Research in Pharmaceutical Sciences, December 2022; 17(6): 665-676
Received: 15-02-2022
Peer Reviewed: 30-04-2022
Revised: 01-07-2022
Accepted: 12-10-2022
Published: 29-10-2022

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

In vivo anti-inflammatory activities of *Plantago major* extract and fractions and analysis of their phytochemical components using a high-resolution mass spectrometry

Asih Triastuti[1,2,*], Dimas Adhi Pradana[3], Iwang Davi Setiawan[2], Nanang Fakhrudin[4,5], Setiawan Khoirul Himmi[6], Sitarina Widyarini[7], and Abdul Rohman[8]

[1]*Laboratory of Pharmaceutical Biology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia.*
[2]*Herbal Research Center, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia.*
[3]*Laboratory of Pharmacology, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia.*
[4]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[5]*Medicinal Plants and Natural Products Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[6]*Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Bogor, Indonesia.*
[7]*Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
[8]*Center of Excellence Institute of Halal Industry and Systems, Universitas Gadjah Mada, Yogyakarta, Indonesia.*

Abstract

Background and purpose: *Plantago major* has been applied as a herbal remedy for centuries. However, studies on anti-inflammatory activities and their chemical ingredients are limited. The objective of this study was to investigate the anti-inflammatory properties of *P. major* in three animal models and its phytochemical contents.

Experimental approach: Dichloromethane extract (DCM) of *P. major* was fractionated with *n*-hexane to yield the soluble (SHF) and insoluble (IHF) fractions. The anti-inflammatory activities of DCM, SHF, and IHF were evaluated using rat's paw edema induced by carrageenan, thioglycolate-induced leukocyte emigration in the mice, and rheumatoid arthritis (RA) induced by complete Freund's adjuvants in rats. The chemical constituents were analyzed using a high-resolution mass spectrometer (HRMS).

Findings / Results: The DCM, SHF, and IHF inhibited paw edema in the rats and reduced the leukocyte migration in the mice. At dose 560 mg/kg, the percentage of inhibitory was 47.33%, 55.51%, and 46.61% for the DCM, IHF, and SHF, respectively. In the RA animal model, IHF at 280 and 560 mg/kg reduced osteoclast formation and COX-2 expression compared to diclofenac. Some compounds namely oleic acid, linoleic acid, palmitic acid, and oleamide identified in the DCM, IHF, and SHF may be responsible for these activities.

Conclusion and implications: This study showed that *P. major* has several *in-vivo* anti-inflammatory activities.

Keywords: Anti-inflammatory; Anti-rheumatoid arthritis; Chemical compounds; HRMS; *Plantago major*.

INTRODUCTION

Identification of metabolites is critical in phytochemical research. Metabolite identification plays an important role in a better understanding of plants' chemical compositions and also to study the active metabolites which are responsible for certain pharmacological activities. *Plantago major* (broadleaf plantain) belongs to the Plantaginaceae family, a perennial plant with rosette leaves. For a long time, *P. major* has been distributed globally and used in society to cure a variety of diseases and to promote human health. It has been reported to be an antioxidant, a wound healer, antidiarrhea, antidiabetic, antibacterial, antiviral, and anti-inflammatory agent (1,2).

*Corresponding author: A. Triastuti
Tel: +62-274896439, Fax: +62-274896439
Email: asih.triastuti@uii.ac.id

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.359433

The chemical composition of the *P. major* has been studied using a variety of methodologies involving different extraction systems and instrumental identification from easy and rapid analysis to precise and sophisticated ones such as high-performance liquid chromatography-tandem with mass spectrometry (HPLC-MS/MS) or gas chromatography combined with MS (GC-MS). Jamilah *et al.* discovered that major compounds in *P. major* using GC-MS include organic acids (fumaric acid, syringic acid, vanillic acid, p-hydroxybenzoic acid, ferulic acid, p-coumaric acid, gentisic acid, traces of salicylic acid, benzoic acid), phytol, and benzofuranone (3). Bioassay-guided separation of a dichloromethane extract performed by Ringbom *et al.* resulted in the isolation of several fatty acids including linoleic acid, R-linolenic acid, myristic acid, and palmitic acid (4). Several studies also reported the metabolites from *P. major* including phenolic compounds, iridoid glycosides, polysaccharides, vitamins, flavonoids, terpenoids, alkaloids, and ursolic acid (2,5).

The important biological activities of *P. major* have been studied in several animal models. *P. major* seed extract possess wound-healing activity in the burn wound rat model (6). *P. major* showed re-epithelialization and good granulation tissue organization in wounded tissue and an extract of 50% of concentration was reported to be a suitable substitute for silver sulfadiazine. In another study, water extract of *P. major* leaves also has been reported to have an anxiolytic and hypnotic effect on elevated plus-maze test and the sodium pentobarbital-induced hypnosis method using Wistar rats (7). Ethanolic extract of *P. major* possesses antioxidant activity on cisplatin-induced nephrotoxicity and oxidative stress in rats (8). *P. major* reduced nephrotoxicity by reducing urea concentration, creatinine, sodium, and potassium serum concentration. Furthermore, a study by Hussan *et al*. revealed the activity of methanol extract of *P. major* enhanced innate antioxidant activity and ameliorate acetaminophen-induced liver injury by increasing the hepatic glutathione and superoxide dismutase (9). Despite the numerous biological activities of *P. major*, the detailed studies along with investigations of chemical contents, responsible for these activities, are limited.

The anti-inflammatory property of *P. major* has been documented in a limited number of studies, the majority of which were conducted *in vitro*. Zubair *et al.* evaluated the anti-inflammatory of water and ethanol extract *P. major* in the oral epithelial cell lines (1). In other reports, methanol extract of *P. major* showed antioxidant and anticholinesterase activities *in vitro* (10). Ursolic acid and oleanolic acid isolated from the hexane extract of *P. major* showed a significant cyclooxygenase-2 (COX-2) inhibitor effect (11).

No studies have been conducted to demonstrate the efficacy of extracts from this plant as an anti-inflammatory agent with various animal models and the correlation of its chemical compounds. This study aimed to investigate the anti-inflammatory activity of *P. major* extract and fractions in three animal models and to determine the phytochemical ingredients responsible for the activity using ultra-high performance liquid chromatography / high-resolution mass spectrometry (UHPLC-HRMS).

MATERIALS AND METHODS

Chemicals

Carrageenan, indomethacin, complete Freund's adjuvant (CFA), diclofenac sodium, anti-Cox-2 antibody, N-hexane, ethyl acetate, dichloromethane, acetonitrile, and formic acid were purchased from Merck (Singapore).

