Naunyn-Schmiedeberg's Archives of Pharmacology (2020) 393:1281–1291
https://doi.org/10.1007/s00210-020-01851-z

ORIGINAL ARTICLE

Anti-arthritic and anti- inflammatory effects of extract and fractions of *Malva parviflora* in a mono- arthritis model induced with kaolin/carrageenan

Gabriela Belen Martínez-Hernández[1,2,3] · Gabriela Vargas-Villa[1] · Enrique Jiménez-Ferrer[1] · Maribel Patricia García-Aguilar[1,2,3] · Alejandro Zamilpa[1] · Rubén Román-Ramos[3] · Manasés González-Cortazar[1] · Margarita Avilés-Flores[4] · Macrina Fuentes-Mata[4] · Maribel Herrera-Ruiz[1]

Received: 20 November 2019 / Accepted: 6 March 2020 / Published online: 28 April 2020

Abstract

Malva parviflora is used as food in the gastronomy of some regions of Mexico and, also, in Mexican traditional medicine for inflammation-related conditions like rheumatoid arthritis. The objective of this work was to evaluate its antiarthritic activity in a mice model. In ICR, female mice were tested the dichloromethane extract (MpD) and fractions MpF4 (extracted with a dichoromethane:methanol system) and MpFphy (a precipitate by acetone:methanol) by using the mono-arthritis with kaolin/carrageenan model. During the treatment, joint inflammation was measured daily, and hyperalgesia was measured using the hot plate test. The treatments diminished both joint inflammation and pain. At the end of the evaluation, the left joint and spleen were extracted for determination of pro- and anti-inflammatory cytokines. The results showed that the MpD, MpF4, and MpFphy treatments modulated the concentration of these proteins. Specifically, MpFphy at 1.0 mg/kg increased IL-4 and IL-10 and decreased IL-17, IL-1β, and TNF-α. GC-MS analysis showed that MpF4 contained a mixture of a total of nine compounds, three of them newly reported for the species. The studies confirmed the presence of five sterols in the MpFphy fraction, including stigmasterol and β-sitosterol. These results confirm the anti-rheumatoid and anti-inflammatory activities of a fraction rich in sterols from *Malva parviflora.*

Keywords *Malva parviflora* · Inflammation · Arthritis · Kaolin/carrageenan · Sterols

Gabriela Belen Martínez-Hernández and Gabriela Vargas-Villa contributed equally to this work.

✉ Maribel Herrera-Ruiz
cibis_herj@yahoo.com.mx

[1] Centro de Investigación Biomédica del Sur, Instituto Mexicano del Seguro Social (IMSS), Argentina 1, 62790 Xochitepec, Morelos, Mexico

[2] Doctorado en Ciencias Biológicas y de la Salud, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana (UAM), México City, Mexico

[3] Departamento de Ciencias de la Salud, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco No.186, Col. Vicentina, Iztapalapa,, C.P.09340 México D.F, Mexico

[4] INAH-Morelos, Matamoros 14 Acapantzingo, CP 62440 Cuernavaca, Morelos, Mexico

Abbreviations

RA	Rheumatoid arthritis
TNF-α	Tumoral necrosis factor
TGF-β	Transforming growth factor
MTX	Methotrexate
PMFS	Phenylmethylsulphonyl fluoride
IL	Interleukin
TPA	12-O-Tetradecanoylphorbol-13-acetate
LK	Left knee
MpD	Dichloromethane extract from *Malva parviflora*
MpF4	Fraction F4 from *Malva parviflora*
MpFphy	Sterols fraction from *Malva parviflora*

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is associated with progressive disability due to the pain it causes (Zonzini et al. 2018). It is characterized by hyperplasia

and chronic inflammation that affect the joints, connective and fibrous tissues, muscles, and tendons, which increases mortality and morbidity and decreases the quality of daily life (Alkabeya et al. 2019). According to the World Health Organization (WHO 2019), the prevalence of RA varies between 0.3 and 3% and is two to four times more common among women than men (Frisell et al. 2016). The pathogenesis of RA includes the activation of T and B cells as well as macrophages, which invade the synovial membrane, causing joint damage (mainly to cartilage and bone) (Salinas-Sánchez et al. 2017; Chu et al. 2018). Collaborating T cells play an important role in RA, mainly type 17 cells which are involved in bone erosion and osteoclastogenesis. These cells produce cytokines such as IL-17 and tumor necrosis factor alpha (TNF-α), which together promote the activation of chondrocytes and fibroblasts (McInnes and Schett 2011; Burmester et al. 2014), block the activity of regulatory T cells, and promote the inflammatory state and hyperalgesia that are characteristics of the disease (McNamee et al. 2011; Hoxha 2018). Other cytokines, such as IL-6 and IL-1β, together with TNF-α, produce an increase in the enzyme prostaglandin E synthase, which is expressed in the cartilage and in the synovium, leading to a chronic inflammatory state (Huhtakangas et al. 2017; McGonagle et al. 2018). Anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) contribute to counteracting the damage (Hoxha 2018).

Malva parviflora is a species native to Africa and Europe, with a secondary distribution in Mexico; it is commonly known as mallow, and it is used as an alimentary product in several regions of the country (Castro-Lara et al. 2011). In Mexican traditional medicine, it is mainly used as an anti-inflammatory (Afolayan et al. 2010; Rzedowski 2001).

A few pharmacological studies exist for this species, some of which demonstrate anti-inflammatory and antioxidant activity of extracts, as well as in fractions containing flavonoids, sterols, and fatty acids (Argueta et al. 1994; Bouriche et al. 2011). Additionally, it has demonstrated antihypertensive, antioxidant, and anti-inflammatory effects attributed to oleanolic acid, tiliroside, and scopoletin (Lagunas-Herrera et al. 2019). Based on the above, it was proposed to evaluate the extract and fractions of *Malva parviflora* in a sub-chronic model of monoarthritis-induced with K/C and identify the chemical constitution of active fractions. It has been demonstrated that carrageenan is an inductor of local inflammation, provoking swelling or edema, and pain, accompanied with elevated plasma levels of IL-1β, IL-6, and TNF-α, between other inflammation mediators (Gihan et al. 2016; Nouran et al. 2017). Carrageenan, together with kaolin, is used to produced monoarthritis in rodents, and it is a widely used model, that induces articular inflammation, synovitis, synovial fluid exudate, cartilage's damage, hyperemia, and immune cell infiltration (Milind et al. 2016).

Materials and methods

Plant material and obtaining the dichloromethane extract (MpD) and its chromatographic fractionation

Leaves and flowers of *Malva parviflora* were collected from Ozumba de Alzate, Mexico State. A specimen was sent to the Botanic Garden (INAH) in Cuernavaca and identified, with voucher reference number 2088.

