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Simultaneous Supercritical Fluid Extraction of Heather (*Calluna vulgaris* L.) and Marigold (*Calendula officinalis* L.) and Anti-Inflammatory Activity of the Extracts

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Abstract: Heather (*Calluna vulgaris* L.) and marigold (*Calendula officinalis* L.) are two rich sources of bioactive pentacyclic triterpenes. The supercritical carbon dioxide ($SCCO_2$) extraction of these two plants was explored at a pressure range of 25–50 MPa, 50 °C, with or without fractionation, different extraction times (1.5–4.0 h) and using ethanol as a co-solvent (0 and 10% w/w). In order to determine potential synergisms, a combined extraction (heather + marigold 50:50) was also studied. In general, higher extraction yields were achieved when the co-solvent was added. Higher concentrations of total triterpenic acids were obtained in heather extracts, specially using ethanol. The co-solvent did not increase the terpene concentration in marigold extracts. For the combined extraction, an antagonist effect in the triterpene concentration was observed in absence of a co-solvent, whereas a synergistic effect was exhibited in its presence, especially for ursolic acid. In general, the extracts showed a certain anti-inflammatory effect, although a straight correlation with the analyzed triterpenic acids concentration was not exhibited. Moreover, the combined extraction exposed a similar anti-inflammatory activity in comparison with the individual plant extracts.

Keywords: supercritical fluid extraction; anti-inflammatory; marigold; heather; combined extraction; triterpenic acids

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1. Introduction

Numerous plants and herbs have been found to possess pharmacological properties, and have been used as medicines for thousands of years. Among them are heather (*Calluna vulgaris* L.) and marigold (*Calendula officinalis* L.). These two plants contain several phytochemicals with beneficial health properties, such as antioxidant, antidiabetic, antiproliferative, as well as anti-inflammatory properties [1–7].

Inflammation is a complex biological process in which immune cells and different mediators, such as prostaglandins, cytokines and leukotrienes, are involved. They are the principal protective responses to eliminate the cell injuries and tissue damage and to initiate the tissue repair. However, the consequences of chronic inflammation are often associated to metabolic chronical diseases, such as obesity, atherosclerosis and diabetes [8]. Different studies have evaluated the potential anti-inflammatory properties of heather and marigold. Orhan et al. [9] obtained hexane, chloroform, ethyl acetate, butanol and water fractions from heather ethanolic extracts. The ethyl acetate fraction

showed the most active anti-inflammatory activity. Similarly, Parente et al. [10] carried out the fractionation by hexane and dichloromethane from a marigold ethanolic extract. Extracts presented an anti-inflammatory activity that improved the wound healing process. Preethi, Kuttan and Kuttan [11] observed a significant anti-inflammatory activity of ethanol extracts in mice, obtained from marigold by maceration for two weeks. In a similar manner, Ukiya et al. [2] also obtained marigold anti-inflammatory extracts by maceration over one week, and the extracts were obtained using methanol.

To the best of our knowledge, there are no studies reported regarding optimization of the extraction conditions with the aim of obtaining extracts with improved anti-inflammatory activity.

Supercritical carbon dioxide ($SCCO_2$) is widely used for the extraction of natural compounds since $SCCO_2$ is a safe and green solvent and it has moderate critical conditions (31 °C, 7.38 MPa) [12]. Due to its low critical temperature and the absence of oxygen during extraction, the degradation of natural products is minimized or avoided, contributing to the production of high quality plant extracts. The only information available concerning the $SCCO_2$ of heather (*Calluna vulgaris* L.) was reported by García-Risco et al. [13] in which extracts with activity against hepatitis C virus were obtained at different pressures, temperatures and amounts of co-solvent. Moreover, Hunt and Zhao carried out the extraction of triterpenic acids from heather plants with different harvest time in their respective Ph.D. theses [14,15]. In the case of marigold (*Calendula officinalis* L.), several studies have been reported. Palumpitag et al. [16] carried out the extraction of lutein fatty acid esters. Hamburger, Baumann and Adler [17], Campos et al. [18], Danielski et al. [19] and Petrović et al. [20] studied the extraction of essential oil and oleoresin. Baumann et al. [21] performed the extraction of faradiol esters. Hamburger et al. [22] carried out the purification of triterpenoid esters combining $SCCO_2$ with chromatography. Nevertheless, only Della Loggia et al. [23] carried out the $SCCO_2$ of marigold and evaluated the anti-inflammatory activity of the extracts obtained. In this case, just one SFE condition (35 MPa and 50 °C) was studied.