Plant collection

The *P. major* plant was collected in the early dry season in July 2021 from the area of Tawangmangu, Central Java, Indonesia (-7.66536, 111.1349). The plant was identified, authenticated, and collected by PSOH (Herbal Research Center, Universitas Islam Indonesia) and the voucher of the plant specimen (Voucher No. BF-UII-pm-003) was deposited at the Biopharmaceutical Laboratory, Department of Pharmacy, Universitas Islam Indonesia, Yogyakarta, Indonesia.

Plant extracts and fractions preparation

The extract and fractions were prepared according to our previous study (12). Briefly, the dried plant material (2 kg) was macerated in 6 L of dichloromethane for 24 h and re-macerated twice to get dichloromethane extract (DCM). The filtrates were evaporated using a rotary evaporator (Heidolph, Germany). The DCM (340 g) was dissolved in *n*-hexane to yield soluble (SHF) and insoluble fractions (IHF). This method yielded 160.2 g of SHF and 96.1 g of IHF fractions, respectively. The DCM, IHF, and SHF were prepared at 140, 280, and 560 mg/kg, respectively, for the treatment of the animals. These doses were chosen according to the report of Rutland Biodynamics that the tincture of Plantago major was safe up to a dose of 6200 mg/kg (13).

Experimental design

All the animal treatments in this study received approval from the Medical and Health Research Ethics Committee of Universitas Islam Indonesia, Yogyakarta under the reference number KE/FK/0753/EC/2020.

Carrageenan-induced paw edema test in the rats

The methods were performed according to the previous methods with a slight modification (14). A week before the experiment, 60 male Wistar rats (25-30 g) were kept (5 in each cage) for acclimatization. The animals were given a standard commercial diet and water *ad libitum* control. The animals were gavage-treated with DCM, SHF, and IHF. Sodium carboxymethyl cellulose (Na-CMC; 0.1 mL/10 g) was given to the control group, while indomethacin (10 mg/kg) was employed as a positive control. After 30 min, edema was induced in the sub-plantar of the right hind paw by injecting 1% carrageenan (0.1 mL). The paw volume was determined with a plethysmometer (Ugo Basile, Italy), immediately before the carrageenan injection and every 30 min for the next 6 h. The paw edema was calculated by subtracting the final and starting volumes (mL).

Thioglycolate-induced leukocytes migration

This method was carried out following the previous research (15). A control group and a treatment group of mice were used (five mice per group). The DCM, SHF, IHF, and indomethacin were given intraperitoneally (i.p. 0.2 mL) to the treatment groups, while the solvent (DMSO-saline) was given to the control group. After 30 min, sterile thioglycolate was injected to induce leukocyte migration (i.p; 0.5 mL). After 4 h, the mice were sacrificed, and the peritoneal lavage was taken carefully and centrifuged for 10 min at 1200 rpm and 4 °C to get the cell pellets. The cells were stained with methylene blue after being resuspended in 1 mL of phosphate-buffered saline (PBS). A Neubauer chamber and direct microscopic counts were used to determine the number of cells (Assistant; Sondheim, Germany).

Evaluation of anti-rheumatoid arthritis

Arthritis was induced according to the previous study (16), with minor modifications. A control group and a treatment group of rats were used (five rats per group). Group I, rats were the normal control; group II, animals were the RA control group; group III, animals received sodium diclofenac, rats in groups IV, V, and VI were the group treated with IHF at 280, 420, and 56 mg/kg, respectively. In brief, complete Freund's adjuvants (0.15 mL) was injected into the left hind paw plantar area. On day 0 of the experiment, an equal volume of saline was injected into the plantar area of the right paw. Every three days until day 47, the volume of edema was determined with a plethysmometer (Italy's Ugo Basile). An arthritis index was used to validate the existence of rheumatoid arthritis (RA) after three weeks of daily assessments (17). The inflammation of the toes, footpads, and ankles was used to gauge the severity of arthritis. Based on these modifications, a maximum score of two was assigned to each paw. The arthritic index (AI) was calculated by averaging the cumulative values for all paws. When AI was greater than one, rats were considered to have arthritis. From days 16 to 46, the treatment groups were given IHF while the negative and positive control groups received either Na-CMC or Na-diclofenac.

COX-2 staining

The joint tissue was removed for histological investigation after animals were euthanized

with a lethal dose of ketamine. In the immunohistochemistry, assays were performed using the streptavidin-biotin-peroxidase method used according to the previous study with modifications (18). The paraffin-embedded sections were deparaffinized, incubated overnight at 4 °C in a peroxidase-blocking solution, and then incubated with an anti-mouse COX-2 primary antibody (1:250). After three times washing with PBS, the secondary biotinylated universal antibody (anti IgG; 1:200) was applied, incubated for 5 min at room temperature, and then a conjugated streptavidin peroxidase complex was added. The sections were stained with peroxidase substrate solution diethyl amino benzidine (DAB), and counter-stained with hematoxylin. Mounting media was added and sections were covered by deck glass for examination using a light microscope. COX-2 (brown color) was expressed in the cytoplasm of inflammatory cells. The expression of COX-2 was quantified using Image Raster and OptiLab viewer.

Chemical constituents' detection using UHPLC-HRMS

All extracts were profiled using a Dionex™ Ultimate 3000 RSLC nano UHPLC coupled with Thermo Scientific™ Q Exactive™ HRMS. A total of 2 µL samples in methanol were injected into a phenyl hexyl 100 mm × 2.1 mm. The analysis was performed at a flow rate of 50 µL/min and using the gradient according to a previous study (19). Mass detection was performed using a heated electrospray ionization source in positive ionization and negative ionization modes at full MS at 70,000 and full width at half maximum (FWHM) resolution data-dependent MS2 at 17,500 full width at half maximum. Compound identification was performed by Thermo Scientific™ Compound Discoverer Software.

Statistical analysis

Data were analyzed using Graph Pad Prism version 9. The result of the treatment groups and controls were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post-test. *P*-values < 0.05 was considered statistically significant.

RESULTS

Anti-inflammatory effect of P. major on a carrageenan-induced paw edema

Rat hind paw volume over time is presented in Fig. 1. The DCM, SHF, IHF, and indomethacin significantly reduced the edema volume compared with the edema control group ($P < 0.05$). The percentage of the inflammatory activity of *P. major* is presented in Fig. 2.

The DCM, IHF, and SHF reduced the volume of edema in the rats induced by carrageenan. In the dose of 560 mg/kg, the inhibitory percentage is 47.33, 55.51, and 46.61% for DCM, IHF, and SHF, respectively, although the activity was less compared to the indomethacin (63.81%).

