounds involved and their precipitation behavior. Nevertheless, observing the solubility behavior of pure compounds in SC-CO_2, it can be established that in the case of more polar compounds (e.g. hydroxycinnamic acids) an increase of pressure from 10 to 15 MPa resulted in 4.4-5.4 solubility increase (Murga, Sanz, Beltran & Cabeza, 2003), while for less polar compounds (e.g. quercetin) this ratio was about 3.0-3.5 (Chafer, Fornari, Berna &Stateva, 2004). Thus, according to the pure component solubility behavior, an increase of the precipitation pressure could increase the recovery of aglycones in precipitation vessel.

Thus, the higher antioxidant activity found in P10 and P15, compared to original extract, could be related with the significant increase in several phenolic compounds found in these fractions, mainly luteolin-7-*O*-glucoside, 3,5-DCQA, luteolin (only in P15), apigenin-7-*O*-glucoside and 6-hydroxyluteolin-7-*O*-glucoside. Besides, when comparing P10 and P15, it can be observed that P10, with the higher antioxidant activity, also presented the higher quantity of luteolin-7-*O*-glucoside and 3,5-DCQA (representing almost 55% of the fraction). Kim et al. (2011) indicated that 3,5-DCQA presented an important antioxidant activity, significantly higher than chlorogenic acid. Besides, the antioxidant activity of luteolin-7-*O*-glucoside have also been reported (Song & Park, 2014; Antonisamy et al., 2016). However, it must be taken into account that this fact could be also due to synergies among all the phenolic compounds found in P10 fraction.

3.3. Anti-inflammatory activity of yarrow extract and SAF fractions.

First, original extract and SAF fractions were evaluated for cytotoxicity on THP-1/M cells by MTT method. Results showed that, at the higher concentration used in the anti-inflammatory assays, 10 µg/mL, neither original extract nor SAF fractions presented cytotoxicity (cell viability ≥ 95%). Indomethacin at 5 µg/mL also presented no cytotoxicity.

The activation of THP-1/M was carried out with the addition of LPS to the medium. Fig. 1 showed that these LPS treated cells (positive control), after an incubation period of 24h, presented an important increase in the release of TNF-α, IL-1β and IL-6, compared to non-activated controls (negative control). When THP-1/M were activated with LPS in presence of 5 and 10 µg/mL of original extract and SAF fractions, a decrease in TNF-α secreted level was observed (Fig. 1), compared with positive control. Moreover, 5 µg/mL of original extract inhibited TNF-α secretion in a 40%. Regarding P10 and P15 fractions, P15 presented a decrease in TNF-α superior to P10 and similar to that obtained with original extract. However, it should be noted that the greatest decrease in TNF-α secretion was achieved in presence of S10 and S15 fractions. Thus, 5 µg/mL of S15 fraction reduced TNF-α release in a 70%.

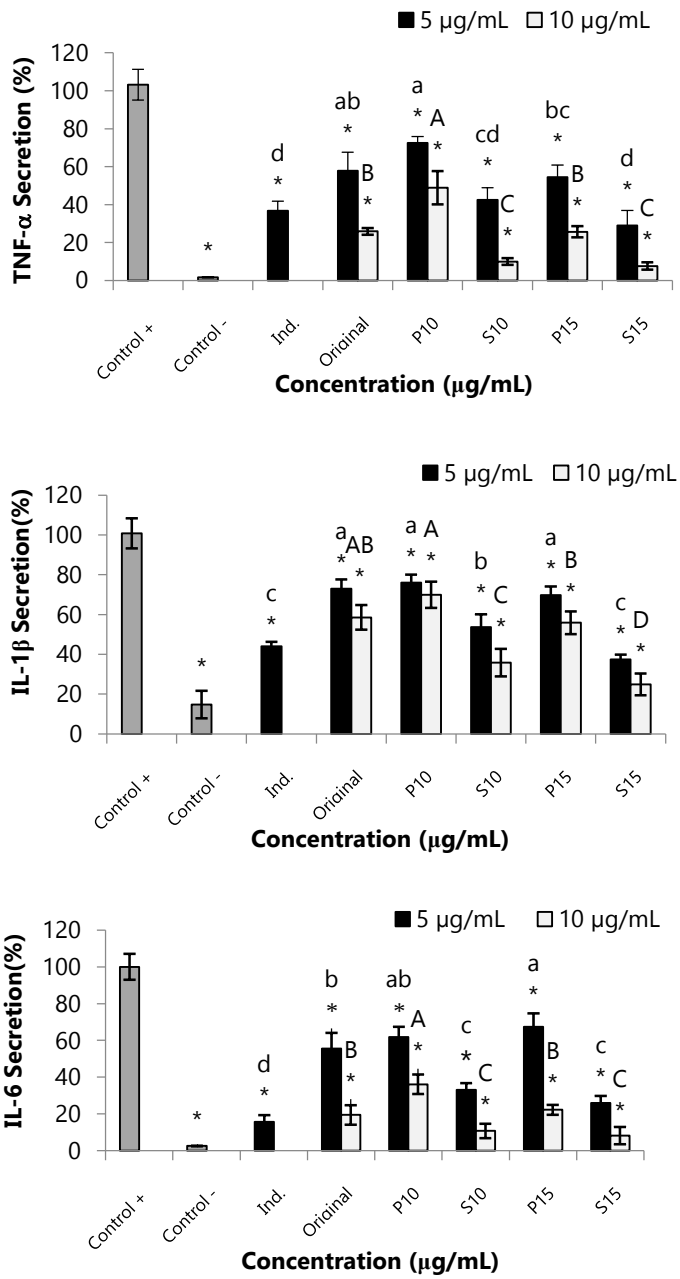

Figure 1. Levels of TNF-α, IL-1β and IL6 secreted by THP-1/M activated with LPS in presence of original extract and SAF fractions. Positive control: cells stimulated with LPS but in absence of extract. Negative control: cells in contact just with RPMI media. Ind.: Indomethacin. Original: original yarrow extract. P10: precipitation vessel fraction at 10 MPa. P15: precipitation vessel fraction at 15 MPa. S10: separator fraction at 10 MPa. S15: separator fraction at 15 MPa. Each bar is the mean of three determinations ± S.D. *Denotes statistical differences when compares with positive control. ^{a,b,c,d,} Different lowercase letters indicate statistical differences between samples at 5 µg/mL. ^{A,B,C,D} Different capital letters indicate statistical differences between samples at 10 µg/mL. Significance level at $p \leq 0.05$ with Fisher's Least Significant Difference (LSD) test.

In the same way, the original extract and SAF fractions inhibited the IL-1β secretion by activated cells, at both concentrations employed, although separator fractions showed the greatest inhibition (Fig. 1). Thus, meanwhile 10 µg/mL of original extract decrease IL-1β release in a 40%, the same concentration of S15 showed an inhibition near to 70%. In addition, 5 µg/mL of S15 reduced IL-1β secretion by a 60%. The obtained results for the IL-6 release in presence of samples (Fig. 1) were similar to those obtained for TNF-α and IL-1β, since S10 and S15 fractions were the most active and produced an important decrease in the IL-6 release (near to basal levels).

These results indicated that all SAF fractions presented anti-inflammatory activity, although separator fractions were much more active than precipitation vessel ones. The anti-inflammatory activity of precipitation vessel fractions could be related to their content in phenolic compounds, more specifically with dicaffeoylquinic acids, luteolin, apigenin and its glycosides, since the anti-inflammatory effects of these compounds have been previously described (Liang & Kitts, 2016; Wang et al, 2014; Wang et al., 2017). Moreover, Francisco et al. (2014) indicated that luteolin-7-*O*-glucoside presented a certain anti-inflammatory activity, but lower than that observed with the luteolin aglycone in LPS-stimulated macrophages. Similarly, Choi et al. (2014) reported that apigenin presented higher anti-inflammatory activity than other naturally occurring *C*-glycosylated derivatives of apigenin. These results could explain the higher inhibition of TNF-α, IL-1β and IL-6 secretion found when using P15 compared with P10, since P15 contained a higher amount of luteolin and apigenin aglycones, whereas P10 presented a higher quantity of luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside.

However, the higher anti-inflammatory activity found in separator fractions, could not be related to its phenolic compounds content, due to the low quantity of these compounds presented in these fractions. Thereby, it´s relevant to notice that conditions used to obtain the separator fractions allowed the enhancement of these fractions in essential oil components. Since essential oils from *A. millefolium* have been reported to present anti-inflammatory activity (Chou, Peng, Hsu, Lin & Shih, 2013; Kazemi, 2015), S10 and S15 were analyzed by GC-MS in order to establish a relationship between the composition and the anti-inflammatory activity of these fractions.

3.4. GC-MS characterization of separator fractions

In order to identify the compounds involved in the anti-inflammatory activity found in separator fractions (S10 and S15), a characterization by GC-MS of these fractions, along with the original extract was performed. The identification of the main volatile compounds presented in the sample (Table 3) was performed based on the comparison of their mass spectra and retention index (RI). As can be observed, 30 identified and 2 non-identified compounds were found in S10 and S15.

In general, a very similar GC-MS profile was obtained for both fractions, being camphor, artemisia ketone, borneol and 2,6-dimethyl-1,7-octadiene-3,6-diol the most abundant compounds in both fractions. Original yarrow extract also presented a similar profile to S10 and S15, although total chromatographic area (expressed as Σ AUC) was much higher for the fractions. As expected, separator fractions have been enriched in essential oil components (1.75 times for S10 and 2.18 for S15), in comparison to the original extract.

Regarding main components, camphor and borneol were shown to reduce TNF-α, IL-1β and IL-6 secretion in THP-1 macrophages stimulated with LPS or ox-LDL (oxidized low-density lipoproteins) (Arranz et al., 2014a; Arranz et al., 2014b). Similarly, Rungqu et al. (2016) reported that an essential oil containing a 37.5% of artemisia ketone presented an important anti-inflammatory activity, evaluated in rats, using egg albumin-induced paw edema.

Therefore, the anti-inflammatory activity exhibited by S10 and S15 fractions could be mainly related to the presence of these three compounds (camphor, borneol and artemisia ketone) that represented approximately 30% of the fractions. However, the contribution to other anti-inflammatory compounds presented in smaller quantities, such as eucalyptol and β-linalool, to this activity cannot be ruled out. Thus, the enrichment of S10 and S15 fractions in compounds that exhibit anti-inflammatory activity, regarding to the original extract, would explain the higher anti-inflammatory activity of these fractions. Accordingly, S15 fraction that presented a higher enrichment in essential oil compounds than S10, also presented a higher anti-inflammatory activity.

Table 3. GC-MS identification, peak area contribution (%), and retention index (RI) of compounds found in original extract and separator fractions. S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa).

RI	Compound	Original	S10	S15
997	Yomogi alcohol	2.1	2.0	2.2
1028	Eucalyptol	4.3	3.5	3.4
1037	γ-Vinyl-γ-valerolactone	1.8	1.6	1.5
1058	Artemisia ketone	11.0	9.9	9.4
1070	1,2-Epoxylinalool	0.9	0.8	0.8
1079	Artemisia alcohol	1.0	0.8	0.8
1084	*cis*-Linalool oxide	0.8	0.6	0.7
1099	β-Linalool	0.9	0.9	0.9
1136	Camphor	13.8	12.5	12.3
1141	*cis*-Verbenol	0.9	0.7	0.7
1160	Borneol	8.7	8.9	8.6
1171	2-Methyl-2-octen-4-ol	1.0	0.9	1.0
1174	Terpinene-4-ol	0.5	0.4	0.5
1182	*p*-Cymen-8-ol	0.7	0.8	0.9
1188	3,7-dimethyl-1,5-Octadiene-3,7-diol	5.9	6.1	6.2
1200	Verbenone	0.5	0.6	0.6
1212	Fragranol	0.4	0.4	0.4
1218	2-Hydroxycineole	0.6	0.6	0.7
1236	*trans*-Chrysanthenyl acetate	2.4	2.5	2.5
1250	Piperitone	0.9	0.9	1.0
1261	(5*E*)-5,9-Dimethyl-5,8-decadien-2-one	1.5	1.4	1.3
1276	2,6-Dimethyl-1,7-octadiene-3,6-diol	8.6	9.3	9.3
1280	Bornyl acetate	0.8	1.0	1.0
1284	n.i.	7.2	8.7	8.5
1393	Jasmone	1.6	1.6	1.5
1412	β-Caryophyllene	1.4	1.6	1.5
1569	Spathulenol	0.9	1.2	1.1
1578	Caryophyllene oxide	4.9	6.0	5.8
1640	β-Eudesmol	1.2	1.2	1.5
1810	Saussurea lactone	3.4	3.2	3.2
1845	Hexahydrofarnesyl acetone	3.0	3.2	3.4
2069	n.i.	6.5	6.2	6.7
Σ AUC		**24.41 10^6**	**42.80 10^6**	**53.40 10^6**

n.i.: no identified. AUC, area under curve.

4. CONCLUSION

Supercritical anti-solvent fractionation of an ethanolic yarrow extract resulted an adequate method to improve its antioxidant and anti-inflammatory activities. Thus, a selective precipitation of phenolic compounds increased its antioxidant activity twice, compared to original extract, especially when fractionation was carried out at 10 MPa. Regarding anti-inflammatory activity, separator fractions presented higher anti-inflammatory activity than precipitation vessel ones. This fact was related to separator fractions enrichment in essential oil compounds with anti-inflammatory activity. Being more active, in this case, the separator fraction obtained when pressure was 15 MPa.

Therefore, this study pointed out the feasibility of SAF process as a green technology in order to achieve a fractionation of compounds with different biological activities. This fact could be a useful tool for food or nutraceutical products design.

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ABBREVIATIONS

SAF; Supercritical anti-solvent fractionation; DCQA, Dicaffeoylquinic acid; SC-CO_2 Supercritical CO_2; UAE, Ultrasound-Assisted extraction; GAE; Gallic acid equivalents; THP-1/M, Human THP-1 monocytes differentiated to macrophages.

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5. DISCUSIÓN GENERAL

5. Discusión General

En las últimas décadas se ha producido un notable incremento en el número de investigaciones relacionadas con el uso de las plantas como fuente de compuestos beneficiosos para la salud (Premkumar y Vasudevan, 2018). No obstante, con tal fin es necesario establecer qué metabolitos concretos, de todos los generados por las plantas, son los responsables de su posible acción beneficiosa. De entre estos metabolitos, los compuestos fenólicos se han estudiado ampliamente poniéndose de manifiesto su actividad antimicrobiana, antiproliferativa, antiinflamatoria, entre otras, y en particular antioxidante (Ferreira y col., 2017); asociándose estas actividades con sus características estructurales (Pan y col., 2010; Del Río y col., 2013). Así, el uso de los compuestos fenólicos como ingredientes bioactivos es uno de los temas que mayor interés han despertado dentro del desarrollo de alimentos o complementos alimenticios.

Dentro de la gran variedad de fuentes naturales de compuestos fenólicos destacan las familias Lamiaceae y Asteraceae, de las cuales se distinguen algunas especies por sus características aromáticas y saborizantes, además de ser reconocidas ampliamente como plantas medicinales (Bessada y col., 2015; Costa y col., 2015). Por otra parte, de cara a la obtención de los compuestos fenólicos a partir de matrices vegetales, hoy en día se está potenciando el uso de técnicas sostenibles de extracción, dentro de los principios de la llamada *química verde*, aplicable sobre todo al uso de disolventes y tecnologías alternativas (Chemat y col., 2012).

En este contexto, en el ***Capítulo 1, Publicación 1***, se propuso el empleo de tecnologías sostenibles como la extracción con fluidos supercríticos y la extracción asistida con ultrasonidos, para la obtención de extractos con propiedades antioxidantes y/o antiinflamatorias de cuatro plantas seleccionadas: *Melissa officinalis* L. y *Origanum majorana* L. (melisa y mejorana, familia Lamiaceae), y *Calendula officinalis* L. y *Achillea millefolilum* L. (caléndula y milenrama, familia Asteraceae). Los extractos obtenidos por SFE presentaron una baja actividad antioxidante, al igual que un bajo contenido en compuestos fenólicos totales. Por su parte, los extractos UAE, en particular aquellos obtenidos con etanol:agua (50:50,

v/v), se diferenciaron por un mayor TPC y, por consiguiente, una mayor actividad antioxidante, destacando los de mejorana seguida de la melisa.

En el análisis de los compuestos fenólicos individuales de los extractos UAE-50% etanol (UAE-50) de mejorana y melisa, se observó que ambas plantas se caracterizaron por la abundancia del ácido rosmarínico. Sin embargo, en la mejorana se encontraron además una gran variedad de flavonoides, mayoritariamente derivados glucosilados de luteolina, como la 6-hidroxiluteolina-7-*O*-glucósido. Por lo que, la alta actividad antioxidante de los extractos UAE-50 de mejorana se relacionaría al gran contenido en compuestos fenólicos, pero específicamente a su abundancia en RA y 6-hidroxiluteolina-7-*O*-glucósido (López-Lázaro, 2009; Miron y col., 2013).

También se ha evidenciado que los extractos UAE 100% etanol, presentaron una destacada actividad antiinflamatoria, sobre todo los obtenidos a partir de milenrama. Así, con respecto al extracto UAE 100% etanol de milenrama, su composición presentaba un contenido muy importante del ácido 3,5-DCQA seguido de luteolina-7-*O*-glucósido, lo cual podría relacionarse con el efecto antiinflamatorio observado en este extracto (Hong y col., 2015). Además, se puede pensar que la presencia de agliconas de otros flavonoides como la luteolina, casticina y centaureidina, igualmente con reconocida actividad antiinflamatoria, podrían contribuir a dicha bioactividad (Jachak y col. 2011; Francisco y col., 2014; Chan y col., 2018). Por otra parte, teniendo en cuenta que ciertos compuestos del aceite esencial de milenrama se han relacionado también con su actividad antiinflamatoria (Chou y col., 2013; Abdossi y Kazemi, 2016), se realizó el análisis de la fracción volátil del extracto etanólico de milenrama. Así, en dicha fracción volátil, se obtuvo una cantidad importante de borneol y alcanfor que también podrían contribuir a la actividad anti-inflamatoria de este extracto (Arranz y col., 2014b; Abdossi y Kazemi, 2016).

Por lo tanto, en este primer estudio se determinó que tanto la mejorana como la milenrama eran las plantas con el mayor potencial antioxidante y/o antiinflamatorio, las cuales se eligieron para continuar los posteriores estudios en esta tesis doctoral.

El ***Capítulo 2*** de esta tesis está dedicado al desarrollo de estrategias y formulaciones que permitan mejorar la biodisponibilidad de los compuestos

fenólicos presentes en la mejorana. En un primer paso, con el fin de optimizar la extracción del RA, uno de los compuestos bioactivos mayoritarios en mejorana (Jungbauer y Medjakovic, 2012), la técnica UAE se comparó con otra técnica sostenible de extracción, la extracción con líquidos presurizados (***Capítulo 2, Publicación 2***). Así, la PLE bajo condiciones moderadas de temperatura (100 ºC) y el uso de etanol:agua (70:30, v/v), permitió la obtención de un extracto de mejorana con aproximadamente 3,4% de RA. Esta técnica resultó además favorable en términos del rendimiento de extracción y actividad antioxidante, por lo que los posteriores estudios se realizaron con este extracto óptimo de mejorana (PLE-70).