In the present work, the $SCCO_2$ extraction of heather and marigold was studied with varying pressure, temperature, amount of co-solvent and with or without fractionation, with the aim of evaluating the obtained extracts for their in vitro anti-inflammatory activity. In order to determine potential synergisms, the simultaneous extraction of the two plants was also carried out. Furthermore, the concentration of ursolic, oleanolic and betulinic acid was determined for a better understanding of their role with respect to the anti-inflammatory activity of the extracts, considering that these triterpenic acids have been previously recognized in the literature as anti-inflammatory compounds [24,25].

2. Materials and Methods

2.1. Samples and Chemicals

Oleanolic acid (≥99%), ursolic (≥98%) and betulinic acid (≥98%) standards were purchased from Extrasynthese (Genay, Lyon, France). Acetonitrile (HPLC grade) was from LabScan (Gliwice, Poland) and absolute ethanol was purchased from Panreac Química S.A.U. (Barcelona, Spain).

Heather and marigold samples consisted of dry leaves and flowers (8.4% mass water content) purchased from an herbalist's producer (Murcia, Spain). The vegetal material was ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain) and sieved to sizes between 500–100 µm.

2.2. Supercritical Fluid Extractions

Supercritical fluid extraction (SFE) was carried out using a pilot-plant supercritical fluid extractor (model SF2000; Thar Technology, Pittsburgh, PA, USA), comprising of a 2 L cylinder extraction cell with independent control of temperature and pressure, and two separators (S1 and S2), each of 0.5 L capacity. The extraction device also includes a recirculation system where CO_2 is condensed, pumped and heated up to working conditions. A detailed explanation of the experimental device employed can be found elsewhere [26]. For each experiment, the cell was loaded with 500 g of plant. In the case

of the simultaneous extraction (50:50 heather + marigold) the mass loaded was 500 g (250 g for each plant). The experimental conditions employed are listed in Table 1.

Table 1. Experimental conditions for the supercritical fluid extraction of heather, marigold and combined heather + marigold (50:50) extraction. (P) pressure, (T) temperature, (t) time, (F) flow rate, (P S1) pressure in separator 1, (P S2) pressure in separator 2, (T S1) temperature in separator 1, (T S2) temperature in separator 2, (EtOH) mass of ethanol (w/w) used as a co-solvent.

Run	P (MPa)	T (°C)	t (h)	F (g/min)	P S1 (MPa)	P S2 (MPa)	T S1 (°C)	T S2 (°C)	EtOH (%)
E1	50	50	4	50	30	6	35	35	0
E2 [a]	30	50	4	50	2	0	10	10	10
E3-1	25	50	1.5	50	6	6	35	35	0
E3-2	30	50	2.5	50	2	0	10	10	10

[a] Notes: Extraction carried out in duplicates at this experimental condition.

For Run 1 (E1), each separator (S1 and S2) was kept at the desired pressure and temperature to conduct the online fractionation of the extract. Run 2 (E2) was performed with no fractionation and by using ethanol as a co-solvent. This extraction was carried out by duplicate for each plant matrix (heather, marigold and simultaneous extraction of heather + marigold). Finally, Run 3 (E3) was carried out by two sequentially extraction steps. The first one (Run E3-1) was performed with pure CO_2. During the extraction, both separators were kept at the recirculation system pressure (6 MPa) and fractions collected were blended. The second one (Run E3-2) was conducted using a co-solvent (ethanol), CO_2 was not recycled and the extract was recovered from both separators (extract from second separator represented less than 0.2% of the material recovered in the first separator).

The fractions precipitated in the separators were collected using ethanol, which was eliminated by rotary evaporation at 30 °C. Samples were kept at −20 °C under darkness until analysis.