From the method used by Ramírez-Serrano et al. 2019, the leaves and flowers were dried at room temperature for 2 weeks. Dry material was milled in a grinder (Pulvex) obtaining particles of approximately 5 mm in diameter. Milled material was macerated with dichloromethane (10 L) for 24 h at room temperature; this process was carried out in duplicate. The solvent was removed with a high vacuum rotary evaporator (Laborota 4000, Heidolph, Germany) to obtain 184.5 g of dichloromethane extract (MpD), which was submitted (200 g) to open column chromatography (3.0 × 60 cm), packed with silica gel (260 g, 70-230 mesh, Merck). The elution system was *n*-hexane and ethyl acetate, resulting in 19 fractions that were grouped according to the similarity of the compounds into seven fractions (MpF1-MpF7). Based on previous reports (Ramírez-Serrano et al. 2019), MpF3 and MpF4 were chosen for biological evaluation in a mono-arthritis model.

Chemical identification of MpF3 and MpF4

In the MpF3 fraction, a precipitate was obtained by adding a mixture of acetone and methanol (7:3). This precipitate (MpFphy) consisted of five compounds, principally sterols. The MpF4 fraction contained nine compounds. Identification of these two fractions was done using GC-MS analysis.

GC-MS analysis of MpFphy and MpF4 fractions

The chemical compositions of the MpFphy and MpF4 fractions were analyzed on a gas chromatograph (GC) equipped with a quadruple mass detector in electron impact mode at 70 eV. Volatile compounds were separated on a HP 5MS capillary column (25 m long, 0.2 mm i.d., with 0.3-μm film thickness). Oven temperature was set at 40 °C for 2 min, then programmed from 40 to 260 °C at 10 °C/min and maintained at 260 °C for 20 min. Mass detector conditions were as follows: interphase temperature 200 °C and mass acquisition range, 20–550. Injector and detector temperatures were set at 250 and 280 °C, respectively. Spitless injection mode was carried out with 1 μL of each fraction (3 mg/mL solution). The carrier gas was helium at a flow rate of 1 mL/min. Volatiles were identified by comparing their mass spectra with those of the National Institute of Standards and Technology (NIST) 1.7 Library and data from the literature.

Main reagents and kits

The aluminum silicate (kaolin) was obtained from Merk (USA). The carrageenan and methotrexate (98%) were purchased from Sigma Chemical Co (USA). The origin of the IL-1β, TNF-α, IL-6, IL-4, and IL-10 cytokine kits was BD Biosciences, Inc. The IL-17 cytokine was obtained from BioLegend (San Diego, CA, USA).

Animals

Female ICR mice weighing 30–35 g were purchased from Production and Experimentation Unit of Laboratory Animals (UPEAL) at the Autonomous Metropolitan University at Xochimilco (UAM, Xochimilco), Mexico. The animals remained in their cages under standard laboratory conditions (12 h light/dark cycle, constant temperature of 22 ± 3 °C, humidity of 70 ± 5%, food, and water ad libitum). The experiments were performed according to official Mexican Norm 062-ZOO-1999 (Technical Specifications for the Production, Care and Use of Laboratory Animals) and the international ethical guidelines for the care and use of laboratory animals. The experimental protocol was authorized by the local Health Research Committee [Mexican Institute of Social Security IMSS], with approval number R-2014-1701-26.

Arthritis model induced by kaolin/carrageenan (K/C)

The experiment lasted 10 days. There were 7 groups of 12 mice each; 6 groups received a kaolin/carrageenan (K/C) challenge by the intra joint pathway on the first day of experiment. The groups were as follows:

Negative control (mice without any treatment, VEH group); mice that only received sterile saline solution (SS, animals without-induced arthritis, both intra-joint and as a diary treatment); mice treated with a disease-modifying drug, methotrexate, MTX at 1 mg/kg; mice with MpD at 50 mg/kg; mice with MpF4 at 10 mg/kg; mice treated with MpFphy 1 mg/kg; and finally mice with MpFphy to 2 mg/kg; doses were selected based in several works in the laboratory, and according to the chemical complexity of the treatment (Salinas-Sánchez et al. 2017). Each mouse received its treatment by oral pathway (o.p.) beginning on the second experimental day and until day 10.

For the induction of mono-arthritis, the base of the left knee joint was measured prior to intervention as a reference in all groups using a Mitutoyo digital micrometer (Micrometric calibration MDC-1"SB, Mitutoyo Products). Then, the animals were anesthetized with sodium pentobarbital intraperitoneally (i.p.) at a dose of 55 mg/kg, and groups 1–6 were administered with kaolin solution (40%, 40 μl) in the left knee joint cavity; and consecutively, flexions and extensions were performed during 15 min. Then, the carrageenan solution (2%, 40 μl) was injected into the joint cavity and once again the push-ups and extensions were made for 5 min. Animals from group 7 were handled similarly, but were injected with sterile saline solution rather than kaolin and carrageenan solutions. The articular edema was monitored once daily. The treatments were administered 24 h after the kaolin/carrageenan administration. The mice were sacrificed on day 10, to dissect the left knee and spleen, which were stored at − 70 °C for later homogenization.

Spleen index

The spleen was weighed, and the "spleen index" was calculated with respect to the total weight of the animal and represented as a percentage.

Thermal hyperalgesia evaluation

Thermal hyperalgesia was evaluated using a hot plate. During the test, the animals were placed one by one onto a surface at 50 °C ± 2 °C. The latency to leave the surface was recorded on days 2, 6, and 9 using a manual chronometer with a maximum of 20 s per session (Sluka et al. 1998; Langford and Mogil 2008).

Homogenization of spleen and joint tissues

The joint tissue was put into a mortar with dry ice and ground until the dry ice was gone. The joint and spleen tissues were put on a vial with 2 mL of phosphate buffer solution (PBS, pH = 7.4) and phenyl-methyl-sulfonyl fluoride (PMFS) at 0.01% dissolved in isopropyl alcohol. For the complete homogenization of the organs, a homogenizer (Dragon Lab D-500 Pack 1, 10–500 mL) was used for 10 to 15 s. Later, the samples were centrifuged at 12000 RPM for 5 min. Aliquots of 300 μl were stored at − 70 °C for later quantification of different cytokines using the ELISA method ($n = 4$, represent a mixture of tissue for 3 animals).

Measurement of anti-inflammatory and pro-inflammatory cytokines in supernatant

Each cytokine measurement was carried out by the ELISA method using a kit (OptEIATM ELISA sets; BD Biosciences, Franklin Lakes, NJ, USA) and following the manufacturer's instructions. Briefly, in 96-well plates, we added 100 μL/well of the antibody uptake; the plates were incubated for 12 h at 4 °C. Once this time had elapsed, the plate was washed with PBS buffer (0.05% of Tween-20, 300 μL/well × 3 times). We added 100 μL of PBS with fetal bovine serum (FBS) at 10%, pH 7.0, during 1 h at room

temperature. The contents were discarded, and the plate was washed with PBS buffer (0.05% of Tween-20, 300 μL/well × 3 times). To the corresponding wells, we added 100 μL of the standard, the target (PBS with FBS), and the test samples. The plate was incubated for 2 h at room temperature. The contents were discarded, and the plate was washed with PBS buffer (0.05% of Tween-20, 300 μL/well × 5 times). For TNF-α, IL-6, IL-4, and IL-10, we added 100 μL/well of detection antibody and streptavidin-horseradish peroxidase (HRP) enzyme. These plates were incubated for 1 h, and washed with 300 μL/well × 7 times, with a PBS solution (added with 0.05% of Tween-20).