En este trabajo también se realizó un estudio para evaluar la encapsulación del extracto PLE-70 en diferentes matrices de tal manera que permitiesen su posible uso como ingrediente funcional. Así, se seleccionaron matrices proteicas utilizadas en formulaciones dirigidas a consumo humano como las caseínas de la leche y a los aislados de proteína de soja (Livney, 2010; Tang, 2017). La eficiencia de encapsulación del extracto, medida respecto al RA por ser el compuesto más abundante, fue mayor cuando se utilizaron los SPI que con las dispersiones de caseína. Este resultado coincide con el mostrado por otros autores, donde la encapsulación de un compuesto fenólico, como la curcumina, con el uso de SPI resultó más eficaz que la caseína (Chen y col., 2015). Por otra parte, al evaluarse el efecto inmunomodulador de los complejos formulados, se observó que ambas matrices proteicas no alteraban la acción ejercida por el extracto. Así, estos resultados podrían estar relacionados, al menos en parte, con el efecto antiinflamatorio del RA observado también en diferentes líneas celulares (Jiang y col., 2009; Lembo y col., 2014). De esta manera, se puede considerar que las matrices proteicas seleccionadas en este estudio podrían representar una opción segura como portadores del extracto de mejorana con un potencial uso para la formulación de alimentos con propiedades saludables (Arranz y col., 2015; Chen y col., 2015).

En un segundo estudio ***(Capítulo 2, Publicación 3)*** se abordó el uso de estrategias de enriquecimiento en compuestos fenólicos con el fin de incrementar la actividad biológica del extracto seleccionado PLE-70 de mejorana. Este trabajo se centró en el RA por ser uno de los compuestos fenólicos más abundantes y con mayor actividad biológica (Hossain y col., 2014). Dicho enriquecimiento se llevó a cabo mediante el uso de resinas de adsorción XAD-7HP. En este sentido, el uso de resinas macrospóricas de adsorción se ha planteado previamente como una

estrategia adecuada para la obtención de fracciones enriquecidas en compuestos fenólicos; asimismo, las resinas de adsorción representan una técnica sostenible y fácilmente escalable (Pérez-Larrán y col., 2018). De esta manera, el uso de la resina XAD-7HP permitió incrementar el contenido en RA, entre otros compuestos fenólicos, de aproximadamente 1,5-1,8 veces en comparación con el extracto PLE, observándose el mismo efecto en la actividad antioxidante.

Teniendo en cuenta que para que los compuestos fenólicos ejerzan su acción beneficiosa en el organismo, deben ser biodisponibles, se llevó a cabo la digestión *in vitro* de los extractos de mejorana (PLE-70 y el enriquecido en compuestos fenólicos), seguida de su absorción intestinal usando el modelo de células Caco-2 diferenciadas (Soler-Rivas y col., 2010). Así, tras el proceso de digestión, el RA, en ambos extractos, sufrió una pérdida aproximada del 50%, demostrando que su estabilidad se vería comprometida tras la digestión, como se ha mostrado previamente por otros autores (Gayoso y col., 2016; Zoric y col., 2016). Igualmente, los compuestos flavonoides mayoritarios, la 6-hidroxiluteolina-7-*O*-glucósido y luetolina-*O*-glucósido, se vieron afectados incluso en mayor medida que el RA. Al tratarse de una matriz compleja, otros compuestos fenólicos apenas registraron cambios e incluso se detectaron nuevos metabolitos, como el ácido neoclorogénico y el ácido *p*-cumárico, relacionados posiblemente con degradaciones e isomerizaciones de los ácidos hidroxicinámicos presentes en el extracto, como el RA y el ácido criptoclorogénico (Baba y col., 2005; Xie y col., 2011).

Tras los ensayos de absorción realizados con las células Caco-2, el RA se presentó como el compuesto más abundante en el compartimiento basolateral, seguido de la luteolina-7-*O*-glucósido y la 6-hidroxiluteolina-7-*O*-glucósido. Se ha estudiado con anterioridad la absorción del RA, proponiéndose distintos mecanismos en su transporte celular. Así, se ha demostrado su transporte por difusión pasiva, mediante la vía paracelular (Qiang y col., 2011), pero también se ha descrito que ciertas proteínas transportadoras podrían estar involucradas en su absorción (Falé y col., 2013).

Finalmente, se determinó la bioactividad en la fracción basolateral (biodisponible), observándose que dicha fracción presentaba actividad antioxidante y antinflamatoria. Estos resultados podrían relacionarse con los compuestos

fenólicos presentes en la fracción biodisponible donde destaca el RA (Hossain y col., 2011; Lembo y col., 2014).

El ***Capítulo 3*** de la presente tesis doctoral, se orientó al desarrollo de estrategias y formulaciones que permitieran mejorar la biodisponibilidad de los compuestos fenólicos presentes en la milenrama. Un enfoque parecido al *Capítulo 2*, respecto a la optimización de una composición fenólica con actividad biológica relevante, mediante el uso de técnicas de extracción sostenible y su posterior enriquecimiento en compuestos fenólicos, se siguió con la planta de milenrama. Así, derivado de la comparación entre las técnicas UAE y PLE, se eligió como extracto óptimo el obtenido con UAE y 100% de etanol (UAE-100), principalmente por sus propiedades antiinflamatorias destacadas (***Capítulo 3, Publicación 4***). En el fraccionamiento de los compuestos fenólicos, mediante resinas de adsorción XAD-7HP, se utilizaron distintas mezclas de etanol y agua, como eluyente, dando lugar a distintas fracciones. En la fracción recuperada con el 80% de etanol (F-80) se logró un enriquecimiento de la gran mayoría de los compuestos fenólicos individuales de milenrama, siendo el 3,5-DCQA el más abundante, seguido de luteolina-7-*O*-glucósido y 4,5-DCQA. Dicha fracción destacó por presentar la mayor actividad antioxidante y antiinflamatoria que podría relacionarse con la presencia de estos compuestos (Park y col., 2013; Hong y col., 2015); sin embargo, es probable que otros compuestos, como las agliconas de flavonoides, casticina, centaureidin y luteolina también contribuyan con esta bioactividad (Jachak y col., 2011; Liou y col., 2014; Wang y col., 2017). Asimismo, tras el análisis de los compuestos volátiles de F-80 se encontraron, en una gran proporción, algunos sesquiterpenos con reconocida actividad antiinflamatoria, tales como el camazuleno y β-eudesmol (Seo y col., 2011; Zargaran y col., 2014).

En el ***Capítulo 3, Publicación 5*** se determinó la biodisponibilidad tanto del extracto etanólico de milenrama obtenido por UAE, como de la F-80 mediante una digestión *in vitro* y posteriorabsorción intestinal en células Caco-2. En la primera etapa de este estudio, se analizó la estabilidad de los diferentes compuestos fenólicos durante las tres fases de digestión: oral, gástrica e intestinal; evidenciándose que en las últimas dos fases los compuestos fenólicos se mostraron más susceptibles a modificaciones, principalmente en la intestinal. Uno de los compuestos más afectados, tras la etapa intestinal, fue el 3,5-DCQA, degradándose aproximadamente un 63%, aunque hay que destacar que tras esta etapa también se

produjo un incremento de sus isómeros 4,5-DCQA y 3,4-DCQA. Asimismo, un efecto similar se observó para el ácido clorogénico, dando lugar a un incremento de los ácidos neoclorogénico y criptoclorogénico tras la etapa intestinal. Por otra parte, la luteolina-7-*O*-glucósido, uno de los compuestos mayoritarios en los extractos de milenrama, apenas se vio disminuido tras la digestión intestinal. Estos cambios detectados en algunos compuestos fenólicos, especialmente en los derivados del ácido dicafeoilquínico, podrían estar ocasionados principalmente por el pH de la fase intestinal (Bouayed y col., 2012; D'Antuono y col., 2015). Después de la digestión, también se registró una disminución de la actividad antioxidante en ambos extractos de milenrama (entre 38-25%), que se podría explicar tanto por las pérdidas, como por las isomerizaciones de compuestos fenólicos con un alto poder antioxidante registrados tras la digestión, como los derivados del ácido clorogénico y de la luteolina (Shang y col., 2010; Song y Park; 2014)

Para continuar con la evaluación de la biodisponibilidad de los compuestos fenólicos de milenrama, una vez finalizada la digestión, se midió su absorción en las células Caco-2. De los compuestos originalmente con mayor abundancia en el extracto digerido, los ácidos 4,5-, 3,5- y 3,4-DCQA, así como la luteolina-7-*O*-glucósido, apenas una pequeña cantidad se logró detectar en la fracción biodisponible (0,6-5%) a las 2h del ensayo. Sin embargo, las agliconas de flavonoides, como la apigenina, casticina, diosmetina y metoxiacacetina, demostraron una mayor absorción en este tiempo (21-43%). En general, los compuestos fenólicos glicosilados, que son más hidrofílicos que sus correspondientes agliconas, no son capaces de atravesar la membrana celular de los enterocitos vía difusión pasiva, por lo que requieren otros mecanismos para su absorción, como su interacción con proteínas transportadoras y procesos de desconjugación mediados por enzimas (Velderrain-Rodríguez y col., 2014). Conforme avanzó el tiempo de incubación (4h y 6h) del extracto en las células Caco-2, se detectó una acumulación de las agliconas, pero una disminución en los demás compuestos fenólicos, relacionándose esto último posiblemente con los diversos procesos metabólicos que tienen lugar en las células intestinales, o con los propios mecanismos de eflujo del que pueden ser susceptibles estos compuestos (D'Antuono y col., 2015; Bowles y col., 2017).

La fracción biodisponible recogida tras las 6h de ensayo con las Caco-2, demostró una cierta actividad antiinflamatoria. Estos resultados podrían relacionarse

en parte con la presencia de casticina, diosmetina y centaureidina, principales componentes de dicha fracción, los cuales han sido reconocidos por sus propiedades antiinflamatorias (Jachak y col., 2011; Patel y col., 2013; Liou y col., 2104). Sin embargo, la posible acción sinérgica de otros metabolitos derivados de los fenólicos de milenrama, tanto los detectados como los no detectados en el estudio, podrían influenciar en dicha actividad.

Debido a los cambios observados en los compuestos fenólicos de milenrama durante su digestión grastointestinal, se consideró oportuno realizar un estudio que permitiera la inclusión del extracto UAE-100% etanol en matrices proteicas formuladas como nanoemulsiones (NEs), usando caseinato de sodio como estabilizante, y geles lácteos acidificados (MGs) (***Capítulo 3, Publicación 6***). Entre estas formulaciones, las NEs y los MGs que contenían 1,0 mg/mL del extracto de milenrama, demostraron una mayor eficiencia de encapsulación. Cuando estas matrices se sometieron a un proceso de digestión, se observó que las NEs solo consiguieron una protección parcial de los compuestos fenólicos, mientras que, al emplearse los MGs, se registró una composición fenólica muy similar a la que presentaban los extractos de milenrama antes de su digestión. Uno de los resultados más evidentes obtenidos con los MGs, se correspondió con una menor degradación del 3,5-DCQA, el principal compuesto en el extracto. Además, la encapsulación del extracto en los MGs evitó la pérdida de actividad antioxidante que sufrieron los extractos no encapsulados durante el proceso de digestión. Estos resultados podrían estar relacionados con las interacciones ocurridas entre las proteínas de la leche y los compuestos fenólicos, que pueden preservar la estructura y función de estos compuestos durante su paso por el tracto gastrointestinal (Lamothe y col., 2014; Adrar y col., 2019).

Finalmente, se llevó a cabo un método alternativo de fraccionamiento del extracto UAE-100% etanol de milenrama (***Capítulo 3, Publicación 7****),* utilizándose la tecnología supercrítica de precipitación antisolvente. Es técnica representa una alternativa sostenible y escalable para la obtención de fracciones enriquecidas en compuestos fenólicos según la afinidad su afinidad por el CO_2 supercrítico que actúo como antisolvente. Así, empleándose 10 y 15 MPa de presión (Villanueva-Bermejo y col., 2017), se fraccionaron, por un lado, los compuestos fenólicos con poca afinidad por el CO_2, correspondiéndose con aquellos compuestos más polares, recuperados en la celda de precipitación (P10 y P15). Por otro lado, los compuestos menos

polares, se concentraron en su mayoría en el separador una vez que se despresurizó el CO_2 (S10 y S15). Así, las fracciones P10 y P15 duplicaron su actividad antioxidante, respecto al extracto de partida, lo que se relacionó principalmente con el enriquecimiento en luteolina-7-*O*-glucósido y 3,5-DCQA en ambos extractos, y de luteolina en P15 (Kim y col., 2011; Song y Park., 2014). Respecto a las propiedades antiinflamatorias, si bien las fracciones P10 y P15 demostraron una cierta acción inhibitoria en la secreción de interleuquinas proinflamatorias, los resultados fueron similares al extracto original UAE-100. Por lo que, el efecto inhibidor observado en estas fracciones (P10 y P15) podría estar relacionado con el contenido en compuestos fenólicos, pero específicamente con los ácidos DCQA (Liang y Kitts, 2016) y luteolina y apigenina, tanto en su forma aglicona como glucosilada (Choi y col., 2014; Francisco y col., 2014). Por otro lado, las fracciones recuperadas en el separador, S10 y S15, demostraron una baja actividad antioxidante pero una remarcada actividad antiinflamatoria, en comparación con las fracciones de la celda de precipitación y UAE-100. Curiosamente en las fracciones S10 y S15, la presencia de agliconas de flavonoides resultó evidente, aunque el contenido en compuestos fenólicos totales fue mínimo, por lo que la actividad antiinflamatoria en este caso no podría explicarse solo por el contenido de dichos compuestos. Considerando las condiciones para la obtención de las fracciones en el separador mediante la técnica del SAS, estas se podrían asimilar a las utilizadas para la obtención de aceites esenciales (Chinnarasu y col., 2015); por lo que las fracciones S10 y S15 podrían contener compuestos volátiles parecidos al aceite esencial de milenrama. En este estudio, tras el análisis por GC-MS de S10 y S15, se corroboró un enriquecimiento, del casi el doble, en los componentes de la fracción volátil, en comparación con UAE-100. Así, la mayor abundancia de alcanfor, artemisia cetona y borneol, podría relacionarse también con la actividad antiinflamatoria observada en las fracciones S10 y S15 de milenramana (Arranz y col., 2014a; Arranz y col., 2014b; Rungqu y col., 2016).

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6. CONCLUSIONES

6. Conclusiones

En línea con los objetivos parciales planteados en la tesis doctoral, se presentan las siguientes conclusiones:

Objetivo 1. *Identificar las plantas pertenecientes a las familias Lamiaceae y Asteraceae que presentan un mejor potencial como fuente compuestos con actividad antioxidante y/o antiinflamatoria mediante el uso de técnicas de extracción sostenibles. Optimizar el proceso de extracción: técnica y solvente empleado.*

- De las plantas estudiadas, la mejorana (*O. majorana*) presentó el mayor potencial como fuente de compuestos fenólicos antioxidantes, mientras que la milenrama (*A. millefolium*) destacó por su potencial antiinflamatorio.
- La extracción con líquidos presurizados (PLE) y la extracción asistida con ultrasonidos (UAE), demostraron ser las técnicas más efectivas para la obtención de extractos con propiedades antioxidantes y antiinflamatorias. Así, el uso de mezclas etanol:agua permitió la extracción de un mayor contenido de compuestos con actividad antioxidante, mientras que el empleo de etanol 100% favoreció la obtención de extractos con mayor actividad antiinflamatoria.

Objetivo 2. *Investigar la relación existente entre la composición de los extractos obtenidos y su actividad antioxidante y/o antiinflamatoria*.

- La actividad antioxidante del extracto de mejorana se relaciona con su alto contenido en compuestos fenólicos, y en particular con la elevada cantidad presente de ácido rosmarínico y 6-hidroxiluteolina-7-O-glucósido.
- En la composición fenólica de los extractos de milenrama destaca la presencia de ácido 3,5-dicafeoilquínico (DCQA) y luetolina-7-*O*-glucósido, lo que se relaciona con la importante actividad inflamatoria de los mismos. Sin embargo, esta actividad también se podría relacionar con la abundancia en borneol y alcanfor de la fracción volátil.

Objetivo 3. *Aplicar el uso de técnicas de fraccionamiento sostenibles altamente escalables, como las resinas de absorción y la tecnología supercrítica de precipitación antisolvente, para la obtención de fracciones enriquecidas en compuestos con actividad antioxidante y/o antiinflamatoria.*

- La técnica de fraccionamiento con resinas de adsorción XAD-7HP, empleando etanol al 80% como disolvente de elución, resulta una estrategia adecuada para la obtención de extractos enriquecidos en compuestos fenólicos bioactivos a partir de extractos de mejorana y milenrama.
- La tecnología supercrítica antisolvente con el uso de CO_2 supercrítico hace posible el fraccionamiento de los compuestos del extracto de milenrama, permitiendo la obtención de una fracción rica en compuestos antioxidantes y otra en compuestos con alta actividad antiinflamatoria.

Objetivo 4. *Estudiar la biodisponibilidad de los compuestos fenólicos presentes en los extractos seleccionados mediante un modelo de digestión gastrointestinal in vitro y su posterior absorción intestinal en células Caco-2.*

- El proceso de digestión gastrointestinal provoca la degradación y transformación estructural de los compuestos fenólicos antioxidantes presentes en los extractos de mejorana, para los que se registraron pérdidas aproximadas del 50% en los compuestos mayoritarios.
- La actividad antioxidante y antiinflamatoria de la fracción biodisponible del extracto de *mejorana* obtenido tras su absorción intestinal *in vitro*, podría relacionarse con la abundancia del ácido rosmarínico en esta fracción, aunque el resto de compuestos fenólicos detectados también podrían contribuir en dichas actividades.
- La digestión gastrointestinal de los extractos de milenrama muestra que las principales transformaciones de los compuestos fenólicos se producen en la etapa intestinal, destacando las isomerizaciones sufridas por los ácidos DCQAs y el ácido clorogénico.
- Los compuestos fenólicos más abundantes detectados en la fracción biodisponible del extracto de milenrama posterior a su absorción intestinal fueron la casticina, diosmetina y centaureidina, las cuales podrían contribuir, al menos en parte, a la actividad antiinflamatoria detectada en dicha fracción.

Objetivo 5. *Mejorar la biodisponibilidad y/o favorecer la incorporación en matrices alimentarias de los extractos seleccionados mediante el desarrollo de distintas formulaciones.*

- Las matrices formuladas con proteínas, caseína y proteína aislada de soja, resultan ser una propuesta eficiente para la encapsulación del extracto de mejorana rico en ácido rosmarínico, sin por ello mermar su eficacia biológica.
- La inclusión del extracto de milenrama en geles lácteos acidificados, se presenta como una estrategia adecuada para mejorar la estabilidad de los compuestos fenólicos con actividad antioxidante durante el proceso de digestión gastrointestinal *in vitro*, mejorando así su bioaccesibilidad.

