Trends Phytochem. Res. 6(3) 2022 247-258

Trends in Phytochemical Research (TPR)

Journal Homepage: http://tpr.iau-shahrood.ac.ir

*Original Research Article*

# Anti-inflammatory potential of aqueous extract and ethyl acetate fractions of *Munronia pinnata* (Wall) Theob. and the isolated compound, senecrassidiol

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**ABSTRACT**

This study was designed to evaluate *in vivo* and *in vitro* anti-inflammatory potential of the different extracts of *Munronia pinnata* and compare it with the isolated compound, Senecrassidiol by using the carrageenan-induced paw edema animal model in Wistar rats. *Munronia pinnata* (Wall) Theob (Meliaceae) has been traditionally practiced for hundreds of years by traditional physicians as a home remedy for the treatment of inflammatory conditions in folk medicine in Sri Lanka. The tested ethyl acetate fractions of methanol extract and its isolated compound senecrassidiol exhibited significant anti-inflammatory activity in the tested models. It demonstrated the scientific rationale for the use of an anti-inflammatory agent in folk medicine. The MPaq showed a significant ($p < 0.5$) inhibition of infiltration of rat peritoneal cells, inhibition of NO production by rat peritoneal cells, and inhibition of membrane stabilization as well as the anti-histamine activity which are the probable anti-inflammatory mechanisms.

**ARTICLE HISTORY**

Received: 24 June 2022
Revised: 16 September 2022
Accepted: 18 September 2022
ePublished: 25 September 2022

**KEYWORDS**

Anti-inflammatory
Folk medicine
Membrane stabilization
*Munronia pinnata*
Senecrassidiol
Wistar rats

**dor: 20.1001.1.25883623.2022.6.3.6.6**

## 1. Introduction

Inflammatory processes have received a great deal of interest in the field of medical research as inflammation underlies almost every disease condition. This is a complex physiological reaction of the immune cells of vascular tissues to adverse stimuli like toxins and pathogenic organisms, resulting in irritation caused by the secretion of strong chemicals and physical damage. A healing process is initiated to eradicate injurious stimuli (Sugita et al., 2016). Acute inflammation begins within a short duration following injury to tissues. The exudation of fluid and plasma proteins (oedema) and the migration of leukocytes, predominantly neutrophils are the specific features of acute inflammation. Non-steroidal and steroidal anti-inflammatory drugs in allopathic medicine have shown only limited success against all types of inflammatory conditions. These drugs can cause gastric or intestinal ulceration that can lead to many secondary conditions. Hence, gastrointestinal problems associated with the use of anti-inflammatory drugs are still an enduring dilemma in the medical world. There is an ever-increasing interest and demand for the potential use of new phytomedicines consisting of potent herbs which possess comparatively safer anti-inflammatory activity with lesser or no side effects. Such novel therapies will open up a new panorama for chronic life-threatening inflammatory disorders. *Munronia pinnata* (Wall) Theob (Meliaceae); locally called "Binkohomba" in Sinhalese and "Nilawembu" in Tamil. is an important medicinal plant in Sri Lanka. This plant is used as a substitute for *Kiratha* or *Kiratha thiktha* (*Swertia chiriyata*, family-Gentianaceae) as an ingredient in the decoctions and powders used for the treatment of fever and upper respiratory tract inflammations (Jayasinghe et al., 1976; Jayaweera and Senarathne, 1982; Dassanayake et al., 1995). There are several synonyms for *M. pinnata* described in the Flora of China (*M. delavayi* French., *M. hainanensis* How et T.

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Chen., *M. hainanensis* How et T. Chen. var. *microphylla* X.M. Chen., *M. henryi* Harms., *M. heterochicha* H.S.LO., *M. heterophylla* Merr., *M. neilgherrica* Weight., *M. pumila* Wight., *M. sinica* Diels., *M. timoriensis* Baill., *Turraea pinnata* Wall., and *M. wallichii* Wight.). In Sri Lankan Flora also, several synonyms are found (*M. hainanensis* How et T. Chen, *M. henryi* Harms., *M. pumila* Wight., *Munronia sinica* Diels.). However, some scholars in China have described these names as species of the genus *Munronia* (Li et al., 2012). Phytochemical analysis and some biological investigations have been carried out by Chinese researchers in Yunnan province on *M. delavayi, M. henryi,* and *M. sinica*. giving rise to the identification of six novel tetranortriterpenoid derivatives in the methanol extract of the whole plant, from *M. henryi* (Qi et al., 2003). These compounds involve munronins A and B ($C_{26}H_{30}O_7$), munronin C ($C_{28}H_{32}O_9$), munronin D ($C_{28}H_{33}NO_8$), munronin E ($C_{26}H_{30}O_7$), and munronin F ($C_{25}H_{29}O_9$).

Li et al. (2012) have reported the main chemical constituents are tetranotriterpenoids and triterpenoids in *M. delavayi* and *M. henryi* among which tetranotriterpenoids in *M. delavayi* have exhibited antibacterial and antifungal activities (Lin et al., 2010). Two new compounds, namely musinisins A(1) and B (2), together with five known compounds have been identified by Li et al. (2012). Furthermore, these compounds have been tested for their potential antiangiogenic activities using a zebrafish model and antiproliferative activities using A 549 lung cancer cells. To date, there have been only a few sporadic scientific investigations recorded in Sri Lanka on chemical constituents from methylene chloride fraction of ethanolic extracts of *M. pinnata* such as one fatty acid containing 15 carbon atoms ($R_f$ = 0.52) and one triterpenoid containing 34 carbon atoms ($R_f$= 0.36). However, their structures had not been elucidated (Munasinghe, 2002).

Recently, several researchers have studied the chemical profile of *M. pinnata* for the identification of phytosterol, fatty acids, sesquiterpenes, and some chemical constituents from the *n*-hexane extract of this plant (Dharmadasa et al., 2013; Napagoda et al., 2014). In addition, previous *in vivo* studies conducted by our research team was able to establish significant hypoglycaemic (Hapuarachchi et al., 2011a, Hapuarachchi et al., 2011b) and anti-inflammatory activities of the natural plant of *M. pinnata* (Hapuarachchi et al., 2012) in Wistar rats. Recently, biologically active compounds with anti-dengue viral activity have been reported in *M. pinnata* (Jayasekara et al., 2022). Traditional physicians have been using this plant in the folk medical practice of Sri Lanka for hundreds of years for inflammatory conditions. However, scientific reports were confirming this activity (Jayasinghe et al., 1976; Dassanayake et al., 1995). The purpose of the present study was to evaluate the anti-inflammatory activity of the aqueous extracts (MPaq) and ethyl acetate fractions of *M. pinnata* in Wistar rats.

