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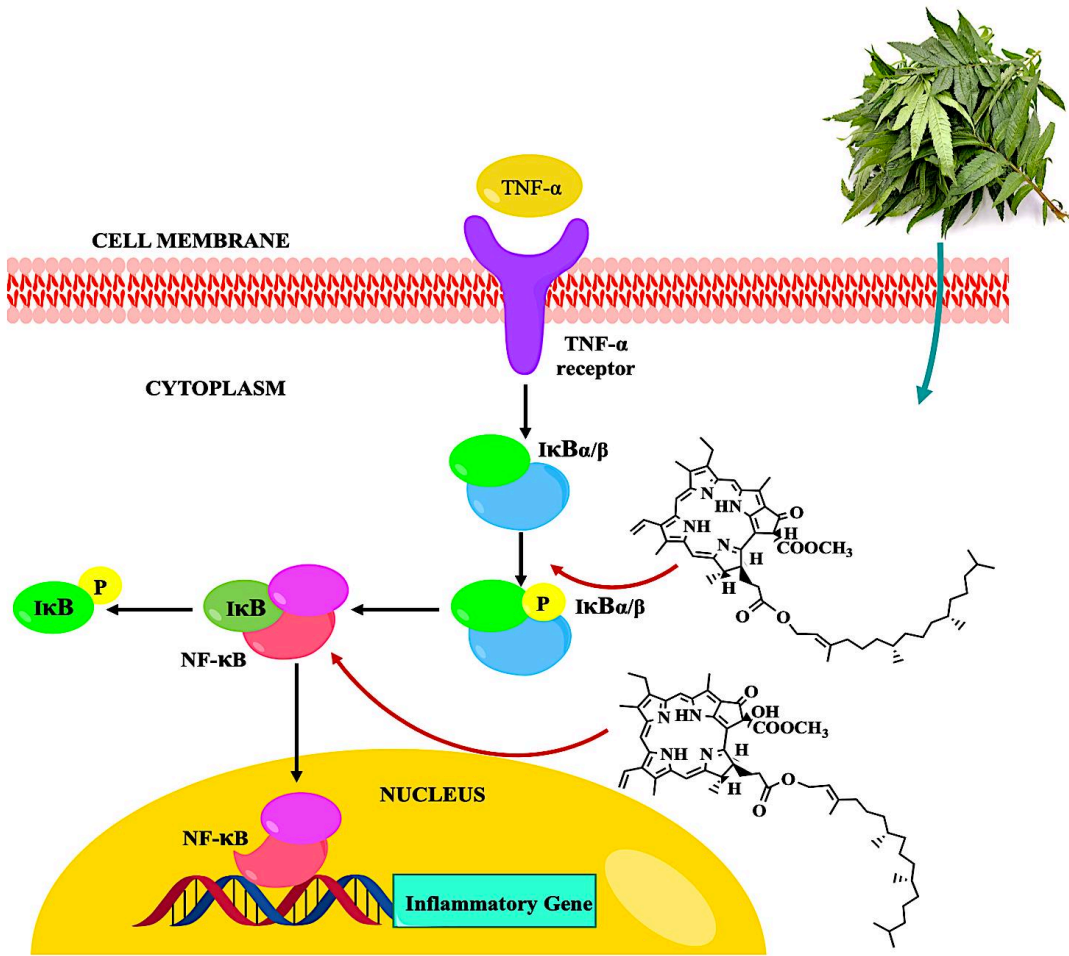
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Hydroalcoholic extract of *Tagetes minuta* L. inhibits inflammatory bowel disease through the activity of pheophytins on the NF- κ B signalling pathway



Hydroalcoholic extract of *Tagetes minuta* L. inhibits inflammatory bowel disease through the activity of pheophytins on the NF- κ B signalling pathway

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Abbreviations:

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DSS: Dextran sulphate sodium

ELISA: Enzyme-linked immunosorbent assay

26	ERK:	Extracellular signal-regulated kinase
27	FBS:	Fetal bovine serum
28	IBD:	Inflammatory bowel disease
29	JSH-23:	4-methyl- <i>N</i> 1-(3-phenylpropyl)-1,2-benzenediamine
30	LPS:	Lipopolysaccharide
31	NF- κ B:	Nuclear factor kappa-light-chain-enhancer of activated B cells
32	NOS2:	NO synthase-2
33	PBMCs:	Peripheral blood mononuclear cells
34	PBS:	Phosphate buffered saline
35	PMS:	<i>N</i> -methyl dibenzopyrazine methyl sulphate
36	QTOF:	Quadrupole time-of-flight
37	TLC:	Thin-layer chromatography
38	TNBS:	2,4,6-Trinitrobenzenesulfonic acid
39	TNF- α :	Tumour necrosis factor alpha
40	TPA:	12- <i>O</i> -tetradecanoylphorbol 13-acetate
41	STAT-1:	Signal transducer and activator of transcription 1
42	XTT:	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-
43		carboxanilide inner salt

44

45 **ABSTRACT**

46 *Ethno-pharmacological relevance:* Species of the genus *Tagetes* are well known for their
47 anti-inflammatory properties. *Tagetes minuta* "Huacatay" is an endemic species of South
48 America that has been used in traditional medicine since ancient times as a remedy for
49 stomach and intestinal discomfort.

Aim of the study: The aim of this study is to investigate the anti-inflammatory activity of the aqueous and hydroalcoholic extracts of the Huacatay, identifying the compounds responsible for this activity.