Así, de manera **general,** podemos concluir que tanto la mejorana como la milenrama, constituyen una fuente importante de ingredientes antioxidantes y/o antiinflamatorios con un posible uso en el diseño de alimentos y/o complementos alimenticios destinados a la mejora de la salud. Sin embargo, la utilización de estrategias dirigidas al enriquecimiento y desarrollo de formulaciones, resultan imprescindibles para mejorar su biodisponibilidad y, por tanto, su potencial actividad biológica *in vivo*.

6. Conclusions

According to the partial objectives established in this PhD thesis, the next conclusions can be drawn:

Objective 1. *To identify the plants belonging to Lamiaceae and Asteraceae families that have a better potential as a source of compounds with antioxidant and/or anti-inflammatory activities through the use of sustenible extraction techniques. To optimize the extraction process: the technique and the solvent used.*

- From the plants studied, marjoram (*O. majorana)* presented the greatest potential as a source of antioxidant phenolic compounds, while yarrow (*A. millefolium)* stood-out for its anti-inflammatory potential.
- Pressurized liquid extraction (PLE) and ultrasonic assisted extraction (UAE) proved to be the most effective techniques for obtaining extracts with antioxidant and anti-inflammatory properties. Therefore, the use of ethanol: water mixtures allowed the extraction of a higher content of compounds with antioxidant activity, whereas the use of 100% ethanol favoured the obtantion of extracts with greater anti-inflammatory activity.

Objective 2. *To investigate the relation between the composition of the obtained extracts and their antioxidant and/or anti-inflammatory activity.*

- The antioxidant activity of marjoram extract relates to its high content of phenolic compounds, and in particular to the high amount of rosmarinic acid and 6-hydroxyluteolin-7-*O*-glucoside
- The presence of 3,5-dicafeyolquinic acid (DCQA) and luteolin-7-*O*-glucoside stands out in the phenolic composition of yarrow extracts, which is related to their important ant-inflammatory activity. However, this activity could also be related to the abundance of borneol and camphor in the volatile fraction.

Objective 3. *To apply the use of highly scalable and sustainable fractionation techniques, such as adsorption resins and supercritical anti-solvent fractionation, to obtain fractions with enriched compounds with antioxidant and/ or anti-inflammatory activity.*

- The fractionation technique with XAD-7HP adsorption resins, using 80% ethanol as elution solvent, is a suitable strategy for obtaining extracts enriched in bioactive phenolic compounds from marjoram and yarrow extracts.
- Supercritical anti-solvent technology with the use of supercritical CO2 makes it possible to fractionate the compounds of yarrow extract, obtaining a fraction rich in antioxidant compounds and another one rich in compounds with high anti-inflammatory activity.

Objective 4. *To study the bioavailability of the phenolic compounds, which are present in the selected extracts by means of an in vitro gastrointestinal digestion model and its subsequent intestinal absorption in Caco-2 cells.*

- The gastrointestinal digestion process causes the degradation and structural transformation of the antioxidant phenolic compounds present in marjoram extracts, for which 50% losses, approximately, were recorded in the majority compounds.
- The antioxidant and anti-inflammatory activity of the bioavailable fraction of the marjoram extract obtained after its *in vitro* intestinal absorption could be related to the abundance of rosmarinic acid in this fraction, although the rest of the phenolic compounds detected could also contribute to these activities.
- The gastrointestinal digestion of yarrow extracts shows that the main transformations of phenolic compounds occur in the intestinal stage. The isomerization processes suffered by DCQAs and chlorogenic acid are to be highlighted.
- The most abundant phenolic compounds detected in the bioavailable fraction of the yarrow extract, after intestinal absorption, were casticin, diosmetin and centaureidin, which could contribute, at least in part, to the anti-inflammatory activity detected in that fraction.

Objective 5. *To improve the bioavailability and/or facilitate the incorporation of the selected extracts in food matrices through the development of different formulations.*

- Protein matrices formulated with casein and soy protein isolate, prove to be an efficient proposal for the encapsulation of marjoram extract, rich in rosmarinic acid, without reducing its bioefficacy.
- The inclusion of yarrow extract in acidified milk gels, is an adequate strategy to improve the stability of phenolic compounds with antioxidant activity during the *in vitro* gastrointestinal digestion process, thus improving their bioaccessibility.

Thus, in **general**, we can conclude that both marjoram and yarrow are an important source of antioxidant and/or anti-inflammatory ingredients with a possible use in the design of food and/or food supplements intended for health improvement. However, the use of strategies aimed at the enrichment and development of formulations are essential to improve their bioavailability and, therefore, their potential biological activity *in vivo.*

ANEXOS

ANEXO I

Otras contribuciones del doctorando además de las mencionadas en esta tesis.

Publicaciones en revistas SCI:

1. Morales, D., Rutckeviski, R., **Villalva, M.,** Abreu, H., Soler-Rivas, C., Santoyo, S., Iacomini, M., & Smiderle, F. R. (2019). Isolation and comparison of α-and β-D-glucans from shiitake mushrooms (Lentinula edodes) with different biological activities. *Carbohydrate Polymers*, 115521.

2. Arranz, E., Corrochano, A. R., Shanahan, C., **Villalva, M.,** Jaime, L., Santoyo, S., Callanan, M.J., Murphy, E., & Giblin, L. (2019). Antioxidant activity and characterization of whey protein-based beverages: Effect of shelf life and gastrointestinal transit on bioactivity. *Innovative Food Science & Emerging Technologies*, *57*, 102209.

3. Morales, D., Smiderle, F. R., **Villalva, M.,** Abreu, H., Rico, C., Santoyo, S., ... & Soler-Rivas, C. (2019). Testing the effect of combining innovative extraction technologies on the biological activities of obtained β-glucan-enriched fractions from Lentinula edodes. *Journal of Functional Foods*, *60*, 103446.

4. Villanueva-Bermejo, D., Vázquez, E., **Villalva, M.,** Santoyo, S., Fornari, T., Reglero, G., & Rodriguez García-Risco, M. (2019). Simultaneous Supercritical Fluid Extraction of Heather (*Calluna vulgaris* L.) and Marigold (*Calendula officinalis* L.) and Anti-Inflammatory Activity of the Extracts. *Applied Sciences*, *9*(11), 2245.

5. Villanueva-Bermejo, D., Zahran, F., Troconis, D., **Villalva, M.,** Reglero, G., & Fornari, T. (2017). Selective precipitation of phenolic compounds from *Achillea millefolium* L. extracts by supercritical anti-solvent technique. *The Journal of Supercritical Fluids*, *120*, 52-58.

6. Martin, D., Salas-Perez, L., **Villalva, M.,** Vazquez, L., Garcia-Risco, M. R., Jaime, L., & Reglero, G. (2017). Effect of alkylglycerol-rich oil and rosemary extract on oxidative stability and antioxidant properties of a cooked meat product. *European Journal of Lipid Science and Technology*, *119*(7), 1600412.

ANEXO II

Research Article

SCI

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Protein matrices ensure safe and functional delivery of rosmarinic acid from marjoram (*Origanum majorana*) extracts

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Abstract

BACKGROUND: To understand the interactions between carriers and functional ingredients is crucial when designing delivery systems, to maximize bioefficacy and functionality. In this study, two different protein matrices were evaluated as means to protect the extract isolated from marjoram leaves (*Origanum majorana*), casein micelles from fresh skim milk and soy protein isolate (SPI).

RESULTS: Marjoram extract was obtained from pressurization of ethanol and water solvent. Protein dispersions of casein and SPI (5 g L^{-1} each) with or without marjoram extract (0.1–3 mg mL^{-1}) were prepared and homogenized. The physicochemical characterization of charge and entrapment efficiency were conducted. The results demonstrated that entrapment efficiency was highly dependent on the carrier itself where SPI formulations showed 20% higher affinity when compared to casein micelles. To investigate the physiological behaviour of the marjoram–protein dispersions, human macrophages were employed. A non-specific inflammatory response of macrophages stimulated with bacterial lipopolysaccharide was measured for TNF-α, IL-1β and IL-6 cytokine secretion.

CONCLUSION: Casein and SPI protein formulations warranted high bioefficacy of marjoram extract, showing their potential as safe carriers.

Keywords: marjoram; rosmarinic acid; entrapment; soy protein isolate; caseins; anti-inflammatory activity

INTRODUCTION

Health promoting benefits of aromatic plants and spices have been extensively described. Among them marjoram (*Origanum majorana* L.) is recognized for being used as a food additive with flavouring properties in addition to promoting digestive system well-being. Marjoram extract (ME) is known for its antioxidant and antimicrobial properties[1,2] and for its anti-inflammatory activity.[3] This activity is contributed to the composition of the ME mainly phenolic acids like rosmarinic, caffeic, carnosic and gallic, as well as other phenolic compounds such as luteolin, apigenin or carnosol.[4] Rosmarinic acid has been reported as the main compound detected in hydroalcoholic MEs.[5]

Environmental clean technologies for extraction and concentration of compounds from plant origin are well established and offer reduced toxicity usually associated with traditional solid–liquid extractions where methanol, hexane or acetone is used as a solvent. More advanced techniques have become available aiming to reduce losses on the bioactive quantities extracted from plants and increase their purity and maximize their functionality *per se*. Consequently, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are techniques that offer high reproducibility in short time, simple manipulation and low energy input.[6] Potential industrial application of UAE to plant materials has been previously described providing higher extraction yield than classic methods (e.g. solid–liquid extraction).[7] Pressurized liquid extraction (PLE) is also employed in food technology and is considered a safe and clean technique. Extraction process using PLE occurs at solvent temperature between 50–200 °C and pressure around 6–12 MPa.[8] Optimization of extraction process

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has been achieved using higher temperatures to increase solubility and extraction rate, for example, water can behave as an organic solvent at certain temperature that affects its dielectric constant.[9]

Despite the advantages of applying these techniques in ingredient development, they are mainly and widely used in pharmaceutical applications. Nevertheless their incorporation in food products represent a challenge due to low solubility of final products in water or complex matrices and the level of solvent traceability in the final product. Therefore, protein-based nanostructures are ideal alternative carriers of bioactive compounds including carotenoids or phenolic compounds. Compounds, especially poor water-soluble molecules enclosed in milk or plant-based proteins have shown improved solubility and stability during processing or transit through the gastro intestinal tract.[10,11] Milk components, including milk proteins, have been described as delivery carriers for biologically active molecules. Caseins represent more than 80% of total milk proteins where about 95% is present as colloidal aggregates, so called casein micelles, due to the presence of calcium and phosphate linked to serine-phosphate residues in native milk. It is described that the hydrophobic environment of the core of casein micelles offers the possibility to entrap non-polar molecules.[12] Examples of successful delivery of low water-soluble molecules in casein micelles are curcumin or vitamin D.[13]

Noteworthy, plant-based proteins are an abundant low-cost source of bioactive peptides, such as soy proteins. Soy proteins exposure to alkali solubilization and acid precipitation process outcomes protein denaturation, and as a result aggregation in isolates with an average size of nanometres and low water solubility, highly dependent of preparation conditions is achieved.[14] Soy protein isolate (SPI) is the most commercially available soy protein and commonly recognized for good ability to adsorb and stabilize the interphase of oil-in-water emulsions, owing to the amphiphilic properties of isolates. Moreover, SPI has been described as carrier of lipophilic compounds (e.g. β-carotene or curcumin).[15,16]

The objective of this study was to evaluate the bioefficacy and functionality of ME entrapped in protein matrices, respectively, casein micelles and SPI. Physicochemical characterization of the formulations was conducted and entrapment efficiency was determined by means of high-performance liquid chromatography (HPLC). To determine the potential bioefficacy of the encapsulated ME an *in vitro* model of inflammation was employed. Immuno-modulatory response was conducted in human macrophages to confirm the hypothesis that ME entrapment in protein matrices may ensure safe delivery and therefore functionality.

MATERIALS AND METHODS

Marjoram samples preparation

Dried marjoram leaves (*Origanum majorana* L.) were obtained from Herboristeria Murciana company (Murcia, Spain), certified ISO 9001:2008. The sample was ground in a knife mill (Grindomix GM 200, Restch, Llanera, Spain) and the particle size was determined by sieving the ground plant material to the appropriate size (< 500 μm).

Pressurized liquid extraction

Extraction of marjoram was performed in a Dionex ASE 350 (Dionex Corporation. Sunnyvale, CA, USA) system equipped with a solvent controller unit. Three different ratios of ethanol/water (*v*/*v*) solutions were applied (50:50, 70:30, 100:0) as extraction solvent. Powdered marjoram sample (1.0 g) was mixed with sea sand (4.0 g) and placed into an 11 mL Dionex (ASE 350) stainless-steel cell. The extraction was performed at 100 °C for 10 min at 10.34 MPa, in duplicates. Prior to freeze-drying (Labconco Corporation, Kansas City, MO, USA) the extracts, the solvent was evaporated in a Rotovapor IKA RV 10 (VWR International, Barcelona, Spain). All the lyophilized samples were stored at −20 °C until use.

Ultrasound-assisted extraction (UAE)

Ground marjoram (40 g) with the corresponding concentration of ethanol/water solution (50:50, 70:30, 100:0) in a ratio 1:10 (bark/solvent) were submitted to ultrasound extraction for 30 min using a $^1/_2$ diameter disruptor horn probe at 70% amplitude (maximum power output of 400 W at 60 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury, CT, USA) maintaining the temperature at 35 °C with an ice bath and assisted with a stir plate. After sonication, the samples were filtrated, evaporated and freeze-dried. All samples were stored under −20 °C prior to analysis.

Rosmarinic acid quantification, total phenolic content and antioxidant activity determination

HPLC-photodiode array detection (PAD) analysis of rosmarinic acid in marjoram extracts was performed as previously described.[17]

Total phenolic content (TPC) was determined using the Folin–Ciocalteu's colorimetric method developed by Singleton *et al.*[18] A standard curve was calculated using gallic acid, and results were expressed as gallic acid equivalents (GAE) (milligrams of gallic acid per gram of dried extract). The antioxidant activity of the ME was determined by the $ABTS^{\bullet+}$ assay. This method was applied according to Re *et al.*[19] protocol. The results were expressed as TEAC values (millimoles of Trolox equivalents per gram of dried extract).

Entrapment of marjoram pressurized liquid extract in protein aggregates

Two different protein matrices, caseins and SPI were employed as carriers of marjoram pressurized liquid extract. Preliminary studies were performed to choose a SPI concentration with minimum insoluble fraction (less than 10%). A range of SPI concentrations were prepared in 50 mM sodium phosphate buffer pH 7.4, stirred for 1 h at 40 °C and stored overnight at 4 °C for complete hydration. Conventional homogenization was then performed using the protein solutions at 450 kPa for four passes followed by low speed centrifugation (100×*g* for 5 min) (Eppendorf, Brinkmann Instruments, Westbury, NY, USA). Supernatant aliquots were collected and protein content was determined by Lowry assay (DC Protein Assay, BioRad Laboratories, Mississauga, ON, Canada), using BSA as standard.

Caseins were isolated from skim milk by centrifugation at 62 000×*g* for 30 min and 20 °C (OptimaTM LE-80 K, with a Ti-45 rotor, Beckman–Coulter, Mississauga, ON, Canada). Protein analysis of the pellets was measured using a Dumas combustion method nitrogen analyser (FP-528, Leco Inc., St Joseph, MI, USA). Casein pellets were dissolved at 5 g L^{-1} (based on protein) in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride to ensure the isotonic environment using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Scientific, Mississagua, ON, Canada). ME stock solutions were dissolved in ethanol/imidazole buffer (1:3), final volume 1 mL, and added dropwise to achieve 0.1,

Table 1. Evaluation of extraction yield [% dry weight ± standard deviation (SD)], total phenolic content [TPC (mg GAE g^{-1} dry extract ± SD)], antioxidant activity [TEAC (mmol TE g^{-1} dry extract ± SD)] and quantification of rosmarinic acid (RA) (mg RA g^{-1} of dry extract ± SD) for marjoram extracts obtained by ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) using different percentages of ethanol (% *v/v*) during the extraction process[A,B]

Extraction technique	Ethanol/ water (% *v/v*)	Extraction yield (%)	TPC (mg GAE g^{-1} dry extract)	TEAC (mmol TE g^{-1} dry extract)	RA (mg RA g^{-1} dry extract)
UAE	50:50	$11.56 \pm 0.7^{b\,2}$	$233.2 \pm 2.1^{b\,1}$	$1.44 \pm 0.02^{b\,2}$	$33.62 \pm 1.18^{b\,1}$
	70:30	$15.6 \pm 0.3^{a\,2}$	$256.6 \pm 3.4^{a\,1}$	$1.52 \pm 0.04^{a\,2}$	$35.87 \pm 1.89^{a\,1}$
	100:0	$5.86 \pm 0.4^{c\,2}$	$143.4 \pm 1.7^{c\,2}$	$0.54 \pm 0.02^{c\,2}$	$23.36 \pm 0.82^{c\,2}$
PLE	50:50	$22.9 \pm 0.1^{a\,1}$	$237.5 \pm 2.2^{b\,1}$	$1.49 \pm 0.02^{b\,1}$	$31.48 \pm 0.26^{c\,1}$
	70:30	$23.3 \pm 0.1^{a\,1}$	$265.9 \pm 4.8^{a\,1}$	$1.81 \pm 0.04^{a\,1}$	$33.94 \pm 0.75^{a\,1}$
	100:0	$11.1 \pm 0.34^{b\,1}$	$201.2 \pm 4.9^{c\,1}$	$0.81 \pm 0.02^{c\,1}$	$32.36 \pm 0.39^{b\,1}$

[A] Within an extraction technique, different superscript lowercase letters indicate statistical differences between ethanol/water composition at $P < 0.05$.
[B] Within the same ethanol/water composition, different superscript numbers indicate statistical differences between extraction technique at $P < 0.05$.

0.25, 0.5, 1, 2 and 3 mg mL^{-1} in the casein solution. The mixtures were further kept for 1 h on a magnetic stirrer at 37 °C. Casein formulations were then submitted to high-pressure homogenization at 475 kPa for four passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA).

Protein solutions containing 5 g L^{-1} SPI were chosen to incorporate ME. Stock extract solutions were dissolved in ethanol/sodium phosphate buffer (1:3), final volume 1 mL, to achieve 0.1, 0.25, 0.5, 1, 2 and 3 mg mL^{-1} in the SPI formulations. Protein solutions were prepared as described earlier and after overnight storage at 4 °C, extract solutions were added dropwise. The mixtures were further kept for 1 h on a magnetic stirrer at 37 °C. High-pressure homogenization was then performed at 475 kPa for four passes using a microfluidizer (model M-110Y).

Zeta (ζ)-potential of the fresh casein and SPI formulations was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK). Casein formulations were diluted in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride (1:1000) while SPI formulations were diluted in 50 mM sodium phosphate buffer pH 7.4 (1:100).

