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Research article

LC/MS, GC/MS screening and *in vivo* anti-inflammatory activity of Malaysian *Moringa oleifera* Lam leaf extracts and fractions against carrageenan -induced paw oedema in rats

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Key words: Carrageenan, *Moringa oleifera,* Anti-inflammatory, LC/MS and GC/MS.

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Abstract

The anti-inflammatory activity of three extracts of Moringa leaf and the fractions of most active extracts was evaluated. Carrageenan-induced paw oedema in rats was used to evaluate the anti-inflammatory activity and to determine the effective dose of *M. oleifera* leaf extract and its fractions. In addition to that, LC/MS and GC/MS analysis of most active fraction were used to identify the phytoconstituents. 95% ethanol extract, at dose of 250 mg/kg body weight in rat, and its dichloromethane fraction found to be the most active as anti-inflammatory. LC/MS identified 18 compounds and GC/MS identified 8 compounds including flavinoids, phenol glucosides, amino acids and vitamin. A more detailed studies, including in human studies, to identify the phytochemical(s) and to establish the mechanism of action responsible for anti-inflammatory activity are highly recommended.

Introduction

The World Health Organization (WHO) reported that about 80% of the population in many third world countries still uses and rely on traditional medicine (e.g., medicinal plants) for their primary health care [1,2], due to poverty, lack of access to modern medicine, acceptability or awareness of adverse effects of synthetic modern medications [3,4]. Nowadays, there is a considerable increase in medicinal plant based industries which is growing at a rate of 7 to 15% annually [5].

Moringa oleifera Lam is a very well known and widely used plant for its nutritional and medicinal properties all around the world. *M. oleifera* is the most widely cultivated species of a mono-generic family, the *Moringaceae* , that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh, Afghanistan and Malaya [6,7]. All parts of Moringa tree possess medicinal properties [8], but the leaves, with its exceptional richness of biologically active phytoconstituents like flavonoids, polyphenols, alkaloids, carotenoids, glycosides in addition to high content of amino acids, minerals and vitamins, is the most used plant part [9,10]. This unique rare combination of bioactive phytoconstituents leads to a wide diversity of both traditional and scientifically approved uses of Moringa in the treatment of various diseases, malnutrition and health conditions such as antimicrobial, antihyperlipidemic, anticancer, antiulcer, antidiabetic, analgesic, antihypertension, antifertility, anticonvulsant, hepatoprotective, prostrate problems, syphilis and many others [11,12]. Among other therapeutic effects of Moringa, its anti-inflammatory properties have been positively highlighted in fruit, seed and pod [13] but only few *in vivo* studies on anti-inflammatory activity of leaves are available in the literature [14]. Coppin *et al.,* [15] have studied the flavonoids content and *in vitro* anti-inflammatory activity of different Moringa varieties collected from Ghana, Senegal and Zambia. The results showed a variation in flavonoids content, variation in anti-inflammatory activity and one Moringa sample from Zambia showed no anti-inflammatory activity. This finding advocates the need to assess the phytoconstituents and the biological activity of local Moringa varieties. To the best of our knowledge, no *in vivo* study of anti-inflammatory activity of 95% ethanolic extract of Moringa cultivated in Malaysia.

Inflammation is the key pathophysiological component of a wide range of diseases, including rheumatoid arthritis, asthma, inflammatory bowel disease atherosclerosis, cancer and others [16] since many of the inflammatory mediators are not specific for a particular tissue target [17]. Most of available anti-inflammatory medications like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, *etc* associated with serious adverse effects and not suitable for long term uses. These facts motivate the researchers to find out a safe and may be more effective alternative. Natural

sources like plants consider being very attractive for such researches.

Carrageenan is commonly used method to induce inflammatory oedema in experimental animals without any injury or damage to the inflamed paw and to evaluate the anti-inflammatory activity of the substances under investigation [18]. A number of inflammatory mediators and pro-inflammatory cytokines were suggested to be involved in the inflammatory responses to carrageenan includes, but not exclusive, histamine, serotonin, TNF-α, COX-2, IL-6, IL-1β, and NF-κB [19, 20,21].