2.3. Chemical Analysis

The concentration of ursolic, oleanolic and betulinic acid in the extracts was measured in the Varian ProStar HPLC equipment (Varian, Palo Alto, USA), which was equipped with a diode array detector (DAD). The column employed was KromaPhase C18 (250 mm × 4.6 mm and 5 µm). The analytical method was based on what was reported by Martelanc, Vovk and Simonovska [27], which comprised of 30 min isocratic elution using a mixture of acetonitrile and water (90:10 vol. %) as mobile phase. The flow rate was constant at 1 mL/min and the injections (20 µL injection volume) were carried out by duplicate. The detection of compounds was carried out at 210 nm and the quantification of the three triterpenic acids was accomplished by calibration curves using commercial standards.

2.4. Anti-Inflammatory Activity Assays

2.4.1. Cell Culture and Treatment

Human THP-1 monocytes (American Type Culture Collection, ATCC, Barcelona, Spain) were cultured in RPMI 1640 culture medium (Gibco, Madrid, Spain) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM β-mercaptoethanol at 37 °C in 95% humidified air containing 5% CO_2. Cells were collected and plated at a density of 5 × 105 cells/mL in 24 wells plates. Differentiation to macrophages (THP-1/M cells) was induced by maintaining the THP-1 cells for 48 h in presence of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma, Madrid, Spain). After differentiation, cells were washed with PBS and incubated with 0.05 µg/mL lipopolysaccharide (LPS) (Sigma, Madrid, Spain), in the presence of different concentrations of supercritical extracts for 24 h in a fetal bovine serum (FBS) free medium. Then, the supernatant was frozen at −80 °C.

2.4.2. Cytotoxicity Assays

The cytotoxic effect of the extracts on THP-1/M cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, Madrid, Spain), according to a published method [28]. THP-1/M cells in 24-multiwell plates were incubated with RPMI containing different concentrations of the extracts for 24 h at 37 °C. Cells were then washed with phosphate buffered saline (PBS), and 0.5 mg/mL of MTT were added to each well and incubated 4 h at 37 °C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulphate in a mixture of dimethyl formamide and water (1:1 v/v), adjusted to pH 4.7 with acetic acid) overnight at 37 °C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan, Barcelona, Spain), and the extraction solution was considered as a blank.

2.4.3. Quantification of Cytokines by ELISA

The release of IL-1β, IL-6 and TNF-α was measured in the supernatants of THP-1/M cells treated with LPS or in the presence of different concentrations of extract using ELISA kits (BD biosciences, Madrid, Spain), according to the manufacturer's instructions. The color generated was determined by measuring the optical density at 450 nm using a multiscanner autoreader (Sunrise, Tecan, Barcelona, Spain).

2.4.4. Statistical Analysis

All data were expressed as the mean of three determinations ± SD. 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 $p < 0.05$. Statistical analyses were performed using Statgraphics Centurion XVI software for Windows (Statpoint Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Effect of the Process Parameters on Overall Extraction Yield

Experiments showed in Table 2 illustrate the influence of the extraction conditions on the overall extraction yield (mass of extract / mass of plant) obtained from the SFE of heather, marigold and heather + marigold (50:50). Regarding the extraction of each plant separately, as expected, higher extraction yields were achieved by using a co-solvent (Run E2 and E3-2) due to the extraction of more polar phytochemicals. In general, values around 4.5%–7.0% for both, heather and marigold, were obtained when 10% (w/w) ethanol and 30 MPa were applied. When neat $SCCO_2$ was used, the yield obtained at 50 MPa and 4 h for marigold was 3.4% (sum of yield obtained from both separators, that is, S1 + S2), while the yield obtained at 25 MPa and 1.5 h for the same material was 2.8% (E3-1). Moreover, the value reached from heather at 25 MPa was 2.6%, which is even higher than the obtained at the highest pressure studied (2.2% extraction yield for S1 + S2). Accordingly, the amount of extract recovered did not considerably vary as pressure increased from 25 MPa to 50 MPa. A possible explanation could be formulated on the basis of compound solubility. Despite the increase of pressure leading to a higher CO_2 density and, therefore, a greater solvation power, the majority of extractable compounds from these plants could be appreciably soluble in $SCCO_2$ at 25 MPa. It could be the reason for the low extraction yields obtained from the first separator (Run E1-S1) by the fractionation of the extracts. In this case, the yield of the fractionated extracts from the first separator (30 MPa) was lower than 0.4% for both plants. Just as for pressure, long extraction times did not seem to exert a strong effect on the extraction yield. Taking into account the extraction yields mentioned for 50 and 25 MPa (Run E1-S1 + S2 and run E3-1), the extraction time prolonged from 1.5 h to 4 h did not result in a considerable increase in the amount extracted. In the case of the combined extraction (heather + marigold 50:50), a comparison between the obtained experimental and theoretical extraction yields (calculated as the