Rosmarinic acid entrapment efficiency

Entrapment efficiency of rosmarinic acid in casein and SPI formulations was measured after homogenization. Samples were priory filtered (0.45 µm PVDF filters, Fisher Scientific) and aliquots of 500 µL were centrifuged in concentrator microcentrifuge tubes (Spin-x UF 500 10 K MWCO PES 500 µL, Corning, NY, USA), for 15 min at 3000×*g* (benchtop Eppendorf centrifuge 5415D, Brinkmann Instruments). Collected permeate was further analysed for rosmarinic acid quantification by means of HPLC-PAD as previously described.[17]

In vitro immunomodulatory activity of marjoram formulations

Human THP-1 monocytes (American Type Culture Collection, ATCC, CEDARLANE Corporation, Burlington, ON, Canada) were cultured in RPMI 1640 culture medium supplemented with 10% foetal bovine serum (FBS), 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin, 2 mM L-glutamine and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada) at 37 °C in 95% humidified air containing 5% CO_2. Cells were plated at a density of 5×10^5 cells mL^{-1} in 24 well plates. Differentiation to macrophages was induced by incubating the cells with 100 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h.

The toxic effect of the marjoram formulations (50, 100 and 200 µL) on differentiated macrophages was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich) following Mosmann's method.[20]

For immunomodulatory assay, cells were washed with phosphate-buffered saline (PBS) solution and incubated with 0.05 µg mL^{-1} lipopolysaccharide (LPS) (Sigma-Aldrich) in the presence of either 100 µL of casein or SPI formulations containing, respectively, 0, 0.5 and 1 mg mL^{-1} of ME for 24 h. Formulations were tested along with a control of ME 100 and 50 µg mL^{-1}. Then, the supernatants were kept frozen at −80 °C. The release of cytokines IL-1β, IL-6 and TNF-α was measured in the supernatants of macrophages cells using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Bio-Techne Corporation, Minneapolis, MN, USA), according to the manufacturer's instructions.

Statistical analysis

For each sample, duplicate extractions were performed and the analysis of phenolic compounds was carried out in triplicate expressed as mean values and standard deviation. The results were analysed using one-way analysis of variance (ANOVA) followed by LSD (least significant difference) test with a $P \leq 0.05$ using Statgraphics Centurion XVI (Statpoint Inc., Washington, DC, USA) software.

RESULTS AND DISCUSSION

Comparison of marjoram extracts and rosmarinic acid determination

Two extraction techniques (PLE and UAE) were used to obtain a ME with a high quantity of rosmarinic acid and a significant antioxidant activity using three different concentrations of ethanol/water like solvent extraction. Both ethanol and methanol have been widely used to extract phenolic compounds from plant material due to their polarity and good solubility, although, for industrial purposes ethanol is preferable since it is considered GRAS.[9,21] The effect of ethanol on yield extraction, TPC and TEAC value of marjoram extract for both techniques are presented in Table 1. The higher extraction yield occurred when a mixture of ethanol/water was used as solvent extraction, instead of absolute ethanol. Moreover, the values were significantly higher when the extraction was carried out by the PLE technique, in comparison with UAE, up to 23% of yield when 50% and 70% ethanol was used.

For the TPC determination, the values ranged from 143.4 to 265.9 mg GAE g^{-1} of dry extract for both techniques, PLE and UAE. However, at absolute ethanol as extraction phase, PLE yields the highest activity of ME (201.2 ± 4.9 versus 143.4 ± 1.7 mg GAE g^{-1} of dry extract). The highest TPC value was achieved for a mixture of ethanol/water (70:30) (*v*/*v*). Meanwhile, the antioxidant activity of MEs, expressed as Trolox equivalent (TE) per gram of dry matter, ranged from 0.54 to 1.52 mmol TE g^{-1} for those obtained with the UAE technique and from 0.81 to 1.81 mmol TE g^{-1} for the PLE technique. Curiously, the effect of ethanol in the TEAC values in both techniques, was higher for those extractions performed with an ethanol/water of 70:30 composition rather than 100% ethanol. Particularly in this condition, the use of PLE allowed a slightly elevated value than UAE. In addition, in this study, a strong correlation between the TPC and the antioxidant activity is exhibited, as the higher TPC values corresponding to the higher TEAC values. Other researchers have reported a positive correlation between the TPC and antioxidant activity of herbs.[22]

Rosmarinic acid quantification and its potential antioxidant activity in MEs is shown in Table 1. It can be observed that the values were similar in ME with 50% and 70% of ethanol, unlike for the absolute ethanol condition where PLE showed a better result. Thereby, PLE and UAE seem to represent an appropriate approach to obtain MEs with optimum quantity of rosmarinic acid, although a better extraction yield was obtained by the PLE.

Based on the earlier results we selected the PLE technique as an extraction technique using 70% ethanol, due to the advantages that PLE presents, like a remarkable higher extraction yield and a slightly higher antioxidant activity when compared with UAE in the studied conditions.

Entrapment of rosmarinic acid from marjoram pressurized extract in delivery systems

A number of delivery systems were designed to maximize entrapment of rosmarinic acid from marjoram pressurized extract. Preliminary experiments were conducted in oil-in-water emulsions. Previous studies have demonstrated that tea polyphenols are able to associate at the interface of sodium caseinate stabilized soybean oil emulsions.[23] Different concentrations of ME were studied in 10% soybean oil and 0.5% sodium caseinate formulated emulsions, however less than 10% rosmarinic acid was adsorbed at the interface. In addition, low solubility of the extract was observed in soybean oil and emulsions were not further considered as carriers of marjoram pressurized extract. Rosmarinic acid has low solubility in water and low partition coefficient, which complicates its formulation.[24]

Since marjoram pressurized extracts showed slight solubility in water, entrapment of PLE ME was assessed in protein carriers, caseins and SPI. Complexation of low water-soluble compounds with SPI has been described to improve water dispersibility and stability to processing treatments.[15] Previous research from our group demonstrated that the commercial SPI employed in this study has lower water solubility than that reported in the literature.[25] A range of SPI solutions in water (0.1–200 g L^{-1}) were prepared to determine protein insoluble fraction using the Bradford protein assay.[26] Results showed that protein concentrations below 5 g L^{-1} assure an insoluble fraction lower than 10%. As for the caseins dispersions, higher solubility in water was observed. Hence, protein dispersions of caseins and SPI were employed at 5 g L^{-1} along the study.

Table 2 shows ζ-potential results of casein and SPI formulations determined by dynamic light scattering. Furthermore, ζ-potential of formulations with SPI were not affected by incorporation of ME. Similar values were obtained in the presence of the highest concentration of ME (−14.56 ± 1.01 mV) and without extract (−13.77 ± 0.61 mV). The same effect was found in casein formulations, no differences in surface charge caused by the addition of extract (−19.96 ± 2.08 mV) and (−20.55 ± 1.49 mV) for 0 and 1 mg mL^{-1} of ME, respectively. Hence, ζ-potential results without the extract are consistent with those previously reported in the literature for SPI (−13.40 mV) and casein micelles (−21.7 mV).[10,27] Therefore, the presence of ME did not compromise physical stability of SPI and casein.

Table 2. Measurements of ζ-potential of protein suspensions (5 g L^{-1}), caseins (CAS) or soy protein isolate (SPI), containing 0, 0.5 or 1 mg mL^{-1} of marjoram extract (ME)[A]

Sample	ζ-Potential (mV)
CAS (0 mg mL^{-1} ME)	−19.96 ± 2.08[a]
CAS (0.5 mg mL^{-1} ME)	−20.06 ± 1.05[a]
CAS (1 mg mL^{-1} ME)	−20.55 ± 1.49[a]
SPI (0 mg mL^{-1} ME)	−13.77 ± 0.61[a]
SPI (0.5 mg mL^{-1} ME)	−14.70 ± 0.30[a]
SPI (1 mg mL^{-1} ME)	−14.56 ± 1.01[a]

[A]Within the same protein suspension, different superscript lowercase letters indicate statistical differences between 0, 0.5 and 1 mg mL^{-1} of marjoram at $P < 0.05$.

Entrapment efficiency of rosmarinic acid in caseins and SPI solutions was determined by means of HPLC analysis. Rosmarinic acid concentration was measured in permeate samples obtained after centrifugation in concentrator tubes. Figure 1 illustrates the results obtained for entrapment efficiency in casein (Fig. 1A) and SPI (Fig. 1B) formulations. Caseins micelles entrapped 56.40 ± 4.82% of rosmarinic acid contained in 0.1 mg mL^{-1} of ME. Similar results were found with 0.25 mg mL^{-1} of the extract (57.40 ± 10.02%), however the entrapment efficiency rapidly dropped to 20% at 1 mg mL^{-1} of extract that remained stable at 2 and 3 mg mL^{-1}. Our previous studies also demonstrated successful delivery of aromatic plant extracts in casein micelles, particularly the two main compounds, carnosic acid and carnosol, presented in rosemary supercritical extracts.[10] Encapsulation of hydrophobic pure compounds as curcumin and vitamin D in casein micelles has also been described.[13,28] Moreover, encapsulation in casein micelles provides protection from degradation of β-carotene exposed to common industrial treatments as pasteurization, sterilization or baking.[29]

From the SPI results obtained, it is interesting to point out that at the lowest concentrations of ME (0.1 mg mL^{-1}), the entrapment efficiency of rosmarinic acid in SPI reached the highest value (87.11 ± 8.51%). As the extract concentration increased, the entrapment progressively decreased and the amount detected in the aqueous phase increased to 67.54 ± 2.58% at the highest analysed concentration of 3 mg mL^{-1}. However, at 1 mg mL^{-1} of marjoram in SPI an entrapment efficiency of 45.07 ± 6.79% rosmarinic acid was detected. Similarly, decay in encapsulation efficiency of curcumin in SPI solutions while the concentration of curcumin was increased was also described.[11] In the study by Chen *et al.*,[11] complexation of curcumin was assessed using 50 g L^{-1} SPI solution and the maximum encapsulation efficiency was obtained at 0.0315 mg mL^{-1} of curcumin. Teng *et al.*[30] described the same trend of encapsulation efficiency that increased with decreasing

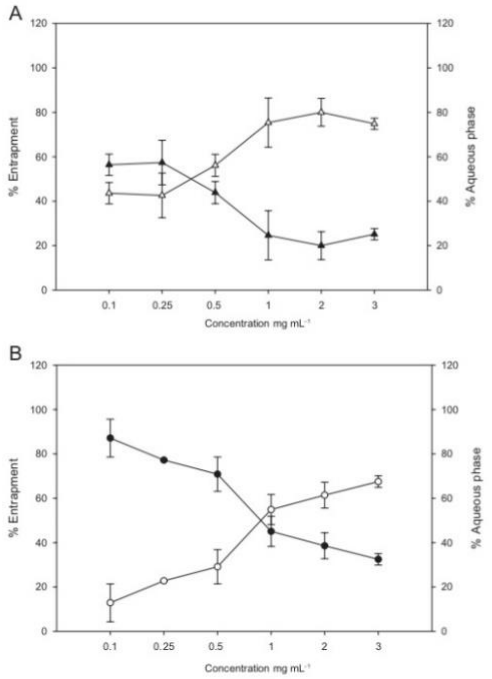

Figure 1. Percentage of rosmarinic acid entrapment (black triangles and circles) and detected in the aqueous phase (white triangles and circles) in caseins (A) and soy protein isolate (B) formulations at different marjoram pressurized extract concentrations. Entrapment was calculated based on the amount of rosmarinic acid detected in the aqueous phase. Results are the average of three independent experiments, with bars representing standard deviation.

curcumin and protein ratio. A ratio of 10 g curcumin kg^{-1} protein provided an encapsulation efficiency of 97.2% while when increased to 50 g curcumin kg^{-1} protein, the encapsulation efficiency decreased to just 52.8%.

When comparing entrapment efficiency of ME using caseins and SPI, SPI noted 20% to 30% higher entrapment efficiency than caseins at the studied concentrations. SPI nanoparticles seemed to provide a more favourable environment for rosmarinic acid than casein micelles. A similar effect was previously reported in a comparison of caseins and SPI as delivery carriers for curcumin. Chen *et al.*[11] noted in their study higher encapsulation of curcumin in SPI nanoparticles than casein micelles by spray-drying, 96% of encapsulation efficiency compared to 83.1% reported by Pan *et al.*[31]

Immunomodulatory activity of marjoram–protein formulations

To evaluate the bioefficacy and functionality of marjoram–protein formulations, their *in vitro* immunomodulatory activity was assessed using human macrophages differentiated from THP-1 monocytes cell line. Preliminary data was obtained to determine cytotoxicity of protein formulations containing ME (10, 50, 100 and 200 µL) and 100 µL was the maximum volume that did not induced cytotoxicity on the cells (data not shown). Figure 2 illustrates the results for TNF-α, IL-1β and IL-6 secretion. As shown, the incorporation of LPS (Control+) increased the secretion of the three measured cytokines compared with basal levels of secretion in untreated cells (Control−). Formulated marjoram protein carriers, caseins and SPI, and ME solution significantly reduced TNF-α secretion. In particular, caseins formulations showed a significant higher effect in reduction of TNF-α secretion compared to SPI formulations. Marjoram solutions (100 and 50 µg mL^{-1}) reduced TNF-α secretion to similar levels than those obtained with SPI formulations containing 1 and 0.5 mg mL^{-1} of ME. Secretion of the pro-inflammatory cytokine IL-1β was only reduced in marjoram solution treated cells up to 50% with 100 µg mL^{-1}. Neither casein alone formulations or SPI solutions with or without marjoram encapsulated showed any effect on suppressing IL-1β secretion. Both protein formulations showed elevated level of the cytokine from 130 to 180%. The presence of marjoram triggered reduction of IL-1β secretion caused by casein and SPI solutions alone. Similar to TNF-α secretion, IL-6 secreted levels were reduced in cells treated with marjoram solutions, casein and SPI formulations, compared to activated cells (Control+). However, the SPI empty solution seemed to reduce the secretion of IL-6 (83% secretion), no statistical differences were found. When comparing casein and SPI marjoram formulations, only at the highest concentration of marjoram (1 mg mL^{-1}), casein formulation showed a higher reduction of IL-6 secretion. Studies have shown the potential of rosmarinic acid to induce anti-inflammatory effects on different cell lines. Thus, Jiang *et al.*[32] showed evidence of rosmarinic acid down regulating the levels of TNF-α, IL-6 and high mobility box 1 protein in LPS induced RAW264.7 cells, indicating that rosmarinic acid might inhibit activation of the nuclear factor-κB pathway by inhibiting IκB kinase activity. Accordingly, rosmarininc acid inhibited LPS-induced up-regulation of IL-1β, IL-6, TNF-α and suppressed expression of iNOS in human gingival fibroblasts.[33] Further, Lembo *et al.*[34] indicated that rosmarinic acid produced a significant reduction in IL-1β, IL-6, IL-8 and TNF-α gene expression in HaCat cells after UVB irradiation.

Our results showed that empty casein and SPI suspensions reduced TNF-α and IL-6 secretion. Anti-inflammatory properties of sodium caseinate has also been described in cell models.[35] TNF-α activated Caco-2 cells reduced IL-8 secretion after exposure with sodium caseinate hydrolysates for 24 h. In addition, casein derived peptides as glycomacropeptide are described in the literature for their immunomodulatory properties.[36] Lunasin, known as a bioactive polypeptide identified in soybean with chemopreventive properties, has also been described as anti-inflammatory in RAW 264.7 macrophages.[37,38] Similar to our study, lunasin reduces secretion of TNF-α and IL-6 in LPS activated RAW 264.7 macrophages.[39] Peptides obtained from pepsin and pancreatin hydrolysates of soy products also showed anti-inflammatory activity by means of inhibition of NO production, TNF-α and IL-1β secretion.[40]

CONCLUSION

The findings indicated that PLE and UAE are adequate techniques to obtain MEs with a high content of rosmarinic acid and consequently antioxidant activity. Among extracts, PLE with a solvent mixture of 70:30 (*v*/*v*) ethanol/water presented the highest yield and antioxidant activity. Entrapment of PLE MEs in SPI provided 20% to 30% higher entrapment efficiency than caseins. The complexes of ME with caseins or SPI did not alter

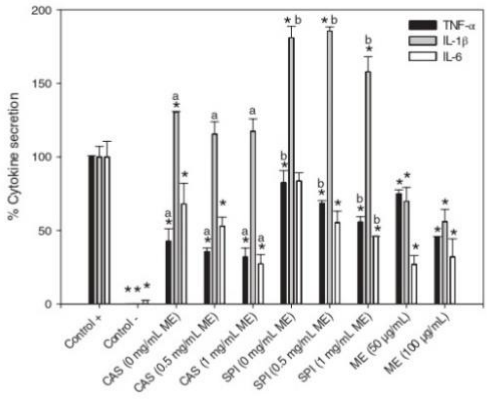

Figure 2. Effect of casein (CAS) and soy protein isolate (SPI) formulations versus marjoram extract (ME) in cytokines secretion determined by enzyme-linked immunosorbent assay (ELISA). Percentage of secretion was determined after 24 h incubation. A control lipopolysaccharide (LPS) activated macrophages (Control+) was used for comparison. TNF-α (black bars), IL-1β (grey bars) and IL-6 (white bars). Results are the average of three independent experiments, with bars representing standard deviation. Asterisk (*) denotes statistical differences between Control+ and each other sample per cytokine analysed $P < 0.05$. Lowercase letters a and b indicate statistical differences between pair comparison of casein and SPI formulation at the same concentration $P < 0.05$.

the immunomodulatory response of the extract itself. The results of this study would suggest that SPI and caseins could be safely used as carriers of herb extracts for applications in food product development.

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Anti-Inflammatory and Antioxidant Activities from the Basolateral Fraction of Caco-2 Cells Exposed to a Rosmarinic Acid Enriched Extract

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ABSTRACT: The potential use of *Origanum majorana* L. as a source of bioavailable phenolic compounds, specifically rosmarinic acid (RA), has been evaluated. Phenolic bioavailability was tested using an *in vitro* digestion process followed by a Caco-2 cellular model of intestinal absorption. The high-performance liquid chromatography−photodiode array detector-tandem mass spectrometry (HPLC−PAD-MS/MS) analysis showed the main components in the extract were 6-hydroxyluteolin-7-*O*-glucoside and rosmarinic acid, followed by luteolin-*O*-glucoside. After digestion process, the amount of total phenolic compounds (TPC) only decreased slightly, although a remarkable reduction in RA (near 50%) was detected. Bioavailable fraction contained 7.37 ± 1.39 mg/L digested extract of RA with small quantities of lithospermic acid and diosmin and presented an important antioxidant activity (0.89 ± 0.09 mmol Trolox/L digested extract). Besides, this bioavailable fraction produced a significant inhibition in TNF-α, IL-1β, and IL-6 secretion, using a human THP-1 macrophages model. Therefore, RA content in the basolateral compartment could play an important role in the antioxidant and anti-inflammatory activities found.

KEYWORDS: *anti-inflammatory activity, antioxidant activity, rosmarinic acid, in vitro digestion, Caco-2 cells*

INTRODUCTION

Origanum majorana L. (marjoram) is a culinary herb often used in foods. Its essential oil and extracts have been indicated to possess antioxidant, antimicrobial, anticancer, and anti-inflammatory activities.[1,2] These activities have been attributed to the presence of a high percentage of phenolic acids and flavonoids in marjoram leaves,[3] particularly to rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid naturally occurring in marjoram.[4,5] Antioxidant activity of RA has been generally admitted, since this compound may act as a free radical scavenger.[6,7] Related to RA anti-inflammatory effects, Jiang et al.[8] showed that this compound down regulated the levels of TNF-α, IL-6, and high mobility box 1 protein in bacterial lipopolysaccharide (LPS) induced RAW264.7 cells, indicating that RA might inhibit activation of the nuclear factor-κB pathway by inhibiting IκB kinase activity. Accordingly, Zdarilová et al.[9] reported that RA inhibited LPS-induced up-regulation of Il-1β, IL-6, TNF-α and suppressed expression of iNOS in human gingival fibroblasts. Further, Lembo et al.[10] indicated that RA produced a significant reduction in IL-1β, IL-6, IL-8 and TNF-α gene expression in HaCat cells after UVB irradiation.