In this study, the anti-inflammatory activity of *M. oleifera* leaf extracts and fractions of the most active extract were evaluated using carrageenan-induced paw oedema in rats. In an attempt to identify the active phytoconstituent(s), the most active fraction was analysed by LC/MS and GC/MS.

Experimental

Material and Methods

Materials and equipments

All chemicals and solvents used were analytical grade reagents (AR). Carrageenan- λ (Sigma, California, USA). Chloroform, Dichloromethane, Ethyl acetate, 95% Ethanol, n-Hexane, Acetic acid and Formic acid were obtained from Fisher Scientific, Selangor, Malaysia. Acetonitrile and methanol Fisher Optima LCMS Grade; Rotary evaporator, EYEL4, China; Water bath and drying oven, Memmert, Germany; digital micrometer (Mitutoyo, ID-C1012EXBS, Kawasaki, Japan); Agilent 1290 Infinity LC system coupled to Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source, Agilent 5977A GC/MSD system (Santa Clara, CA, USA).

Plant collection and preparation

A fresh leaves of *M. oleifera* Lam were collected from Butterworth area, Penang, Malaysia in November, 2015. These were identified and authenticated by Dr. Rahmad Zakaria, Plant Sciences, and a voucher specimen (voucher No. 11626) reserved in the herbarium, School of Biological Sciences, Universiti Sains Malaysia (USM). The fresh leaves were cleaned out and dried in oven at 40 ˚C for 3 to 5 days to get a constant weight dry mass. The dry leaves was pulverized using grinding mill to a particle size of about 0.5 mm then packed in a polyethylene bag and stored in sealed container at dark cool room until use.

Preparation of extract and fractions

Three extracts of Moringa dried leaves using different extraction solvent, i.e. 95% ethanol, 50% ethanol and water. The extracts were prepared by maceration of 100 g of dried Moringa leaves in 500 mL either one of extraction solvents at 45℃±2 for 48 h with occasional shaking shake. At the end of maceration period the extracts were filtered first with muslin cloth then with Whatman No.1 filter paper and concentrated by rotary evaporator to about 10% of the original volume. Thereafter, the concentrated extracts were dried in drying oven at 45℃±2 until a constant weight of dry mass was obtained. The collected dried extracts were stored at -20℃ until use in evaluation of anti-inflammatory activity. The extract which showed best anti-inflammatory activity was selected for determination of minimum effective dose and fractionated by liquid-liquid fractionation in to four fractions namely, n-Hexane, Chloroform, Dichloromethane and Water fraction, dried first by rotary evaporator then in drying oven at 45 C±2 until a constant weight of dry mass was obtained and the anti-inflammatory activity of the fractions was evaluated.

Animals

Animal experimental protocol was approved by Animal Ethics Committee USM (AECUSM) of Univeristi Sains Malaysia, School of Pharmaceutical Sciences (No.: USM/Animal Ethics Approval/2016/ (103)(764)). Healthy Sprague-Dawley male rats of approximately the same age, weighing about 150- 200 gm were used in the study. They were fed with standard pellet diet and free access to water (*ad libitium*). They were housed in polypropylene cages (3 animals per cage) maintained under standard condition (12 hour light, 12 hour dark cycle; 25 ± 3 ℃, 60-70 % relative humidity) and allowed to acclimatised for 7 days before starting the experiment.