Materials and methods: Anti-inflammatory activity of the compounds, fractions and extracts was evaluated in Hs 746T (stomach), HIEC-6 (intestine) and THP-1 (monocytes peripheral blood) cells by measuring their inhibitory capacity against the NF- κ B production.

Results: Aqueous and hydroalcoholic extracts of *Tagetes minuta* displayed anti-inflammatory activity *in vitro*, the hydroalcoholic extract being the most active (IC₅₀ between 59.72-66.42 μ g/mL) in all cell lines. Bio-guided hydroalcoholic extract fractionation led to the isolation and characterisation of two pheophytins, pheophytin a (**1**) and 13²-hydroxy pheophytin a (**2**). Both compounds inhibited the production of NF- κ B with IC₅₀ values in the low micromolar range, with an IC₅₀ between 12.32-16.01 μ M for compound **1** and 7.91-9.87 μ M for compound **2**.

Conclusions: The two pheophytins isolated in this study inhibit the production of NF- κ B, thus showing that the traditional anti-inflammatory use of *Tagetes minuta* can be proved through pharmacological assays. This contributes to understanding the anti-inflammatory activity of the Huacatay extracts and their use in the treatment of stomach and intestinal discomfort.

Key words:

Tagetes minuta, pheophytins, anti-inflammatory, TNF- α , NF- κ B.

1. Introduction

Inflammatory bowel disease (IBD) comprises a group of autoimmune disorders of

the digestive tract with a chronic nature and unknown origin (Sairenji *et al.*, 2017). Dysregulated cytokine production and signalling mechanisms by intestinal epithelial cells, lymphocytes and macrophages have been involved in the pathogenesis of IBD, and the NF- κ B transcription factor is one of the major regulators in this complex scenario (Neurath, 2014).

The analysis of the macrophages and epithelial cells isolated from inflamed gut samples from IBD patients showed increased levels of NF- κ B p65 (Neurath *et al.*, 1996). Moreover, the amount of activated NF- κ B was significantly correlated with the severity of intestinal inflammation (Rogler *et al.*, 1998). In addition to macrophages and epithelial cells, lamina propria fibroblasts have been described as playing an NF- κ B-mediated pro-inflammatory role in IBD (Gelbmann *et al.*, 2003).

Likewise, it has been shown that the increased NF- κ B expression in mucosal macrophages in IBD patients is accompanied by an increased capacity of these cells to produce and secrete TNF- α , IL-1 and IL-6 p65 (Neurath *et al.*, 1996). This finding reflects the central function of NF- κ B in monocytes, which is the induction and control of pro-inflammatory cytokines. Beside TNF- α , IL-1 and IL-6, NF- κ B is also able to regulate the expression of IL-12 and IL-23 (Becker *et al.*, 2001; 2003). Thus, these pro-inflammatory cytokines are directly involved in the mucosal tissue damage typically occurring in IBD.

Currently, many of the already established immunosuppressive drugs used against IBD (corticosteroids, sulfasalazine, methotrexate and anti-TNF- α antibodies) have anti-inflammatory effects by inhibiting the NF- κ B activity (Thiele *et al.*, 1999; Majumdar & Aggarwal, 2001; Weber *et al.*, 2001; Guidi *et al.*, 2005). However, these established drugs do not specifically target NF- κ B, so much effort has been made in recent years to develop selective inhibitors of nuclear import systems to prevent the translocation of NF- κ B into the nucleus, target the IKK complex or try to stabilise I κ B proteins by developing

ubiquitylation or proteasome inhibitors (Li & Verma, 2002). For this reason, alternatives such as herbal therapies may have a better risk-benefit ratio (Bent, 2008). It has been observed that plants act through various mechanisms reducing the overproduction of some cytokines that modify ion transport and epithelial permeability (Triantafyllidi *et al.*, 2015; Tasneem *et al.*, 2019).

In this context, the *Tagetes* genus belonging to the Compositae family, which comprises 53 recognised species (Senatore *et al.*, 2004), has been shown to contain a wide variety of compounds with anti-inflammatory activity (Gongadze *et al.*, 2019; Meurer *et al.*, 2019; Monterrosas-Brisson *et al.*, 2019). *Tagetes minuta* L. (Compositae), commonly known as "Huacatay", is a native species from the mountainous regions of South America (Perkins, 1912; McVaugh, 1943). Traditionally, its decoction (aqueous and hydroalcoholic solutions) is used as a remedy for stomach and intestinal diseases (Neher, 1968), for gastric pain (Oblitas, 1982; De Feo, 1992), and for diarrhoea (De Lucca & Zalles, 1992).

The methanolic extract of *Tagetes minuta* has shown a high anti-inflammatory potential in human PBMCs (Peripheral Blood Mononuclear Cells) (Ibrahim *et al.*, 2018). Moreover, its essential oils have reduced significantly the expression of TNF- α in J774.1A murine macrophages (Karimian *et al.*, 2014).

Relating the anti-inflammatory activity of *T. minuta* with its chemical composition, it has been determined that most of its compounds are essential oils (Senatore *et al.*, 2004; Shahzadi *et al.*, 2010; Wanzala & Ogoma, 2013; Shirazi *et al.*, 2014), thiophenes and, to a smaller extent, flavonoids (Ibrahim *et al.*, 2018). However, given that the species of this genus have other types of compounds with a promising anti-inflammatory potential, it is highly relevant to identify and characterise the compounds present in the aqueous and hydroalcoholic extracts of *Tagetes minuta*, as a source of compounds with possible anti-

inflammatory activity for the treatment of IBD, thus testing the previously mentioned traditional use of this plant species.