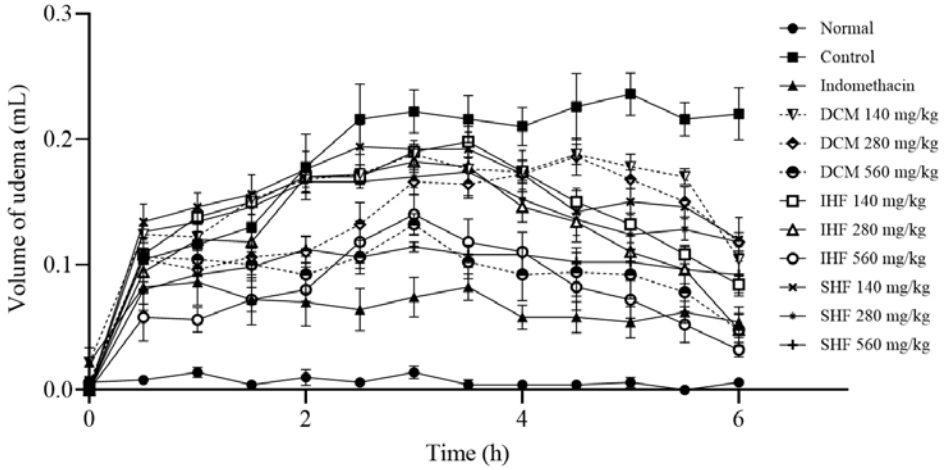

Fig. 1. The anti-inflammatory properties of *P. major* in rats with carrageenan-induced paw edema. DCM, Dichloromethane extract of the *P. major*; SHF, *n*-hexane-soluble fraction; IHF, *n*-hexane-insoluble fraction.

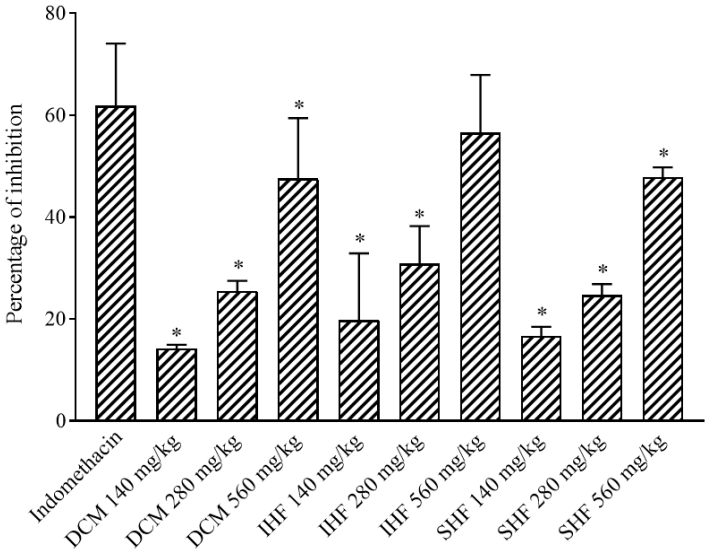

Fig. 2. The inflammatory inhibition following indomethacin, DCM, IHF, and SHF treatment group (n = 5). Data are presented as mean ± SEM. $^{*}P < 0.05$ Indicates significant differences compared with indomethacin. DCM, Dichloromethane extract of the *P. major*; SHF, n-hexane-soluble fraction; IHF, *n*-hexane-insoluble fraction.

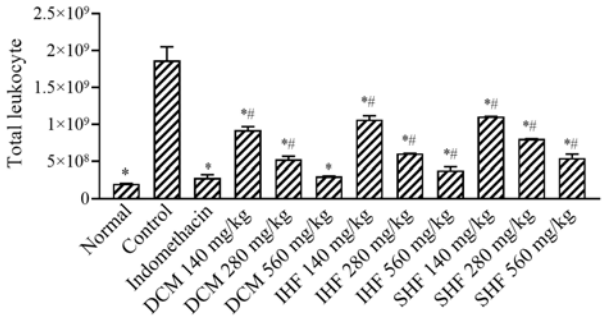

Fig. 3. The total leukocyte migration of indomethacin, DCM, IHF, and SHF treatment group (n = 5). Data are presented as mean ± SEM. $^{*}P < 0.05$ Indicates significant differences compared to the control group; $^{\#}P < 0.05$ versus indomethacin. DCM, Dichloromethane extract of the *P. major*; SHF, n-hexane-soluble fraction; IHF, n-hexane-insoluble fraction.

The activity of inflammation inhibition increased along with the dose increment. For example, in DCM groups, the activity was 13.86, 25.15, and 47.33%, respectively for doses of 140, 280, and 560 mg/kg. In the IHF group, the activity was 17.84, 29.17, and 55.51% and in the SHF group was 14.68, 22.89, and 46.61%, respectively for the doses of 140, 280, and 560 mg/kg.

Anti-inflammatory effect of P. major on thioglycolate-induced peritonitis

The solvent group had a similar number of leukocytes to the normal group indicating that the solvent did not stimulate a leukocyte migration in the test animals. Meanwhile, thioglycolate injection increased the number of leukocytes ten folds when compared to the normal group showing a large migration of leukocytes to the peritoneal area (Fig. 3).

Indomethacin was able to dramatically limit leukocyte migration. Although the efficiency of the IHF was much greater than that of the negative control group, it was still less effective than the indomethacin. Additionally, the higher the extract/fractions level, the greater the possibility of inhibiting the leukocyte migratory activity (Table 1).

Anti-inflammatory effect of IHF of P. major on CFA-induced RA

The IHF was chosen for chronic anti-inflammation using a CFA-induced RA as the most active fraction of the two *in-vivo* anti-inflammatory models. In this test, the doses used were 280, 420, and 560 mg/kg. Every four days until day 47, the AI was used to determine arthritic incidence and severity in CFA-induced rats. The AI scale was utilized to determine RA in the rats. In RA rats, the toes swelled, and the soles changed form. The AI on day 47 of IHF for doses of 280, 420, and 560 mg/kg were 1.50 ± 0.15, 1.42 ± 0.13, and 0.93± 0.06, respectively, and the AI of diclofenac was 1.22 ± 0.12. (Fig. 4).

Table 1. The reduction of leukocyte migration in thioglycolate-induced mice with DCM, IHF and SHF *P. major*.