In order to extrapolate results found in the *in vitro* to *in vivo* situations, it is important to know the bioavailability of bioactive compounds. Therefore, the use of an *in vitro* digestion/Caco-2 cell culture model has been proposed by several authors as an economical and useful alternative to study the bioavailability of these compounds.[11,12] Thus, although the transepithelial transport of RA in intestinal Caco-2 cells monolayers has been studied,[13] it is crucial to investigate the effect that plant matrix plays on its bioavailability, since the plant material matrix may alter absorption and bioavailability of phytochemicals.[14] For that matter, it would be very interesting to determine the anti-inflammatory and antioxidant effect of the bioavailable fractions of marjoram extracts enriched in RA, in order to corroborate the biological activities described for these extracts.

Solid−liquid extraction (SLE) is the most traditional technology used to extract active compounds from the plant matrix. It is widely known that higher temperatures enhance the solubility of the solute in the solvent and thus improve its recovery. Nevertheless, the SLE temperature is limited by solvent boiling and, in some cases, by the loss of volatile compounds. In this regard, pressurized liquid extraction (PLE) allows the use of solvents in a liquid state at higher temperatures. Furthermore, a compression effect is made on vegetal particles, which also contributes to improve extraction yield; moreover, a lower amount of solvent is required, extraction is faster, and volatiles loss is minimized.[15] Thus, several studies proposed PLE extraction as an alternative to conventional solid/liquid extraction in order to obtain phenolic compounds from herbs and spices.[16,17]

However, it is hard to obtain a highly concentrated extracts in phenolic compounds using only PLE, due to the complexity and the presence of impurities in crude extracts of herbal raw materials. Nowadays, the use of adsorption resins has been proposed as one of the most useful tools for selective enrichment of phenolic compounds from plant material, such

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as naringenin recovery from orange juice[18] or anthocyanins from grapes.[19]

For this purpose, some of the most commonly employed resins are XAD-2, XAD-7, XAD-16, and Oasis HLB that have been successfully used for phenolic compounds enrichment from natural extracts.[20,21] Among them, XAD-7 has been proposed for rosmarinic acid enrichment from *Lavandula vera*[22] or *Rabdosia serra*.[23]

The aim of this work was to study the anti-inflammatory and antioxidant properties of the bioavailable fraction of PLE extracts enriched in rosmarinic acid. In order to increase the quantity of RA in PLE extracts of marjoram, an Amberlite XAD-7HP resin was employed. The bioavailability of both, original and enriched extracts, was determined by using an *in vitro* digestion/Caco-2 cell culture model. Thus, the anti-inflammatory and antioxidant activity of the basolateral fraction was measured.

MATERIALS AND METHODS

Chemicals. Ethanol of analytical grade and Folin-Ciocalteu's reagent were obtained from Panreac (Madrid, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), gallic acid for titration (97.5%), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Madrid, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). Reference substances (analytical standard or HPLC purity ≥95%) for phenolic compounds identification such as cryptochlorogenic acid, neochlorogenic acid, and rosmarinic acid were purchased from Sigma-Aldrich (Madrid, Spain). Apigenin, apigenin 7-*O*-glururonide, caftaric acid, diosmin, lithospermic acid, luteolin, salvianolic acid, and vicenin 2 were from Phytolab (Madrid, Spain). Ethyl gallate, apigenin 7-*O*-glucoside, caffeic acid, luteolin 7-*O*-glucoside, *p*-coumaric acid, and protocatechuic acid were obtained from Extrasynthese S.A. (Genay, France). Finally, luteolin-7-*O*-glucuronide was from HWI Analytic GmbH (Rülzheim, Germany). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain).

Marjoram Samples and PLE Extraction. Marjoram sample consisted of dried leaves obtained from an herbalist shop (Murciana herboristería, Murcia, Spain). The sample was ground in a knife mill (Grindomix GM 200, Retsch, Llanera, Spain), and the particle size was determined by sieving the ground plant material to the appropriate size (<500 μm). The whole sample was stored at −20 °C until use.

Extractions were carried out in an ASE 350 system from Dionex Corporation (Sunnyvale, CA) equipped with a solvent controller unit. Each extraction cell (11 mL of capacity) was filled with a mixture of 1 g of solid sample and 4 g of sea sand. Then, the cell was filled with the solvent (a mixture of ethanol−water, 70:30) up to a pressure of 1500 psi and heated to 100 °C. Static extractions were performed for 10 min. The extracts were recovered in glass vials, ethanol was eliminated by evaporation, and extracts were lyophilized. The dried samples were stored at 4 °C in the dark until analysis.

Enrichment in Phenolic Compounds by Resin Column. Enrichment experiments were carried out in a glass column (3 cm × 50 cm) packed with XAD-7HP resin (Sigma-Aldrich, St. Louis, MO). In order to remove the monomers and pyrogenic agents trapped inside the pores during the synthesis process, a pretreatment of this resin was realized following the method described by Lin et al.[23]

After the pretreatment process, the resin was rehydrated overnight with ethanol at 4 °C and packed with a bed volume (BV) of 174 mL. In order to remove the ethanol, a distillated water washing was placed (4 BV) at constant flow of 4 BV/h. PLE extract was dissolved in acid water (pH 3) (15 mg/mL), and 45 mL was applied onto the column. After absorption equilibrium was reached (1 h), the column was washed with 2 BV of distilled water and eluted with 3 BV of 80% ethanol at a constant flow of 2 BV/h. The eluted fraction was collected, evaporated to remove methanol, freeze-dried, and stored at −20 °C until evaluation.

Determination of Total Phenolic Content (TPC) and Antioxidant Activity. In order to determine the TPC, the Folin-Ciocalteau reagent method was applied as described by Singleton et al.[24] The results were expressed as mg of gallic acid equivalents (GAE)/g extract. Antioxidant activity was determined by the DPPH method. This method was applied according to Brand-Williams et al.[25] protocol. The results were expressed as the TEAC value (mmol trolox/g extract or L of digested extract). All analyses were done in triplicate.

Chemical Characterization of Samples. High-performance liquid chromatography−photodiode array detector (HPLC−PAD) analysis of phenolic compounds was performed by using an Agilent HPLC 1260 Infinity series system with a photodiode-array detector (Agilent Technologies Inc., Santa Clara, CA). A reverse phase ACE Excell 3 Super C18 column (150 mm × 4.6 mm, 3 μm particle size) protected by a guard column ACE 3 C18-AR (10 mm × 3 mm) was used at 35 °C. A volume of 20 μL of sample (extract, digested extract, apical, or basolateral fractions) was injected previously filtered by a 0.45 μm PVDF filter.

Chromatographic separation was achieved using solvent A (99.9/0.1 water/formic acid v/v) and solvent B (ACN) at a flow rate of 0.5 mL/min as follows: 0 min, 0% B; 1 min, 0% B; 6 min, 15% B; 21 min, 25% B; 26 min, 35% B; 36 min, 50%; 41 min, 50% B; 44 min, 100% B; 49 min, 0% B. Chromatograms were recorded at 280, 320, and 360 nm. Peaks were tentatively identified according to its retention time and UV−vis spectrum by comparison with analytical standards. Ethyl gallate was added as internal standard in each analyze sample before it was filtered.

Confirmation of the identified compounds was carried out by HPLC−MS analyses. HPLC 1100 (Agilent Technologies Inc., Santa Clara, CA) coupled to a hybrid quadrupole-time-of-flight mass spectrometer (QTOF, QSTAR pulsar i, ABSciex) equipped with a turbo ion electrospray source was used. MS experiments in negative mode were carried out in TOF/MS and MS/MS mode. The instrumental parameters were set as follows: mass range, 50−2000 Da; ion spray voltage (IS), −4500 V; ion source gas pressure (GS1), 65 psi; (GS2), 65 psi; curtain gas pressure (Cur), 20 psi; declustering potencial (DP), 30 V; focusing potential (FP), 210 V; declustering potential 2 (DP2), 15 V; collision gas, 3 psi in MS experiments and 5 psi in MS/MS experiments. In TOF/MS experiments, just before separation, an external calibration in the mass spectrometer was performed with a mixture of phosphazenes and verified after the assays. The maximum error accepted to calibrate in the whole range of mass was 5 ppm. In MS/MS product ion experiments, the ion precursor was selected and the collision energy was fixed to 35 eV. Just before each MS/MS experiment, the instrument was calibrated with taurocholic acid and verified after the experiment. The accurate masses obtained were processed using the elemental composition calculator incorporated in the Analyst Software (Applied Biosystems). A margin of error up to 5 ppm for unknown compounds was allowed. Chromatographic conditions were similar as for HPLC−PAD analysis.

Quantification of identified compounds was carried out by using calibration curves of its authentic standard (Extrasynthese S.A., Genay, France) at five levels in triplicate by HPLC−PAD. Moreover, 6-hydroxyluteolin-7-*O*-glucoside, luteolin-*O*-glucoside, lithospermic acid isomer, and salvianolic acid isomer were quantified by the calibration curve of luteolin-7-O-glucoside, lithospermic acid and salvianolic acid, respectively. Validation of the chromatographic method (LOD, LOQ, precision, repeatability, stability, and recovery) was previously done (data not shown).

***In Vitro* Digestion.** The digestion process was carried out following a previously published protocol[12] slightly modified. Briefly, each extract (100 mg) was dissolved in 5 mL of ethanol−water (50:50), mixed with 0.1 mL of α-amylase from human saliva type XIII-A (Sigma-Aldrich, St. Louis, MO) (9.3 mg in Cl_2Ca 1 mM) and shaking for 2 min at 37 °C (oral phase). Stomach and intestinal phases were carried out employing a titrator Titrino Plus 877 (Methrom AG, Herisau, Switzerland). Thus, oral phase was mixed with 25 mL of a

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gastric solution (127 mg of porcine pepsin from porcine mucosa, 536 U/mg, (Sigma-Aldrich, St. Louis, MO) at pH 2 (adjusted with 0.1 M HCl) and shaking for 1 h at 37 °C. After gastric digestion, samples were adjusted to pH 7 with 1 M NaOH prior to the pancreatic step. Next, a pancreatic-bile extract containing 9.3 mg of pancreatin (Sigma-Aldrich, St. Louis, MO) and 115.7 mg of bile salts in 2.8 mL of 10 mM trizme-maleate buffer was incorporated and incubated for 2 h at 37 °C. At the end of digestion, the enzyme reaction was stopped immediately by cooling the samples in ice and samples were kept at −20 °C until analysis.

Caco-2 Experiments. Caco-2 cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 1% nonessential amino acids, and 2 mM L-glutamine (Gibco, Paisley, U.K.) at 37 °C in at humidified atmosphere containing 5% CO_2. The cytotoxic effect on Caco-2 cells of the extracts after digestion process was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay following the Mosmann[26] method. Briefly, Caco-2 cells were plated in 24-well plates until cell monolayers were obtained. Then the medium was aspirated and cells were treated with different concentrations of digested extracts for 6 h. After the medium was removed and cells washed and incubated with MTT for 2−3 h. The medium was removed, and 500 μL of DMSO were added. The absorbance was measured at 570 nm.

For transport experiments, Caco-2 cells were seeded onto six-wells Transwell plates (0.4 μm pore size, inserts of 24 mm diameter, Costar, Corning, Madrid, Spain) at a density of 3×10^5 cells per insert. The cells were maintained along 21 days, once the monolayer was formed, during which time culture medium was replaced every 3 days. The Caco-2 monolayer was used when transepithelial electrical resistance (TEER) (EVOM2, World Precision Instruments, Hitchin, U.K.) values were larger than 350 Ω cm^2. Apical and basolateral compartments were washed once with PBS and then incubated with 1.5 and 2.6 mL of supplement DMEM without FBS. A volume of 150 μL of digested extracts were incorporated in the apical compartment and incubated for 6 h at 37 °C. The TEER value was measured twice before and after the experiment to monitor the integrity of the Caco-2 monolayer. Then apical and basolateral samples were freeze-dried and stored at −20 °C prior to analysis.

Anti-Inflammatory Activity of Basolateral Samples from Caco-2 Experiments. Human THP-1 monocytes (ATCC, Manassas, VA) were cultured in RPMI 1640 culture medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Gibco, Paisley, U.K.), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain) at 37 °C in 95% humidified air containing 5% CO_2. Cells were plated at a density of 5×10^5 cells/mL in 24 wells plates. Differentiation of monocytes to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 48 h. The viability of THP-1/M cells in the presence of basolateral medium from Caco-2 experiments was tested used the MTT assay following the Mosmann[26] method. The assays were performed in triplicate.

After differentiation, cells were washed with PBS and incubated with 0.05 μg/mL of LPS from *E. coli* O55:B5 (Sigma-Aldrich, Spain) in the presence of the basolateral medium from Caco-2 experiments for 24 h. Then, the supernatant was frozen at −20 °C. Positive controls represented THP-1/M cells incubated with LPS but without basolateral medium and negative controls indicated cells non-stimulated with LPS and without basolateral medium.

The release of TNF-α, IL-1β, and IL-6 was measured in the supernatants of THP-1/M cells using ELISA kits (BD Biosciences, Aalst, Belgium), according to the manufacturer's instructions. The generated color was quantified by measuring the optical density at 450 nm with substrate correction at 570 nm using a multiscanner autoreader (InfiniteM200 Tecan, Barcelona, Spain). The results were expressed as the mean of three determinations ± standard deviation (SD).

Statistical Analysis. Experimental results are expressed as means ± SD. One-way analysis of variance (ANOVA) followed by least significant difference test was used at $p \leq 0.05$ to look for differences between the means. Statistical analyses were performed using Statgraphics v. Centurion XVI software for Windows (Statpoint Inc., Warranton, VA).

RESULTS AND DISCUSSION

Phenolic and Functional Characterization of PLE and Enriched Extracts. PLE extracts from marjoram were carried out using 70% ethanol, 100 °C and 10 min. These extraction conditions were supported by previous studies (data not shown) developed to obtain extracts with a high content of TPC and an important antioxidant activity. The PLE obtained extract presented a TPC of 265.9 mg GAE/g extract and a TEAC value of 1.81 mmol Trolox/g extract.

For the purpose to achieve extract enrichment in phenolic compounds, XAD-7HP resin was used. The effectiveness of this kind of resins for rosmarinic acid enrichment has been previously reported.[23] As Table 1 shows; the use of this resin achieved a TPC 1.5-fold superior to the original PLE extract. Thus, the amount of phenolic compounds presented in the new extract represented 39% of the total extract. Moreover, this enriched extract also presented significantly higher antioxidant activity (Table 1).

Table 1. TPC Content and Antioxidant Activity in Original and Enriched Extracts[a]

		TPC (mg GAE/g)	TEAC value (mmol Trolox/g)
original extract	initial	266 ± 4.80 a2	1.81 ± 0.04 a2
	after digestion	2220 ± 12.6 b2	1.71 ± 0.05 b2
enriched extract	initial	389 ± 38.6 a1	2.81 ± 0.01 a1
	after digestion	312. ± 4.86 b1	2.67 ± 0.03 b1

[a]Data shown represent means ± SD ($n = 3$). Different letters denote significant differences within the same extract before and after digestion ($p \leq 0.05$). Different numbers denote significant differences between both extracts in the same condition (before or after digestion) ($p \leq 0.05$).

The HPLC−PAD-MS-QTOF analysis of the phenolic compounds (Table 2) resulted in the identification of 17 compounds in the extracts (original and enriched), whereas *p*-coumaric and neochlorogenic acids were also identified in the digested extracts. All the identified compounds in the extracts had been previously described in marjoram extracts.[27−29]

Moreover, according to its specific λ_{max} and accurate mass, MS/MS fragments 6-hydroxyluteolin-7-*O*-glucoside, luteolin-*O*-glucoside, lithospermic acid isomer, and salvianolic acid isomer were tentatively identified in the extracts.[28,30,31]

Both extracts showed a similar qualitative composition, although important quantitative differences were found (Table 3). Therefore, enriched extract presented, in general, an increase between 1.8 and 1.5 times in the quantity of all compounds in relation to the original extract. Accordingly, the main components in both extracts were 6-hydroxyluteolin-7-*O*-glucoside and rosmarinic acid, followed by a luteolin-*O*-glucoside and an isomer of salvianolic acid.

This phenolic composition was consistent with other research works where marjoram extracts had been characterized by an extended composition in phenolic acids and flavonoids.

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Table 2. Phenolic Compounds Identified in the Samples Using HPLC−PAD-MS/MS

peak	compound	R_t (min)	λ_{max} (nm)	accurate mass (m/z)	error (ppm)	MS^2 (m/z)
1	neochlorogenic acid[a]	12.9	300, 326	353.0878	+1.3	191(100), 179(75), 135(30)
2	protocatechuic acid[a]	13.4	260, 294	153.0193	+1.2	153(30), 109(100)
3	caftaric acid[a]	13.7	300, 328	311.0408	+2.5	149(100)
4	cryptochlorogenic acid[a]	15.1	300, 326	353.0878	+4.0	191(100), 179(75), 135(30)
5	vicenin II[a]	15.9	272, 336	593.1511	−1.8	503(20), 473(100), 383(20), 353(30)
6	caffeic acid[a]	17. 8	299, 324	179.0349	+2.6	135(100)
7	6-hydroxyluteolin-7-*O*-glucoside	19.8	282, 344	463.0882	−4.1	463 (20), 301(100)
8	*p*-coumaric[a]	22,9	310	163.0400	+2.1	119(100)
9	luteolin-*O*-glucoside	23.4	282, 334	447.0932	−3.4	285(100), 151(20)
10	luteolin 7-*O* glucoside[a]	23.8	254, 266, 348	447.0932	−2.6	285(100), 151(20)
11	luteolin 7-*O*-glucuronide[a]	24.0	254, 266, 348	461.0725	−4.7	285(100), 151(20)
12	diosmin[a]	27.1	352, 266, 346	607.1668	−2.7	607(10), 299(100), 284(10)
13	apigenin 7-*O*-glucoside[a]	27.6	266, 336	431.0983	−4.6	431(20), 269(100)
14	apigenin 7-*O*-glucuronide[a]	28.1	266, 336	445.0776	−4.3	445(20), 269(100)
15	rosmarinic acid[a]	28.9	288, 330	359.0772	+0.7	197(20), 161(100), 179(30), 135(20)
16	lithospermic acid isomer	29.7	288, 310, 334	537.1038	−4.9	493(10), 359(30), 295(30), 197(20), 161(100), 135(80)
17	salvianolic acid isomer	30.4	288, 310, 334	717.1461	−2.8	717(10), 519(30), 475(10), 359(100), 295(10), 179(10)
18	luteolin[a]	33.7	254, 266, 348	285.0404	−3.0	285(20), 217(35), 175(100), 151(50)
19	apigenin[a]	37.0	266, 336	269.0444	−3.8	269(20), 225(30), 151(100), 119(50)

[a]Comparison with standards.