2. Materials and methods

2.1. Plant material

Sample of *T. minuta* was collected from the Pongo community (Murillo province, La Paz, Bolivia), in August 2018, at an altitude of 3700 m (16°20'03.3"S and 67°57'33.6"W). The botanical identification was confirmed by the National Herbarium of Bolivia (No. 13609).

2.2. General experimental procedures

First grade organic solvents (ethanol, methanol, ethyl acetate, dichloromethane, *n*-heptane) were used for isolating the compounds and they were purchased from Sigma-Aldrich. TLC (Thin-layer chromatography) was performed using Merck Silica gel 60-F₂₅₄ plates. Chromatograms thus obtained were visualised by UV absorbance (254 nm) and through heating a plate stained with phosphomolybdic acid. Column chromatography was performed with silica gel 60 (0.2-0.5 mm, Merck). NMR experiments were performed on the Bruker Advance DRX 300 spectrometers operating at 300 MHz (¹H) or 75 MHz (¹³C). The deuterated solvents were CDCl₃-*d*₁, MeOD-*d*₄ and D₂O-*d*₂. Spectrums were calibrated by assignment of the residual solvent peak to δ_{H} 7.26; δ_{H} 3.31; δ_{H} 4.79 and δ_{C} 77.16; δ_{C} 49.00, for CDCl₃, MeOD and D₂O respectively. The complete assignment of protons and carbons was done by analysing the correlated ¹H-¹H COSY, HSQC and HMBC spectra. Mass spectra were performed on a mass spectrometer with QTOF (Quadrupole time-of-flight) hybrid model QSTAR pulsar i analyser from the

commercial company AB Sciex. The samples were analysed using the electrospray ionisation technique in positive ion detection mode. They were introduced into the mass spectrometer by direct infusion at a flow of 10 μ L/min using a syringe pump.

2.3. Extraction and isolation

The air-dried aerial parts of *Tagetes minuta* L. (500 g) were extracted by decoction (30 minutes at boil) with distilled water (1000 g), and by maceration (in triplicate) at room temperature (25°C) with a hydroalcoholic mixture (ethanol 96°GL 700 g and distilled water 300 g). The aqueous (300 g) and hydroalcoholic (250 g) extracts were subjected to an evaluation of their biological activity (viability and inhibition of NF- κ B production) and hydroalcoholic extract was selected as the more active one.

Subsequently, the hydroalcoholic extract was further extracted with *n*-heptane, dichloromethane/methanol (1:1) and distilled water. As a result, three fractions of 85 g, 48 g, and 86 g, respectively, were obtained. Each fraction was evaluated for its viability and its influence on the inhibition of NF- κ B production.

The dichloromethane/methanol fraction was selected as the most active one and was fractionated with vacuum column chromatography (silica gel 60; 40-63 μ m) starting with *n*-heptane/ethyl acetate (5:1), obtaining twelve fractions (**I-XII**). Fractions **II** (2 g), **III** (3 g) and **IV** (1 g) were observed to be the most active fractions (inhibition NF- κ B production). Finally, the **II-III** (these fractions were pooled for their similar chromatographic profile) and **IV** fractions were purified by a microcolumn using silica gel (20-45 μ m) as stationary phase and *n*-heptane/ethyl acetate, (3:0 to 0:3) as mobile phase, obtaining compounds **1** (100 mg) and **2** (35 mg), respectively.

2.4. Cell lines and culture conditions

Two cell lines were used in this study. Human stomach (Hs 746T, ATCC® HTB-135) and Human small intestine (HIEC-6, ATCC® CRL-3266) cell lines were selected to carry out the assays on the extracts and compounds from *Tagetes minuta*. We have selected these cell lines according to their physiological link with the IBD and the use in traditional medicine of *Tagetes minuta* as a remedy for stomach and intestinal discomfort. In addition, peripheral blood monocyte (THP-1, ATCC® TIB-202) cells were used to assess the safety of the compounds. Cells were cultured in DMEM (Dulbecco's modified eagle medium, Sigma-Aldrich St. Louis, USA) supplemented with L-glutamine (PanReac AppliChem, Barcelona, Spain), 10% FBS (Fetal bovine serum, Summit Biotechnology Ft. Collins, CO), 100 U/mL penicillin and 100 µg/mL streptomycin (Fisher Scientific, Pittsburgh, USA) at 37°C in an atmosphere containing 5% CO₂.

2.5. Cytotoxicity assay

Hs 746T, HIEC-6 and THP-1 cells proliferation was determinate using the commercial kit (XTT; Boehringer Mannheim, Germany). Cells were sub cultured in 96-well culture plates at a density of 5×10^4 cells/well in 100 µL of DMEM medium and incubated for 24 h at 37°C. After 24 h incubation, the old medium was removed and the cells were filled with 100 µL of fresh medium treated with various concentrations (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 and 0.2 µg/mL or µM) of the samples.