## 2. Experimental

### 2.1 Ethical clearance

The Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (No: 474/09) approved the study protocol. International guidelines and recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) were followed in the handling of these animals and the 3R principle (reduction, replacement, and refinement) has been adhered. All experiments were carried out at the Animal House, Department of Biochemistry, Faculty of Medical Sciences of the University of Sri Jayewardenepura, Sri Lanka, and the Immunology Laboratory of the Institute of Biochemistry, Molecular Biology, and Biotechnology, University of Colombo, Sri Lanka.

### 2.2. Collection and authentication of plant material

Plants of *Munronia pinnata* (3 leaflets) were collected from the medicinal plant nursery at Haldummulla, Sri Lanka. Plant specimens were taxonomically identified and authenticated by the National Herbarium, Department of National Botanic Gardens, Peradeniya, Sri Lanka where a voucher specimen was deposited (PDA/MP 01).

### 2.3. Preparation of aqueous extract of *M. pinnata*

According to the conventional preparation of decoctions in traditional medical practice (Jayasinghe et al., 1976), 60.0 g of air-dried coarsely powdered whole plant of *M. pinnata* was extracted with 1920.0 mL of drinking water in an earthen vessel over moderate heat for 4 h. The filtered aqueous extract (240.0 mL) of MPaq was freeze-dried and stored at -4 ˚C until use.

### 2.4. Successive extraction and isolation of compounds from different fractions

Air-dried and powdered whole plants of *M. pinnata* (970.0 g) were extracted successively with hexane (500.0 mL x 3), chloroform (1000.0 mL x 3), and methanol (1000.0 mL x 3) at room temperature (Fig. 1). The extracts were then dried using a rotary evaporator under reduced pressure to obtain hexane (MPh; 170.0 g), chloroform (MPc; 206.0 g), and methanol (MPm; 470.0 g) extracts. The methanol extract (MPm) which gave the highest yield was selected for further partitioning between water and ethyl acetate (Qi et al., 2003; Li et al., 2012). ethyl acetate fractions (MPE) (200.0 mL x 3) were pooled and concentrated (MPE; 340.0 g). Ethyl acetate fraction (MPE) was subjected to column chromatography on Sephadex-LH20 (22.0 cm x 1.5 cm) using methanol as the eluent to obtain four fractions, namely MPE1, MPE2, MPE3, and MPE4. Following a comparison of TLC fractions, three subfractions were prepared (MPE2-1, MPE2-2, MPE 2-3) from MPE2 which were subsequently subjected to column chromatography on Sephadex-LH20. The semi-solid, brown colored MPE 2-1 (94.0 g) fraction was selected for further purification in a silica column (12.0 cm x 1.5 cm), eluted with ethyl acetate-hexane (4:6) solvent mixture to yield MPE 2-1a (3.0 g) MPE 2-1b (82.0 g, 0.082 w/w%) and MPE 2-1c (2.8 mg). The purity of the MPE 2-1b was examined by TLC (Fig.

2) and then subjected to NMR analysis.