For IL-1β, we added 100 μL/well of antibody detection; this was incubated for 1 h and washed with 300 μl/well × 5 times, with a solution of PBS (added with 0.05% of Tween-20) followed by the streptavidin-HRP enzyme (100 μL/well). These plates were incubated for 1 h, and washed with 300 μL/well × 7 times, with a PBS solution (added with 0.05% of Tween-20).

To each well, we added 100 μL of previously prepared o-phenylenediamine (OPD) substrate (one tablet of OPD and one of urea dissolved in 20 mL of distilled water). This was incubated for 30 min at room temperature in total darkness. We then added a stop solution (2N H2SO4). The plate was read using a Stat Fax 2100 spectrophotometer (Awareness Technologies, Bellport, NY, USA) at a 450-nm wavelength at 37 °C.

For IL-17, we added 50 μL/well of RD1–38 and 50 μL/well of a standard into the corresponding wells. The plate was gently shaken for 1 min and was incubated at room temperature for 2 h. The contents were then discarded, and the plate was washed with PBS buffer 5 times. We added 100 μL of polyclonal antibody peroxidase to each well, incubated this again for 2 h at room temperature, then washed five times.

Then, we added 100 μL of substrate solution and incubated this for 30 additional min in total darkness. Finally, we added stop solution (100 μL, 2N H_2SO_4) to each well and shook gently. The plate was read at a 450-nm wavelength at 37 °C.

Statistical analysis

The data are expressed as mean ± SD. Statistical analysis was conducted in SPSS 22.0 software (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc., Chicago, IL, USA). Data on the spleen index, thermal hyperalgesia, and temporal course of inflammation were analyzed by significant differences by one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test; and Student's *t* test was used to analyze the results of cytokines ($n = 4$). Statistical difference was considered when $^*p \leq 0.05$ in comparison with the control group (VEH).

Results

Profile of MpF4 by GS-MS

The chemical content of the MpF4 fraction obtained by GC-MS is shown in Table 1. It identified nine compounds, listed in the order of elution.

Profile of MpFphy by GS-MS

The GC-MS analysis of MpFphy identified 5 compounds, all of them of sterol type. Table 2 lists these compounds in order of elution: (M) campesterol (12.3%), (M) stigmasta-5, 22-den-3-ol (33.3%), (R) β-sitosterol (47.6%), (M) stigmastan-3-ol, (3β,5α) (4.0%), and (M) sitosterol acetate (2.6%).

Anti-inflammatory effect of extract, fraction, and phytosterol mixture from *Malva parviflora*

A previous screening for anti-inflammatory properties of *M. parviflora* extracts by our research group was using ear edema induced with the phorbol ester TPA (Ramírez-Serrano et al. 2019). Based on these results, we decided to evaluate the extract (MpD), fractions (MpF4), and a sterols mixture (MpPhy) in a model of arthritis induced with K/C.

Figure 1 shows the time course of the joint inflammation in each group. The first measurement, on the second day after the administration of K/C, has shown that the SS group was different to VEH group ($^*p < 0.05$, Fig. 1), and all treatments were similar to this last ($p > 0.05$, Fig. 1).

While the joint measurement of SS group remained constant throughout the experiment, as expected, in the VEH group, K/C administration provoked an inflammation along all experiment.

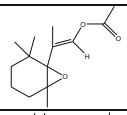
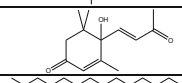
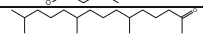
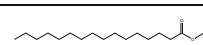
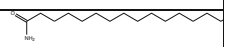
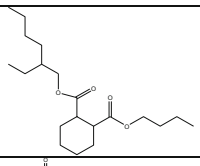
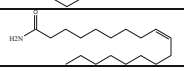
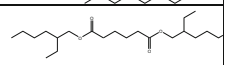
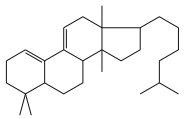
The MTX group, and the treatments from *M. parviflora* (MpF4 and MpFphy at 1 and 2 mg/kg), showed a recuperation of the inflammation induced by K/C; the activity was variable in each group; however, on the day 3 of measure, all of them were statistically different to the VEH group, and in the last experimental day, MpFphy at 1 mg/kg induced the best activity ($^*p < 0.05$, Fig. 1).

In the spleen index, all *M. parviflora* treatments decreased the percentage significantly compared to the VEH group (Fig. 2).

Effect of *M. parviflora* on thermal hyperalgesia

Figure 3 shows the effect of treatments on the time that each group of animals spent on the hot plate on the 3 days they were evaluated. On day 2, all groups presented similar levels of hyperalgesia (no statistical difference between treatments and the VEH group, $p > 0.05$). On day 6, only the SS group, spending an augmented time on the hot plate, different from the VEH group ($^*p < 0.05$). By day 10, the *M. parviflora* treatments MpD, MpF4, and MpFphy (1.0 mg/kg) provoked

Table 1 Chemical composition of MpF4 obtained from *Malva parviflora*

#	Retention time (min)	Molecular weight (a.m.u.)	Compound	Amount (%)	Structure
1	17.298	238	Acetic acid,2-(2,2,6-trimethyl-7-oxa-bicyclo [4.1.0] heptyl)-propenyl ester	7.027	
2	17.495	222	2-Cyclohexen-1-one,4hydroxy-3.5.5-trimethyl-4(3-oxo-1-butenyl)-	1.929	
3	17.895	268	2-Pentadecanone,6,10,14 trimethyl-	20.605	
4	18.697	270	Hexadecanoic acid, methyl ester	7.039	
5	21.154	255	Hexadecanamide	3.930	
6	21.870	334	1,2-benzenedicarboxylic acid, butyl 2-ethylhexyl ester	3.930	
7	22.901	281	9-Octadecenamide, (z)-	14.947	
8	23.085	370	Hexanedioic acid, bis (2-ethylhexyl) ester	31.467	
9	36.353	410	3-(1,5-Dimethyl-hexyl)-3a,10,10,12b-tetramethyl-1,2,3, 3a,4,6,8,9,10,10a,11,12,12a,12b-tetradecahydro-benzo[4,5]cyclohepta[1,2-E]inde Lanostadiene Cycloheptaindanos	9.123	

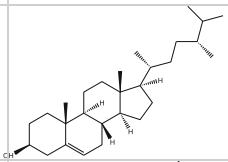
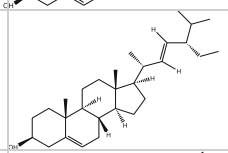
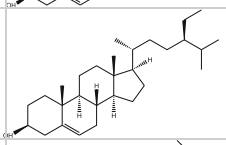
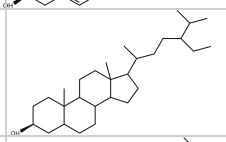
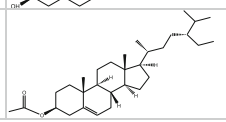
an increase in the time spent in the hot plate, all of which are statically different from VEH (*$p < 0.05$).