Stock solutions of the samples were prepared by dissolving them in DMSO at a concentration of 20 mg/mL for the fractions and 10 mM for the compounds. Subsequently, from the stock solutions, a series of dilutions were performed until a final DMSO concentration of 0.1% was obtained in each of the wells of the plate for each of the tested concentrations (Park *et al.*, 1992). Actinomycin D (≥95% Sigma-Aldrich, CAS

Number 50-76-0) was used as a positive control at a concentration of 0.008 μ M, showing cell death.

The plates were incubated in a humidified incubator at 37°C under a 5% CO₂ atmosphere for 24 h. At the end of the incubation, the medium was discarded and the cells were washed with PBS. 50 μ L of XTT were prepared by mixing 5 mL of XTT-labelling reagent and 100 μ L of electron coupling reagent, and were added to each well. After 4 h of incubation in an incubator at 37°C and 5% CO₂, the absorbance was measured at 450 nm (with a 630 nm reference filter) in a spectrophotometric ELISA plate reader (SpectraMax® i3, Molecular Devices, CA, USA).

2.6. *NF- κ B inhibition assay*

Hs 746T, HIEC-6 and THP-1 cells (5×10^4 cells/well) were transfected using a lipofectamine plus transfection reagent (Thermo Fisher Scientific, Spain) with 0.3 μ g of the NF- κ B-promoted luciferase reporter gene plasmid (pGL2-NF- κ B-Luc) (Promega, USA), and 0.03 μ g of the Renilla luciferase reporter plasmid (pTK-Renilla) (transfection normalisation vector, Promega). After 1 day, the cells were incubated with LPS (0.20 μ M) in the absence or presence of the isolated compounds at different concentrations (the same concentrations were used as in the viability assay) for 12 h. Concerning the inhibition of NF- κ B, the results were compared to that of JSH-23 (4-methyl-N1-(3-phenylpropyl)-1,2-benzenediamine), used as the positive control (IC₅₀=7.1 μ M). The choice of the used concentration in the assay was based on previous work (Kumar *et al.*, 2011). Then, the luciferase activity was measured by Dual-Luciferase Reporter Assay Kit (Promega, USA), following the manufacturer's instruction.

2.7. Statistical analysis

CC₅₀ (Cytotoxic concentration 50%) and IC₅₀ (Inhibitory concentration 50%) values were determined by non-linear regression. All the experiments were performed in triplicate. One-way ANOVA statistical analysis (Tukey's multiple comparisons test, * p <0.05; *** p <0.001) was performed to evaluate the significant differences among values. All the analysis was performed using GraphPad Prism, version 8.4.3.

3. Results and discussion

3.1. Extraction, isolation, and characterisation of compounds

Tagetes minuta was extensively extracted with water (decoction) and hydroalcoholic solution (maceration). The obtained extracts were concentrated (evaporated or lyophilised) and chemically analysed (Figures S1 and S2). Moreover, their cytotoxicity was evaluated (Table 1S), as well as their anti-inflammatory activity (Table 2S), calculating their CC₅₀s and IC₅₀s values.

The results show that the aqueous (CC₅₀=96.01-91.49 μ g/mL) and hydroalcoholic (CC₅₀=89.79-84.03 μ g/mL) extracts did not present a statistically significant cytotoxicity when compared to the Actinomycin D (CC₅₀=0.01 μ M) in any of the cell lines. Regarding the inhibition of NF- κ B production, the hydroalcoholic extract showed a statistically significant activity with an IC₅₀ between 59.72-66.42 μ g/mL when compared to the aqueous extract that showed an IC₅₀ between 81.96-88.65 μ g/mL, in all cell lines. The most active extract (hydroalcoholic extract) was chosen for subsequent fractionation by using solvents of different polarity.

Further fractionation of the hydroalcoholic extract of *Tagetes minuta* with *n*-heptane, dichloromethane/methanol and distilled water produced three fractions with

different composition and anti-inflammatory activity. The dichloromethane/methanol fraction was selected as the most active fraction (IC_{50} between 46.23-44.25 $\mu\text{g/mL}$) (Table S4). Likewise, the dichloromethane/methanol fraction (CC_{50} between 76.92-74.19 $\mu\text{g/mL}$) did not show a decrease in the viability when compared to the Actinomycin D ($CC_{50}=0.01 \mu\text{M}$) (Table S3).

The dichloromethane/methanol fraction was selected as the most active fraction, being subjected to separation through column chromatography, obtaining 12 fractions (**I-XII**) that were subjected to a viability (Table 1) and anti-inflammatory activity evaluation (Table 2).

Table 1.

The results suggest that fractions **I-IV** did not decrease the viability of the tested cell lines when compared to the Actinomycin D ($CC_{50}=0.01 \mu\text{M}$). Fractions **V-XII** showed a statistically significant decrease in viability when compared to the negative control (untreated cells) (Table 1). Regarding the inhibition of NF- κ B production, fractions **II**, **III** and **IV** showed a statistically significant activity in all cell lines when compared to the JSH-23 (Table 2).

Table 2.

Consequently, we proceeded to purify fractions **II-III** and **IV**. This purification led to the identification of two compounds; pheophytin a (**1**) and 13²-hydroxy pheophytin a (**2**) (Figure 1). Structural data are reported in the supplementary material.

Figure 1.