In vivo Anti-inflammatory assay

Anti-inflammatory activity of *M. oleifera* crude extracts and fractions was assessed by carrageenan-induced rat hind paw oedema as described by Raj *et al.,* [22]. Animals were fasted 12 hours before the experiment; however they were given access to water *ad libitum.* Rats were divided into groups of six animals each (n=6), weighed and numbered. *In vivo* anti-inflammatory activity of Moringa leaves was carried out first with crude extracts (95% ethanol, 50% ethanol and water extract) at a dose of 1000 mg/kg body weight, then with three different doses (i.e. 500, 250 and 125 mg/kg) of the most active extract suspended in 1% CMC solution; finally, with fractions of the most active extract (calculated according to % yield of each fraction). At each experimental stage, treatment groups consisted of rats given extracts or fractions, a positive control group given Indomethacin (5 mg/kg body weight) and a control group given 1% carboxymethyl cellulose (CMC) in distilled water. After 1 hour of oral doses, inflammatory paw oedema was induced by injection of 0.1 ml of 1%(w/v) freshly prepared carrageenan in saline into the sub-plantar region of left hind paw, while control group inject with0.1 ml saline. The thickness of left hind paw was measured using digital micrometer just before carrageenan injection and at hour 1, 2, 3, 4 and 5 after carrageenan injection. The anti-inflammatory activity of the extracts/fractions and reference drug was calculated from the formula:

$$\% \text{ increase oedema} = \frac{A - B}{B} \times 100$$

where A is the paw thickness at respective hours, and B is the paw thickness at hour 0 of the experiment.

Thin layer chromatography (TLC) profile of extracts and fractions

The TLC profile of the three extracts and fractions of the selected extract were used to evaluate the efficiency of extraction solvent and identification of some phytochemicals present in Moringa extract. After a preliminary TLC runs with various mobile phases, a mobile phase consisting of ethyl acetate: formic acid: acetic acid: water (90:3.5:1.5:9) was used which reveal best separation of components. Ten µl of 10 mg/mL extracts sample in methanol and 10 µl of 100 µg/mL of Kaempferol-3-*O*-glucoside (Astragalin), Quercetin-3-o-glucoside (Isoquercetin) and 4-*O*-Caffeoylquinic acid (Cryptochlorogenic acid) standards were separately applied in the form of band (5 × 0.45 mm) 1 cm from the bottom using TLC Silica gel 60 F_{254} pre-coated plate. The plate was developed up to the distance of 8 cm from bottom, air dried, heated at 100 ℃ for 5 minutes, sprayed with 1% (w/v) diphenylboryloxyethylamine in methanol (NP) then sprayed with 5% (w/v) poly ethylene glycol 4000 (PEG4000) in ethanol, air dried and visualized by viewing in UV-cabinet under long wavelength (365nm).

Screening of phytochemicals present in *M. oleifera* 95% ethanol leaf extract

A qualitative screening of Phytochemicals for detection of presence of alkaloids, glycoside, steroid, tannins, saponins and reducing sugar, was performed using the crude extract. For detection of alkaloids Mayer's test and Dragendroff's test were used. Tests for steroids was done according to Salkowski tests; tests for tannins using a Ferric chloride test; test for glycosides Kellar killani's test and test for reducing sugars was done using Benedict's reagent (CuSO4).

Liquid chromatography-mass spectrometry (LC/MS) conditions

HPLC separation was performed with the mobile phase containing solvent A and B in gradient, where A was 0.1% (v/v) formic acid in water and B was 0.1% (v/v) formic acid in acetonitrile for the following gradient: 5% B for 5min and 5-100% B in 15 min and 100% B for 5min at a flow rate of 0.5 ml/min. Column was Agilent Zorbax Eclipse XDB-C18 (2.1x150mm x 3.5 µm), oven temperature 25 ℃ and the injection volume was 1 µl and sample concentration was 18 mg/ml in methanol. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) under positive ion mode and scanned from 100 to 1000 m/z. ESI was conducted by using a fragmentor voltage of 125 V, skimmer 65 V. High-purity nitrogen (99.999%) was used as drying gas and at a flow rate of 10 L/min, nebulizer at 45 psi and capillary temperature at 350℃. As a blank, 0.1% formic acid in methanol (v/v) was use. Data analysis was processed with Agilent Mass Hunter Qualitative Analysis B.05.00 software and compounds were identified by METLIN_AM_PC database.