Table 3. Effect of *in Vitro* Gastrointestinal Digestion Process in Extracts Composition

	original extract (mg/g extract)		enriched extract (mg/g extract)	
compound	initial	after digestion	initial	after digestion
neochlorogenic acid	N.D.[a]	0.57 ± 0.01[b]	N.D.[a]	0.88 ± 0.02[b]
protocatechuic acid	0.17 ± 0.01	0.19 ± 0.00[b]	0.30 ± 0.01	0.18 ± 0.00[b]
caftaric acid	0.06 ± 0.00	0.04 ± 0.00[b]	0.10 ± 0.01	0.09 ± 0.00
cryptochlorogenic acid	0.86 ± 0.00	0.53 ± 0.00[b]	1.49 ± 0.01	0.76 ± 0.00[b]
vicenin II	2.32 ± 0.02	2.24 ± 0.03[b]	4.00 ± 0.01	3.73 ± 0.01[b]
caffeic acid	0.93 ± 0.07	0.57 ± 0.01[b]	1.32 ± 0.01	0.88 ± 0.01[b]
6-hydroxyluteolin-7-*O*-glucoside	33.9 ± 0.09	14.5 ± 0.18[b]	60.9 ± 0.04	21.0 ± 0.23[b]
p-coumaric	N.D.[a]	0.36 ± 0.00[b]	N.D.[a]	0.52 ± 0.00[b]
luteolin-*O*- glucoside	25.2 ± 0.03	10.5 ± 0.12[b]	44.7 ± 0.02	15.3 ± 0.17[b]
luteolin 7-*O* glucoside	15.5 ± 0.08	14.6 ± 0.02[b]	25.5 ± 0.01	21.8 ± 0.04[b]
luteolin 7-*O*-glucuronide	5.69 ± 0.02	5.38 ± 0.02[b]	9.29 ± 0.01	8.17 ± 0.04[b]
diosmin	6.48 ± 0.04	6.46 ± 0.03	10.3 ± 0.01	10.8 ± 0.06[b]
apigenin 7-*O*-glucoside	2.45 ± 0.02	3.22 ± 0.03[b]	4.69 ± 0.01	4.90 ± 0.04[b]
apigenin 7-*O*-glucuronide	3.64 ± 0.49	5.74 ± 0.06[b]	7.20 ± 0.01	8.70 ± 0.02[b]
rosmarinic acid	33.9 ± 0.05	19.0 ± 0.11[b]	57.2 ± 0.05	29.0 ± 0.34[b]
lithospermic acid isomer	9.10 ± 0.02	6.91 ± 0.11[b]	15.9 ± 0.05	11.0 ± 0.11[b]
salvianolic acid isomer	17.2 ± 0.35	2.60 ± 0.14[b]	24.9 ± 0.16	4.27 ± 0.09[b]
luteolin	1.39 ± 0.08	0.55 ± 0.03[b]	2.07 ± 0.01	0.73 ± 0.02[b]
apigenin	0.45 ± 0.01	0.22 ± 0.00[b]	0.75 ± 0.01	0.31 ± 0.04[b]

[a]N.D. = non detected. [b]Denotes significant difference when compares initial and after digestion concentration within same extract ($p \leq 0.05$). Data represent means ± SD ($n = 4$).

These works reported that, among phenolic acids, RA was the main compound detected. In addition, luteolin and its glucosil derivatives have been also detected as the main flavonoids.[3,16]

Phenolic Composition and Antioxidant Activity of the Extracts during *in Vitro* Digestion. Digestion effect in TPC and antioxidant activity for both extracts is shown in Table 1. After the digestion process, the amount of TPC only decreased slightly for both extracts as well as antioxidant activity. Notwithstanding, digestion step produced a remarkable reduction of RA (approximately 50%) in both original and enriched extracts (Table 3). This decrease in RA content during gastrointestinal digestion was in agreement with other studies that reported RA degradation or transformation into other compounds during *in vitro* gastrointestinal digestion.[32,33] Moreover, Zoric et al.[34] showed that gastrointestinal stability of RA was highly influenced by the plant matrix, indicating that the presence of some flavonoids such as luteolin or apigenin enhances the stability rate of RA during the digestion process.

Moreover, the quantitative analysis of phenolic composition after the digestion process (Table 3) showed that most compounds, presented in both extracts, were affected by digestion step to a lesser or greater extent. For that matter, although RA was reduced after digestion, it represented the main compound in both digested extracts, followed by luteolin

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Table 4. TPC Content and Antioxidant Activity in Original and Enriched Extracts after Caco-2 Absorption Experiments[a]

		TPC (mg GAE/L digested extract)	TEAC value (mmol Trolox/L digested extract)
original extract	after digestion	502 ± 18.1 a2	3.41 ± 0.16 a2
	apical fraction	358 ± 26.7 b2	1.77 ± 0.21 b2
	basolateral fraction	68.5 ± 6.40 c2	0.73 ± 0.01 c2
enriched extract	after digestion	684 ± 31.1 a1	4.78 ± 0.58 a1
	apical fraction	556 ± 40.5 b1	3.74 ± 0.36 b1
	basolateral fraction	84.8 ± 7.86 c1	0.89 ± 0.09 c1

[a]Data shown represent means ± SD (n = 3). Different letters denote significant differences within the different fractions of the same extract ($p \leq 0.05$). Different numbers denote significant differences among the same fractions of both extracts ($p \leq 0.05$).

Table 5. Extracts Phenolic Composition after Caco-2 Absorption Experiments[a]

	original extract (mg/L digested extract)			enriched extract (mg/L digested extract)		
compound	initial	apical	basolateral	initial	apical	basolateral
neochlorogenic acid	1.45 ± 0.41	0.94 ± 0.04	0.31 ± 0.03	2.09 ± 0.03	1.77 ± 0.09	0.41 ± 0.07
protocatechuic acid	0.45 ± 0.03	0.37 ± 0.00	N.D.[b]	0.43 ± 0.03	0.39 ± 0.04	N.D.[b]
caftaric acid	0.06 ± 0.00	N.D.[b]	N.D.[b]	0.15 ± 0.03	0.05 ± 0.00	N.D.[b]
cryptochlorogenic acid	1.27 ± 0.00	0.81 ± 0.08	0.13 ± 0.03	1.75 ± 0.02	1.39 ± 0.09	0.21 ± 0.07
vicenin II	4.81 ± 0.54	4.07 ± 0.52	0.63 ± 0.09	8.35 ± 0.14	7.74 ± 0.42	1.00 ± 0.40
caffeic acid	1.51 ± 0.17	1.08 ± 0.10	0.37 ± 0.03	2.39 ± 0.01	1.86 ± 0.07	0.54 ± 0.09
6-hydroxyluteolin-7-*O*-glucoside	17.1 ± 1.04	2.66 ± 0.35	0.47 ± 0.29	20.5 ± 0.43	6.69 ± 1.09	1.01 ± 0.62
p-coumaric	0.80 ± 0.15	0.50 ± 0.03	0.30 ± 0.01	1.38 ± 0.05	0.81 ± 0.01	0.45 ± 0.06
luteolin-*O*-glucoside	12.8 ± 0.84	2.81 ± 0.10	0.42 ± 0.21	15.9 ± 0.41	6.61 ± 0.89	1.02 ± 0.58
luteolin 7-*O* glucoside	17.2 ± 1.35	2.69 ± 0.41	0.54 ± 0.32	23.84 ± 0.58	8.34 ± 1.22	1.03 ± 0.05
luteolin 7-*O*-glucuronide	7.03 ± 0.99	2.26 ± 0.04	0.74 ± 0.06	8.36 ± 0.23	4.71 ± 0.28	1.26 ± 0.03
diosmin	10.5 ± 0.19	8.56 ± 0.02	1.06 ± 0.21	15.7 ± 0.17	18.6 ± 1.36	1.96 ± 0.62
apigenin 7-*O*-glucoside	4.25 ± 0.10	1.66 ± 0.02	0.17 ± 0.06	6.20 ± 0.08	3.72 ± 0.14	0.46 ± 0.21
apigenin 7-*O*-glucuronide	9.03 ± 0.36	3.23 ± 0.38	0.75 ± 0.16	13.4 ± 0.24	7.33 ± 0.19	1.40 ± 0.47
rosmarinic acid	32.2 ± 2.30	23.1 ± 4.05	4.45 ± 0.54	51.6 ± 2.61	42.4 ± 5.63	7.37 ± 1.39
lithospermic acid isomer	11.4 ± 1.02	8.53 ± 1.37	1.45 ± 0.29	18.3 ± 0.76	14.4 ± 0.08	2.13 ± 0.42
salvianolic acid isomer	4.27 ± 0.41	3.20 ± 0.33	0.61 ± 0.08	6.44 ± 0.10	4.82 ± 0.10	0.87 ± 0.10
luteolin	0.23 ± 0.02	1.92 ± 0.75	0.15 ± 0.01	0.45 ± 0.00	3.82 ± 0.69	0.61 ± 0.47
apigenin	0.04 ± 0.00	0.70 ± 0.39	N.D.[b]	0.07 ± 0.00	1.48 ± 0.37	0.17 ± 0.00

[a]Data represent means ± SD (n = 4). [b]N.D. = non detected.

7-*O*-glucoside, 6-hydroxyluteolin-7-*O*-glucoside, and luteolin-*O*-glucoside. Among these compounds, 6-hydroxyluteolin-7-*O*-glucoside and luteolin-*O*-glucoside were most affected by the digestion step, meanwhile, luteolin 7-*O*-glucoside and glucuronide were much less affected, even more apigenin 7-*O*-glucoside and glucuronide increased after digestion. It should be also noted the appearance of neochlorogenic and *p*-coumaric acids in both digested extracts, compounds not detected in the nondigested extracts and whose presence could be attributed to isomerization and degradation processes of initial phenolic acids. Accordingly, Xie et al.[35] noted isomeric transformations of chlorogenic acids, where cryptochlorogenic acid was mainly turned into chlorogenic acid at pH 7 and 37 °C, in agreement with intestinal conditions. Moreover, some studies have shown the presence of coumaric acid as a related metabolite of rosmarinic acid in human and animal plasma.[36,37]

Caco-2 Cell Transport Experiments. Transport experiments of digested extracts were carried out using an *in vitro* model of the intestinal barrier: Caco-2 cells differentiated to enterocytes.[38] Prior to transport experiments, the cytotoxicity of the digested extracts was evaluated at 6 h. The results showed that 150 μL of the digested extracts was the maximum concentration that did not significantly affected cell viability (data not shown). In addition, the integrity of the Caco-2 monolayer during exposure experiments was evaluated as a TEER-value.

Total phenolic compounds content recovered in apical and basolateral fractions for both digested extracts was measured to determine the quantity of these compounds unabsorbed and bioavailable, respectively (Table 4). For both extracts, only a small amount of the phenolic compounds presented in the digested extracts was detected in the bioavailable fraction. Thereby, the bioavailable fraction from enriched extract presented a quantity of 84.8 mg GAE/L digested extract, meanwhile for original extract only 68.5 mg GAE/L digested extract was detected in basolateral fraction.

The phenolic compounds recovered in apical and basolateral fractions for both digested extracts were also analyzed by HPLC in order to determine the bioavailability of individual components (Table 5). The results indicated that, for both extracts, the main component detected in basolateral fraction was RA, although enriched extract presented a quantity 1.6 times superior. Besides RA, lithospermic acid isomer and diosmin were the major components of basolateral fraction in both extracts, meanwhile 6-hydroxyluteolin-7-*O*-glucoside and luteolin 7-*O*-glucoside, compounds with an important presence after digestion process, only were presented in less than 5% in that fraction.

The permeability of RA across Caco-2 cells monolayer has been studied by several authors. Accordingly, Konishi and Kobayashi[13] reported that RA transport throughout Caco-2 cells was mainly via paracellular diffusion and its intestinal

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absorption efficiency was low. These authors supported this idea with the fact that RA transport increased linearly with the concentration and did not reach a plateau even at 30 nM. However, more recently, Qiang et al.[39] suggested that RA was absorbed across Caco-2 cells via both paracellular and transcellular diffusion. Moreover, Falé et al.[40] reported that the transport of RA across these cells was increased in the presence of a mixture of luteolin and apigenin, since these flavonoids may interfere in several mechanisms involved in the permeation of RA, such as uptake and efflux mechanisms, indicating that the flux of RA may be mediated by transport systems. Data obtained in this work for RA were consistent with the idea that RA transport throughout Caco-2 cells was mainly via paracellular diffusion, since in this case the transport increased linearly with the concentration. Thus, enriched extract (after digestion) with a quantity of rosmarinic acid 1.6 times higher than original extract, also presented in basolateral fraction 1.6-fold of RA than the original extract. However, it could not be discarded the influence of the flavonoid content in the absorption efficiency of RA obtained in this work (approximately 14%).

In this work, the bioavailability of luteolin and apigenin derivatives was, in general, lower than for RA, although, in this case the transport also increased linearly with the concentration. However, in this study is noteworthy that, after 6 h of experiment, the amount of luteolin and apigenin aglycone in the apical compartment substantially increased in relation to the amount of these components at $t = 0$. According to those results, Yasuda et al.[41] suggested that luteolin glucoside is partially hydrolyzed by LPH (lactase-phlorizin hydrolase), and moreover, only a fraction of the released aglycone is absorbed inside the cells, where it is converted to its glucuronide conjugate and subsequently secreted to basolateral compartment. In this study, glucoside and glucuronide forms of luteolin and apigenin were detected in basolateral solution after 6 h, inferring that a portion of glycoside derivatives are also transported in an unchanged way across Caco-2 cells.[42,43] Nevertheless, for a better comprehensive absorption path for flavones, further analysis should be submitted.

In addition, antioxidant activity for both digested extracts was also measured in apical and basolateral compartments and data are shown in Table 4. As could be observed, the antioxidant activity detected in basolateral fraction from enriched extract was significantly higher than that measured in this fraction when original extract was used. This result was in agreement with the greater amount of phenolic compounds detected in the basolateral chamber for this extract. Thus, mainly RA, lithospermic acid and diosmin presented in the bioavailable fraction could be responsible for its antioxidant activity.

Anti-Inflammatory Activity of Basolateral Samples from Caco-2 Experiments. The activation of THP-1/M was carried out by LPS incorporation into the medium. After 24 h of incubation, LPS treated cells showed an important increase in all pro-inflammatory cytokines measured (TNF-α, IL-1β, and IL-6), compared to nonactivated controls (Figure 1). These activated cells were considered as positive controls for all the cytokines tested. The cytotoxicity assays (data not shown) indicated that 20 μL of the basolateral fraction was the maximum concentration that did not affected THP-1 viability. Thus, when the activation of THP-1/M was carried out in the presence of 20 μL of basolateral medium from Caco-2 experiments, an important decrease in TNF-α secreted level was observed compared with levels obtained in the absence of

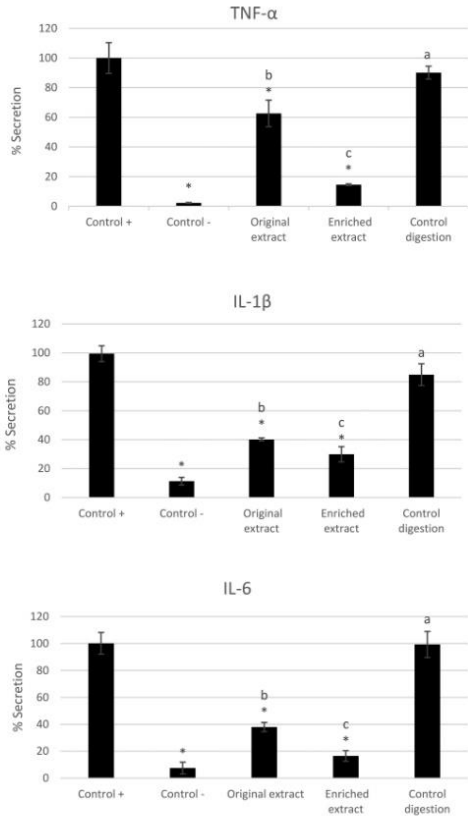

Figure 1. Levels of TNF-α, IL-1β, and IL-6 secreted by THP-1/M, activated with LPS, in the presence of bioavailable fractions from extracts. Positive control (cells stimulated with LPS but in absence of extract), negative control (cells in contact just with RPMI media), control digestion (bioavailable fraction from a digestion without extract, only digestion juices). Each bar is the mean of three determinations ± SD. *Denotes statistical differences when compares with positive control. a,b,c, Different letters indicate statistical differences among the original extract, enriched extract, and digestion control. Significance level at $p \leq 0.05$ with LSD procedure.

extracts (positive control). Moreover, basolateral samples from enriched extract after digestion achieved an 80% of inhibition in TNF-α secretion, higher to that obtained with the original extract (40%). IL-1β and IL-6 secretion were also reduced (60%) in the presence of 20 μL of basolateral fractions of the original digested extracts (with respect to positive control). For both interleukins, the enriched extract decreases its release in a greater extent than the original extract, 70% for IL-1β and an 85% for IL-6. Besides, the basolateral fraction from controlled digestion did not reduce significantly the secretion of any of the pro-inflammatory cytokines studied, compared to the positive control.

Considering these results, basolateral fractions of both digested extracts presented an important anti-inflammatory

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activity, although enriched extract showed a higher inhibition in the release of all pro-inflammatory cytokines studied. In this regard, several studies have reported that RA, either as pure standard or incorporated into a vegetable matrix, inhibited LPS-induced up-regulation of IL-1 β, TNF-α, and IL-6 in different cells lines.[11,12] Besides, luteolin and its derivatives have also been reported to be able to inhibit the production of pro-inflammatory cytokines, such as TNF-α and IL-1β.[44] However, none of these studies measured the inhibition in the production of these cytokines by extracts or pure compounds after digestion and absorption processes.

In conclusion, this study showed the potential use of marjoram extracts as a source of bioavailable compounds with an important antioxidant and anti-inflammatory activities. Thus, the results indicated that PLE extracts from *Origanum majorana* L. represented a rich source of bioavailable RA, especially when using the enriched extract. Besides RA, luteolin derivatives, lithospermic acid isomer, and diosmin were also detected in the basolateral fraction in both extracts. Moreover, the bioavailable fractions of both extracts showed remarkable antioxidant and anti-inflammatory activities, being more prominent when using enriched extract. Thus, RA could have an important role in these activities, although other phenolic compounds detected in the basolateral fractions could also interact synergistically.