Although pheophytins are well known in natural chemistry, this is the first time they have been identified in the *Tagetes minuta* species. Reports mention that **pheophytin a** was isolated in the *Lonicera hypoglauc*a (Wang *et al.*, 2009), *Syringodium isoetifolium* (Sathyanathan *et al.*, 2016), *Ocimum labiatum* (Kapewangolo *et al.*, 2017) and *Suaeda vermiculata* species (Mohammed *et al.*, 2019). Regarding **13²-hydroxy pheophytin a**, reports indicate that it was isolated from the *Amaranthus tricolor* (Jerz *et al.*, 2007), *Clinacanthus nutans* (Sakdarat *et al.*, 2009), *Wissadula periplocifolia* (Teles *et al.*, 2014) and *Sidastrum micranthum* species (Albuquerque *et al.*, 2015).

3.1.1. Identification of compound 1

Regarding compound **1**, the ¹H NMR spectrum shows three singlet signals at δ_H 8.56, 9.20 and 9.38, integrating each signal for an aromatic or heterocyclic class proton. Likewise, an ABX system corresponding to a vinyl residue is observed showing the signals δ_{HX} 7.87 (dd, 1H, $J_{AX}=17.7$, $J_{BX}=11.6$ Hz), δ_{HA} 6.21 (dd, 1H, $J_{AX}=17.7$, $J_{AB}=1.2$ Hz) and δ_{HB} 6.12 (dd, 1H, $J_{BX}=11.6$, $J_{AB}=1.2$ Hz). Additionally, three singlets appear in the ¹H NMR spectrum, corresponding to three methyls linked to a C_{sp2} at δ_H 3.08 (s, 3H), 3.36 (s, 3H), 3.67 (s, 3H), and a methoxy group at δ_H 3.88 (s, 3H). In this region, the presence of two multiplets is also observed at δ_H 4.47-4.58 (m, 3H) and 4.25 (m, 1H). Likewise, in the region comprising δ_H 2.26-2.68, four diastereotopic protons of complex multiplicity are observed. In the upper part of the spectrum two doublets appear, corresponding to three methyl δ_H 1.86 (d, 3H, $J=7.3$ Hz) and 0.88 (d, 6H, $J=6.6$ Hz). Likewise, the presence of two methyl at δ_H 0.88 indicates the presence of an aliphatic chain terminal isopropyl residue, which present coupling with CH at δ_H 1.53 (sept, 1H,

$J=6.6$ Hz). Finally, in the region between δ_{H} 1.0-1.4 (m, 24H), a large congestion of aliphatic signals that correspond to aliphatic alkyl chain CH_2 can be detected. The signal congestion and the presence of a large number of methyls attached to a C_{sp^2} show that at least one fragment comes from the mevalonate route. Finally, the presence of a singlet signal in the negative field δ_{H} -1.72 (s, 1H), characteristic of organic molecules with a porphyrinic NH group, can be observed.

Regarding the mass spectrum of compound **1**, the $[\text{M}+\text{H}]^+$ m/z 871.5729 and $[\text{M}+\text{Na}]^+$ m/z 893.5642 ions corresponding to the molecular formula $\text{C}_{55}\text{H}_{74}\text{N}_4\text{O}_5$ with an unsaturation degree of 21 can be observed.

Analysing the degree of unsaturation, the molecular formula, and the NMR spectroscopic analysis (^1H and ^{13}C) led us to the conclusion that the molecule has a pheophytin-like structure. However, small variations in chemical shift can be observed, especially in the ^1H NMR spectrum. This effect is due to the fact that the pheophytins, by not presenting the chelated magnesium, have NMR spectra that strongly depend on the concentration. In other words, as the concentration increases, the π - π type interactions between the porphyrin rings are amplified, which generates changes in chemical shifts, especially accentuated in the ^1H NMR spectra. In this sense, comparing the spectroscopic data of compound **1** with the existing pheophytins, it can be determined that compound **1** corresponds to pheophytin a. Data was compared with the references (Smith *et al.*, 1984).

3.1.2. Identification of compound 2

Regarding compound **2**, the NMR spectra showed a spectroscopic profile similar to that of compound **1** with some important variants. In the ^1H NMR spectrum the most significant change is the disappearance of the singlet signal at δ_{H} 6.31, corresponding to the H-10 position of compound **1**, and in the ^{13}C NMR spectrum the CH corresponding

to C-10 can not be observed. Instead, a signal appears at δ_C 89.4, characteristic of a quaternary carbon with a hydroxyl group. In this sense, the presence of the hydroxyl group would justify the deshielding of the α , β -unsaturated ketone (δ_C 192.1), due to the formation of an intramolecular hydrogen bond. Regarding the HRESIMS spectrum, two ions are observed at $[M+H]^+$ m/z 887.5695 and $[M+Na]^+$ m/z 909.5529, corresponding to the molecular formula $C_{55}H_{74}N_4O_6$. Analysing the spectroscopic and spectrometric data with the reported pheophytins, we can conclude that compound **2** is 13²-hydroxy pheophytin a. Data was compared with the references (Jerz *et al.*, 2007).

3.2. Cytotoxic effects of compounds

As shown in the previous screening of *Tagetes minuta* extracts and fractions, they showed anti-inflammatory activity on different cell lines, due to the presence of a mixture of structurally different compounds. In relation to the isolated compounds, we have first evaluated the viability of THP-1, Hs 746T and HIEC-6 cells treated with the isolated compounds (Figure 2). Compound **1** showed moderate toxicity in all the cells lines with CC_{50} of 83.97 μ M (THP-1), 81.49 μ M (Hs 746T) and 72.50 μ M (HIEC-6). Regarding compound **2**, it showed slight toxicity in all the cells lines with CC_{50} of 97.17 μ M (THP-1), 89.63 μ M (Hs 746T) and 89.18 μ M (HIEC-6), when compared to the Actinomycin D (CC_{50} =0.01 μ M).