arithmetic mean of the experimental extraction yield obtained from single plants) was done. As can be observed from Table 2, the overall experimental extraction yields obtained from the combined extraction were 1.1–1.6 times higher than that of theoretical values, except for sequential extraction (E3-1 and E3-2).

Table 2. Overall extraction yield (mass of extract/mass of plant × 100) obtained from SFE of heather, marigold and combined heather + marigold (50:50) extraction. Theoretical extraction yields are expressed as the arithmetic mean of the experimental extraction yield obtained from each individual plant.

	Heather	Marigold	Heather + Marigold (50:50)	
Run	Experimental	Experimental	Experimental	Theoretical
E1-S1	0.26	0.33	0.49	0.30
E1-S2	1.94	3.04	2.64	2.49
E2	1.71 [a]	6.94 [a]	6.06 [a]	4.33
E3-1	2.65	2.76	2.04	2.71
E3-2	5.28	4.48	3.43	4.88

Notes: [a] Mean value between duplicate experiments. The coefficient of variation obtained was 7.0%, 9.4% and 6.3% for heather, marigold and heather + marigold, respectively.

3.2. Effect of the Process Parameters on Triterpenic Acids Content

Table 3 shows the concentration of betulinic, oleanolic and ursolic acid in the heather and marigold extracts. In the case of heather, the use of the co-solvent (Run E2 and E3-2) promoted the extraction of the three acids (Run E2 and E3-2), mainly oleanolic acid, despite ursolic acid being the largest quantity of triterpenic acid in heather. This effect was also observed by García-Risco et al. [13]. A possible explanation can be formulated considering the solubility of these compounds. To our knowledge, no solubility data of these acids in $SCCO_2$ has been reported. Nevertheless, the solubility of oleanolic acid in ethanol is higher in comparison with ursolic acid [29]. In the case of the ursolic acid, the highest concentration in the heather extracts was obtained by fractionating at 50 MPa (Run E1-S1). Pressure above 30 MPa was necessary for the efficient extraction and concentration of this compound without using a co-solvent. Unlike heather, the use of a co-solvent did not increase the concentration of the triterpenic acids in the marigold extracts. The highest concentrations were obtained when pure $SCCO_2$ was used, reaching extracts with high concentration of these compounds at 30 MPa. The main triterpenic acid obtained was oleanolic acid regardless of the use of co-solvent, whereas betulinic acid was only obtained in heather extracts. This finding agrees with the finding reported by Garcia-Risco et al. [13].

Table 3. Concentration (mg/g extract) of oleanolic, ursolic and betulinic acid in heather and marigold extracts obtained by SFE.

	Heather			Marigold [a]	
Run	Oleanolic	Ursolic	Betulinic	Oleanolic	Ursolic
E1-S1	13.50 ± 1.65	98.56 ± 10.20	3.89 ± 0.37	36.79 ± 3.87	19.46 ± 1.20
E1-S2	2.53 ± 0.22	2.95 ± 0.05	3.29 ± 0.21	42.96 ± 2.44	22.79 ± 1.98
E2	54.04 ± 3.21	38.67 ± 1.23	17.78 ± 1.58	29.90 ± 1.25	15.54 ± 1.33
E3-1	1.44 ± 0.15	2.19 ± 0.10	2.11 ± 0.39	59.96 ± 1.08	30.80 ± 0.90
E3-2	73.14 ± 2.87	51.62 ± 1.52	28.13 ± 3.10	8.97 ± 0.87	3.59 ± 0.35

Notes: [a] Amount of betulinic acid in marigold extracts below quantification limit.