Gas chromatography- mass spectrometry (GC/MS) conditions

GC/MS analysis of n-hexane fraction of *M. oleifera* leaf extract was performed on a Agilent 5977A GC System, fitted with a HP-5MS capillary column (30 m X 0.25 mm inner diameter, 0.25 µm film thickness; maximum temperature, 350 °C), coupled to Agilent 5977A Series MSD System. Ultra-high purity helium (99.99%) was used as carrier gas at a constant flow rate of 1.2 ml/min. The injection, transfer line and ion source temperatures were all at 280 °C. The ionizing energy was 70 eV. The oven temperature was programmed from 70 °C (hold for 2 min) to 280 °C at a rate of 2 °C/min. Injection volume was 1 µl with a split ratio 50:1. All data were obtained by collecting the full-scan mass spectra within the scan range 35 -650 amu. The identification and characterization of chemical compounds was based on GC retention time and best hit fragmentation. The mass spectra were computer matched with those of standards available in NIST-02 and WILEY-275 mass spectrum libraries (MassHunter GC/MS Acquisition).

Data analysis

The results were expressed as mean (± SEM), and were compared to control by using one way analysis of variance (ANOVA) test, followed by Dunnett t (2-sided) post hoc multiple comparison test using IBM–SPSS software version 20. Difference between treatment groups and control group were considered significant at $P < 0.05$ and $p < 0.01$

Results and Discussion

Results

After drying of the three extracts, the percent yield of 95% ethanol, 50% ethanol and water extract was 25.02, 38.19 and 37.80% respectively. Of the three extracts, 95% ethanol extract significantly inhibit paw oedema ($P<0.05$) comparing to control in which the effect started at the first hour post carrageenan injection with a maximum activity at hour 3 post injection, with % increase oedema 33.377 ±0.377, and persisted until hour 5 post injection (Figure1). Indomethacin effect also started at the first hour, showed a maximum inhibition of paw oedema at hour 2 post injection and the effect diminished after hour 4 post injection. For 50% ethanol and water extracts the results showed a non-significant inhibition of paw oedema. Accordingly, 95% ethanol extract was selected for determine the minimum effective dose and thereafter, liquid-liquid fractionation into four fractions. Administration of different doses of 95% ethanol Moringa leaf extract showed that the minimum

effective dose with significant effective ($p< 0.05$) was 250 mg/kg body weight. No considerable difference in the activity was detected between the dose 750 and 500 mg/kg body weight (Figure 2). Two fractions i.e. n-Hexane and Dichloromethane (Figure 3), significantly inhibit paw oedema ($P<0.05$ and $P<0.01$respectively) while chloroform and water fractions showed no significant effect. Dichloromethane fraction appeared to be more active with lower % increase paw oedema of 28.32 ±7.130 and earlier starting of the oedema inhibitory effect at hour 1 post injection of carrageenan, while for n-Hexane fraction the % increase paw oedema was 33.22 ±7.619 and the oedema inhibitory effect started at hour 2 post injection. Administration of 95% ethanol extract at doses 125, 250, 500 and 750 mg/kg body weight showed unusual result that best inhibitory effect was at dose 250 mg/kg higher than at doses of 500 and 750 mg/kg.

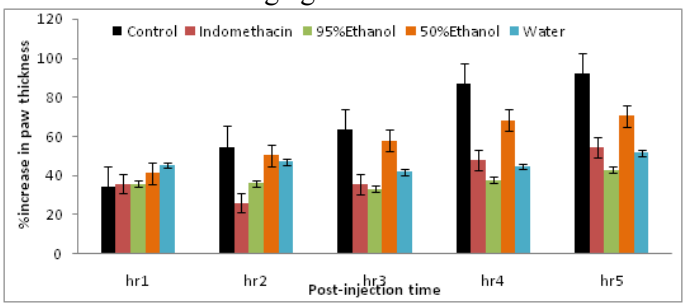

Figure 1. Effect of Moringa leaf 95%ethanol, 50%ethanol, water extract at dose 1000 mg/kg and Indomethacn at dose 5 mg/kg on Carrageenan-induced paw.