Figure 2.

3.3. Inhibition of production NF- κ B

Subsequently, it was determined whether compounds **1** and **2** affect the production of NF- κ B, through the NF- κ B translocation (mechanism performed by the positive

control JSH-23), where NF- κ B plays an important role in the regulation of the expression of TNF- α during inflammation.

Through our results, we observed that NF- κ B is translocated to the nucleus when treated with LPS, but treatment with compounds **1** and **2** suppresses nuclear translocation, with an IC₅₀ of 12.32 μ M (THP-1), 14.47 μ M (Hs 746T), 16.01 μ M (HIEC-6) for compound **1** and 7.91 μ M (THP-1), 9.86 μ M (Hs 746T), 9.87 μ M (HIEC-6) for compound **2** (Figure 3). The results show that the compounds decreased LPS-stimulated transcriptional activation of NF- κ B in a dose-dependent manner. However, only compound **2** shows a similar activity to the JSH-23 (IC₅₀ of 7.1 μ M) (^{ns} $p>0.999$).

Figure 3.

Previous studies have indicated that the anti-inflammatory activity of pheophytins is due to the fact that they act on the inhibition of the NOS2 (NO synthase-2) promoter, blocking ERK (Kinase regulated by extracellular signals) and STAT-1 (Signal transducer and activator of the transcription 1) pathways, but they did not report an influence on the production of NF- κ B (Islam *et al.*, 2013; Lin *et al.*, 2014).

The extracts (decoction and hydroalcoholic) of *Tagetes minuta* showed anti-inflammatory activity, with the hydroalcoholic extract being the most potent inhibitor. Two pheophytins were isolated from this active extract: pheophytin a (**1**) and 13²-hydroxy pheophytin a (**2**). Both compounds showed anti-inflammatory activity *in vitro* with an IC₅₀ in the low micromolar range, inhibiting the production of NF- κ B, possibly through the translocation of NF- κ B (mechanism performed by the positive control JSH-23). But, only 13²-hydroxy pheophytin a (**2**) showed similar activity to the positive control.

4. Conclusions and future perspectives

In this work we have tested the anti-inflammatory activity of *Tagetes minuta* extracts and its compounds (pheophytins), through the inhibition of the production of NF- κ B. The activity of these compounds on NF- κ B explain why extracts (decoction and hydroalcoholic solutions) of this plant can be used to treat stomach and intestinal discomforts.

Furthermore, the activity of these compounds on multiple targets is receiving much more attention, for example as xanthine oxidase inhibitors (Xu *et al.*, 2017), anti-tumour (Higashi-Okai *et al.*, 1998) and anti-HIV (Kapewangolo *et al.*, 2017).

Disclosure statement

The authors declare no conflict of interest.

Author contributions

L.A.T., A.R.S., performed phytochemical analyses and compound isolation. G.I.L., assisted in manuscript writing and figure preparation. A.M.S., conducted biological experiments and statistical analysis. L.A.T., conceived and supervised the study, and wrote and edited the manuscript. All authors read and approved the final manuscript.

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Supplementary material

^1H and ^{13}C NMR, ^1H - ^1H COSY, HSQC, HMBC, and MS spectra for compound **1**

and **2** (Figures 1S-13S); viability and anti-inflammatory activity data of the extracts and fractions (Tables 1S-4S) is provided as supporting information.

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Figure captions

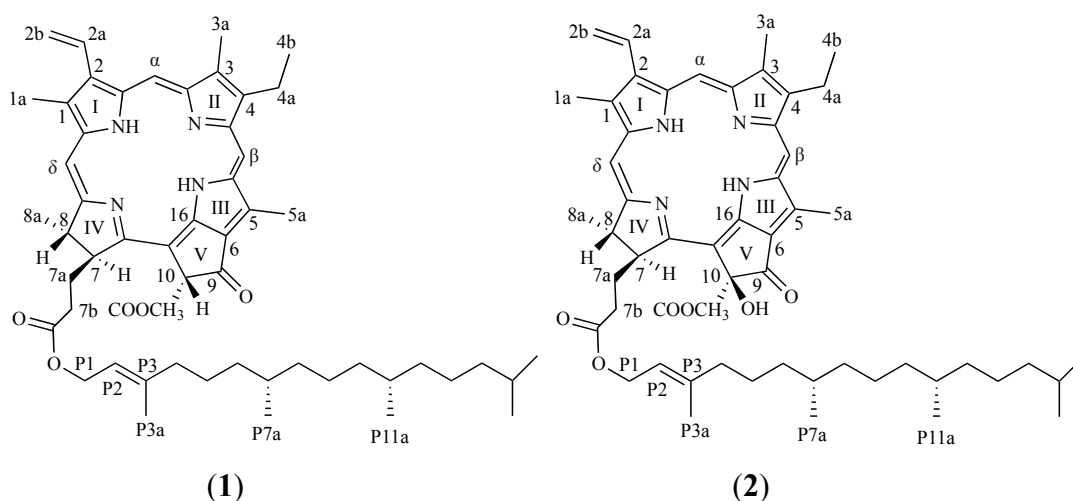


Figure 1. Chemical structure of pheophytin *a* (1) and 13²-hydroxy pheophytin *a* (2)