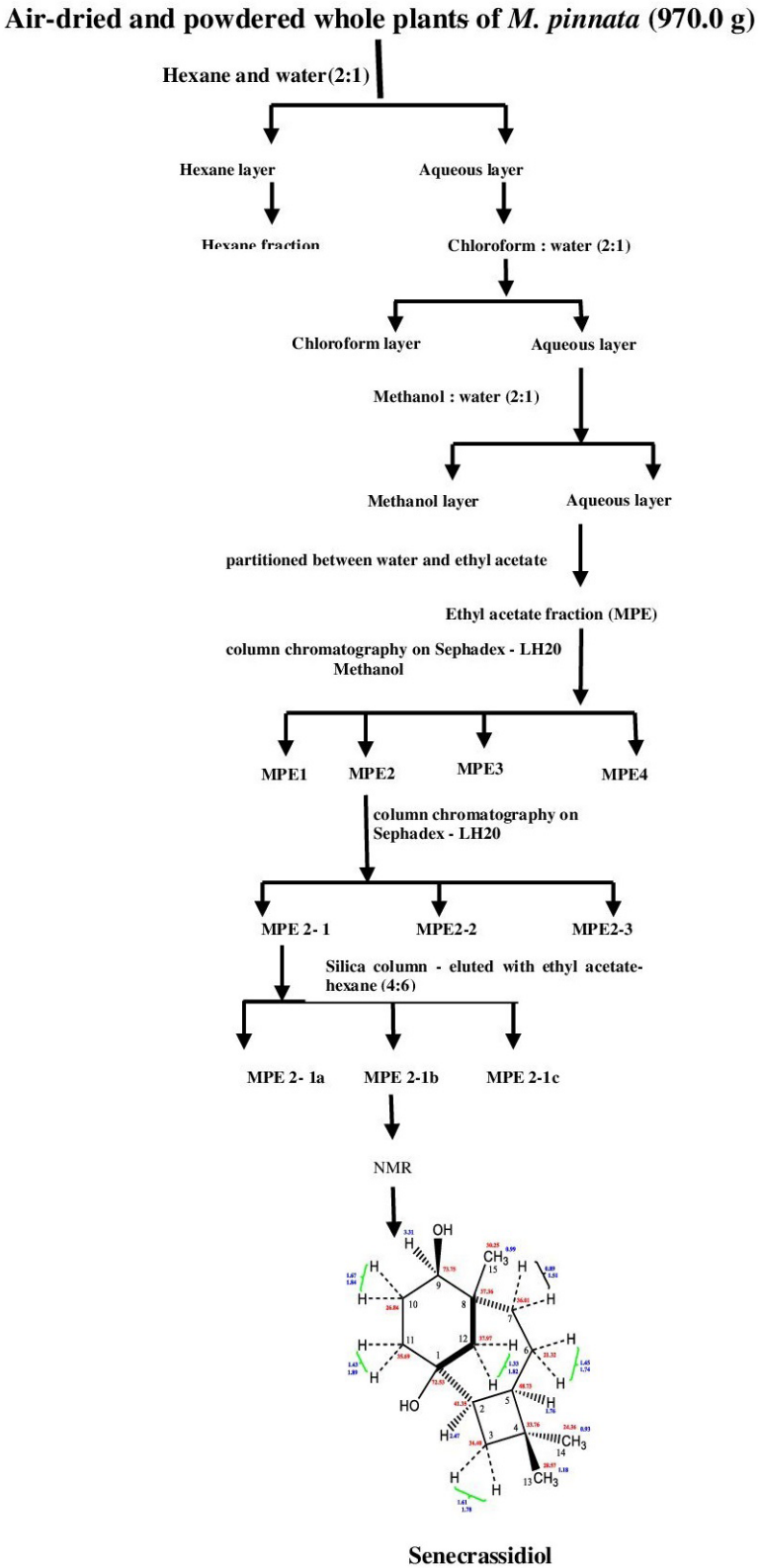


**Fig. 1.** Scheme of the extraction and bio guided isolation of *M. pinnata*.

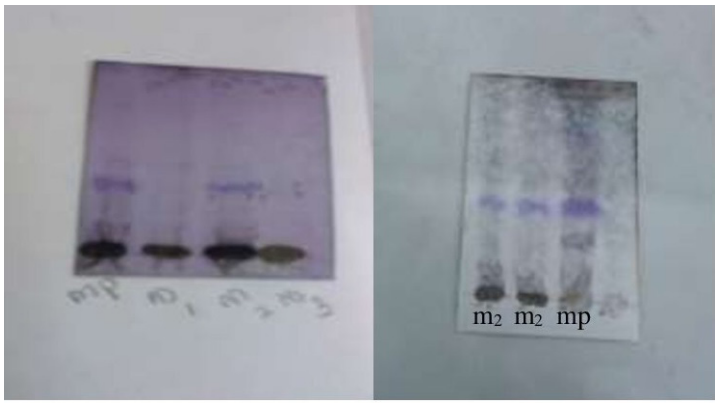


**Fig. 2.** TLC of MPE 2-1b fraction ($m_2$) compared with MPE 2-1fraction of *M. pinnata* (mp-MPE2-1 fraction, $m_1$-MPE2-1a, $m_2$-MPE2-1b, $m_3$-MPE2-1c).

### 2.5. Preliminary phytochemical analysis

Phytochemical screening of the extracts of natural plants was carried out based on procedures by Trease and Evans (2008). The phytochemicals assayed included alkaloids, terpenoids, glycosides, flavonoids, and tannins with qualitative analysis and specific spraying reagents for alkaloids, and terpenoids namely dragendorff and vanillin, respectively.

### 2.6. *In vivo* assays

#### 2.6.1. Experimental animals

Healthy adult male Wistar rats (150.0-200.0 g) were purchased from the Medical Research Institute, Colombo 8, Sri Lanka. Male rats were used for all experiments except the toxicity study. Animals were individually housed at 29 ± 2 °C in a well-ventilated room with free access to food and tap water until the day of the experiment. Following the acquisition, rats were allowed to acclimatize to the local environment for at least one week before commencing experiments. Six animals per group (n = 6) were used for all experiments.

#### 2.6.2. Standard drugs

The standard drugs involving indomethacin (10.0 mg/kg), prednisolone (10.0 mg/kg), chlorpheniramine (0.67 mg/kg) and aspirin (200 µg/mL) were purchased from State Pharmaceutical Corporation, Sri Lanka. Tablets were ground and the powders were dispersed in distilled water for administration to rats.

### 2.7. Evaluation of anti-inflammatory activity in healthy rats

#### 2.7.1. Anti-inflammatory activity of MPaq in carrageenan-induced paw oedema in healthy Wistar rats

Rats were divided into six groups and carrageenan-induced paw oedema was produced according to the method described by previous researchers (Handunnetti et al., 2009; Kumari et al., 2014). Initially, baseline values of the left hind paw volumes were taken at zero hours using a plethysmometer (Letica Scientific Instruments, Barcelona, Spain). The test groups were treated with 100.0, 200.0, 300.0, and 500.0 mg/kg of MPaq as a single dose respectively. The negative group was treated orally with 1.0 mL of tween 20 (Kumari et al., 2014) dissolving 1.0 mL of tween 20 in NaCl (0.9%)). The rats in the positive control group 2 received 10.0 mg/kg of indomethacin (the reference drug), at a dose comparable to the normal therapeutic dose of an adult human. After 1 hour, 0.1mL of carrageenan suspension (1.0% carrageenan suspended in phosphate-buffered saline, pH 7.4) was injected subcutaneously into the plantar surface of the left hind paw of all these rats under mild ether anesthesia. Thereafter, the left hind paw volumes of these rats were measured using the plethysmometer at hourly intervals up to 5 hours. The degree of swelling was calculated by the increase in paw volume ($V_t$ -$V_o$) where $V_t$ and $V_o$ are the volumes of the hind paw after and before the carrageenan injection, respectively.

The percentage of inhibition of inflammation at each hour compared to control was calculated for each group as follows:

[($V_t$-$V_o$) in control rats - ($V_t$- $V_o$) in treated rats/($V_t$-$V_o$) in control rats] x 100 (Eqn. 1)

#### 2.7.2. Estimation of an optimum effective dose of ethyl acetate fractions for acute anti-inflammatory action

Rats were divided into six groups. The same protocol (2.7.1) was applied to measure the percentage inhibition of paw oedema. The treated groups received the extracts at the doses of 100, 200, 300, and 400 mg/kg b.w. of each ethyl acetate fraction (MPE) in 1.0 mL of tween 20, respectively.

#### 2.7.3. Acute anti-inflammatory activity of ethyl acetate

fractions (MPE) of MP

The acute anti-inflammatory activity of the ethyl acetate fractions (MPE1, MPE2, MPE3, and MPE4) of MPE was also evaluated as described earlier in subsection 2.7.1. The test groups were treated with MPE1, MPE2, MPE3, and MPE4 in T20 at the dose of 200 mg/kg b.w.

### 2.7.4. Determination of the comparative effect of MPE2 fractions on carrageenan-induced rat paw oedema

The aforementioned method (subsection 2.7.1) was applied to evaluate the activity of the sub-fractions (MPE2-1, MPE2-2, MPE2-3, and MPE2-1b) of MPE2 in T20 at the dose of 100.0 mg/kg b.w.

## 2.8. Determination of the mechanisms of the acute anti-inflammatory activity of MPaq

### 2.8.1 Assessment of carrageenan-induced infiltration of peritoneal cells of rats

Peritoneal cells were isolated as described in the previous protocol (Adnan et al., 2014). Rats were divided into four groups. Each group was orally treated with 1.0 mL of each extract as follows; MPaq (500.0 mg/kg) for group 1, prednisolone (10.0 mg/kg) for group 2, and distilled water for group 3 as a single dose, respectively. After 1 h, carrageenan (1.0 mg/mL in PBS, pH 7.4) was injected into the peritoneal cavity at a dose of 5.0 mg/kg under ether anesthesia. Two hours later, sterile PBS (40.0 mL) was injected intraperitoneally and after 5 minutes, 35.0 mL of peritoneal fluid was drained using an 18 G cannula into the sterile tubes with gentamycin (50.0 µg/mL). The peritoneal fluid was centrifuged at 150 g for 10 minutes at 40 ˚C and the pellet was resuspended in 1.0 mL of PBS after discarding the supernatant layer. A 50.0 µL aliquot of cell suspension in PBS was mixed with 10.0 mL of neutral red (1.0%) to visualize the macrophage cells. Total cell count and phagocyte/macrophage cell count were determined using a haemocytometer.