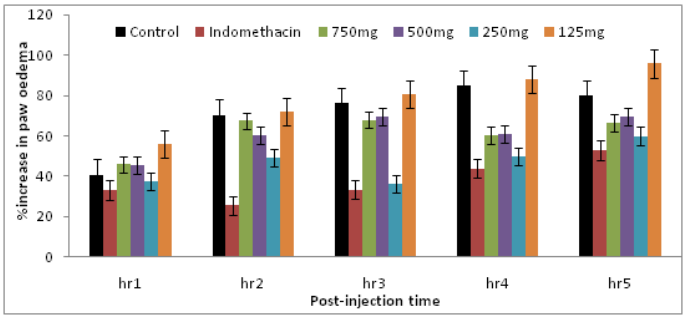

Figure 2. Effect of 95% ethanol extract of Moringa leaf at doses 750, 500, 250 and 125 mg/kg on carrageenan-induced paw oedema in rats.

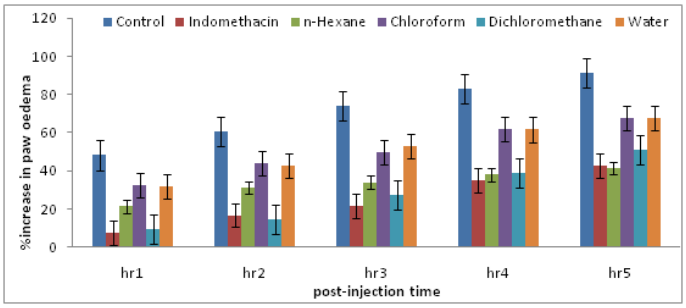

Figure 3. Effect of n-Hexane, Chloroform, Dichloromethane and water fractions of 95% ethanol extract of Moringa leaf on carrageenan- induced paw oedema in rats.

The TLC profile of 95% ethanol, 50% ethanol and water extract of Moringa leaf and fractions of 95% ethanol extract in addition to crypto chlorogenic acid, isoquercetin and astragalin as reference standards with retention factors of 0.243, 0.294 and 0.371 respectively(Figure 4). The TLC profile illustrated that 95% ethanol has extracted more phytoconstituents than other extraction solvent used here.

Phytochemical profile of Moringa leaves extract showed that 95% ethanol extract contain an abundant tannins flavonoids and phenolic compounds than 50% ethanol extract and water extract (Table 1). Dichloromethane fraction of 95% ethanol extract contain higher concentration of flavonoids and phenolic compounds than other fractions.

Analysis of LC/MS chromatogram of Dichloromethane fraction (Figure 5) depending molecular feature extraction (MFE) of the best hits of not less than 7. The identification of selected compounds was done by comparing the molecular mass and fragment of both cation and anion entries of each individual compound with that available database METLIN_AM_PC and confirmed by compares with data available in NBCI. The identities of 18 compounds were determined along with their retention time, chemical formula, protonated molecular ions and the characteristic fragment ions for each individual peaks. The identified compound includes amino acids, flavonoids, flavin and fatty acids (Table 2).

Analysis of GC/MS total ion chromatogram (Figure 6), based on selection of most abundant peaks with larger % area and highest quality identification of not less than 85% with matching of ion fragments for each selected compounds depending on database of NIST-02 and WILEY-275 library, revealed identification of 8 compounds (Table 3). The identified compounds represent 72.11% of total compounds detected by GC/MS and involves fatty acids, fatty acid ester, diterpene alcohol and vitamin.

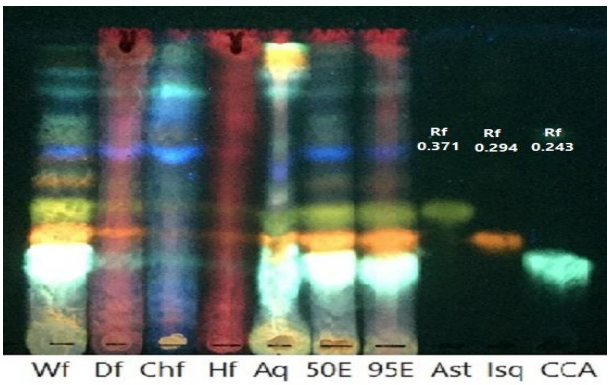

Figure 4. TLC profile of three M. oleifera extracts, four fractions of 95% ethanol extractand three reference standards sprayed with NP reagent; CCA: crypto chlorogenic acid, Isq: Isoquercetin, Ast: Astragalin, 95E: 95%ethanol extract, 50E: 50%ethanol extract, Aq: aqueous extract, Hf: n-Hexane fraction, Chf: Chloroform fraction, Df: Dichloromethane fraction and Wf: Water fraction.