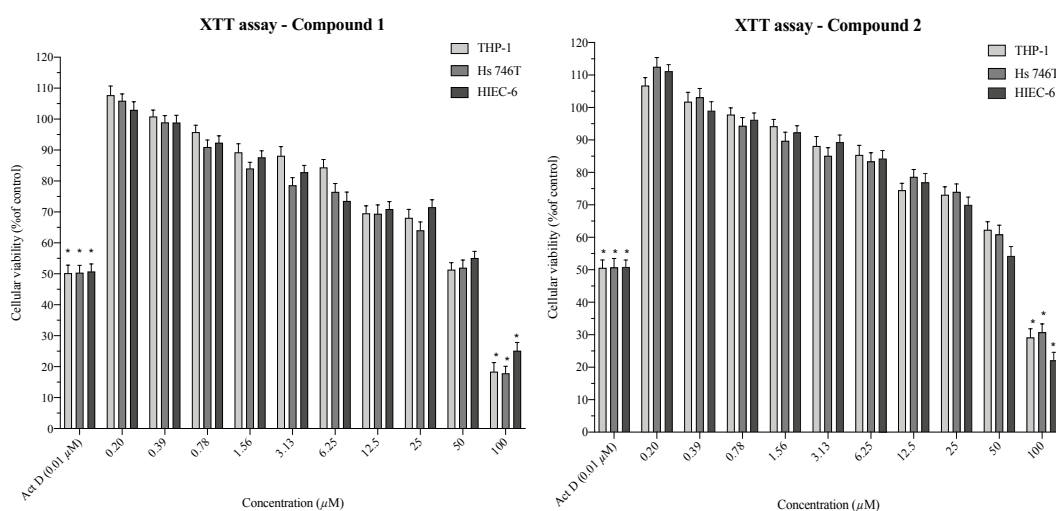


Figure 2. Viability of THP-1, Hs 746T and HIEC-6 cells treated at different concentrations of the compounds pheophytin *a* (1) and 13²-hydroxypheophytin *a* (2) after 12 h of treatment. Act D (Actinomycin D, CC₅₀=0.01 μM) was used as a positive control. Significant diff. among means (***)/*p*<0.001/Tukey's multiple comparisons test.

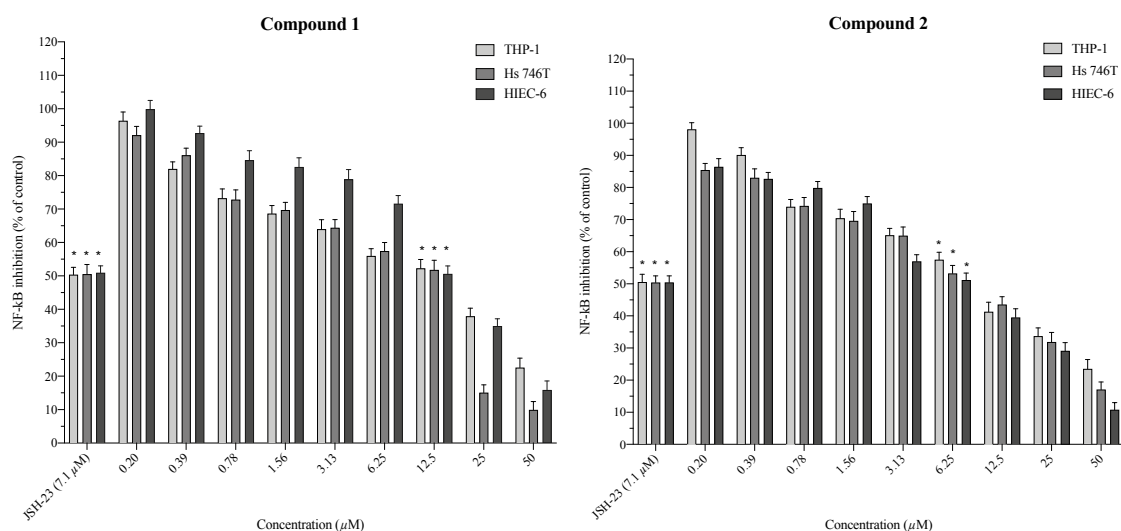


Figure 3. Effect of pheophytin *a* (**1**) and 13²-hydroxy pheophytin *a* (**2**) on TNF- α -induced NF- κ B activation in THP-1, Hs 746T and HIEC-6 cells at different concentrations after 12 h of treatment. JSH-23 (4-methyl-*N*-1-(3-phenylpropyl)-1,2-benzenediamine, IC₅₀=7.1 μ M) was used as a positive control. Significant diff. among means (***)/Tukey's multiple comparisons test.

Tables captions

Table 1. CC₅₀s of the XTT (Viability) tests calculated for the fractions obtained from the dichloromethane/methanol fraction from *Tagetes minuta*. CC₅₀ was calculated using Prism v8.4.3 (GraphPad Software) using non-linear regression, dose-response curves. CI95%: Confidence interval 95%/Tukey's multiple comparisons test ($p<0.001^{***}$).