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Supercritical anti-solvent fractionation for improving antioxidant and anti-inflammatory activities of an *Achillea millefolium* L. extract

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ABSTRACT

Achillea millefolium L. is a plant widely used in traditional medicine. Nowadays, there is a growing concern about the study of its bioactive properties in order to develop food and nutraceutical formulations. Supercritical anti-solvent fractionation (SAF) of an *A. millefollium* extract was carried out to improve its antioxidant and anti-inflammatory activities. A selective precipitation of phenolic compounds was achieved in the precipitation vessel fractions, which presented an antioxidant activity twice than original extract, especially when fractionation was carried out at 10 MPa. The main phenolic components identified in this fraction were luteolin-7-*O*-glucoside, 3,5-dicaffeoylquinic acid, 6-hidroxyluteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside. However, separator fractions presented higher anti-inflammatory activity than precipitation vessel ones, particularly at 15 MPa. This fact could be related to separator fractions enrichment in anti-inflammatory compounds, mainly camphor, artemisia ketone and borneol.

Therefore, SAF produced a concentration of antioxidant and anti-inflammatory compounds that could be used as high-added valued ingredients.

1. Introduction

Achillea millefolium L. (yarrow) is a flowering plant widely used in folk medicine in Europe. Aqueous and alcoholic extracts from dried upper parts of yarrow have been employed in the treatment of digestive problems, hepato-biliary disorders and externally, for the treatment of skin and mucous membrane inflammation (Dias et al., 2013). The study of this plant, both its composition and biological activities, has awakened a growing interest in order to develop pharmaceutical, food and nutraceutical products. Vitas, Cvetanović, MaškoviĆ, Švarc-Gajić, and Malbaša (2018) produced kombucha beverages from a yarrow infusion and extracts. In addition, nowadays there are on the market several herbal tea mixtures (containing yarrow), mainly indicated for digestive problems.

Certain naturally occurring bioactive compounds present in *A. millefolium*, such as phenolic compounds, particularly chlorogenic and dicaffeoylquinic acids (DCQA) and flavonoids, as well as those belonging to the volatile oil fraction have been associated with health benefits (Mohammadhosseini, Sarker, & Akbarzadeh, 2017). Moreover, recent reports indicated that *Achillea* genus presents an important antioxidant activity, related to its flavonoids and total phenolic content (Giorgi, Mingozzi, Madeo, Speranza, & Cocucci, 2009; Mohammadhosseini et al., 2017). In addition, Trumbeckaite et al. (2011) reported that the radical-scavenging properties of a hydroalcoholic extract of *A. millefolium* were related to the presence of luteolin and chlorogenic acid in the extract, an in a lesser extent, to the presence of rutin and luteolin-7-*O*-glucoside. *A. millefolium* extracts have also been reported to present anti-inflammatory activity (Tadíc et al., 2017). Moreover, Kazemi (2015) showed that an *A. millefolium* essential oil, with high quantities of thymol and borneol, was able to inhibit nitric oxide production in macrophages stimulated with LPS (lipopolysaccharide).

Different approaches have been carried out in order to obtain fractions with high concentrations of phenolic compounds or essential oils components than original plants extracts; such as applying anion exchange resins (Kammerer, Boschet, Kammerer, & Carle, 2011), high pressure techniques (Fernández-Ponce, Casas, Mantell, & de la Ossa, 2015), membrane separation (Cissé, Vaillant, Pallet, & Dornier, 2011), supercritical fluid extraction with fractionation (Reverchon & de Marco, 2006) or chromatography methods (Pedan, Fischer, & Rohn, 2016;

Abbreviations: SAF, Supercritical anti-solvent fractionation; DCQA, Dicaffeoylquinic acid; SC-CO_2, Supercritical CO_2; UAE, Ultrasound-Assisted extraction; GAE, Gallic acid equivalents; THP-1/M, Human THP-1 monocytes differentiated to macrophages)

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Shaheen, Lu, Geng, Shao, & Wei, 2017). Recently, supercritical antisolvent fractionation (SAF) has been proposed for the fractionation of complex plants extracts. In addition, the use of carbon dioxide as a supercritical fluid offers many advantages, such as the low critical temperature of CO_2 and the absence of oxygen during extraction, which allows minimize or avoid the degradation of solutes, as well as the possibility of recovering a free-solvent fraction (Wijngaard, Hossain, Rai, & Brunton, 2012). In the SAF process, a polar liquid solution of a plant extract, containing several families of compounds, is sprayed continuously in a co-current with supercritical CO_2 (SC-CO_2), which acts as antisolvent. This contact allows the precipitation of more polar components from the liquid solution, insoluble in SC-CO_2, whereas the remaining compounds, that are mainly less polar components, remained dissolved and are recovered by downstream pressure reduction (Meneses, Caputo, Scognamiglio, Revenchon, & Adami, 2015).

For that matter, SAF technique has recently used to fractionate phenolic compounds from plants extracts. Therefore, Natolino, Da Porto, Rodríguez-Rojo, Moreno, and Cocero (2016) used this technique to obtain fractions enriched in polyphenols from a grape marc extract. Operating at 12 MPa, 45 °C and 0.99 CO_2 molar fraction, they obtained fractions with a relative enrichment of 350% of total polyphenols and a proanthocyanidins enrichment between 300 and 450%. Visentín, Cismondi, and Maestri (2011) also applied the SAF to improve carnosic acid (CA) recovery from an ethanolic extract of rosemary leaves, obtaining two different fractions, one insoluble with low concentration in CA (< 5%) and another resinous extract with 33% of CA. Moreover, Villanueva et al. (2015) carried out the fractionation of green tea extracts obtaining decaffeinated fractions with high concentration in catechins.

Nevertheless, there are only few studies relating the enrichment in phenolic compounds or essential oil components of the fractions obtained by this technique, with their biological activities. Chinnarasu et al. (2015) obtained precipitates with high antioxidant activity from the fractionation of olive leaves extracts and Marqués, Porta, Reverchon, Renuncio, and Mainar (2013) concentrated antioxidants from a defatted grape seed waste extract, achieving concentrations up to 2.7 higher than those in the starting solution. Sánchez-Camargo et al. (2016) also enhanced the antiproliferative activity of a rosemary fraction enriched in carnosic acid and carnosol.

In this context, and based on a previous work (Villanueva-Bermejo et al., 2017), the aim of the present study was to improve the antioxidant and anti-inflammatory activities of an *A. millefollium* extract using SAF technology. Besides, the original extract and fractions obtained were analyzed in order to relate its chemical composition with the biological activities found.

2. Materials and methods

2.1. Reagents and chemicals

Ethanol (99.5% purity) and Folin-Ciocalteu's reagent were obtained from Panreac (Barcelona, Spain). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid for titration (> 97.5%), potassium persulfate (99.9%) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (Madrid, Spain). Formic acid (99%) was obtained from Acros Organics (Madrid, Spain) and acetonitrile HPLC grade from Macron Fine Chemicals (Madrid, Spain). Reference standards for phenolic compounds, such as chlorogenic acid, cryptochlorogenic acid, diosmetin, ferulic acid, neochlorogenic acid, rosmarinic acid and vitexin (all analytical standard or HPLC purity ≥95%) were purchased from Sigma Aldrich (Madrid, Spain). 1,5-Dicaffeoylquinic acid (DCQA), 3,4-DCQA, 3,5-DCQA, 4,5-DCQA, apigenin, caftaric acid, casticin, luteolin, orientin, schaftoside and vicenin II were obtained from Phytolab (Madrid, Spain). Finally, amentoflavone, apigenin-7-*O*-glucoside, caffeic acid, homoorientin, luteolin-7-*O*-β-D-glucoside, quercetin and rutin were obtained from Extrasynthese S.A. (Genay, France) and luteolin-7-*O*-β-D-glucuronide from HWI Analytic GmbH (Rülzheim, Germany). The water used in this study was ultrapure type 1 (Millipore, Madrid, Spain). CO_2 (N38) was purchased from Carburos Metalicos (Madrid, Spain).

2.2. Yarrow samples and ultrasound-assisted extraction (UAE)

A. millefolium from Bulgaria was supplied by a local herbalist (Murcia, Spain). According to supplier specifications, the sample included inflorescences and upper dried leaves of the plant (harvested in spring) and sun-dried (water content < than 5% wt). The plant was ground using a Premill 250 hammer mill (Leal S.A., Granollers, Spain) and sieved (particle size < 500 μm). UAE plant extraction was carried out by using an ultrasonic device (Branson Digital Sonifier 250, Danbury, USA) with a power of 200 W and frequencies of 60 kHz. Extraction conditions employed were ethanol as extraction solvent (1:10 plant/solvent ratio), 30 min time, 40 °C temperature and an output of 70% with respect to the nominal amplitude. Finally, the extract was concentrated, until the final volume contained 17.9 mg/mL of total solid concentration (2.3% wt.) by rotary evaporation and stored at −20 °C until its use in the SAF process.

2.3. SAF process

A detailed explanation of the device and the process design employed can be found elsewhere (Villanueva-Bermejo et al., 2017). Briefly, fractionation of UAE yarrow solution (concentration of 17.9 mg/mL) was carried out at two different pressures (10 and 15 MPa), 40 °C and CO_2/extract flow ratio of 31.3 g/g (50 g/min for CO_2 and 1.6 g/min for UAE extract). The experiment started by pumping SC-CO_2 into the precipitation vessel until the pressure and temperature conditions were attained. Then, the UAE yarrow solution was pumped into the precipitator. After mixing, the yarrow extract components that were not soluble in SC-CO_2 + ethanol mixture precipitated in the precipitation vessel and were collected (precipitation vessel fraction). The fraction soluble in SC-CO_2 + ethanol went to separators where reduced pressure turned CO_2 into a gas and this fraction together with ethanol was also collected. The samples obtained in both separators were combined in a single fraction and ethanol removed by rotary evaporation under vacuum (separator fraction). Fractions were kept at −20 °C under darkness until analysis.

2.4. HPLC-PAD-ESI-QTOF-MS analysis

Phenolic compounds were analyzed by HPLC following the chromatographic method developed by Villalva et al. (2018). An Agilent HPLC 1260 Infinity series system (Agilent Technologies Inc., Santa Clara, CA, USA) was used for that purpose. Chromatographic separation was carried out by using a reverse phase ACE Excell 3 Super C18 column (150 mm × 4.6 mm, 3 μm particle size) from Advanced Chrmatography Technologies (Aberdeen, Scotland) protected by an ACE 3 C18-AR (10 mm × 3 mm) guard column. Dry samples were dissolved in ethanol to reach a 5 mg/mL concentration and filtered by 0.45 μm polyvinylidene fluoride (PVDF) filter before injection (20 μL). Retention time and UV–Vis spectrum of each chromatographic peak was compared with analytical standards for identification purpose; moreover, accurate mass from HPLC-ESI-QTOF-MS in negative mode analysis was used for compounds assignment.

Compounds quantification was carried out by using calibration curves from analytical standard, as previously described in Villalva et al. (2018). In addition, luteolin-6,8-di-*C*-glucoside, 6-hydroxyluteolin-7-*O*-glucoside and non-identified flavones were quantified by the calibration curve of orientin, luteolin-7-*O*-glucoside and luteolin respectively. Likewise, schaftoside and vicenin II calibration curves

were used for schaftoside isomer and apigenin-*C*-hexoside-*C*-pentoside quantification.

2.5. GC–MS analysis of the separator fractions

The analysis of the UAE extract and SAF fractions collected from the separator was carried out in an Agilent 7890A system (Agilent Technologies, Santa Clara, CA, USA). The unit comprised a split/splitless injector, a FID detector and a mass spectrometer detector (5975C triple-axis). The analysis was performed using an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm phase thickness) and the following chromatographic method: 40 °C initial temperature, from 40 °C to 150 °C at 3 °C min^{-1}, isothermal at 150 °C for 10 min, then increased from 150 to 300 °C at 6 °C min^{-1} and finally isothermal at 300 °C for 1 min. Samples were dissolved in ethanol (at 5 mg/mL), filtered by 0.45 μm filters and inyected (1 μL) in splitless mode. Helium (99.99%) was employed as carrier gas (1 mL/min flow rate). The temperatures used were 250 °C for the injector and 230, 280 and 150 °C for the mass spectrometer ion source, interface and quadrupole, respectively. The mass spectrometer operated under electron impact mode (70 eV) and it was used in total ion current (TIC) mode (mass range from 40 to 500 *m/z*). The identification of compounds was performed by matching the mass spectral fragmentation patterns with the Wiley 229 mass spectral library, as well as comparing their corresponding retention index to those reported in the literature. Analyses were done in triplicate.

2.6. Determination of total phenolic content (TPC) and antioxidant activity

TPC determination was carried out according to Folin-Ciocalteau reagent method as described by Singleton, Orthofer, and Lamuela-Reventos (1999) using gallic acid as standard. In brief, 10 μL of samples (5 mg/mL for all samples, except for P10 and P15 which working solution was 3 mg/mL) were mixed with 50 μL of Folin-Ciocalteau reagent and 790 μL of deionized water. After 3 min, 150 μL of sodium carbonate solution (20% *w/v*) were added and mixed. After 2 h, the absorbance at 760 nm was recorded. The results were expressed as mg of gallic acid equivalents (GAE)/g dry sample. Analyses were performed at least in triplicate.

The antioxidant activity was measured using the $ABTS^{\cdot+}$ radical scavenging assay as described in Re et al. (1999). The reaction was placed with 990 μL of the diluted $ABTS^{\cdot+}$ radical solution and 10 μL of plant extract dilutions in order to achieve a 20% to 80% of radical inhibition (sample concentration varying from 5 mg/mL to 20 mg/mL). The reaction was allowed to stand until the absorbance reached a plateau, and the absorbance was recorded at 734 nm. Results were expressed as mmol Trolox equivalent/g dry sample (TEAC value). Analyses were done at least in triplicate.

2.7. Anti-inflammatory activity

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were plated at a density of 5×10^5 cells/mL in 24 wells plates. Culture medium consisted in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Gibco, Paisley, UK) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, Madrid, Spain) at 37 °C in 95% humidified air containing 5% CO_2. Monocytes differentiation to macrophages (THP-1/M cells) was induced by maintaining the cells with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 48 h. THP-1/M cells viability, in presence of yarrow extract or fractions, was tested by MTT assay following Mosmann (1983) method. The assays were performed in triplicate.

After differentiation, THP-1/M cells were washed with PBS and incubated with 0.05 μg/mL of LPS from *E. coli* O55:B5 (Sigma-Aldrich, Spain) in presence of yarrow extract or fractions for 24 h. Then, the supernatants were collected and frozen at −20 °C. An anti-inflammatory drug, indomethacin (5 μg/mL), was used as a reference.

ELISA kits (BD Biosciences, Aalst, Belgium), according to manufacturer's instructions, were used to measure the release of TNF-α, IL-1β and IL-6 in the supernatants of THP-1/M cells. The quantification was carried out at 450 nm with substrate correction at 570 nm using a multiscanner autoreader (InfiniteM200 Tecan, Barcelona, Spain). The results were expressed as the mean of three determinations ± standard deviation.

2.8. Statistical analysis

Statistical analysis was performed using Statgraphics v. Centurion XVI package for Windows (Statpoint Inc., Warrenton, VA, USA). Statistical differences between samples were analyzed by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) procedure was applied to determine significant differences between means at $p \leq .05$.

3. Results and discussion

3.1. TPC content and antioxidant activity of yarrow extract and SAF fractions

UAE extracts from yarrow were carried out using ethanol as extraction solvent, at 40 °C and 30 min. Previous studies carried out in the research group (data not shown) showed that those conditions were the most appropriated to obtain extracts with a high content of TPC and an important antioxidant activity. Thus, this UAE extract (called original extract) presented a yield of 5.7 ± 0.9% (% dry wt.), a TPC of 54.30 ± 1.07 mg GAE/g extract and a TEAC value of 0.173 ± 0.004 mmol Trolox/g extract.

SAF process was carried out in order to enhance the antioxidant activity of this extract. Consequently, two different experiments, at 10 and 15 MPa of pressure, 40 °C and CO_2/extract flow ratio of 31.3 g/g, were developed. These conditions were based in a previous work (Villanueva-Bermejo et al., 2017).

Fractions obtained at 10 and 15 MPa from the precipitation vessel (called P10 and P15) and separators (called S10 and S15) were collected and its yield value, TPC content and antioxidant activity were determined (Table 1). Results indicated that SAF process achieved an important phenolic compounds enrichment in the precipitation vessel fractions. It is worth to mention that this enrichment was significantly higher when used pressure was 10 MPa, where P10 fraction presented an antioxidant activity 2-fold superior than the original extract. Regarding separator fractions, S10 and S15 showed a similar antioxidant activity and near 5-fold lower than original extract. This result was related to the lower quantity of TPC presented in these fractions.

Therefore, phenolic composition of the original extract and fractions

Table 1
Yield values (mass precipitated/mass of solid pumped), TPC content and antioxidant activity (TEAC value) in original extract and SAF fractions: P10 (precipitation vessel fraction at 10 MPa), P15 (precipitation vessel fraction at 15 MPa, S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa). Data shown represents mean ± S.D. ($n = 3$).

Sample	Yield (%)	TPC (mg GAE/g)	TEAC value (mmol Trolox/g)
Original extract	5.7 ± 0.9	54.30 ± 1.07 ^{c}	0.173 ± 0.004 ^{c}
P-10	6.6 ± 1.1	136.44 ± 1.28 ^{a}	0.345 ± 0.002 ^{a}
S-10	52.1 ± 5.3	30.30 ± 0.25 ^{d}	0.035 ± 0.002 ^{d}
P-15	13.3 ± 2.4	119.20 ± 2.23 ^{b}	0.317 ± 0.003 ^{b}
S-15	42.6 ± 4.2	23.83 ± 0.85 ^{e}	0.033 ± 0.002 ^{d}

^{a,b,c,d,e}Different superscript letters denote statistical differences between samples within the same column. Significance level at $p \leq .05$ with Fisher's Least Significant Difference (LSD) test.

Table 2
Phenolic composition of original extract and SAF fractions (mg compound/g dry fraction). P10 (precipitation vessel fraction at 10 MPa), P15 (precipitation vessel fraction at 15 MPa), S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa). Data shown represents mean ± S.D. (n = 3).