Effect of *M. parviflora* on pro- and anti-inflammatory cytokines

The intra-articular application of K/C provoked a significant increase in the concentration of TNF-α, IL-1β, IL-6, and IL-7 in spleen and LK of animals without treatment, compared to mice from the SS group (Table 3). MTX and all of the *M. parviflora* derivates induced a decrease of TNF-α levels in both organs compared to the VEH group (*$p < 0.05$, Table 3). While the levels of IL-1β, in all groups, was decreased in LK in comparison with VEH; but for the spleen, only MpF4 and MpFphy at 1 and 2 mg/kg decreased this parameter, with MpFphy having a stronger effect in both organs. Neither MTX nor MpD showed differences in comparison with VEH. For IL-6, MTX decreased the concentration in both organs; and the different treatments with *M. parviflora*, extracts and fraction, and MpFphy at both doses, also significantly reduced this protein in both organs. The concentration of IL-17, in animals with MTX, MpD, or MpFphy (1, 2 mg/kg) had significant differences with VEH in LK and spleen. But MpF4 only decreased the concentration of this cytokine in LK (*$p < 0.05$, Table 3); in the spleen, the levels were similar to the VEH group ($p > 0.05$, Table 3).

In the case of IL-10, being an anti-inflammatory cytokine, the levels in VEH were diminished in the spleen and LK, compared to SS (*$p < 0.05$). MTX and treatments from *M. parviflora*, MpD, and MpFphy induced an increment of IL-10 on spleen and LK, which were statistically different to VEH. The administration of MpF4, provoked a decrement of this cytokine on the spleen, while in LK showed a significative increment. The negative control group, VEH, had low concentrations of IL-4, in both organs in relation with SS (*$p < 0.05$). MTX had no effect on the concentration compared to VEH. The treatment MpD has highly significant levels in the spleen in comparison with VEH, but there was no change in LK ($p > 0.05$).

Table 2 Chemical composition of MpFphy obtained from *Malva parviflora*

Retention time (min.)	Molecular weight (a.m.u.)	Compound	% in the sample	Structure
34.875	400	(M) Campesterol	12.304	
35.637	412	(M) Stigmasta-5,22-den-3-ol	33.349	
36.912	420	(R) τ-Sitosterol	47.650	
36.997	416	(M) Stigmastan-3-ol, (3β,5α)-	4.030	
39.546	456	(M) Sitosterol acetate	2.667	

MpF4 demonstrated a smaller but still significant increase in both organs. Finally, the two doses of MpFphy were different with VEH, with an increase in the levels of IL-4 (*$p < 0.05$, Table 4).

Discussion

Malva parviflora is a widely distributed species in Mexico that is used for several inflammation-related ailments

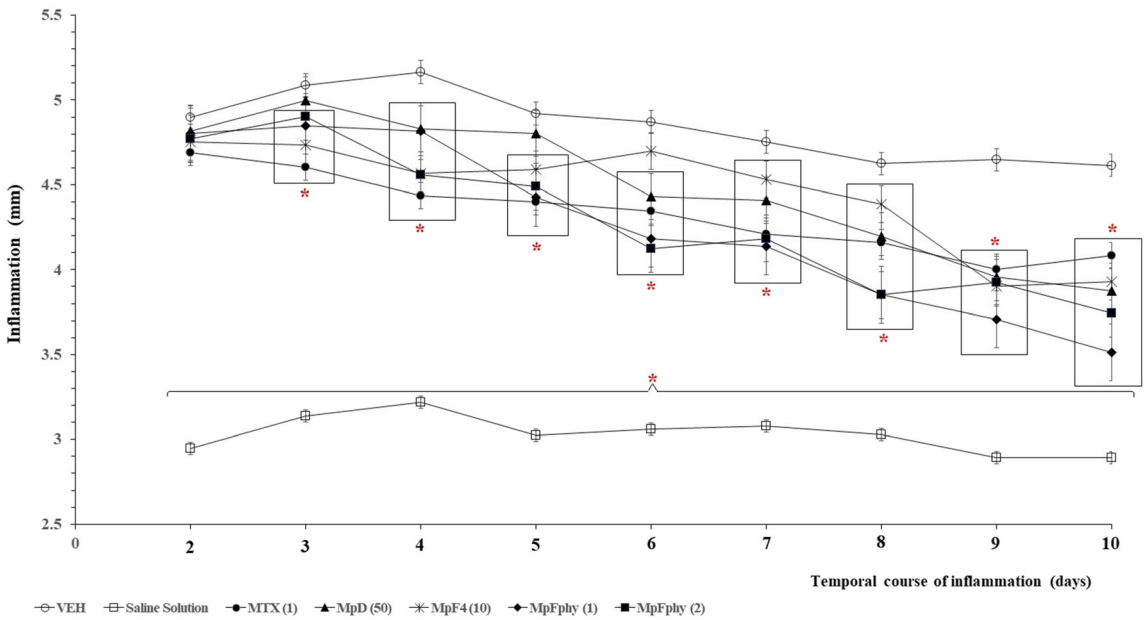


Fig. 1 Effect of different treatments of *Malva parviflora* on the temporal course of joint inflammation of mice with mono-arthritis induced with K/C. Saline solution, MTX (methotrexate), MpD (dichloromethane extract), MpF4 (Fraction 4), MpFphy (phytosterol mixture). Points show the mean, and standard deviation, for each group ($n = 12$ mice per group). The statistical difference between VEH (vehicle group, Tween 20, 1%) and treatments is shown for those points into the box which have a *$p \leq 0.05$, by using ANOVA with post hoc Dunnet in each day of measuring

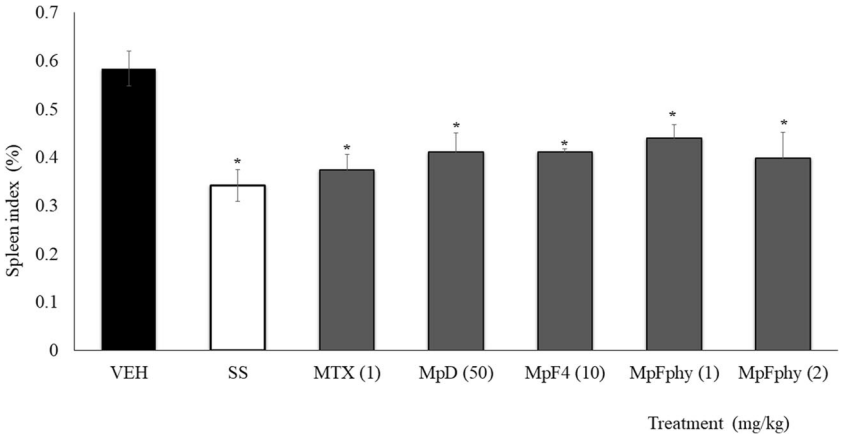


Fig. 2 Effect of different treatments of *Malva parviflora* on spleen index of mice with mono-arthritis induced with K/C. VEH (Vehicle Tween 20%), saline solution, MTX (methotrexate), MpD (dichloromethane extract), MpF4 (Fraction 4), MpFphy (phytosterols mixture). Data represent the mean ± SD of each group ($n = 12$), ANOVA with post hoc Dunnet against VEH $^*p \leq 0.05$

(Rzedowski 2001). Recently, anti-inflammatory activity of this plant has been shown using several different assays. These include reports that the hydroalcoholic extract had activity in 5XFAD mice, a model of Alzheimer's disease, that a fraction and daucosterol isolated from this plant had activity in TPA-induced local ear edema induced in mice, and that it decreased neuroinflammation and the activation of macrophages induced by LPS (Ramírez-Serrano et al. 2019). Also, there is a study about the anti-inflammatory activity of extracts of different polarity obtained from leaves or root from this species, which were collected in different places. In this assay carried out through the measure of the inhibition of cyclooxygenase (COX) enzyme type 1 and 2, the authors of that work showed that there was a variability in that activity with respect to not only of the type of extract, but also of the area from which the specimens came from. Their explanation is based on the difference in the variation in the chemical content, and although they do not indicate the compounds in each treatment, they did measure the activity when different fractions are combined, finding with this strategy a synergistic effect (Shale et al. 2005).