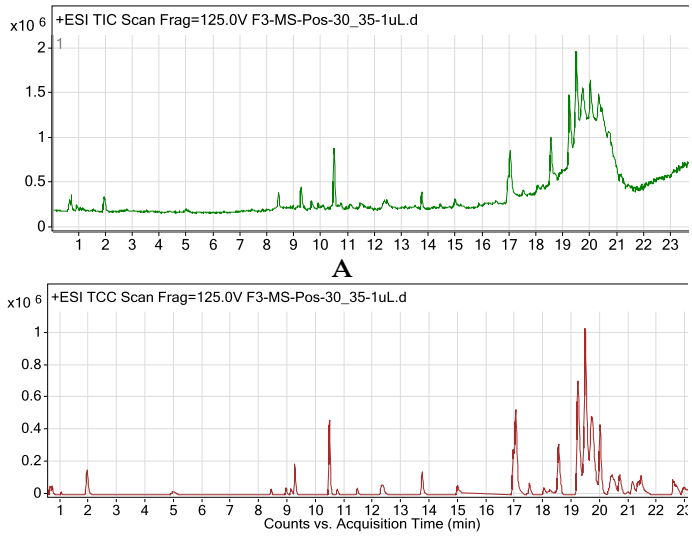

Figure 5. MS chromatogram of Dichloromethane fraction of M. oleifera leaf 95% ethanol extract. A: total ion chromatogram (TIC) and B: total compound chromatogram (TCC).

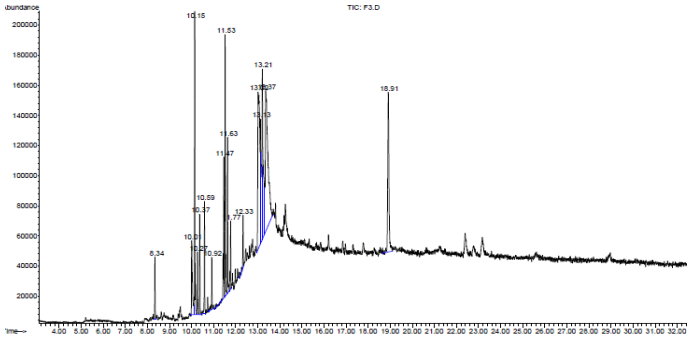

Figure 6. GC/MS total ion chromatogram of dichloromethane fraction of M. oleifera leaf extract.

The prevailing compounds identified in dichloromethane fraction was α-Tocopherol, which is known for its strong anti-oxidant activity, with 20.12%, limonene, a cyclo-terpene known for its anticancer and gallstone dissolving activity [28], with12.02%. other interesting compounds identified here was phytol which is found to be effective in prevention and treatment of arthritis at different stages of the disease [29].

Table 1. Phytochemicals profile of *Moringa oleifera* leaf extracts and fractions

Test Extract	Alkaloids	Tannins	Flavonoids	Phenolic	Saponins	Steroids	Glycosides	Reduced sugar
95%EtoH	+	+++	+++	+++	++	+	++	-
50%EtoH	-	+	++	+	++	+	+++	-
Water	-	-	+	+	+	-	++	++
Fractions of 95% Ethanol extract								
n-H	-	++	-	++	-	+	-	-
Ch	-	+++	-	+	-	-	-	-
D	+	+	+++	+++	-	-	+	-
Water	+	-	+	-	+	-	+	++

(+) sign indicate intensity of reaction, (+++): high; (++): medium; (+): low; (-): absence of phytochemicals; (n-H):n-hexane fraction; (Ch): chloroform fraction and (D): dichloromethane fraction.