### 2.8.2. *In vivo* assay for NO production by peritoneal cells

The Griess reagent was used for the determination of nitrite concentration in the culture supernatant. Three groups of rats (n = 6 per group) were treated with MPaq (500.0 mg/kg), prednisolone (10.0 mg/kg), and distilled water, respectively. Peritoneal cells were collected after 1 hour of the oral administration of the respective doses as per the above-described method from each animal. The collected peritoneal cells were plated in 96 well tissue culture plates at 1 x 106 cells/mL in RPMI culture medium (GIBCO BRL, Life Technologies, Scotland) supplemented with bovine serum albumin (1.0%) (BSA-Sigma Chemicals Company, St Loius, Mo, USA). They were then incubated at 37 ˚C in a CO2 (5.0%) ± 95% air, incubator (Sanyo Electric Co. Ltd., Osaka, Japan). After 24 h incubation, the supernatant was aspirated from the plate and centrifuged at 10,000 g for 10 minutes and the clear supernatant was assessed for nitrite production. An equal volume (100.0 µL) of culture supernatant and freshly prepared Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl) and ethylenediamine hydrochloride in distilled water mixed at a 1:1 ratio) were mixed and kept at room temperature (25 ˚C) for15 minutes. The relevant optical density (OD) was determined at 540 nm in an ELISA plate reader (ELX 800, Bio Tek Instruments INC, Winooski, VT, USA). The NO concentration was calculated using the calibration curve of a dilution series (0.7-100.0 µM) of $NaNO_2$.

### 2.8.3. Assay for antihistamine activity

The fur on the left lateral side of the rats was shaved. After twenty-four hours, these rats were randomly divided into four groups. Group 1 was treated with (500.0 mg/kg) of MPaq, chlorpheniramine (0.67 mg/kg) was given as the reference drug for group 2 (positive control group) and 1.0 mL of distilled water was administered to the negative control group. After 1 hour, 50 µL of 200 µg/mL histamine dihydrochloride (Fluka, Buchs, Switzerland) was subcutaneously injected into the skin where the fur had been shaved in each animal. After two minutes, the area of wheal that appeared in the shaved area was measured (Handunnetti et al., 2009; Kumari et al; 2014).

## 2.9. *In vitro* assay

### 2.9.1 Assay for NO production by peritoneal cells

The *in vitro* cytotoxicity assay was performed as described by Maccioni et al. (2003) using different concentrations of MPaq. The viability of cells was assessed by trypan blue exclusion assay (Handunnetti et al., 2009) after 30 minutes of incubation at 37 ˚C in a $CO_2$ (5.0%) incubator. Peritoneal cells harvested after the administration of 5 mg/kg carrageenan intraperitoneally were plated in 96-well tissue culture plates at a concentration of 0.2 x 106 cells/well. They were treated with the MPaq (7.5 to 500 µg/mL) in RPMI 1640 medium supplemented with 1.0% bovine serum albumin in a $CO_2$ (5.0%) incubator for 30 minutes at 37 ˚C. The treated 1mM *N*-monoethyl-L-arginine acetate salt (NMMA; nitric oxide synthase inhibitor) was used as positive control and cells which were not treated with MPaq were used as a negative control. The cell suspension was centrifuged at 150 g for 2 min, 180 µL of the supernatant aspirated and 20 µL of trypan blue (0.2%) added and incubated at room temperature for 5 min. After 24 h incubation, the supernatant was aspirated from the plate and centrifuged at 10,000 g for 10 minutes and the clear supernatant was assessed for NO production.

### 2.9.2. Assay for membrane stabilizing activity

This assay was performed using a modification of the heat-induced haemolysis of rat erythrocytes as described previously (Adnan et al., 2014). A tenfold dilution series of MPaq and aspirin was made using PBS for concentrations from 1 mg/mL to 0.001 µg/mL. One mL of PBS was used as a control. Ten µL of rat blood was added to each tube containing 500 µL of test, reference drug, and negative control samples. All dilutions of

MPaq and aspirin were made in triplicates. Samples were incubated at 37 ˚C for 15 min. The modifications included an additional centrifugation step after this initial incubation. Cell suspensions were centrifuged at 1500 g for 3 min, the supernatants were removed and the cells were resuspended in 500 µL of PBS. Samples were incubated at 54 °C for 25 min to initiate heat-induced haemolysis and centrifuged at 1500 for 5 min. The OD value was taken at 540 nm after the transfer into an ELISA plate. Percent inhibition of haemolysis was calculated concerning the controls and $EC_{50}$ values were derived by the graph plotted with percentage inhibition vs concentration of the extract of the reference drug aspirin (Eqn. 2).

Percent inhibition of haemolysis = [(OD) control -OD sample /OD control] x100 (Eqn. 2)

### 2.10. Statistical analysis

The data were presented as mean ± SEM and analyzed using the statistical package for social sciences (SPSS 17.0). Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test (Katz, 2006), and $p < 0.05$ was considered statistically significant.

## 3. Results and Discussion

### 3.1. Characterization of phytochemical classes of extracts/fractions of MP

The phytochemical screening of ethyl acetate extract of the successful extraction of *M. pinnata* showed strong positive observations for terpenoids with anisaldehyde sulphate and vanillin test. In addition, it revealed the presence of alkaloids, flavonoids, tannins, glycosides, and unsaturated sterols in this extract. This was comparable with earlier reports (Munasinghe, 2002; Dharmadasa et al., 2013). The isolated compound was identified by its proton-nuclear magnetic resonance ($^1$H-NMR), carbon-nuclear magnetic ($^{13}$C-NMR), two-dimensional NMR, mass spectroscopy (MS), UV spectra, and crystallography. Fractionation of the ethyl acetate extract of the natural plant yielded (MPE 2-1b) pure compound (Fig. 2) which was identified as senecrassidiol ($C_{15}H_{26}O_2$); a known sesquiterpene (Arciniegas et al., 2013). Interpretation of NMR spectral data of senecrassidiol with $^{13}$C NMR and $^1$H NMR spectroscopy and compared with the published data (Arciniegas et al., 2013) established that the major sesquiterpene white crystals isolated from the extract of *M. pinnata* as senecrassidiol (Fig. 3).