Our study further explores the anti-inflammatory activity of this plant using a model of mono-arthritis induced by kaolin/carrageenan for evaluating the extract and fractions of this medicinal species; the treatments were selected based on recent assays by our research group, in which the dichloromethane extracts decreased inflammation.

The kaolin/carrageenan model is used to emulate several characteristics of rheumatoid arthritis, including joint inflammation and increase in pro-inflammatory cytokines. It has been shown that the injection of these two chemical agents provokes histopathological features similar to the inflammation that develops in humans, which includes hyperplasia and exudation of synovial liquid, mononuclear cells like macrophages, lymphocytes, and monocytes which in turn provoke damage and destruction, mainly of bone and cartilage (Kannan et al. 2005; Suh et al. 2016; Eman et al. 2018). The daily administration of different treatments from *M. parviflora* induced a gradual recuperation of the joint. Over the temporal course, all treatments were statistically better than the VEH group. These results are consistent with the previous reports, indicating that *M. parviflora* has relevant anti-inflammatory activity.

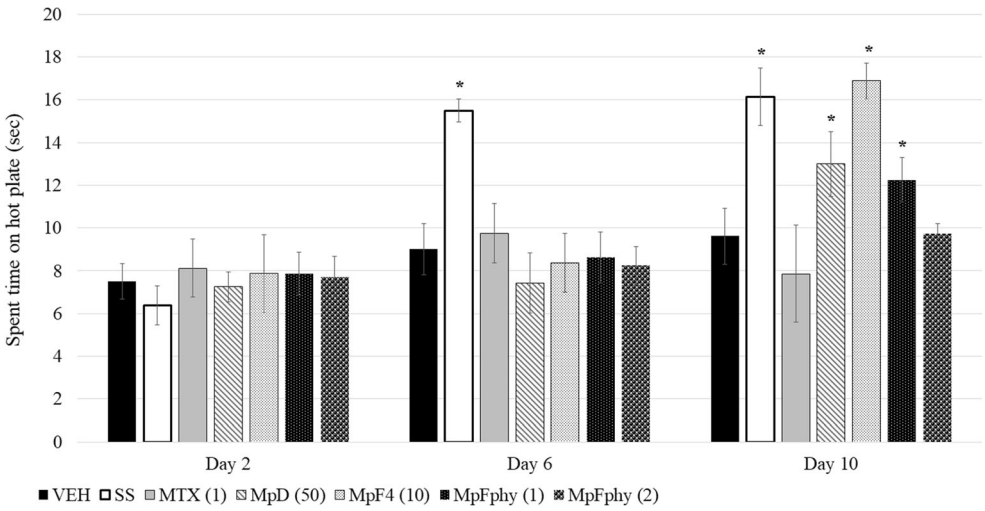


Fig. 3 Effect of different treatments of *Malva parviflora* on thermal hyperalgesia of the inflamed joint of mice with mono-arthritis induced with K/C. VEH (Vehicle Tween 20%), SS (saline solution), MTX (methotrexate), MpD (dichloromethane extract), MpF4 (Fraction 4), MpFphy (phytosterols mixture). Data represent the mean ± SD of each group ($n = 12$), ANOVA post hoc Dunnet $^*p \leq 0.05$ in comparison with VEH group on each evaluation day

Table 3 Concentration of pro-inflammatory cytokines in mice with mono-arthritis induced by K/C

	TNF-α		IL-1β		IL-6		IL-17	
	pg/mg of protein							
	Spleen	LK	Spleen	LK	Spleen	LK	Spleen	LK
SS	1972.39 ± 92.9*	3651.72 ± 507.0*	2581.92 ± 821.3*	768.53 ± 301.7*	63.99 ± 2.1*	94.59 ± 10.6*	7605.6 ± 176.3	8869.9 ± 1245.0
VEH	*3691.56 ± 330.2*	*12,293.01 ± 1129.3*	*6038.06 ± 711.5*	*138,535.85 ± 7660.3*	*126.46 ± 2.1*	*481.07 ± 50.4*	*9970.4 ± 334.4*	*25,407.7 ± 532.5*
MTX (1)	314.57 ± 11.1*	1382.65 ± 288.8*	6208.7 ± 1377.8*	16,263.28 ± 2970.4*	23.99 ± 4.5*	97.45 ± 4.9*	6754.9 ± 1564.9*	9100.5 ± 764.2*
MpD (50)	990.32 ± 121.0*	1814.35 ± 566.1*	6209.60 ± 773.5*	9957.31 ± 1719.7*	20.10 ± 4.1*	58.43 ± 17.9*	7529.8 ± 1646.6*	17,084.5 ± 1888.4*
MpF4 (10)	161.94 ± 20.0*	684.86 ± 213.02*	4667.45 ± 803.5*	31,859.60 ± 4087.8*	11.60 ± 0.7*	25.55 ± 8.7*	12,161.9 ± 76.6*	11,721.7 ± 298.5*
MpFphy(1)	1813.96 ± 354.8*	5692.99 ± 673.02*	1158.86 ± 100.3*	3492.99 ± 632.2*	28.50 ± 0.5*	67.41 ± 1.06*	4868.6 ± 425.3*	3357.6 ± 363.3*
MpFphy(2)	795.15 ± 199.8*	7746.30 ± 643.7*	707.73 ± 66.8*	3280.86 ± 267.5*	28.21 ± 0.06*	127.06 ± 2.1*	4406.7 ± 444.5*	6188.05 ± 614.3*

Data represent the mean ± SD of each group ($n = 12$), ANOVA post hoc Dunnet *$p \leq 0.05$ in comparison with VEH

The exudation of synovial liquid and the participation of monocytes and macrophages contribute to the maintenance of rheumatoid arthritis (RA). The antigen-presenting T cells interact with the synovitis (type A) and allow the activation of these cells as well as the production of cytokines leading to an extravasation of lymphocytes (Fox et al. 2010). The increase in the liberation of pro-inflammatory cytokines like IL-1β, IL-6, IL-8, and the tumoral necrosis factor (TNF-α) affects articulation (Sakaki et al. 2004; Sur et al. 2019). TNF-α generates mediators like metalloproteinases, cytokines, nitric oxide, and E2 prostaglandins, while IL-1β mediates mainly bone and cartilage destruction. These two cytokines at the same time power the IL-6 activity; they work synergistically and are the main components of the inflammatory process (Sánchez-Ramón et al. 2011).