Table 2. Peak assignment for the LC/MS analysis of dichloromethane fraction of *M. oleifera* leaf ethanol extract identified by METLIN_AM_PC database

N0.	R_t(min)	Formula	$[M+H]^+$(m/z)	Mass	Identities
1	0.643	$C_5H_{13}NO$	104.1068	103.0995	*DL*-Valinol
2	0.672	$C_{18}H_{20}O_6S$	365.1066	364.0992	Butyl 4-[(4-methoxyphenyl) sulfonyl] oxy benzoate
3	0.726	$C_5H_{11}NO_2$	118.0864	117.079	*L*-Valine
4	1.047	$C_6H_{13}NO_2$	132.1017	131.0948	Isoleucine
5	1.934	$C_7H_{13}NO_2$	144.1018	143.0941	1-Aminocyclohexane carboxylic acid
6	8.947	$C_{21}H_{20}O_{12}$	465.1045	464.0973	Quercetin-3-*O*-glycoside
7	9.115	$C_{16}H_{12}N_2O_2$	265.0975	264.0904	2-(b-Carbolin-1-yl)-5-hydroxy methylfuran (β-carbolines)
8	10.469	$C_6H_8O_3$	129.0547	128.0477	2-dehydropantolactone (flavin)
9	12.296	$C_{16}H_{35}NO_2$	274.2742	273.2669	Hexadeca dihydrosphingosine
10	13.724	$C_{31}H_{50}N_6O_{12}$	699.356	698.3489	Isotriornicine
11	15.062	$C_{18}H_{28}O_2$	277.2172	276.2101	8,11-octadecadiynoic acid
12	17.010	$C_{16}H_{22}O_4$	279.1602	278.1531	3-Benzylidenyl-levulinic acid
13	18.515	$C_{18}H_{30}O_2$	279.2324	278.2251	Elaidolinoleic acid
14	18.532	$C_{21}H_{38}O_4$	377.2679	354.2784	Cryptochlorogenic acid
15	19.432	$C_{18}H_{32}O_2$	281.2487	280.2414	Isolinoleic acid
16	19.472	$C_{19}H_{38}O_4$	353.2674	330.2788	1-Palmitoylglycerol
17	20.953	$C_{18}H_{37}NO$	284.2948	283.2875	Octadecanamide
18	22.956	$C_{40}H_{77}O_{13}P$	797.5178	796.5105	1-hexadecanoyl-2-penta decanoyl-glycero-3-phospho-(1'-myo-inositol)

Table 3. Peak assignment for the GC/MS analysis of dichloromethane fraction of *M. oleifera* leaf ethanol extract identified by NIST-02 and WILEY library database

Peak No.	R_t (min)	Formula	Mass	Identities
1	4.70	$C_{10}H_{16}$	136.240	Limonene
2	8.13	$C_{15}H_{24}$	204.351	(1S,6R)-γ-himachalene
3	10.34	$C_{14}H_{24}$	208.340	(4aS,7S)-7-Isopropyl-4a-methyloctahydro-2(1H)-naphthalenone
4	10.66	$C_{18}H_{36}O_2$	284.477	Hexadecanoic acid, ethyl ester
5	11.53	$C_{20}H_{40}O$	296.531	Phytol
6	11.85	$C_{18}H_{30}O_2$	278.2251	9,12,15-octadecatrienoic methyl ester
7	13.44	$C_{12}H_{15}N_5O$	245.280	4-(2-Furyl)-6-(1-piperidinyl)-1,3,5-triazin-2-amine
8	19.19	$C_{29}H_{50}O_2$	430.706	α-Tocopherol