Group of treatment	Percentage of leukocyte migration
Normal	10.88 ± 0.09*
Control	100.00
Indomethacin	15.18 ± 1.08*
DCM 140 mg/kg	49.59 ± 1.07*#
DCM 280 mg/kg	28.16 ± 1.01*#
DCM 560 mg/kg	16.39 ± 0.06*
IHF 140 mg/kg	57.02 ± 1.24*#
IHF 280 mg/kg	32.43 ± 0.07*#
IHF 560 mg/kg	20.57 ± 0.99*#
SHF 140 mg/kg	59.29 ± 0.02*#
SHF 280 mg/kg	43.10 ± 0.05*#
SHF 560 mg/kg	29.24 ± 1.23*#

The number of leukocyte migrations in the DMSO-saline treated group was set as 100% upon the induction of thioglycollate. The mice (5 per group) were pre-treated (i.p.) for 30 min with the indomethacin (10 mg/kg). DCM, Dichloromethane extract of *P. major*; SHF, n-hexane-soluble fraction; IHF, n-hexane-insoluble fractions; or solvent (DMSO-saline) before being stimulated with thioglycollate (i.p.). Data are presented as mean ± SEM, n = 5. *$P < 0.05$ indicates significant differences in comparison with rheumatoid arthritis control animal; #$P < 0.05$ versus indomethacin.

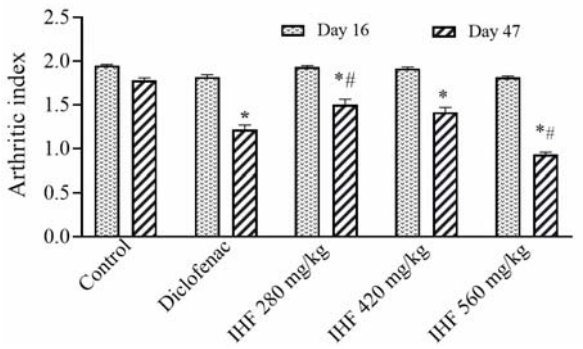

Fig. 4. The Arthritic index of treated groups (n = 5) on days 16 and 47. Data are presented as means ± SEM. *P < 0.05 indicates significant differences in comparison with rheumatoid arthritis control; #P < 0.05 versus sodium diclofenac. DCM, Dichloromethane extract of P. major; SHF, n-hexane-soluble fraction; IHF, n-hexane-insoluble fraction.

Table 2. Comparison of the osteoclast formation, inflammatory cells, and COX-2 expression on CFA-induced RA animals on day 47.

Group of treatment	Osteoclast formation (%)	Inflammatory cells	COX-2 expression
Normal	0	7.4 ± 1.12*	7.8 ± 0.58*
Rheumatoid arthritis control	100	378.6 ± 3.91	71.4 ± 1.60
Diclofenac	22.22 ± 0.55*	330.0 ± 7.87 *	60.6 ± 1.57*
IHF 140 mg/kg	22.22 ± 0.55*	332.6 ± 3.53*	36.2 ± 2.20*#
IHF 280 mg/kg	11.11 ± 0.55*#	316.4 ± 5.82 *	30.20 ± 1.93*#
IHF 560 mg/kg	11.11 ± 0.55*#	313.6 ± 3.12 *	22.0 ± 1.14*#

Data are presented as mean ± SEM, n = 5. *$P < 0.05$ indicates significant differences in comparison with rheumatoid arthritis control; #$P < 0.05$ versus sodium diclofenac. IHF, n-Hexane-insoluble fraction.

The histopathological data showed the potency of the IHF to suppress the course of RA in the left leg of the rats induced with CFA. The evaluation was accomplished by observing the histopathology by accessing the osteoclast formation, the inflammatory cells, and the COX-2 expression (Fig. 5). IHF at 280, 420, and 560 mg/kg decreased inflammatory cell infiltration and osteoclast formation in RA rats (Table 2).

The negative control group exhibited higher inflammatory cells and COX-2 expression. These findings demonstrated that CFA induction is effective at inducing RA. The observation of Na-diclofenac and treatment with IHF revealed the non-existence and existence of the osteoclasts in numerous visual fields. IHF at 420 and 560 mg/kg lowered osteoclast formation and COX-2 expressions compared to RA control and diclofenac.

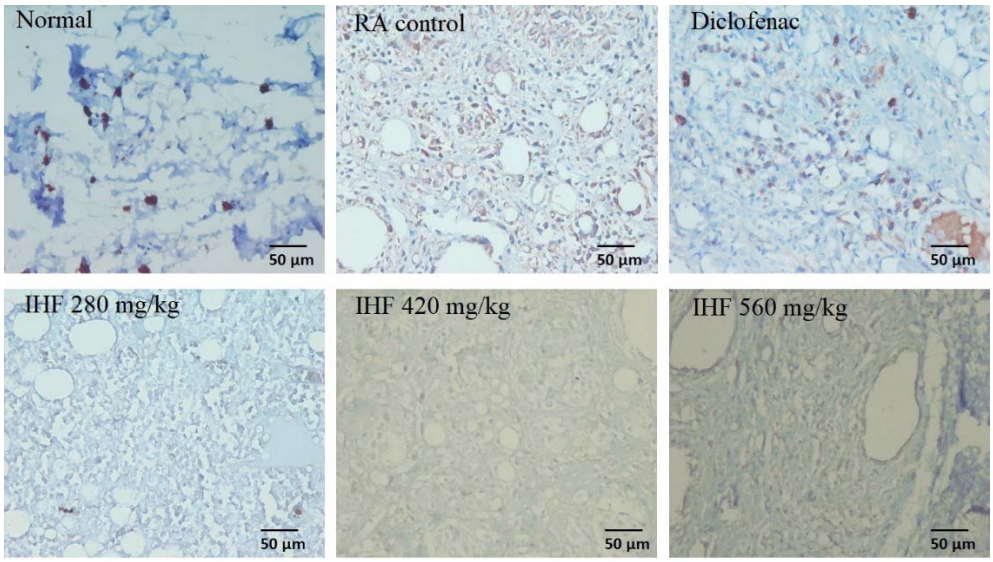

Fig. 5. Histopathological characteristics of joint tissue were magnified 400 times in five separate fields (scale bar: 50 µm). Representative images of cyclooxygenase 2-expressing cells (dark brown stained) in the paw edema. RA, Rheumatoid arthritis; IHF, n-hexane-insoluble fraction.