On the other hand, the Th17 response also plays an important role in the production of proinflammatory cytokines, mainly in RA IL-17, which generates an increase in cytokines and inflammatory mediators (Maddur et al. 2012). The increase of these proinflammatory mediators is due to the increase of proliferative synovium, which also generates joint damage, producing pain (Sur et al. 2019).

The extract (MpD) and the MpF4 and MpFphy fractions evaluated in the mono-arthritis model induced with kaolin/carrageenan showed a decrease in inflammation in the joint. The administration of treatments derived from *M. parviflora* under these conditions significantly decreased the concentrations of proinflammatory cytokines (IL-6, IL-β, and TNF-α) in joints and spleen. The MpF4 fraction decreased cytokine IL-17 only in the joint itself, not in the spleen. Clinical studies have shown that biological therapies anti-interleukin IL-6 and anti-TNF-α are effective for the treatment of RA, but studies are still needed to prove their safety and long-term effectiveness (Davies and Hyrich 2018; Littlejohn and Monrad 2018).

Anti-inflammatory cytokines such as IL-4 and IL-10 through the TH2 response intervene in the differentiation and activation of B lymphocytes (Smith and Haynes 2002). Both cytokines are proteins capable of regulating the synthesis of pro-inflammatory proteins such as IL-1β and TNF-α. In RA, B cells are responsible for the production of IL-10, which participates in the regulation of inflammation and the negative response (Feldmann and Maini 2008; Pan et al. 2010). In the results obtained in this work, we observed that IL-10 levels in the spleen and joint increased compared to the VEH group and LK in all treatments except MpF4, which only increased in the joint. For IL-4, all our treatments presented a significant increase in both organs, with the exception of MpD in the joint.

Both systemic (measured through cytokines in the spleen) and local inflammation (in the joint) were counteracted with *M. parviflora*. The spleen is a secondary immune organ that responds with hyperfunction when the organism is repeatedly exposed to harmful stimuli, which leads the organ to increase in size (Li et al. 2019).

Table 4 Concentration of anti-inflammatory cytokines in mice with mono-arthritis induced by K/C

	IL-10		IL-4	
	pg/mg of protein			
	Spleen	LK	Spleen	LK
SS	182.55 ± 4.7*	779.13 ± 8.5*	221.46 ± 7.7*	363.24 ± 57.1*
VEH	*85.08 ± 4.2*	*345.19 ± 27.8*	*35.11 ± 3.7*	*144.63 ± 7.0*
MTX (1)	152.87 ± 12.2*	742.10 ± 125.1*	43.51 ± 3.3	168.99 ± 27.1
MpD (50)	204.98 ± 1.9*	617.32 ± 41.9*	117.91 ± 4.4*	144.77 ± 18.1
MpF4 (10)	67.21 ± 1.6*	2281.16 ± 30.5*	50.80 ± 0.6*	198.99 ± 5.6*
MpFphy (1)	200.28 ± 90.2*	722.14 ± 15.2*	103.82 ± 2.2*	217.99 ± 7.9*
MpFphy (2)	272.26 ± 42.6*	2175.06 ± 137.6*	92.34 ± 3.2*	471.67 ± 25.1*

Data represent the mean ± SD of each group ($n = 12$), ANOVA post hoc Dunnet $*p \leq 0.05$ in comparison with VEH

The persistent inflammation in RA is generated not only by cytokines, but also by the recruitment of small molecules such as ATP and prostaglandins that act on the nociceptors in the area of the lesion, producing pain, allodynia and hyperalgesia (Smith and Haynes 2002; Rasheed et al. 2018; Eman et al. 2018). Here, we found that the extract (MpD) and the fractions (MpF4 and MpPhy) at 1 mg/kg increased the time animals remained on the hot plate, evidence of decreased hyperalgesia. The SS group, in the first hot plate measurement (second day) showed a behavior similar to that of the other animals, but in the exposure to the hot plate on days 6 and 10, an increase in time was observed. What attracts attention, because the temporal curve (Fig. 1) indicates that these animals do not show significant joint inflammation. It should be noted that the hot plate test represents a general measure of nociceptive reactivity; because it causes a complex behavior in rodents, it is not only a simple reflex, and this test is also sensitive to repeated exposures, and some authors report that the response of animals is due to learning (Espejo and Mir 1994; Plone et al. 1996).

Malva parviflora is not well-studied chemically. Some studies have reported that methanolic and aqueous extracts have anti-inflammatory, antioxidant, and free radical scavenging activities (Bouriche et al. 2010; Bouriche et al. 2011). In this study, the MpF4 fraction of *M. parviflora* contains a mixture of compounds (identified by GM-MS), including hexanedioic acid, bis (2-ethylhexyl) ester (31.467%), 2-pentadecanone, 6,10,14 trimethyl- (20.605%), and the 9-octadecenamide, (z)- (14.947%). Adeosun et al. (2017), showed that the essential oil of *Jatropha curcas* L has anti-inflammatory activity; within its constituents are pentadecanone, 6,10,14 trimethyl- with 12.3% abundance, and 9-octadecenamide, (z)- which have been identified in the leaves of the species *Annona reticulata L* (Rout and Kar 2014). On the other hand, antifungal and antibacterial activity for an ethyl acetate extract obtained from *Diaporthe schini* that contains this kind of compounds (like 3-docosenamide, (Z)-; 2-hexadecene, 3,7,11,15-tetramethyl; 9-octadecenamide and 11-octadecenoic acid), was shown (dos Reis et al. 2019). The MpFphy fraction contains three phytosterols at highest abundance, Stigmasta-5,22-den-3-ol, which is reported to have hypolipidemic activity (Zhao et al. 2019); campesterol, with anti-inflammatory and cytotoxic activity (Moreno-Anzúres et al. 2017; Atolani et al. 2019); and β-sitosterol, which has been identified in the essential oil of *Rhytidium rugosum*, and is one of the most abundant diterpenes (Li et al. 2008). In another study, β-sitosterol was identified as one of the chemical components of an oil ether extract from *Pinellia cordata* that was reported to have anti-inflammatory and analgesic activity in xylene-induced edema and constipation tests induced with acetic acid in mice (Huang et al. 2011).

Conclusion

The immunomodulatory activity of the different treatments of *M. parviflora* is due the chemical composition, based in sterols and other compounds of low molecular weight such as fatty acids. It is necessary to continue with the study of these fractions with the intention of close to the possible action mechanism.