Table 4 shows the concentration of the triterpenic acid in the extracts obtained from the combined heather + marigold extraction. Unlike the extraction of each plant individually, the combined extraction produced extracts with concentrations of ursolic acid higher than oleanolic acid (experimental values). Furthermore, considering the theoretical values of concentration for each compound and operation

condition (calculated as the arithmetic mean of the experimental concentration obtained from singly plants), the combined extraction generally produced an antagonistic effect for the concentration of these acids in the extracts when $SCCO_2$ was used without a co-solvent (experimental values lower than the theoretical ones), whereas a synergistic effect (experimental values higher than theoretical ones) was observed when the co-solvent was used, especially for ursolic acid (experimental concentration value up to 2.9 times higher than the theoretical value in Run E2).

Table 4. Concentration (mg/g extract) of oleanolic and ursolic acid in the extracts obtained from the combined extraction of heather and marigold (50:50) by SFE. Theoretical values are expressed as the arithmetic mean of the experimental concentration in the extracts obtained from each individual plant.

	Oleanolic		**Ursolic**	
Run	**Experimental**	**Theoretical**	**Experimental**	**Theoretical**
E1-S1	13.73 ± 1.22	26.53	21.41 ± 2.10	54.32
E1-S2	6.23 ± 0.25	27.21	12.70 ± 0.52	15.06
E2	43.64 ± 2.32	34.67	58.12 ± 3.21	20.11
E3-1	12.87 ± 1.14	31.29	25.19 ± 1.22	16.79
E3-2	36.35 ± 0.56	43.68	41.95 ± 1.87	29.57

3.3. Anti-Inflammatory Activity of the Extracts

Different concentrations (10 to 60 μg/mL) of supercritical extracts were initially evaluated for their cytotoxicity on THP-1/M cells by the MTT method, with the aim of determining the range of concentrations that can be applied for the assay. The results indicated that 40 μg/mL for heather and heather + marigold combined extracts presented no cytotoxicity, and a value of 20 μg/mL for marigold extracts.

The activation of THP-1/M cells was carried out with the addition of LPS to the medium. These LPS treated cells showed, after an incubation period of 24 h, an important increase in the release of all pro-inflammatory cytokines studied (TNF-α, IL-1β and IL-6), compared to non-activated controls (Figure 1). These activated cells were considered as positive controls for all the cytokines tested. When the activation of THP-1/M cells was carried out in presence of heather (40 μg/mL) or marigold (20 μg/mL) extracts, as well as the extracts from the combined heather + marigold (50:50) extraction (40 μ/mL), no variation was observed in TNF-α secretion, compared with levels obtained in the absence of extracts (positive controls). Regarding IL-1β, only heather extracts containing the highest concentration in triterpenic acids (E3-2), especially oleanolic acid (see Table 3), significantly decreased the IL-1β secretion. With respect to IL-6, most extracts produced a significant decrease in this interleukin secretion. Thus, heather extracts E1-S2, E2 and E3-2 presented a decrease in 30% of the release of this cytokine. However, it is should be noted that although extracts E2 and E3-2 possess an important quantity of triterpenic acids, mainly oleanolic acid, extract E1-S2 only presented a very small concentration in these components. Besides, marigold extract E1-S1 produced the highest inhibition in IL-6 release (45%), meanwhile other extracts with a higher content in triterpenic acids (E3-2) did not inhibited this cytokine secretion. Data obtained from both plants (50:50) combined extraction did not present a synergistic effect, regarding anti-inflammatory activity.