The carrageenan-induced paw oedema model is the standard experimental model for the screening of acute anti-inflammatory agents and has been used since 1962 (Winter et al., 1962; Handunnetti et al., 2009; Arawwawala et al., 2010). The subcutaneous injection of carrageenan into the footpad of rats in the test and control groups produced local oedema in the following 1 h that increased progressively to its peak at the 3$^{rd}$ hour and then began to decline. Carrageenan-induced oedema is a biphasic response. Immediately after, the injection of carrageenan, the first phase will initiate and will diminish in two hours. At the end of the first phase, the second phase initiates and continues for up to five hours. The release of histamine, kinins, and serotonin mediates the first phase, whereas the second phase is linked to the release of prostaglandins and slow-reacting substances which peak in the 3$^{rd}$ hour (Kumar et al., 2009).

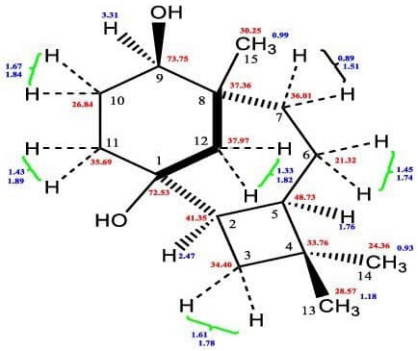


**Fig. 3.** Structure of the isolated compound MPe 2-1b (senecrassidiol-$C_{15}H_{26}O_2$).

These chemical substances produce an increase in vascular permeability, thereby promoting the accumulation of fluid in tissues which causes oedema (Huang et al., 2014). It has been reported that the second phase of oedema is sensitive to drugs like hydrocortisone, phenylbutazone, and indomethacin (Mohan et al., 2012). It is well-known that most anti-inflammatory drugs inhibit the production of prostaglandins through the cyclooxygenase (COX) pathway of arachidonic acid metabolism. However, long-term continuous use of NSAIDs is a tendency to develop side effects due to inhibition of COX-1 as well COX-2 during the inflammation. COX-1 and COX-2 are maximal in the 1$^{st}$ phase and 2$^{nd}$ phase of carrageenan-induced paw oedema in animal models, respectively (Arawwawala et al., 2010).

In the present study, all the extracts effectively inhibited the inflammatory condition in a dose-dependent manner compared with the control group.

### 3.2. Anti-inflammatory effect of MPaq in healthy rats

The freeze-dried MPaq (yield 15.6%, dry weight basis) was used for the anti-inflammatory activity in the acute experimental rat model presented in Table 1. The local oedema was produced by subcutaneous injection of carrageenan (Handunnetti et al., 2009). Then, it progressively increased up to 3$^{rd}$ hour and then began to decrease. The selected doses of MPaq of *M. pinnata* and indomethacin group exhibited statistically significant inhibition of the inflammation from the 2$^{nd}$ hour to the 5$^{th}$ hour. These results revealed that both extracts had a statistically significant ($p < 0.05$, $p < 0.001$) inhibition of inflammation and they were comparable to the reference drug, indomethacin (10.0 mg/kg). MPaq exhibited a significant dose-dependent effect and the highest dose of MPaq (500 mg/kg) showed the maximum inhibition of oedema (85.5%) which exerted a similar pattern of activity along to the reference drug.

**Table 1**
Effect of MPaq of *M. pinnata* against carrageenan-induced paw oedema.

| Treatment (mg/kg) | Mean paw volume increase (ΔV mL) ± SEM | | | | |
|---|---|---|---|---|---|
| | 1 h | 2 h | 3 h | 4 h | 5 h |
| Distilled water | 0.27 ± 0.02 | 0.38 ± 0.06 | 0.59 ± 0.02 | 0.61 ± 0.07 | 0.62 ± 0.07 |
| Indomethacin 10 | 0.12 ± 0.04 (50.9) | 0.14 ±0.02 (67.8)' | 0.14 ± 0.02 (77.7)** | 0.12 ± 0.03* (83.6) | 0.12 ± 0.02 (85.9)** |
| 500 | 0.15 ± 0.04 (53.1)** | 0.08 ± 0.02 (65.8)' | 0.15 ± 0.04 (77.3)** | 0.12 ± 0.03** (82.7) | 0.12 ± 0.01 (85.5)** |
| 300 | 0.16 ± 0.05 (50.1)* | 0.14 ± 0.02 (59.5)** | 0.18 ± 0.02 (71.1)** | 0.18 ± 0.03** (74.2) | 0.12 ± 0.02 (77.1)** |
| 200 | 0.16 ± 0.02 (39.8)* | 0.19 ± 0.03 (48.1)* | 0.24 ± 0.03 (60.5)** | 0.22 ± 0.04** (63.1) | 0.19 ± 0.02 (68.1)** |
| 100 | 0.19 ± 0.02 (30.2)** | 0.23 ± 0.01 (41.1)* | 0.26 ± 0.02 (56.4)** | 0.24±0.02** (60.0) | 0.24 ± 0.02 (61.2)** |

Values are expressed as mean ± SEM, Statistically significantly different from each column of the control group (*$p < 0.05$, **$p < 0.001$). Each value in parenthesis represents the percentage inhibition rate (%) following a single administration of each extract.

The selected doses of MPaq showed a statistically significant inhibitory effect ($p < 0.05$, $p < 0.001$) on paw oedema. These values indicated that there is an incremental anti-inflammatory activity with increasing doses of MPaq, showing a dose-dependent increase with the tested doses. Among the tested doses, the highest dose (500 mg/kg) showed the maximum percentage of inhibition (82.7%) at 4th h. The group treated with indomethacin exhibited the maximum inhibitory effect on paw oedema which was not statistically different from the MPaq. These results showed that the anti-inflammatory activity of the extract was both times- and dose-dependent.

### 3.3 Anti-inflammatory effect of ethyl acetate fractions of MP

#### 3.3.1 Effect of different doses of ethyl acetate fractions of MP

The results of the dose-response study of the tested doses (100.0, 200.0, 300.0, and 400.0 mg/kg b.w.) of MPE on carrageenan-induced oedema in the rat paw are summarized in Table 2. All the MPE fractions showed statistically significant inhibition of paw oedema compared to the control group ($p < 0.05$ and $p < 0.001$) and exhibited maximum inhibition at 4$^{th}$ hour. The dose of 100.0 mg/kg and 200 mg/kg of MPE2 fractions exhibited the highest inhibitory effect of the other two doses (300.0 and 400 mg/kg). These results indicated a reciprocal anti-inflammatory effect with increased doses which was in contrast to what was observed with the MPaq doses. Therefore, both 200 mg/kg and 100 mg/kg may be used as the optimum doses for further studies.