Author contribution statement Animal experiments: M-HGB, V-VG, G-AMP; experimental design, statistical analysis: H-RM, J-FE, R-RR; manuscript preparation: H-RM, M-HGB, V-VG, J-FE; chemical analysis: ZA, G-CM; taxonomic preparation: A-FM, F-MM;

Funding information This work was partially financially supported by FIS/IMSS/PROT/G15/1426

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Adeosun TE, Ogunwande IA, Avoseh ON, Rajj IP, Lawal OA (2017) Composition and anti-inflammatory activity of essential oil of Jatropha curcas. Nat Prod Commun 12:439–440. https://doi.org/10.1177/1934578X1701200333

Afolayan AJ, Aboyade OM, Adedapo AA, Sofidiya MO (2010) Anti-inflammatory and analgesic activity of the methanol extract f *Malva parviflora* Linn (Malvaceae) in rats. Afr J Biotechnol 9:1225–1229. https://doi.org/10.5897/AJB09.838

Alkabeya HA, Hughes AM, Adams J (2019) Factors associated with hand and upper arm functional disability in people with rheumatoid arthritis: a systematic review. Arthritis Care Res 71:1473–1481 https://www.ncbi.nlm.nih.gov/pubmed/30320976

Argueta A, Cano L, Rodarte M (1994) Atlas de las Plantas de la Medicina Tradicional Mexicana. Instituto Nacional Indigenista, México D.F., pp 943–947

Atolani O, Oguntoye H, Areh ET, Adeyemi ET, Kambizi L (2019) Chemical composition, anti-toxoplasma, cytotoxicity, antioxidant, and anti-inflammatory potentials of Cola gigantea seed oil. Pharm Biol 57:154–160. https://doi.org/10.1080/13880209.2019.1577468

Bouriche H, Mexiti H, Senator A (2010) *In Vivo* anti-inflammatory and antioxidant effects of *Malva parviflora* leaf extracts. Acta Hortic 854:23–30 https://www.researchgate.net/publication/283584995_In_vivo_anti-inflammatory_and_antioxidant_effects_of_Malva_parviflora_leaf_extracts

Bouriche H, Meziti H, Senator A, Arnhold J (2011) Anti-inflammatory, free radical-scavenging, and metal-chelating activities of *Malva parviflora*. Pharm Biol 49:492–496. https://doi.org/10.3109/13880209.2011.558102

Burmester GR, Feist E, Dörner T (2014) Emerging cell and cytokine targets in rheumatoid arthritis. Nat Rev Rheumatol 10:77–88 https://www.ncbi.nlm.nih.gov/pubmed/24217582

Castro-Lara D, Basurto-Peña F, Mera-Ovando L, Bye-Boettler R (2011) Los quelites, tradición milenaria en México. ISBN: 978-607-12-0202-4. Universidad Autónoma Chapingo, Texcoco, Estado de México. http: https://www.gob.mx/cms/uploads/attachment/file/231814/Los_quelites_una_tradicion_milenaria_en_mexico.pdf

Chu J, Wang X, Bi H, Ren M, Wang J (2018) Dihydromyricetin relieves rheumatoid arthritis symptoms and suppresses expression of pro-inflammatory cytokines via the activation of Nrf2 pathway in rheumatoid arthritis model. Int Immunopharmacol 59:174–180. https://doi.org/10.1016/j.intimp.2018.04.001

Davies R, Hyrich K (2018) Biologics registers in rheumatoid arthritis. Medicine 46:243–246. https://doi.org/10.1016/j.mpmed.2018.01.001

dos Reis CM, da Rosa BV, da Rosa GP, do Carmo G, Barassuol LM, Andrade G, Kuhn KR, Farias A, Jahn SL, Kuhn RC (2019) Antifungal and antibacterial activity of extracts produced from *Diaporthe schini*. J Biotechnol 294:30–37. https://doi.org/10.1016/j.jbiotec.2019.01.022

Eman A-S, Haidy AG, Mohamed E-S, Mohamed MA-D, Abdel NS (2018) Anti-inflammatory and analgesic activities of cupressuflavone from *Cupressus macrocarpa*: impact on pro-inflammatory mediators. Drug Dev Res 79:22–28. https://doi.org/10.1002/ddr.21417

Espejo EF, Mir D (1994) Differential effects of weekly and daily exposure to the hot plate on the rat's behavior. Physiol Behav 55:1157–1162. https://doi.org/10.1016/0031-9384(94)90404-9

Feldmann M, Maini SR (2008) Role of cytokines in rheumatoid arthritis: an education in pathophysiology and therapeutics. Immunol Rev 223:7–19. https://doi.org/10.1111/j.1600-065X.2008.00626.x

Fox DA, Gizinski A, Morgan R, Lundy SK (2010) Cell-cell interactions in rheumatoid arthritis synovium. Rheum Dis Clin N Am 36:311–323 https://www.ncbi.nlm.nih.gov/pubmed/20510236

Frisell T, Saevarsdottir S, Askling J (2016) Family history of rheumatoid arthritis: an old concept with new developments. Nat Rev Rheumatol 12:335–343 https://www.ncbi.nlm.nih.gov/pubmed/27098907

Gihan A, Shoubaky E, Mohamed MA-D, Mohamed HM, Essam AS (2016) Isolation and identification of a flavone apigenin from marine red alga *Acanthophora spicifera* with antinociceptive and anti-inflammatory activities. J Exp Neurosci 10:21–29. https://doi.org/10.4137/JEN.S25096

Hoxha M (2018) A systematic review on the role of eicosanoid pathways in rheumatoid arthritis. Adv Med Sci 63:22–29. https://doi.org/10.1016/j.advms.2017.06.004

Huang B, Chu Z, He W, Qin L (2011) Chemical composition, anti-inflammatory analgesic activities of the petrol ether extract of *Pinellia cordata*. Afr J Biotechnol 10:11023–11027. https://doi.org/10.5897/AJB11.332

Huhtakangas JA, Veijola J, Turunen S, Karjalainen A, Valkealahti M, Nousiainen T, Yli-Luukko S, Vuolteenaho O, Lehenkari P (2017) Cytokine data obtained from synovial stromal cells of patients with rheumatoid arthritis or osteoarthritis. Data Brief 12:593–602. https://doi.org/10.1016/j.dib.2017.04.041

Kannan K, Ortmann RA, Kimpel D (2005) Animal models of rheumatoid arthritis and their relevance to human disease. Pathophysiology 12:167–181. https://doi.org/10.1016/j.pathophys.2005.07.011

Lagunas-Herrera H, Tortoriello J, Herrera-Ruiz M, Martínez-Hernández GB, Zamilpa A, Aguilar L, García M, Lombardo-Hearl G, Jiménez-Ferrer E (2019) Acute and chronic antihypertensive effect of fractions, tiliroside and scopoletin from Malva parviflora. Biol Pharm Bull 42:18–25 https://www.ncbi.nlm.nih.gov/pubmed/30606987

Langford DJ, Mogil JS (2008) Pain testing in the laboratory mouse. In: Fish RE, Brown MJ, Danneman PJ, Karas AZ, (Eds.), Anesthesia and Analgesia in Laboratory Animals. Academic Press, pp. 549–560. https://doi.org/10.1016/B978-012373898-1.50027-9

Li L, Wu J, Ting-Han H, Lu-Ping Q (2008) Chemical composition of the essential oil and ether extract from *Rhytidium rugosum*. Chem Nat Compd 44:797–799. https://doi.org/10.1007/s10600-009-9172-x

Li HJ, Zhang CT, Du H, Xu T, Li Q, Wang P, Fang G, Fan G (2019) Chemical composition of Bawei Longzuan granule and its anti-arthritic activity on collagen-induced arthritis in rats by inhibiting inflammatory responses. Chem Biodivers 16:e1900294. https://doi.org/10.1002/cbdv.201900294