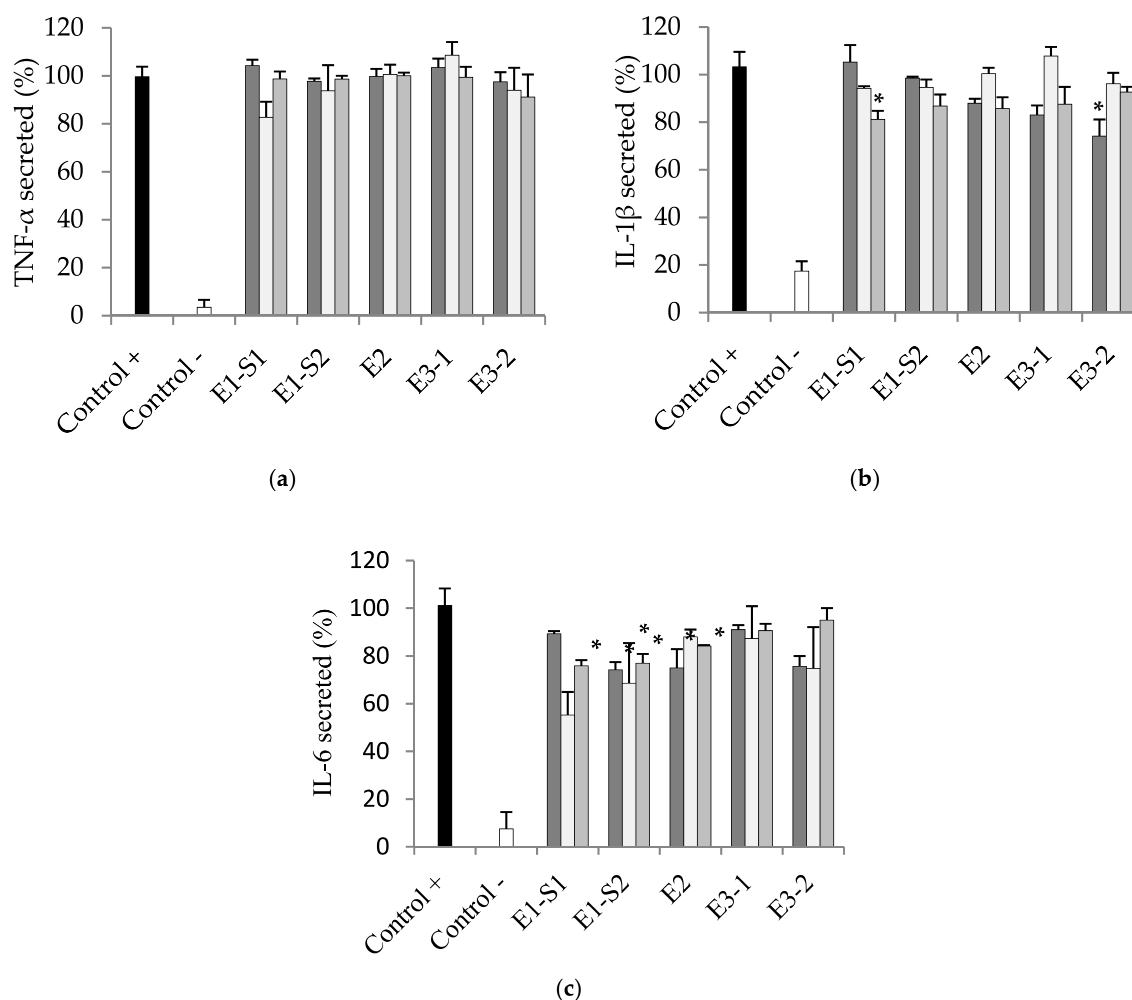

Figure 1. Levels of TNF-α (**a**), IL-1β (**b**) and IL-6 (**c**) secreted by THP-1/M cells activated with LPS in the presence of supercritical fluid extracts from heather (■), marigold (■) and a combined heather + marigold (50:50) extraction (■). Each bar corresponds to the mean of three determinations ± SD. * Denotes statistical differences between control positive and other samples at $p < 0.05$.