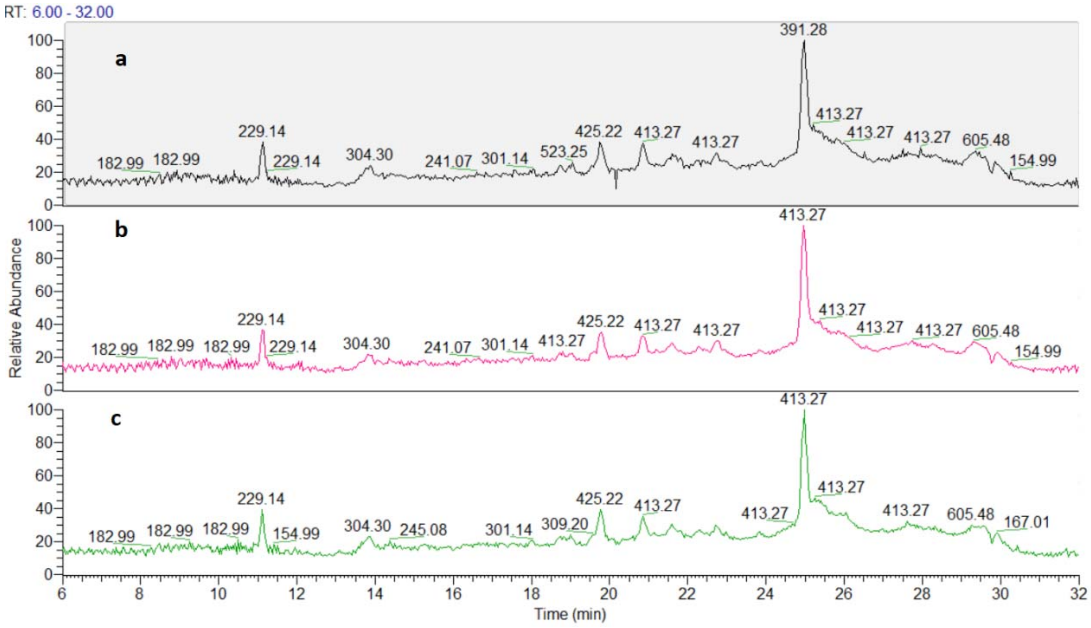

Fig. 6. Total ion chromatograms UHPLC-HRMS (PI) profiles of dichloromethane extract of (a) the *P. major*; (b) insoluble-hexane fraction, and (c) soluble-hexane fraction at retention time 6-32 min. UHPLC-HRMS, Ultra-high performance liquid chromatography / high-resolution mass spectrometry.

UHPLC-HRMS of DCM, IHF, and SHF

The metabolites of extract (DCM) and two fractions (IHF and SHF) were analyzed using a UPLC-HRMS in the positive and negative ionization (Fig. 6). Metabolites detected in the sample were presented in Table 3.

Table 3. The compounds detected in *Plantago major*.

Metabolite name	Formula	Retention time (min)	Observed m/z	Theoretical m/z	Δ mass (ppm)	Expression found in		
						DCM	IHF	SHF
Ostruthin	$C_{19}H_{22}O_3$	15.58	298.1593	298.1574	6.2283	+	+	+
Erucamide	$C_{22}H_{43}NO$	23.84	337.3352	337.3350	0.5217	+	+	+
cis-7-Hexadecenoic acid	$C_{16}H_{30}O_2$	19.78	254.2245	254.2251	2.4742	+	+	+
Oleic acid	$C_{18}H_{34}O_2$	21.58	282.2551	282.2564	4.5668	+	+	+
Vitamin E acetate	$C_{31}H_{52}O_3$	25.09	473.3998	473.3989	1.8124	+	+	+
Palmitoleic acid	$C_{16}H_{30}O_2$	19.76	254.2250	254.2251	0.4288	−	−	+
Linoleic acid	$C_{18}H_3O_2$	20.77	280.2394	280.2408	4.9208	+	−	-
Ethyl palmitoleate	$C_{18}H_{34}O_2$	21.61	283.2640	283.2632	2.9054	+	+	+
9(Z),11(E)-Conjugated linoleic acid	$C_{18}H_3O_2$	20.77	280.2395	280.2408	4.4926	−	+	+
trans-3-Indole acrylic acid	$C_{11}H_9NO_2$	8.96	187.0640	187.0639	0.6041	−	+	+
Hexadecanamide	$C_{16}H_{33}NO$	19.02	255.2568	255.2557	4.2859	+	+	+
Palmitic acid	$C_{16}H_{32}O_2$	11.98	256.2394	256.2397	1.1396	+	+	+
L-Phenylalanine	$C_9H_{11}NO_2$	8.50	165.0796	165.0784	7.2087	+	+	+
DL-Tryptophan	$C_{11}H_{12}N_2O_2$	8.95	205.0978	205.0972	3.1985	+	−	−
Oleamide	$C_{18}H_{35}NO$	19.87	282. 2798	282.2791	3.1985	+	+	+
2,5-di-tert-Butylhydroquinone	$C_{14}H_{22}O_2$	13.92	221.1534	221.1547	5.8466	+	+	+

DCM, Dichloromethane extract; IHF, hexane-insoluble fraction; SHF, hexane-soluble fraction of *P. major;* +, detected in the sample; −, not detected in the sample.

DISCUSSIONS

Inflammation plays a critical role in the progression of several diseases (immune-mediated inflammatory disease), including atherosclerosis, asthma, diabetes, hepatitis, RA, and gout arthritis, as well as central nervous system diseases like Alzheimer's (20). The discovery of anti-inflammatory substances in natural products, particularly medicinal plants, remains difficult and lead to more advanced research. Many plant extracts with a prophylactic or therapeutic efficacy have been developed into pharmaceutical forms. However, the evidence-based research on plants and their correlation with active compounds responsible for their biological activities is limited. An earlier investigation (21) showed that methanol extract of *P. major* had anti-inflammatory activity on carrageenan-induced rat paw edema and hepatoprotective effect on CCl_4-induced hepatotoxicity in rats.

We assessed the anti-inflammatory properties of *P. major* using three animal models of inflammation: carrageenan-induced paw edema in rats and thioglycolate-induced peritonitis in mice. The IHF fractions in the two models were then chosen to be analyzed using the chronic inflammation model, a Freund's complete adjuvant-induced RA in rats.

In the carrageenan-induced paw edema model, the extract and fractions of *P. major* reduced the volume of edema in a dose-dependent manner although the activity was less compared to the indomethacin. In the leukocyte migration model, the DCM at 560 mg/kg had the highest activity to reduce leukocyte migration (activity order DCM > IHF > SHF). Leukocyte migration from the blood to tissue is a critical phase in the inflammatory process. It is driven by a variety of chemokines and cytokines and allows effector cells such as neutrophils, monocytes, and T cells to enter the regions of infection, damage, and stress (22). Controlling the leukocyte migration *via* molecular pathways is one of the prospective therapeutic strategies in the treatment of inflammation and inflammation-mediated illnesses. Thioglycolate is an oxygen-depleting chemical that has been shown to attract neutrophils to the peritoneal cavity. Numerous chemokines, including CXCL1, CXCL2, and CXCL8, are involved in this process (23). *P. major* may be examined further for its efficacy against leukocyte migration-related diseases such as arteriosclerosis, RA, angiogenesis, and inflammatory heart disease.