#### 3.3.2. Effects of different fractions of MPE2 on carrageenan-induced hind paw oedema in Wistar rats

The results of the anti-inflammatory activity of the different fractions of MPE2 in Wistar rats are shown in Table 3. All fractions (MPE2) at the same dose exerted significant acute ant-inflammatory activity ($p < 0.05$; $p < 0.001$) and reduced the paw oedema from 3$^{rd}$ to 5$^{th}$ hours compared to the negative control. The MPE2-1, MPE2-2, and positive control group (indomethacin) exerted higher percentages of inhibition on paw oedema among the other MPE2 fractions. When compared with the MPE2-1, and MPE2-2 fractions, the MPE2-3, and MPE2-4 exhibited a lesser effect on paw oedema though the effects were statistically significant. The MPE2-1 was selected for further fractionation.

#### 3.3.3. Comparison of the effect of MPE2 fractions on carrageenan-induced rat paw oedema

This experiment was carried out to compare the effects of MPE2 fractions with the MPE2-1b on carrageenan-induced rat paw oedema. The results are summarized in Table 4. The reduction of paw oedema of these fractions also exhibited a similar pattern as the previous studies. The subfraction, MPE2-1b was a pure compound and was identified as senecrassidiol ($C_{15}H_{26}O_2$) sesquiterpene. The isolated MPE 2-1b (senecrassidiol) produced a statistically significant anti-inflammatory effect ($p \leq 0.05$ and $p \leq 0.01$) on paw oedema of the rats. We report the isolation and identification of senecrassidiol from *Munronia pinnata* for the first time. The results from this study indicate that senecrassidiol which is isolated from MP shows a similar activity as NSAID. However, the percentage of inhibition of oedema at each hour is less than the reference drug (indomethacin) and the MPE2-1, indicating potential synergism with other active compounds in the aqueous extract. This type of inhibitory effect was reported with some medicinal plants such as *Acacia catechu* (Mehta et al., 2015), *Alostonia scolaris* (Arulmozhi et al., 2012), *Andrographis paniculata* (Chao et al., 2010), *Terminalia chebula* (Reddy et al., 2009), *Trichosanthes cucumerina* (Arawwawala et al., 2010) and *Juniperus sabina* (Zhao et al., 2018). To comprehend the inflammatory process, antagonists of mediators are generally employed in both Ayurveda and Allopathy treatment (Sekhar et al., 2011).

**Table 2**
Effect of different doses of ethyl acetate fractions (MPE) of MP on carrageenan-induced paw oedema in Wistar rats.

| Treated-groups mg/kg | Mean difference of paw oedema (ΔV mL) ± SEM | | | | |
|---|---|---|---|---|---|
| | 1 h | 2 h | 3 h | 4 h | 5 h |
| T20 | 0.27 ± 0.02 | 0.38 ± 0.05 | 0.6 ± 0.02 | 0.61 ± 0.01 | 0.62 ± 22 |
| Indomethacin 10 | 0.03 ± 0.01 (65.4) * | 0.07 ± 0.01 (66.0) * | 0.06 ± 0.01 (72.5) ** | 0.05 ± 0.01 (77.6) ** | 0.07 ± 0.01 (74.3) ** |
| MPE 100 | 0.05 ± 0.01 (45.3) | 0.08 ± 0.01 (61.5) | 0.07 ± 0.01 (67.8) ** | 0.09 ± 0.01 (61) * | 0.11 ± 0.01 (57) ** |
| MPE 200 | 0.05 ± 0.01 (46.3) | 0.38 ± 0.06 (64.5) | 0.06 ± 0.01 (72.3) ** | 0.08 ± 0.01 (67.5) ** | 0.10 ± 0.01 (60.3) ** |
| MPE 300 | 0.13 ± 0.01 (36.2) | 0.29 ± 0.04 (48.6) | 0.35 ± 0.02 (55.0) * | 0.11 ± 0.01 (53) * | 0.12 ± 0.02 (52.4) * |
| MPE 400 | 0.13 ± 0.02 (14.6) | 0.12 ± 0.02 (28.9) | 0.11 ± 0.02 (30.0) * | 0.33 ± 0.02 (34.5) * | 0.15 ± 0.02 (32.0) * |

**Table 3**
Acute anti-inflammatory effect of fractions of MPE2 on carrageenan-induced hind paw oedema in Wistar rats.

| Treated | Mean difference of paw oedema (ΔVmL) ± SEM | | | | |
|---|---|---|---|---|---|
| Groups mg/kg | 1 h | 2 h | 3 h | 4 h | 5 h |
| T20 | 0.27 ± 0.02 | 0.38 ± 0.05 | 0.6 ± 0.02 | 0.61 ± 0.01 | 0.62 ± 22 |
| Indomethacin 10 | 0.03 ± 0.01 (65.4) * | 0.07 ± 0.01 (66.0) * | 0.06 ± 0.01 (72.5) ** | 0.05 ± 0.01 (77.6) ** | 0.07 ± 0.01 (74.3) ** |
| MPE2-1 200 | 0.05 ± 0.01 (46.3)′ | 0.38 ± 0.06 (64.5)′ | 0.06 ± 0.01 (72.3) ** | 0.08 ± 0.01 (67.5) ** | 0.10* ± 0.01 (60.3) ** |
| MPE2-2 200 | 0.05 ± 0.01 (45.3)′ | 0.38 ± 0.06 (61.5)′ | 0.06 ± 0.01 (67.8) ** | 0.08 ± 0.01 (61) * | 0.11 ± 0.01 (57) ** |
| MPE2-3 200 | 0.13 ± 0.01 (36.2)′ | 0.29 ± 0.04 (48.6)′ | 0.35 ± 0.02 (55.0) * | 0.11 ± 0.01 (53) * | 0.12 ± 0.02 (52.4) * |
| MPE2-4 200 | 0.13 ± 0.02 (34.6)′ | 0.12 ± 0.02 (38.9)′ | 0.11 ± 0.02 (50.0) * | 0.33 ± 0.02 (43.5) * | 0.15 ± 0.02 (42.0)* |

Values are expressed as mean ± SEM; n = 6. *$p < 0.05$ and **$p < 0.001$ compared to control.
Each value in parenthesis represents the percentage inhibition rate (%).