In addition to this, two alternative extracts, obtained from mixing the individual heather and marigold extracts (50:50 heather + marigold extract), were assayed (40 μ/mL). The extracts selected for the mixture were E2 and E3-2, due to these fractions containing the highest concentration of triterpenic acids in the heather + marigold (50:50) combined extraction (see Table 4). Results obtained (data not shown) indicated no differences in cytokine secretion between these new extracts and those obtained from the heather + marigold combined extraction. For both extraction conditions, that is, E3-2 and E2, a similar TNF-α, IL-1β and IL-6 secretion was observed for all samples (extracts from combined extraction or mixed extracts).

These data indicated that heather and marigold anti-inflammatory activity could not be only correlated with the concentration of triterpenic acids in the extracts, although the anti-inflammatory activity of ursolic and oleanolic acid has been previously reported in the literature [25,30]. A possible explanation regarding the lack of correlation between the amount of triterpenic acids and the anti-inflammatory activity of this work could be attributed to the relatively high concentration of these compounds required to exert such anti-inflammatory activity. Thus, Tsai and Yin [31] evaluated the anti-inflammatory activity of oleanolic and ursolic acids and observed a considerable reduction on TNF-α and IL-6 secretion in the presence of 20 μM of ursolic acid and 40 μM of oleanolic acid. Regarding the present work, the highest concentration of triterpenic acids (expressed as the sum of the

oleanolic, ursolic and betulinic acid) in the supercritical extracts was 13.3 µM (E3-2 from heather). In addition, ursolic acid has also been associated with a pro-inflammatory activity in a dose-dependent manner in mouse macrophages. In this sense, ursolic acid could promote the production and secretion of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [32]. This fact could in part explain the practically ineffective anti-inflammatory effect of the E1-S1 extract from heather, which presented the highest concentration of ursolic acid (98.6 mg/g) (see Table 3) and the lowest oleanolic/ursolic concentration ratio (0.14). Additionally, the reduction in the cytokine secretion produced by the extracts with a low concentration in these triterpenic acids, such as E1-S2 from heather, could be related to the presence of other bioactive compounds with anti-inflammatory activity. Thus, faradiol- and taraxasterol type compounds presented in marigold extracts have been reported to possess anti-inflammatory activity [10,21,23]. Therefore, additional studies should be carried out with the aim of elucidating the effect on the cytokine secretion of the studied triterpenic acids and their possible synergisms and antagonisms with other bioactive compounds present in the extracts.

4. Conclusions

In the present work, the supercritical fluid extraction of heather, marigold and the combined extraction of both plants (50:50 heather + marigold) was carried out, the concentration of ursolic, oleanolic and betulinic acid analyzed, and the anti-inflammatory activity of the obtained extracts evaluated.

The obtained extraction yields were higher when the co-solvent was used, for both the extraction of each singly plant and the combined heather + marigold extraction. Pressures above 25 MPa and long extraction times did not lead to an increase in the extraction yield. Likewise, the concentration of triterpenic acids, especially oleanolic acid, in the heather extracts was generally higher when the co-solvent was used (110.5–112.9 mg/g expressed as the sum of the three triterpenic acids). Nevertheless, a high concentration of ursolic acid (98.6 mg/g) could be obtained without the use of a co-solvent by fractionating the extract. In the case of marigold, the use of a co-solvent did not involve an increase in the concentration of triterpenic acids. The combined extraction generally produced an antagonistic effect for the concentration of these acids when $SCCO_2$ was used without the co-solvent, whereas a synergistic effect was observed when the co-solvent was used, especially for ursolic acid (experimental concentration value up to 2.9 times higher than the theoretical one).

Regarding the anti-inflammatory activity, it could not be related with the concentration of triterpenic acids in the extracts. This fact could be related with the small concentration of ursolic and oleanolic acids in the extracts, or with the fact that the anti-inflammatory activity could be related to other bioactive compounds presented in the extracts. Therefore, additional studies should be carried out to determine the concentration of other compounds with feasible anti-inflammatory activity, as well as synergisms and antagonisms with other bioactive compounds present in the extracts.

Author Contributions: M.R.G.-R. conceived and supervised the study; E.V., D.V.-B. and M.V. performed the experiments and the data analysis; S.S., T.F. and G.R. analyzed the data. All authors contributed to writing and editing the paper.

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