According to the histopathological results, the administration of IHF is comparable with diclofenac which inhibits osteoclast development in the RA model. Diclofenac

sodium is a selective inhibitor of receptor activator of nuclear factor kappaB (RANK), a protein involved in the process of osteoclast genesis and protection against inflammatory damage in collagen-induced arthritic rats. The study showed that IHF may slow the progression of arthritis by minimizing joint and paw tissue damage. RA is characterized by cartilage deterioration caused by inflammation, which is controlled by a complement, the breakdown of the extracellular matrix, and the production of the pannus (24). Chronic inflammation results in synovial membrane thickness, hypertrophy, restriction of blood flow, necrosis of the cells, and persistent inflammation. Pannus formation is characterized by a granular synovium thickening. Pannus spreads to the synovium, causing inflammation and the production of scar tissue, which results in joint injury and deformity (25). Additionally, IHF therapy reduced COX-2 expression, although the activity was smaller than that of diclofenac. COX-2 is the enzyme that generates pro-inflammatory and anti-inflammatory lipid mediators from ω-3 and ω-6 fatty acids, which are largely responsible for inflammation (26). COX catalyzes the initial step in the synthesis of prostanoids, a wide family of arachidonic acid metabolites that include prostaglandins, prostacyclin, and thromboxane, and is a major target of nonsteroidal anti-inflammatory drugs. This finding was in agreement with prior research (4,11) which showed that *P. major* is a selective COX-2 inhibitor.

Metabolite compounds detected in the DCM, IHF, and SHF are similar except for palmitic acid, conjugated linoleic acid, indole acrylic acid, and DL-tryptophan. *P. major* also contains chain fatty acids such as *cis*-7-hexadecenoic acid, oleic acid, linoleic acid, and amides such as erucamide and oleamide (Fig. 7).

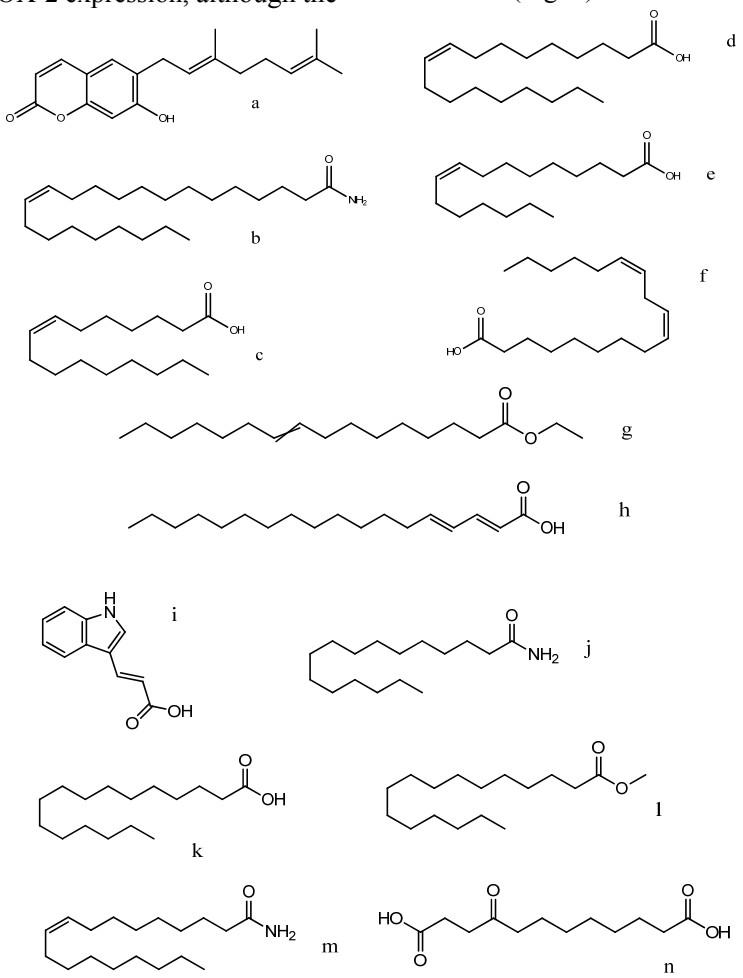

Fig. 7. Chemical structures of compounds detected in *P. major* with UHPLC-HRMS. A, Ostruthin; b, erucamide; c, *cis*-7-hexadecanoic acid; d, oleic acid; e, palmitoleic acid; f, linoleic acid; g, ethyl palmitoleate; h, conjugated linoleic acid; i, *trans*-3-indole acrylic acid; j, hexadecanamide; k, palmitic acid; l, methyl palmitate; m, oleamide; n, 4-oxo dodecanedioic acid; UHPLC-HRMS, Ultra-high performance liquid chromatography / high-resolution mass spectrometry.

This finding is comparable to that of Ringbom *et al*. reporting that the separation of DCM extract from the *P. major* resulted in the isolation of the following fatty acids: linoleic acid, R-linolenic acid, myristic acid, and palmitic acid (4). Ostruthin (6-geranyl-7-hydroxycoumarin) is previously reported to be found in the *Peucedanum ostruthium, Peucedanum decursivum* (27), and *Flacourtia jangomas* (28), but never been reported in *P. major*. It inhibits serum-induced vascular smooth muscle cell proliferation in the rat aorta (27). In another study, ostruthin has been reported to be active against the *Mycobacterium* sp. (29).

Plantago lanceolata, *Plantago ovata, Plantago media*, and *Plantago major* (4,30) have been previously reported to contain fatty acids including palmitic, oleic, linoleic, and palmitoleic acids. Palmitoleic acid enhances insulin sensitivity and reduces hepatic inflammation in rats *via* mechanisms that do not require peroxisome proliferator-activated receptor gamma (PPARγ) (31). Activation of the PPARγ inhibits activation of nuclear factor (NF) κB hence, reducing pro-inflammatory gene expression and cytokine production, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-12 (32). Furthermore, oleic and linoleic acids have been shown to alleviate inflammation in rat macrophages by modulating the production of inflammatory mediators such as IL-6, IL-1, and cytokine-induced neutrophil chemoattractant-2αβ (33). The DCM, IHF, and SHF inhibited leukocyte migration in the thioglycolate-induced peritonitis model. This is in line with a previous study that examined the activity of oleic acid in the peritoneal lavage fluid of sepsis-suffering mice. Oleic acid increased the anti-inflammatory cytokine IL-10 levels and decreased pro-inflammatory cytokines TNF-α and IL-1β (34). Similarly, oleic acid inhibited cell migration induced by the lipopolysaccharide in isolated human neutrophils (35). In the RA animal model induced by a CFA, the IHF decreased the arthritis incident and COX-2 expression. This action may be correlated with the activity of the oleic and linoleic acids as COX-2 inhibitors as described by the previous study (4,36).