A previous study has reported the significant anti-inflammatory activity (30.98%) of senecrassidiol isolated from the hexane extract of roots of *Roldana reticulate* on TPA-induced mouse oedema. However, it also has been less potent than the effect of the reference drug (indomethacin) (Arciniegas et al., 2013).
When comparing the efficacy of the anti-inflammatory effect of senecrassidiol with the aqueous and ethyl acetate extracts of *M. pinnata*, it was observed that this effect is more potent in the extracts than the single compound; senecrassidiol which could be due to synergistic activity together with other compounds present in the extracts. When describing the mechanism of action of many phytomedicines, it has been commonly observed that a total herbal extract exerts a better effect than an equivalent dose of an isolated compound (Reddy et al., 2009).
MPaq significantly ($p \leq 0.05$) reduced the migration of leukocytes in response to a carrageenan-induced inflammatory stimulus. The obtained results showed that the aqueous extract of MP could alter the action of the endogenous factors that are involved in the migration of leukocytes to the site of inflammation, thereby reducing the inflammatory process. The tested extracts with a concentration range of 500.0 to 7.8 µg/mL were not toxic to the peritoneal cells and it was used for the *in vitro* study to detect the nitric oxide (NO) inhibitory effect in rat peritoneal cells. The result of this study implies the inhibitory effect on NO production of peritoneal cells following *in vivo* treatment of MPaq.

**Table 4**
Comparison of the effect of acute anti-inflammatory activity of different fractions of ethyl acetate (MPE2) with MPE2-1b on carrageenan-induced hind paw oedema in Wistar rats.

| Treated | Mean difference of paw oedema (ΔVmL) ± SEM | | | | |
|---|---|---|---|---|---|
| Groups mg/kg | 1 h | 2 h | 3 h | 4 h | 5 h |
| T20 | 0.27 ± 0.02 | 0.38 ± 0.05 | 0.6 ± 0.02 | 0.61 ± 0.01 | 0.62 ± 22 |
| Indomethacin 10 | 0.03 ± 0.01 (65.4) * | 0.07 ± 0.01 (66.0) * | 0.06 ± 0.01 (72.5) ** | 0.05 ± 0.01 (77.6) ** | 0.07 ± 0.01 (74.3) ** |
| MPE2-1 200 | 0.05 ± 0.01 (46.3)′ | 0.38 ± 0.06 (64.5)′ | 0.06 ± 0.01 (72.3) ** | 0.08 ± 0.01 (67.5) ** | 0.10* ± 0.01 (60.3) ** |
| MPE2-2 200 | 0.05 ± 0.01 (45.3)′ | 0.38 ± 0.06 (61.5)′ | 0.06 ± 0.01 (67.8) ** | 0.08 ± 0.01 (61) * | 0.11 ± 0.01 (57) ** |
| MPE2-3 200 | 0.13 ± 0.01 (36.2)′ | 0.29 ± 0.04 (48.6)′ | 0.35 ± 0.02 (55.0) * | 0.11 ± 0.01 (53) * | 0.12 ± 0.02 (52.4) * |
| MPE2-4 200 | 0.13 ± 0.02 (34.6)′ | 0.12 ± 0.02 (38.9)′ | 0.11 ± 0.02 (50.0) * | 0.33 ± 0.02 (43.5) * | 0.15 ± 0.02 (42.0) * |

Values are expressed as mean ± SEM; n=6. *$p$ < 0.05 and **$p$ < 0.001 compared to control.
Each value in parenthesis represents the percentage inhibition rate (%).

Previous studies have shown that NO is produced from L-arginine by inducible NO synthase (iNOS) (Wallace, 2005; Mounnissamy et al., 2007; Kumari et al., 2014). The iNOS is expressed in vascular smooth muscle cells, macrophages, and hepatocytes in response to immune-modulating molecules and pro-inflammatory cytokines. Therefore, this mechanism is a significant therapeutic strategy in the development of anti-inflammatory agents. The observed action can be deemed as a potential contributing factor to the anti-inflammatory effect of selected MPaq extract on carrageenan-induced inflammation in rats.

### 3.4. Effect of *M. pinnata* on rat peritoneal cells infiltration

This *in vivo* assay for carrageenan-induced infiltration of rat peritoneal cells demonstrates the inhibitory effects of the tested doses of MP on the infiltration of rat peritoneal cells (Fig. 4). MPaq showed a statistically significant ($p$ < 0.05) inhibitory effect (41.46%) on the infiltration of rat peritoneal cells compared to the control group. Though the reference drug prednisolone exhibited the highest inhibition 58.29%, this difference was not statistically significant ($p$ > 0.05).

### 3.5. Evaluation of anti-histamine activity

The extract of MPaq has exhibited a significant ($p \leq$ 0.05) reduction (51.48% ) of the area of wheal formed by the subcutaneous injection of histamine. The highest reduction (57.97%) was recorded from the reference drug, chlorpheniramine. The early phase of inflammation is attributed to the release of histamine and serotonin. Histamine released from mast cells causes vasodilation and increased vascular permeability resulting in the exudation of fluids from the blood into the interstitial space and producing oedema.

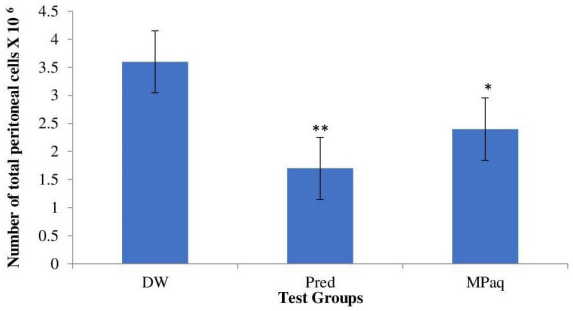


**Fig. 4.** Effect of *M. pinnata* on the infiltration of rat peritoneal cells. Each value represents the mean ± SEM from n = 6 animals in each group. $p \leq 0.05$ compared to the control group (DW). DW- distilled water, Pred-prednisolone 10 mg/kg.

### 3.6. Determination of the nitric oxide production by peritoneal cells

The formation of NO is classically assayed by measuring nitrites ($NO_2^-$) production, a primary, stable, and non-volatile breakdown product of Nitric Oxide (NO). Further, the production of NO by the rat peritoneal cells was calculated using the standard curve for nitrites.
The effect of *M. pinnata* on NO production by rat peritoneal cells is illustrated in Fig. 5. The tested doses of MP showed a statistically significant ($p$ < 0.05) inhibition of NO production in rat peritoneal cells (94.43%). The highest inhibitory effect was obtained by the reference drug, prednisolone (95.56%).