Compound	Original	P-10	S-10	P-15	S-15
Neochlorogenic acid	0.06 ± 0.00	0.18 ± 0.00$_{\zeta}$	–	0.14 ± 0.00$_{\zeta}$	–
Caftaric acid	< L.Q.	0.18 ± 0.00$_{\zeta}$	–	0.22 ± 0.00$_{\zeta}$	–
Chlorogenic acid	0.62 ± 0.00	2.32 ± 0.0 $^{*}_{a}$	0.10 ± 0.00*	1.91 ± 0.04$_{\zeta}$	–
Cryptochlorogenic acid	0.01 ± 0.01	0.03 ± 0.00 $_{a}$	–	0.04 ± 0.01 $_{a}$	–
Vicenin II	0.37 ± 0.01	1.61 ± 0.00$_{\zeta}$	–	1.12 ± 0.01$_{\zeta}$	–
Caffeic acid	0.17 ± 0.00	–	0.19 ± 0.00$_{\lambda}$	–	0.18 ± 0.00$_{\beta}$
Schaftoside isomer	0.26 ± 0.00	1.49 ± 0.05$_{\zeta}$	–	0.90 ± 0.00$_{\zeta}$	–
Schaftoside	0.27 ± 0.00	1.36 ± 0.00$_{\zeta}$	–	0.89 ± 0.00$_{\zeta}$	–
Homoorientin	0.02 ± 0.01	0.32 ± 0.00$_{\zeta}$	–	0.16 ± 0.00$_{\zeta}$	–
Apigenin-*C*-hexoside –*C*-pentoside	0.30 ± 0.00	1.43 ± 0.01$_{\zeta}$	–	0.85 ± 0.00$_{\zeta}$	–
Luteolin-6,8-di-*C*-glucoside	0.47 ± 0.00	2.39 ± 0.01$_{\zeta}$	–	1.51 ± 0.00$_{\zeta}$	–
6-hidroxyluteolin-7-*O*-glucoside	1.45 ± 0.01	7.65 ± 0.02$_{\zeta}$	0. 02 ± 0.00*	4.66 ± 0.00$_{\zeta}$	–
Rutin	0.51 ± 0.01	1.44 ± 0.00$_{\zeta}$	–	1.34 ± 0.02$_{\zeta}$	–
Vitexin	0.13 ± 0.01	0.15 ± 0.00$_{\beta}$	–	0.25 ± 0.01$_{\zeta}$	–
Luteolin-7-*O*-glucoside	7.69 ± 0.08	33.2 ± 0.07$_{\zeta}$	0.39 ± 0.00*	23.9 ± 0.97$_{\zeta}$	–
Luteolin-7-*B*-glucuronide	0.20 ± 0.01	0.56 ± 0.00$_{\zeta}$	–	0.59 ± 0.03$_{\zeta}$	–
Ferulic acid	0.08 ± 0.02	0.09 ± 0.00$_{\zeta}$	–	0.04 ± 0.00$_{\zeta}$	–
3,4-DCQA	0.38 ± 0.05	1.23 ± 0.00$_{\zeta}$	–	0.69 ± 0.00$_{\zeta}$	–
1,5-DCQA	0.69 ± 0.01	2.86 ± 0.01$_{\zeta}$	–	1.80 ± 0.09$_{\zeta}$	–
3,5-DCQA	3.62 ± 0.02	17.8 ± 0.03$_{\zeta}$	0.28 ± 0.01$_{\lambda}$	11.6 + 0.10$_{\zeta}$	0.10 ± 0.00$_{\beta}$
Apigenin-7-*O*-glucoside	1.79 ± 0.01	6.89 ± 0.03$_{\zeta}$	0.32 ± 0.00*	5.88 ± 0.01$_{\zeta}$	–
4,5-DCQA	0.97 ± 0.01	4.30 ± 0.01$_{\zeta}$	0.05 ± 0.00*	3.19 ± 0.00$_{\zeta}$	–
Rosmarinic acid	0.18 ± 0.00	–	–	–	–
Luteolin	4.47 ± 0.01	4.58 ± 0.01$_{\zeta}$	3.18 ± 0.00$_{\lambda}$	13.0 ± 0.00$_{\zeta}$	0.95 ± 0.01$_{\beta}$
Quercetin	0.47 ± 0.00	0.68 ± 0.00$_{\zeta}$	0.32 ± 0.01*	1.44 ± 0.01$_{\zeta}$	–
Flavone n.i.	1.66 ± 0.00	1.15 ± 0.00$_{\zeta}$	1.42 ± 0.00$_{\lambda}$	3.33 ± 0.00$_{\zeta}$	1.16 ± 0.00$_{\beta}$
Apigenin	1.96 ± 0.00	1.00 ± 0.00$_{\zeta}$	1.83 ± 0.00$_{\lambda}$	4.74 ± 0.00$_{\zeta}$	0.93 ± 0.00$_{\beta}$
Diosmetin	0.50 ± 0.00	0.31 ± 0.00$_{\zeta}$	0.53 ± 0.00$_{\lambda}$	0.73 ± 0.00$_{\zeta}$	0.50 ± 0.00$_{\beta}$
Amentoflavone	0.42 ± 0.00	0.16 ± 0.00$_{\zeta}$	0.52 ± 0.00$_{\beta}$	0.41 ± 0.00$_{\zeta}$	0.62 ± 0.00$_{\lambda}$
Flavone n.i.	2.14 ± 0.00	0.57 ± 0.00$_{\zeta}$	2.68 ± 0.00$_{\beta}$	0.57 ± 0.00$_{\zeta}$	3.68 ± 0.00$_{\lambda}$
Casticin	0.29 ± 0.00	–	0.44 ± 0.01$_{\beta}$	–	0.62 ± 0.01$_{\lambda}$

< L.Q.: below limit of quantification. *An asterisk indicates statistical differences between original extract and fractions. ^{a,b} Different lowercase letters denote statistical differences between P10 and P15 fractions. ^{A,B} Different capital letters denote statistical differences between S10 and S15 fractions. Significance level at $p \leq .05$ with Fisher's Least Significant Difference (LSD) test.

were analyzed in order to establish a relationship between its composition and the antioxidant activity found.

3.2. Phenolic characterization of yarrow extract and SAF fractions

The HPLC analysis of phenolic compounds in the original extract and SAF fractions are shown in Table 2. The main compounds identified in the original extract were flavonoids, either in glycosylated or in aglycone form, and phenolic acids. Therefore, the compounds presented in higher concentration corresponded to the flavonoids luteolin and its glycosylated form (luteolin-7-*O*-glucoside), as well as the di-caffeoylquinic acids, where the 3,5-DCQA standed out. These results are in agreement with others previously reported, where the main flavonoids found in several yarrow extracts were luteolin-7-*O*-glucoside, luteolin, apigenin-7-*O*-glucoside and apigenin, while within the di-caffeoylquinic acids, 3,5-DCQA was the one in a greater extent (Benedek, Gjoncaj, Saukei, & Kopp, 2007; Vitalini et al., 2011). Related to precipitation vessel fractions, its principal components were luteolin-7-*O*-glucoside, 3,5-DCQA, luteolin, apigenin-7-*O*-glucoside and 6-hidroxyluteolin-7-*O*-glucoside, representing between 73% (P10) and 60% (P15) of all phenolic compounds identified. Meanwhile, separator fractions contained a reduced quantity of phenolic compounds, where flavonoids aglycones standed out.

Precipitation vessel fractions and the original sample presented a similar phenolic composition, although the compounds concentration in the fractions was, in general, much higher, highlighting luteolin-7-*O*-glucoside and 3,5-DCQA. These results could be related to the low solubility of glycosylated flavonoids and phenolic acids in the SC-CO_2 + ethanol mixture, since these compounds are poorly soluble in low-polar solvents (Chebil et al., 2007). On the other hand, the flavonoids aglycones, as less polar compounds, would be more soluble in the mixture SC-CO_2 + ethanol and therefore they would be dragged to the separator fraction.

However, it should be noted that there were some differences between fractions P10 and P15, since P10 fraction presented a greater amount of luteolin-7-*O*-glucoside and 3,5-DCQA than P15 fraction, whereas P15 contained a higher quantity of luteolin and apigenin than P10. These results could indicate that an increase in pressure during the fractionation process, would increment the presence of aglycones in the precipitation vessel fraction. In order to explain these results, it must be taken into account the complex multicomponent structure of extracts, comprising substances in a wide polarity range and with different solubility in SC-CO_2, which could exert a strong effect regarding the partial solubility of compounds involved and their precipitation behavior. Nevertheless, observing the solubility behavior of pure compounds in SC-CO_2, it can be established that in the case of more polar compounds (e.g. hydroxycinnamic acids) an increase of pressure from 10 to 15 MPa resulted in 4.4–5.4 solubility increase (Murga, Sanz, Beltran, & Cabeza, 2003), while for less polar compounds (e.g. quercetin) this ratio was about 3.0–3.5 (Chafer, Fornari, Berna, & Stateva, 2004). Thus, according to the pure component solubility behavior, an increase of the precipitation pressure could increase the recovery of aglycones in precipitation vessel.

Thus, the higher antioxidant activity found in P10 and P15, compared to original extract, could be related with the significant increase in several phenolic compounds found in these fractions, mainly luteolin-7-*O*-glucoside, 3,5-DCQA, luteolin (only in P15), apigenin-7-*O*-glucoside and 6-hidroxyluteolin-7-*O*-glucoside. Besides, when comparing P10 and P15, it can be observed that P10, with the higher antioxidant activity, also presented the higher quantity of luteolin-7-*O*-

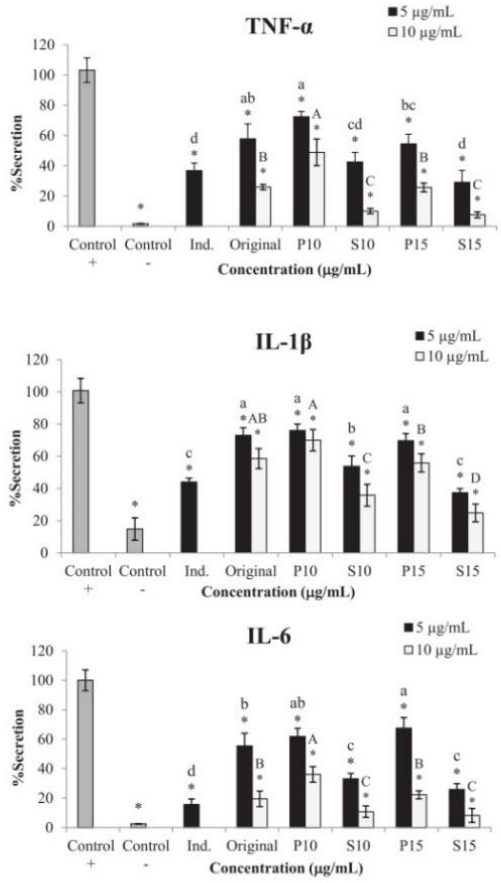

Fig. 1. Levels of TNF-α, IL-1β and IL6 secreted by THP-1/M activated with LPS in presence of original extract and SAF fractions. Positive control: cells stimulated with LPS but in absence of extract. Negative control: cells in contact just with RPMI media. Ind.: Indomethacin. Original: original yarrow extract. P10: precipitation vessel fraction at 10 MPa. P15: precipitation vessel fraction at 15 MPa. S10: separator fraction at 10 MPa. S15: separator fraction at 15 MPa. Each bar is the mean of three determinations ± S.D. *Denotes statistical differences when compares with positive control. ^{a,b,c,d} Different lowercase letters indicate statistical differences between samples at 5 µg/mL. ^{A,B,C,D} Different capital letters indicate statistical differences between samples at 10 µg/mL. Significance level at $p \leq .05$ with Fisher's Least Significant Difference (LSD) test.

glucoside and 3,5-DCQA (representing almost 55% of the fraction). Kim et al. (2011) indicated that 3,5-DCQA presented an important antioxidant activity, significantly higher than chlorogenic acid. Besides, the antioxidant activity of luteolin-7-*O*-glucoside have also been reported (Antonisamy et al., 2016; Song & Park, 2014). However, it must be taken into account that this fact could be also due to synergies among all the phenolic compounds found in P10 fraction.

3.3. Anti-inflammatory activity of yarrow extract and SAF fractions

First, original extract and SAF fractions were evaluated for cytotoxicity on THP-1/M cells by MTT method. Results showed that, at the higher concentration used in the anti-inflammatory assays, 10 µg/mL, neither original extract nor SAF fractions presented cytotoxicity (cell viability ≥95%). Indomethacin at 5 µg/mL also presented no cytotoxicity.

The activation of THP-1/M was carried out with the addition of LPS to the medium. Fig. 1 showed that these LPS treated cells (positive control), after an incubation period of 24 h, presented an important increase in the release of TNF-α, IL-1β and IL-6, compared to non-activated controls (negative control). When THP-1/M were activated with LPS in presence of 5 and 10 µg/mL of original extract and SAF fractions, a decrease in TNF-α secreted level was observed (Fig. 1), compared with positive control. Moreover, 5 µg/mL of original extract inhibited TNF-α secretion in a 40%. Regarding P10 and P15 fractions, P15 presented a decrease in TNF-α superior to P10 and similar to that obtained with original extract. However, it should be noted that the greatest decrease in TNF-α secretion was achieved in presence of S10 and S15 fractions. Thus, 5 µg/mL of S15 fraction reduced TNF-α release in a 70%.

In the same way, the original extract and SAF fractions inhibited the IL-1β secretion by activated cells, at both concentrations employed, although separator fractions showed the greatest inhibition (Fig. 1). Thus, meanwhile 10 µg/mL of original extract decrease IL-1β release in a 40%, the same concentration of S15 showed an inhibition near to 70%. In addition, 5 µg/mL of S15 reduced IL-1β secretion by a 60%. The obtained results for the IL-6 release in presence of samples (Fig. 1) were similar to those obtained for TNF-α and IL-1β, since S10 and S15 fractions were the most active and produced an important decrease in the IL-6 release (near to basal levels).

These results indicated that all SAF fractions presented anti-inflammatory activity, although separator fractions were much more active than precipitation vessel ones. The anti-inflammatory activity of precipitation vessel fractions could be related to its content in phenolic compounds, more specifically with dicaffeoylquinic acids, luteolin, apigenin and its glycosides, since the anti-inflammatory effects of these compounds have been previously described (Liang & Kitts, 2016; Wang et al., 2014; Wang et al., 2017). Moreover, Francisco et al. (2014) indicated that luteolin-7-*O*-glucoside presented a certain anti-inflammatory activity, but lower than that observed with the luteolin aglycone in LPS-stimulated macrophages. Similarly, Choi et al. (2014) reported that apigenin presented higher anti-inflammatory activity than other naturally occurring *C*-glycosylated derivatives of apigenin. These results could explain the higher inhibition of TNF-α, IL-1β and IL-6 secretion found when using P15 compared with P10, since P15 contained a higher amount of luteolin and apigenin aglycones, whereas P10 presented a higher quantity of luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside.

However, the higher anti-inflammatory activity found in separator fractions, could not be related to its phenolic compounds content, due to the low quantity of these compounds presented in these fractions. Thereby, it's relevant to notice that conditions used to obtain the separator fractions allowed the enhancement of these fractions in essential oil components. Since essential oils from *A. millefolium* have been reported to present anti-inflammatory activity (Chou, Peng, Hsu, Lin, & Shih, 2013; Kazemi, 2015), S10 and S15 were analyzed by GC–MS in order to establish a relationship between the composition and the anti-inflammatory activity of these fractions.

3.4. GC–MS characterization of separator fractions

In order to identify the compounds involved in the anti-inflammatory activity found in separator fractions (S10 and S15), a characterization by GC–MS of these fractions, along with the original extract was performed. The identification of the main volatile compounds presented in the sample (Table 3) was performed based on the comparison of their mass spectra and retention index (RI). As can be

Table 3
GC–MS identification, peak area contribution (%), and retention index (RI) of compounds found in original extract and separator fractions. S10 (separator fraction at 10 MPa) and S15 (separator fraction at 15 MPa).

RI	Compound	Original	S10	S15
997	Yomogi alcohol	2.1	2.0	2.2
1028	Eucalyptol	4.3	3.5	3.4
1037	γ-Vinyl-γ-valerolactone	1.8	1.6	1.5
1058	Artemisia ketone	11.0	9.9	9.4
1070	1,2-Epoxylinalool	0.9	0.8	0.8
1079	Artemisia alcohol	1.0	0.8	0.8
1084	*cis*-Linalool oxide	0.8	0.6	0.7
1099	β-Linalool	0.9	0.9	0.9
1136	Camphor	13.8	12.5	12.3
1141	*cis*-Verbenol	0.9	0.7	0.7
1160	Borneol	8.7	8.9	8.6
1171	2-Methyl-2-octen-4-ol	1.0	0.9	1.0
1174	Terpinene-4-ol	0.5	0.4	0.5
1182	*p*-Cymen-8-ol	0.7	0.8	0.9
1188	3,7-dimethyl-1,5-Octadiene-3,7-diol	5.9	6.1	6.2
1200	Verbenone	0.5	0.6	0.6
1212	Fragranol	0.4	0.4	0.4
1218	2-Hydroxycineole	0.6	0.6	0.7
1236	*trans*-Chrysanthenyl acetate	2.4	2.5	2.5
1250	Piperitone	0.9	0.9	1.0
1261	(5*E*)-5,9-Dimethyl-5,8-decadien-2-one	1.5	1.4	1.3
1276	2,6-Dimethyl-1,7-octadiene-3,6-diol	8.6	9.3	9.3
1280	Bornyl acetate	0.8	1.0	1.0
1284	n.i.	7.2	8.7	8.5
1393	Jasmone	1.6	1.6	1.5
1412	β-Caryophyllene	1.4	1.6	1.5
1569	Spathulenol	0.9	1.2	1.1
1578	Caryophyllene oxide	4.9	6.0	5.8
1640	β-Eudesmol	1.2	1.2	1.5
1810	Saussurea lactone	3.4	3.2	3.2
1845	Hexahydrofarnesyl acetone	3.0	3.2	3.4
2069	n.i.	6.5	6.2	6.7
Σ AUC		24.41 10^6	42.80 10^6	53.40 10^6

n.i.: no identified; AUC (area under curve).

observed, 30 identified and 2 non-identified compounds were found in S10 and S15. In general, a very similar GC–MS profile was obtained for both fractions, being camphor, artemisia ketone, borneol and 2,6-dimethyl-1,7-octadiene-3,6-diol the most abundant compounds in both fractions. Original yarrow extract also presented a similar profile to S10 and S15, although total chromatographic area (expressed as Σ AUC) was much higher for the fractions. As expected, separator fractions have been enriched in essential oil components (1.75 times for S10 and 2.18 for S15), in comparison to the original extract. Regarding main components, camphor and borneol were shown to reduce TNF-α, IL-1β and IL-6 secretion in THP-1 macrophages stimulated with LPS or ox-LDL (oxidized low-density lipoproteins) (Arranz et al., 2014; Arranz et al., 2014). Similarly, Rungqu et al. (2016) reported that an essential oil containing a 37.5% of artemisia ketone presented an important anti-inflammatory activity, evaluated in rats, using egg albumin-induced paw edema. Therefore, the anti-inflammatory activity exhibited by S10 and S15 fractions could be mainly related to the presence of these three compounds (camphor, borneol and artemisia ketone) that represented approximately 30% of the fractions. However, the contribution to other anti-inflammatory compounds presented in smaller quantities, such as eucalyptol and β-linalool, to this activity cannot be ruled out. Thus, the enrichment of S10 and S15 fractions in compounds that exhibit anti-inflammatory activity, regarding to the original extract, would explain the higher anti-inflammatory activity of these fractions. Accordingly, S15 fraction that presented a higher enrichment in essential oil compounds than S10, also presented a higher anti-inflammatory activity.

4. Conclusion

Supercritical anti-solvent fractionation of an ethanolic yarrow extract resulted an adequate method to improve its antioxidant and anti-inflammatory activities. Thus, a selective precipitation of phenolic compounds increased its antioxidant activity twice, compared to original extract, especially when fractionation was carried out at 10 MPa. Regarding anti-inflammatory activity, separator fractions presented higher anti-inflammatory activity than precipitation vessel ones. This fact was related to separator fractions enrichment in essential oil compounds with anti-inflammatory activity. Being more active, in this case, the separator fraction obtained when pressure was 15 MPa.

Therefore, this study pointed out the feasibility of SAF process as a green technology in order to achieve a fractionation of compounds with different biological activities. This fact could be a useful tool for food or nutraceutical products design.

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