An interesting finding in this study was the detection of erucamide, oleamide, and hexadecanamide in the DCM, IHF, and SHF. These compounds have never been reported before from *P. major* extracts. Erucamide is the amide of erucic acid, capable of stimulating the growth of blood vessels (37). Oleamide reduced lipopolysaccharide-induced nitric oxide and prostaglandin E2 synthesis, as well as an inducible isoform of nitric oxide synthases and COX-2 expression in BV2 murine microglial cells (38). Hexadecanamide reduces edema and inflammatory hyperalgesia by inhibiting mast cell activation and reducing formalin-induced nociception in animals (39). Our findings support the prior research reporting that *P. major* has anti-inflammatory effects in a variety of preclinical animals. The chemical constituents detected in DCM, IHF, and SHF supported the activity of the *P. major* and the future development of the *P. major* as an anti-inflammatory agent.

CONCLUSION

This investigation established that *P. major* possesses anti-inflammatory properties. The DCM extract, IHF, and SHF fractions possess anti-inflammatory activity with a hexane-insoluble fraction being the most active part. The chemical compounds were detected with a UHPLC-HRMS including oleic acid, palmitic acid, palmitoleic acid, and linoleic acid may be contributed to the anti-inflammatory properties.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Acknowledgments

This research was supported by the Directorate of Research and Community Services and the Directorate of Human Resources Universitas Islam Indonesia with contract number 3354/Rek/10/DSDM/IX/2021.

Authors' contribution

A. Triastuti prepared the extract and fractions of *P. major*, analyzed the UHPLC-HRMS data as well as the putative compounds, and performed the statistical analysis of all the

data in this study. D.A. Pradana performed the anti-inflammatory test on carrageenan-induced edema and I.D. Setiawan performed anti-inflammation in RA animals. N. Fakhrudin investigated the anti-leukocyte migration activity of *P. major* and S. Widyarini analyzed the histopathological test. S.K. Himmi performed the UHPLC-HRMS and A. Rohman analyzed the putative compounds. All authors approved and reviewed the final version of the manuscript.

REFERENCES

1. Zubair M, Widén C, Renvert S, Rumpunen K. Water and ethanol extracts of Plantago major leaves show anti-inflammatory activity on oral epithelial cells. J Tradit Complement Med. 2019;9(3):169-171. DOI: 10.1016/j.jtcme.2017.09.002
2. Adom MB, Taher M, Mutalabisin MF, Amri MS, Abdul Kudos MB, Wan Sulaiman M, *et al*. Chemical constituents and medical benefits of *Plantago major*. Biomed Pharmacother. 2017;96:348-360. DOI: 10.1016/j.biopha.2017.09.152.
3. Jamilah J, Sharifa A, Sharifah N. GC-MS analysis of various extracts from leaf of *Plantago major* used as traditional medicine. World Appl Sci J. 2012;17:67-70.
4. Ringbom T, Huss U, Stenholm A, Flock S, Skattebøl L, Perera P, *et al*. COX-2 inhibitory effects of naturally occurring and modified fatty acids. J Nat Prod. 2001;64(6):745-749. DOI: 10.1021/np000620d.
5. Handjieva N, Spassov S, Bodurova G, Saadi H, Popov S, Pureb O, *et al*. Majoroside, an iridoid glucoside from *Plantago major*. Phytochemistry. 1991;30(4):1317-1318. DOI: 10.1016/S0031-9422(00)95224-5.
6. Amini M, Kherad M, Mehrabani D, Azarpira N, Panjehshahin MR, Tanideh N. Effect of *Plantago major* on burn wound healing in rat. J Appli Anim Res. 2010;37(1):53-56. DOI: 10.1080/09712119.2010.9707093.
7. Caro DC, Rivera DE, Ocampo Y, Franco LA, Salas RD. Pharmacological evaluation of *Mentha spicata* L. and *Plantago major* L., medicinal plants used to treat anxiety and insomnia in colombian caribbean coast. Evid Based Complement Alternat Med. 2018;2018:5921514. DOI: 10.1155/2018/5921514.
8. Parhizgar S, Hosseinian S, Hadjzadeh MAR, Soukhtanloo M, Ebrahimzadeh A, Mohebbati R, *et al*. Renoprotective effect of *Plantago major* against nephrotoxicity and oxidative stress induced by cisplatin. Iran J Kidney Dis. 2016;10(4):182-188. PMID: 27514764.
9. Hussan F, Osman Basah RH, Mohd Yusof MR, Kamaruddin NA, Othman F. *Plantago major* treatment enhanced innate antioxidant activity in experimental acetaminophen toxicity. Asian Pac J Trop Biomed. 2015;5(9):728-732. DOI: 10.1016/j.apjtb.2015.06.013.
10. Kolak U, Boğa M, Uruşak EA, Ulubelen A. Constituents of *Plantago major* subsp. intermedia with antioxidant and anticholinesterase capacities. Turkish J Chem. 2011;35(4):637-645. DOI: 10.3906/kim-1102-990
11. Ringbom T, Segura L, Noreen Y, Perera P, Bohlin L. Ursolic acid from *Plantago major*, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. J Nat Prod. 1998;61(10):1212-1215. DOI: 10.1021/np980088i.
12. Triastuti A, Pradana DA, Saputra DE, Lianika N, Wicaksono HR, DewiAnisari T, *et al*. Anti-rheumatoid activity of a hexane-insoluble fraction from *Plantago major* in female Wistar rats induced by complete Freund's adjuvant. J Tradit Complement Med. 2022;12(3):219-224. DOI: 10.1016/j.jtcme.2021.07.006.
13. Avantor. Safety data sheet plantago major tincture. Rutland; 2015. Available from: https://us.vwr.com/assetsvc/asset/en_US/id/16490607/contents.
14. Shui S, Shen S, Huang R, Xiao B, Yang J. Metabonomic analysis of biochemical changes in the plasma and urine of carrageenan-induced rats after treatment with Yi-Guan-Jian decoction. J Chromatogr B. 2016;1033-1034:80-90. DOI: 10.1016/j.jchromb.2016.08.003.
15. Fakhrudin N, Franyoto YD, Astuti ED, Nurrochmad A, Wahyuono S. The effect of ursolic acid from *Plantago lanceolata* leaves on leukocytes migration and chemokines level. Indonesian J Pharm. 2019;30(4):252-259.
16. Ruckmani A, Meti V, Vijayashree R, Arunkumar R, Rao Konda V, Prabhu L, *et al*. Anti-rheumatoid activity of ethanolic extract of *Sesamum indicum* seed extract in Freund's complete adjuvant induced arthritis in Wistar albino rats. J Tradit Complement Med. 2018;8(3):377-386. DOI: 10.1016/j.jtcme.2017.06.003.
17. Smit HF, Kroes BH, Van Den Berg AJJ, van der Wal D, van den Worm E, J Beukelman C, *et al*. Immunomodulatory and anti-inflammatory activity of *Picrorhiza scrophulariiflora*. J Ethnopharmacol. 2000;73(1-2):101-109. DOI: 10.1016/S0378-8741(00)00268-3.
18. de-Almeida SCX, da-Silva ÂCF, Sousa NRT, Amorim IHF, Leite BG, Neves KRT, *et al*. Antinociceptive and anti-inflammatory activities of a triterpene-rich fraction from Himatanthus drasticus. Braz J Med Biol Res. 2019;52(5):1-12. DOI: 10.1590/1414-431X20197798.
19. Triastuti A, Haddad M, Barakat F, Mejia K, Rabouille G, Fabre N, *et al*. Dynamics of chemical diversity during co-cultures: an integrative time-scale metabolomics study of fungal endophytes *Cophinforma mamane* and *Fusarium solani*. Chem Biodivers. 2021;18(2). DOI: 10.1002/cbdv.202000672.
20. Hopkin SJ, Lewis JW, Krautter F, Chimen M,