### 3.7. Measurement of nitrite in culture supernatants

*In vitro* treatment of the different concentrations (500.0, 250.0, 125.0, 62.5, 31.25, 15.60, and 7.80 µg/mL) of

MPaq (r = 0.873) on infiltrated carrageenan-induced peritoneal cells of rats exhibited a dose-dependent inhibition of NO production compared to the cell-free medium (Fig. 6).

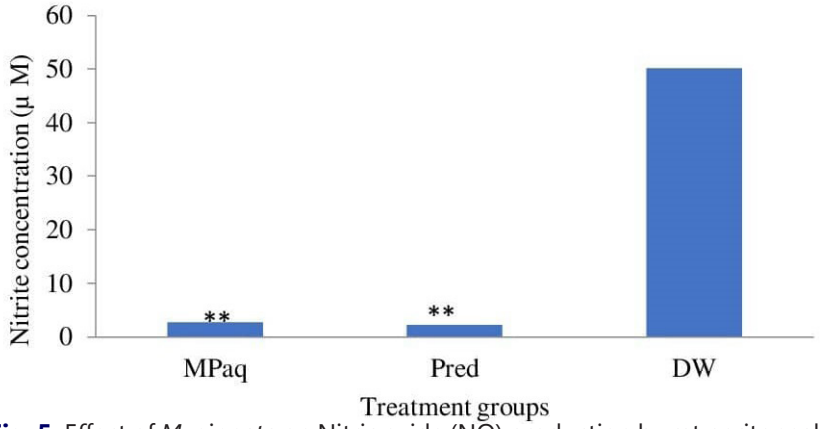


**Fig. 5.** Effect of *M. pinnata* on Nitric oxide (NO) production by rat peritoneal cells. Values are expressed as the mean ± SEM from n = 6 animals in each group. $p < 0.05$ compared to the control group. DW- distilled water, Pred- prednisolone 10.0 mg/kg, MPaq- aqueous extract of natural plants of MP (500.0 mg/kg).

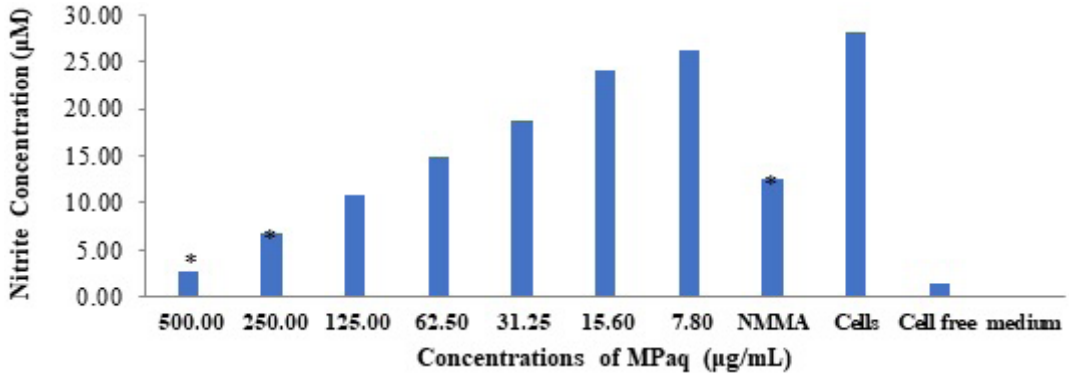


**Fig. 6.** Effect of *M. pinnata* on inhibition of *in vitro* NO production of rat peritoneal cells. Each value represents the mean ± SEM from n = 6 animals in each group. $p < 0.05$ compared with control, NMMA: *N*-monomethyl-L-arginine acetate salt.

### 3.8. Assay for membrane stabilizing activity

The membrane-stabilizing activity of the aqueous extracts of *M. pinnata* (MPaq) on heat-induced haemolysis of rat erythrocytes was assayed. In this study, all the doses of MPaq extract at concentrations of 0.001 µg/mL-1 mg/mL protected the rat erythrocyte membrane against heat-induced lysis in a dose-dependent manner (r = 0.99; $p \leq 0.01$) and it was comparable to the reference drug, aspirin which is a Non-Steroidal Anti-Inflammatory Drug (NSAID). The $IC_{50}$ values for membrane stabilizing activity for MPaq and aspirin were 5.93 ng/mL and 5.48 ng/mL respectively.

During inflammation, there is a lysis of lysosomes which release their component enzymes that produce a variety of disorders. The efficacy of NSAIDs is developed by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes (Mounnissamy et. al., 2007; Chakraborty et al., 2017). There is a close similarity of the RBC membrane system to the lysosomal membrane system; hence exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium, heat, methyl salicylate, or phenylhydrazine results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin (Chioma et al., 2012). The present study also revealed that the selected dose of MPaq possesses an antihistamine activity that is comparable to the reference drug. This antihistamine activity of MPaq could potentially be another mode by which the extract exerts its anti-inflammatory effect. Triterpenes of medicinal plants had been responsible for impairing histamine release from mast cells in

previous studies (José-Luis Ríos, 2010) and this can be applied for MPaq also, according to the observations of the phytochemical study. Further, the total antioxidant capacity (TAC) of *M. pinnata* as reported by Dharmadasa et al. (2013) may have contributed to the inhibition of inflammation during the late phase. Plant extracts that have free radical scavenging properties, act as antioxidants and thereby act as anti-inflammatory agents (Reddy and Urooj, 2013).

## 4. Concluding remarks

The present study provides evidence that the aqueous extract of *M. pinnata* and the isolated compound, senecrassidiol possess potent anti-inflammatory effects in Wistar rats. These findings provide the scientific rationale for the use of *M. pinnata* as an anti-inflammatory agent in folk and Ayurveda medicine. The results obtained revealed that anti-histamine activity, inhibition of cell migration to the site of inflammation, and inhibition of NO production may be the potential mechanisms of the anti-inflammatory activity of *M. pinnata*. Acute and chronic toxicity studies conducted by our team revealed that the plant extract does not cause any mortality or adverse effects in Wistar rats (Hapuarachchi et al., 2020). This is extremely important in the case of anti-inflammatory drugs, which have to be administered over a relatively long period in the therapeutic practice in Ayurvedic as well as traditional medical systems. These findings would pave the way for future clinical trials on the anti-inflammatory activity of this valuable plant in the field of Ayurveda and traditional medicine in Sri Lanka.

## Acknowledgments

This work was supported by the University Grant Commission, Sri Lanka-UGC/ICD/045, and partly by The National Centre for Advanced Studies in Humanities and Social Sciences (NCAS), Sri Lanka.

## Conflict of interest

The authors declare that there is no conflict of interest.

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