Discussion

The inflammatory response after exposure to harmful stimuli is a multi-phases process, changes with time and can be divided into phases [23]. The rapid phase occurs within minutes which characterized by production and release of moderate amounts of inflammatory mediators and induction of inflammatory genes; while the chronic phase occurs when immune system failed to resolve the inflammation response or due to continues exposure to the stimuli and is marked by dramatically increased production of inflammatory mediators [24]. Carrageenan- induced paw oedema in rats is a biphasic response; the first phase (0-1 hr) involves the release of serotonin and histamine while the second phase (over 1 hr) is mediated by prostaglandins, the cyclooxygenase products [25] and the continuity between two phases is provided by cytokines IL-1, IL-6, IL-8, tumour necrosis factor (TNF-α) as well as complement factors C5a and C3b that cause vasodilatation, increased vascular leakage and neutrophil recruitment into inflamed site [26]. The results of control group in this study showed that paw oedema started to appear 30 minutes after sub-plantar carrageenan injection and continuously increase to reach peak at hour 5 post injection then started to decrease and recovered after 48 hr. On the other hand, animal groups treated with 95% ethanol extract of Moringa leaves, and to less extent water extract, showed an inhibitory effect on both phases of inflammation. The inhibitory effect of 95% ethanol extract of Moringa leaf on the first phase of inflammation could be due to inhibition of the serotonin and histamine mediated effect and on the second phase could be due to the suppression of nitric oxide production and inhibition prostaglandins synthesis as suggested by the mechanism of oedema formation by carrageenan. The suggested mechanism of action are in coordinate with finding of Arulselvan *et al.,* and Sudha P *et al.,* [19, 27] which attributed the oedema inhibitory effect and anti-inflammatory activity of Moringa to suppression of nitric oxide and proinflammatory cytokines, like IL-6, IL-1β and TNF-α, inhibited the expression of inflammatory mediators including cyclo-oxygenase-2 (COX-2), inducible nitric oxide synthase, and nuclear factor (NF)-κB p65 through suppression of the NF-κB signalling pathway. This anti-inflammatory activity can be attributed to various phytochemicals like flavonoids which detected in Moringa leaf. Maximum oedema inhibitory effect of 95% ethanol extract was observed at hour 3 post carrageenan injection with activity better than indomethacin, while dichloromethane fractions the effect started much earlier at hour 1 post carrageenan injection.

Low dose of 250 mg/kg of 95% ethanol extract appear to be more active than higher doses of 500 and 750 mg/kg. One suggested reason for that is the presence of phytate and other anti-nutrients which can reduce the bioavailability of certain phytoconstituents by its ability to inhibit the gastric absorption [6]. Another possible explanation is that at higher dose concentration more Moringa extract was suspended in the same vehicle volume leading to higher viscosity of administered extract suspension which may slow down solubility of bioactive phytoconstituent(s), prolong dissolution rate, decrease absorption and eventually, delay the pharmacological activity. In a study conducted by Sun J. [28] using mice shown that a methanol extract of *M. oleifera* leaves given orally at doses of 250 and 750 mg/kg stimulated both cellular and humoral immune responses and low dose was found to be more effective than the high dose of the extract. In the field of natural products drug discovery, plant extractions process with the appropriate solvent system is very essential to identify and isolate the pharmacological compounds from medical plants.

The TLC and phytochemicals screening of Moringa extracts revealed that 95% ethanol is a good extraction solvent since it extracted more phytochemicals and in higher concentration than do water or 50% ethanol.

Of that 18 compounds identified by LC/MS, two compounds, i.e. isoquercetin and cryptochlorogenic acid, were well known for their anti-inflammatory and anti-oxidant activity [29]. The result of GC/MS point out the need of sample pre-treatment like derivatisation or acid hydrolysis in order to clave the glycan and aglycan rings [30]. Many compounds present in *M. oleifera* are glycosides or attached to sugar moiety and need to open or detach the ring to be amenable to GC/MS analysis.

Conclusion

Carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation. The results of this study showed that 95% ethanol extract of *M. oleifera* leaf possesses a considerable anti-inflammatory activity against carrageenan- induced paw oedema and more active than 50% ethanol and water extract. In conclusion, the actions of extract upon the inflammation models tested justified its utility in herbal medicine for treatment or mitigation of inflammation. Furthermore, isolation and identification of the compound responsible for biological activity need to be explored and extensive studies are required to elucidate the exact mechanism for anti-inflammatory activity of *Moringa oleifera* leaf extract so that new potent and safe anti-inflammatory agents can be developed from it.

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