McGettrick HM. Triggering the resolution of immune mediated inflammatory diseases: can targeting leukocyte migration be the answer? Front Pharmacol. 2019;10:1-9.
DOI: 10.3389/fphar.2019.00184.

21. Turel I, Ozbek H, Erten R, Oner AC, Cengiz N, Yilmaz O. Hepatoprotective and anti-inflammatory activities of *Plantago major* L. Indian J Pharmacol. 2009;41(3):120-124.
DOI: 10.4103/0253-7613.55211.
22. Jones DP, True HD, Patel J. Leukocyte trafficking in cardiovascular disease: insights from experimental models. Mediators Inflamm. 2017;2017:9746169.
DOI: 10.1155/2017/9746169.
23. Hermida MDR, Malta R, de Santos MDPC, dos-Santos WLC. Selecting the right gate to identify relevant cells for your assay: a study of thioglycollate-elicited peritoneal exudate cells in mice. BMC Res Notes. 2017;10(1):1-7.
DOI: 10.1186/s13104-017-3019-5.
24. Libby P. Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev. 2007;65(12 Pt 2):S140-S146.
DOI: 10.1111/j.1753-4887.2007.tb00352.x.
25. Kim EY, Sudini K, Singh AK, Haque M, Leaman D, Khuder S, *et al*. Ursolic acid facilitates apoptosis in rheumatoid arthritis synovial fibroblasts by inducing SP1-mediated Noxa expression and proteasomal degradation of Mcl-1. FASEB Journal. 2018;32(11):6174-6185.
DOI: 10.1096/fj.201800425R.
26. Chen C. Lipids: COX-2's new role in inflammation. Nat Chem Biol. 2010;6(6):401-402.
DOI: 10.1038/nchembio.375.
27. Joa H, Vogl S, Atanasov AG, Zehl M, Nakel T, Fakhrudin N, *et al*. Identification of ostruthin from Peucedanum ostruthium rhizomes as an inhibitor of vascular smooth muscle cell proliferation. J Nat Prod. 2011;74(6):1513-1516.
DOI: 10.1021/np200072a.
28. Ahmad J, Wizarat K, Shamsuddin KM, Zaman A, Connolly JD. Jangomolide, a novel limonoid from *Flacourtia jangomas*. Phytochemistry. 1984;23(6):1269-1270.
DOI: 10.1016/S0031-9422(00)80439-2.
29. Schinkovitz A, Gibbons S, Stavri M, Cocksedge MJ, Bucar F. Ostruthin: an antimycobacterial coumarin from the roots of *Peucedanum ostruthium*. Planta Med. 2003;69(4):369-371.
DOI: 10.1055/s-2003-38876.
30. Guil-Guerrero JL. Nutritional composition of *Plantago species* (*P. major* L., *P. lanceolata* L., and *P. media* L.). Ecol Food Nutr. 2001;40(5):481-495.
DOI: 10.1080/03670244.2001.9991663.
31. Souza CO, Teixeira AAS, Lima EA, Batatinha HAP, Gomes LM, Carvalho-Silva M; *et al*. Palmitoleic acid (N-7) attenuates the immunometabolic disturbances caused by a high-fat diet independently of ppar α. Mediators Inflamm. 2014;2014:582197.
DOI: 10.1155/2014/582197.
32. Genolet R, Wahli W, Michalik L. PPARs as drug targets to modulate inflammatory responses? Curr Drug Target Inflamm Allergy. 2004;3(4):361-375.
DOI: 10.2174/1568010042634578.
33. Magdalon J, Vinolo MAR, Rodrigues HG, Paschoal VA, Torres RP, Mancini-Filho J, *et al*. Oral administration of oleic or linoleic acids modulates the production of inflammatory mediators by rat macrophages. Lipids. 2012;47(8):803-812.
DOI: 10.1007/s11745-012-3687-9.
34. Medeiros-de-Moraes IM, Gonçalves-de-Albuquerque CF, Kurz ARM, de Jesus Oliveira FM, de Abreu VHP, Torres RC, *et al*. Omega-9 oleic acid, the main compound of olive oil, mitigates inflammation during experimental sepsis. Oxid Med Cell Longev. 2018;2018:6053492.
DOI: 10.1155/2018/6053492.
35. Hidalgo MA, Carretta MD, Burgos RA. Long chain fatty acids as modulators of immune cells function: contribution of FFA1 and FFA4 receptors. Front Physiol. 2021;12:668330,1-18.
DOI: 10.3389/fphys.2021.668330.
36. Kim H, Youn K, Yun EY, Hwang JS, Jeong WS, Ho CT, *et al*. Oleic acid ameliorates Aβ-induced inflammation by downregulation of COX-2 and iNOS via NFκB signaling pathway. J Funct Foods. 2015;14:1-11.
DOI: 10.1016/j.jff.2015.01.027.
37. Dewick PM. Medicinal Natural Products : A Biosynthetic Approach. 3rd ed. Chichester: John WIley & Sons, Ltd; 2009. pp. 53 .
DOI: 10.1017/CBO9781107415324.004.
38. Oh YT, Lee JY, Lee J, Lee JH, Kim JE, Ha J, *et al*. Oleamide suppresses lipopolysaccharide-induced expression of iNOS and COX-2 through inhibition of NF-κB activation in BV2 murine microglial cells. Neurosci Lett. 2010;474(3):148-153.
DOI: 10.1016/j.neulet.2010.03.026.
39. Roa-Coria JE, Navarrete-Vázquez G, Fowler CJ, Flores-Murrieta FJ, Déciga-Campos M, Granados-Soto V. N-(4-Methoxy-2-nitrophenyl) hexadecanamide, a palmitoylethanolamide analogue, reduces formalin-induced nociception. Life Sci. 2012;91(25-26):1288-1294.
DOI: 10.1016/j.lfs.2012.09.024.