Fractions	Cytotoxicity (CC ₅₀ µg/mL) at 12 h (CI95%, R2)		
	THP-1	Hs 746T	HIEC-6
DMSO	20.44 (15.72 to 25.57, 0.9998)	20.32 (15.96 to 25.66, 0.9998)	20.22 (15.89 to 25.63, 0.9998)
Actinomycin D*	0.012 (0.007 to 0.017, 0.9814)	0.012 (0.007 to 0.017, 0.9814)	0.010 (0.005 to 0.015, 0.9814)
FI	88.92 (83.51 to 93.48, 0.9858)	85.59 (80.26 to 90.95, 0.9858)	82.29 (77.34 to 87.35, 0.9858)
FII	78.76 (73.19 to 83.18, 0.9883)	75.14 (70.35 to 80.58, 0.9883)	72.79 (67.99 to 77.72, 0.9883)
FIII	77.85 (72.34 to 82.95, 0.9833)	75.09 (70.20 to 80.59, 0.9833)	70.79 (65.39 to 75.38, 0.9833)
FIV	75.81 (70.02 to 80.21, 0.9755)	75.31 (70.96 to 80.12, 0.9755)	72.01 (67.22 to 77.82, 0.9755)
FV	67.38 (62.38 to 72.01, 0.9992)	65.47 (60.93 to 70.72, 0.9992)	64.92 (59.85 to 69.15, 0.9992)
FVI	66.04 (61.41 to 71.62, 0.9792)	65.69 (60.39 to 70.96, 0.9792)	64.53 (59.82 to 69.44, 0.9792)
FVII	64.16 (59.32 to 69.70, 0.9829)	63.59 (58.25 to 68.85, 0.9829)	62.44 (57.11 to 67.21, 0.9829)
FVIII	58.24 (53.61 to 63.52, 0.9858)	56.52 (51.55 to 61.98, 0.9858)	56.22 (51.22 to 61.52, 0.9858)
FIX	56.73 (51.31 to 61.01, 0.9811)	54.58 (49.26 to 59.19, 0.9811)	54.57 (49.39 to 59.41, 0.9811)
FX	54.18 (49.36 to 59.44, 0.9845)	53.75 (48.27 to 58.27, 0.9845)	52.83 (47.59 to 57.52, 0.9845)
FXI	49.29 (44.34 to 54.49, 0.9925)	49.12 (44.12 to 54.22, 0.9925)	48.75 (43.95 to 53.43, 0.9925)
FXII	48.82 (43.87 to 53.95, 0.9871)	48.59 (43.04 to 53.28, 0.9871)	45.04 (40.88 to 50.57, 0.9871)

(*): Actinomycin D (CC₅₀=0.01 µM)

Table 2. IC₅₀s of the inhibition of NF-κB production, calculated for the fractions obtained from the dichloromethane/methanol fraction from *Tagetes minuta*. IC₅₀ was calculated using Prism v8.4.3 (GraphPad Software) using non-linear regression, dose-response curves. CI95%: Confidence interval 95%/Tukey's multiple comparisons test ($p<0.001^{***}$).

Fractions	Inhibition of NF-κB production (IC ₅₀ µg/mL) at 12 h (CI95%, R2)		
	THP-1	Hs 746T	HIEC-6
JSH-23*	7.19 (2.06 to 11.18, 0.9880)	7.10 (2.85 to 12.18, 0.9880)	7.12 (2.92 to 12.44, 0.9880)
FI	26.21 (51.99 to 61.58, 0.9692)	24.39 (48.11 to 58.38, 0.9692)	24.15 (50.24 to 60.73, 0.9692)
FII	11.66 (73.03 to 83.62, 0.9868)	12.62 (39.15 to 49.15, 0.9868)	12.71 (74.68 to 84.66, 0.9868)
FIII	11.37 (42.19 to 52.79, 0.9724)	12.98 (41.34 to 51.47, 0.9724)	15.66 (74.65 to 84.32, 0.9724)
FIV	9.05 (80.05 to 90.40, 0.9861)	9.42 (76.27 to 86.82, 0.9861)	9.71 (82.99 to 92.07, 0.9861)
FV	15.34 (92.40 to 102.59, 0.9802)	16.34 (92.69 to 102.49, 0.9802)	16.59 (94.21 to 104.99, 0.9802)
FVI	33.84 (83.81 to 93.82, 0.9892)	35.26 (82.84 to 92.82, 0.9892)	35.83 (81.27 to 91.55, 0.9892)
FVII	37.57 (73.81 to 83.45, 0.9981)	38.18 (73.39 to 83.39, 0.9981)	39.64 (73.94 to 83.41, 0.9981)
FVIII	54.72 (69.43 to 79.62, 0.9917)	55.85 (68.47 to 78.11, 0.9917)	56.08 (69.12 to 79.66, 0.9917)
FIX	57.19 (49.56 to 59.82, 0.9950)	57.72 (48.47 to 58.84, 0.9950)	59.19 (47.59 to 57.63, 0.9950)
FX	73.24 (57.34 to 67.65, 0.9941)	76.05 (58.68 to 68.75, 0.9941)	76.72 (56.99 to 66.86, 0.9941)
FXI	83.06 (59.78 to 69.77, 0.9768)	84.56 (60.28 to 70.52, 0.9768)	84.89 (58.33 to 68.08, 0.9768)
FXII	85.96 (60.28 to 70.52, 0.9796)	88.21 (58.33 to 68.08, 0.9796)	89.07 (59.78 to 69.77, 0.9796)

(*): JSH-23 (IC₅₀=µM)