Littlejohn EA, Monrad SU (2018) Early diagnosis and treatment of rheumatoid arthritis. Prim Care 45:237–255. https://doi.org/10.1016/j.pop.2018.02.010

Maddur MS, Miossec P, Kaveri SV, Bayry J (2012) Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Am J Pathol 181:8–18. https://doi.org/10.1016/j.ajpath.2012.03.044

McGonagle D, Watad A, Savic S (2018) Mechanistic immunological based classification of rheumatoid arthritis. Autoimmun Rev 17:1115–1123. https://doi.org/10.1016/j.autrev.2018.06.001

McInnes IB, Schett G (2011) The pathogenesis of rheumatoid arthritis. N Engl J Med 365:2205–2219 https://www.ncbi.nlm.nih.gov/pubmed/22150039

McNamee KE, Alzabin S, Anand JP, Feldmann M, Williams RO, Inglis JJ (2011) IL-17 induces hyperalgesia via TNF-dependent neutrophil infiltration. Pain 152:1838–1845. https://doi.org/10.1016/j.pain.2011.03.035

Milind MM, Eugene K, Jason JM (2016) Preclinical assessment of inflammatory pain. CNS Neurosci Ther 22:88–101. https://doi.org/10.1111/cns.12486

Moreno-Anzúres NE, Marquina S, Alvarez L, Zamilpa A, Castillo-España P, Perea-Arango I, Torres PN, Herrera-Ruiz M, Díaz ER, García JT, Arellano-García J (2017) Acytotoxic and anti-inflammatory campesterol derivative from genetically transformed

hairy roots of Lopezia racemosa Cav. (Onagraceae). Molecules 22: 118. https://doi.org/10.3390/molecules22010118

Nouran MF, Eman A-S, Mohamed MA-D, Abdel NS (2017) Anti-inflammatory and analgesic activities of Terminalia muelleri Benth. (Combretaceae). Drug Dev Res 78:146–154. https://doi.org/10.1002/ddr.21385

Pan R, Gao XH, Li Y, Xia YF, Dai (2010) Anti-arthritic effect of scopoletin, a coumarin compound occurring in Erycibe obtusifolia Benth stems, is associated decreased angiogenesis in synovium. Fundam Clin Pharmacol 24:477–490. https://doi.org/10.1111/j.1472-8206.2009.00784.x

Plone MA, Emerich DF, Lindner MD (1996) Individual differences in the hot-plate test and effects of habituation on sensitivity to morphine. Pain 66:265–270. https://doi.org/10.1016/0304-3959(96)03048-5

Ramírez-Serrano CE, Jiménez-Ferrer E, Herrera-Ruiz M, Zamilpa A, Vargas-Villa G, Ramírez RJ, Chavarría A, Tortoriello J, Pedraza G, Perez-Martínez L (2019) A Malva parviflora's fraction prevents the deleterious effects resulting from neuroinflammation. Biomed Pharmacother 118:109349

Rasheed H, Afridi R, Khan AU, Ullah MZ, Khalid S, Atiq A, Ahmed MN, Kim YS, Khan S (2018) Anti-inflammatory, anti-rheumatic and analgesic activities of 2-(5-mercapto-1,3,4-oxadiazol-2-yl)-N-propylbenzenesulphonamide (MOPBS) in rodents. Inflammopharmacology 26:1037–1049. https://doi.org/10.1007/s10787-018-0446-4

Rout SP, Kar DM (2014) Identification of chemical compounds present in different fractions of *Annona reticulata* L. leaf by using GC-MS. Nat Prod Res 28:1786–1788. https://doi.org/10.1080/14786419.2014.939084

Rzedowski G (2001) Flora fanerogámica del Valle de México. 2da. Edición. Instituto de Ecología y Comisión Nacional para el Conocimiento y Uso de la Biodiversidad. Pátzcuaro Michoacán

Sakaki H, Matsumiya T, Kusumi A, Imaizumi T, Satoh H, Yoshida H, Satoh K, Kimura H (2004) Interleukin-1beta induces matrix metalloproteinase-1 expression in cultured human gingival fibroblasts: role of cyclooxygenase-2 and prostaglandin E2. Oral Dis 10:87–93. https://doi.org/10.1046/j.1354-523X.2003.00982.x

Salinas-Sánchez DO, Jiménez-Ferrer E, Sánchez-Sánchez V, Zamilpa A, González-Cortazar M, Tortoriello J, Herrera-Ruiz M (2017) Anti-inflammatory activity of polymeric proanthocyanidin from Serjania schiedeana. Molecules 22:863. https://doi.org/10.3390/molecules22060863

Sánchez-Ramón S, López-Longo FJ, Carreño L (2011) Interleukins network in rheumatoid arthritis pathophysiology: beyond proinflammatory cytokines. Reumatol Clín 6:S20–S24. https://doi.org/10.1016/j.reuma.2010.11.010

Shale TL, Stirk WA, van Staden J (2005) Variation in antibacterial and anti-inflammatory activity of different growth forms of *Malva parviflora* and evidence for synergism of the anti-inflammatory compounds. J Ethnopharmacol 96:325–330. https://doi.org/10.1016/j.jep.2004.09.032

Sluka KA, Bailey K, Bogush J, Olson R, Ricketts A (1998) Treatment with either high or low frequency TENS reduces the secondary hyperalgesia observed after injection of kaolin and carrageenan into the knee joint. Pain 77:97–102 https://insights.ovid.com/article/00006396-199807000-00012

Smith JB, Haynes MK (2002) Rheumatoid arthritis- a molecular understanding. Ann Intern Med 136:908–922 https://annals.org/aim/article-abstract/715345/rheumatoid-arthritis-molecular-understanding

Suh HR, Chung HJ, Park EH, Moon SW, Park SJ, Park CW, Kim YI, Han HC (2016) The effects Chamaecyparis obtusa essential oil pain-related behavior and expression of pro-inflammatory cytokines in carrageenan-induced arthritis in rats. Biosci Biotechnol Biochem 80: 203–209. https://doi.org/10.1080/09168451.2015.1075864

Sur B, Kang S, Kim M, Oh S (2019) Inhibition of carrageenan/kaolin-induced arthritis in rats and of inflammatory cytokine expressions in human IL-1β-stimulated fibroblast like synoviocytes by a benzylideneacetophenone derivative. Inflammation 42:928–936. https://doi.org/10.1007/s10753-018-0947-8

World Health Organization, Chronic diseases and health promotion, (2019) (Accessed February 18,2019), https://www.who.int/chp/topics/rheumatic/en/

Zhao CH, Zhao C, Ye HQ, Xi CY, Zou XP, Zhou XH, Hou PP, Zhang TH (2019) Hypolipidemic activity of low-cholesterol ovum oil of *Rana chensinensis* and phytosterol (stigmasterol) in rats. J Zhejiang Univ Sci B 20:613–616. https://doi.org/10.1631/jzus.B1900018

Zonzini JB, Barros BM, Silva NC, de Morais BC, Sachetto Z, Davitt M, de Paiva ME (2018) Disease-related outcomes influence prevalence of fall in people with rheumatoid arthritis. Ann Phys Rehabil Med 62:84–91. https://doi.org/10.1016/j.rehab.2018.09.003