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Assessment of the analgesic, anti-inflammatory and sedative effects of the dichloromethanol extract of *Schinus molle*

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Abstract. – OBJECTIVE: Determination of the active fraction and compounds of the dichloromethanol extract of *Schinus molle* seeds and evaluation of their biological effects.

MATERIALS AND METHODS: Dried seeds of *Schinus molle* were sequentially extracted in hexane, acetyl acetate and dichloromethane. The dichloromethane extract was separated into two fractions (1 and 2) by column chromatography. Fraction 2 was further separated into its two constituent compounds which were characterized as belonging to the lanosteroid group of compounds. Both factions were tested for their analgesic, anti-inflammatory and sedative effects.

RESULTS: The two fractions significantly increased ($p<0.05$) the tail flick latency though fraction 2 provided better and more long lasting protection against thermal pain. On the other hand, the anti-inflammatory effect of ibuprofen, though inferior to the anti-inflammatory effect of fraction 2 was better than the effects of fraction 1. Fraction 2 significantly ($p<0.01$) reduced rat paw oedema compared to the saline treatment group throughout the experiments while fraction 2 compared to fraction 1 showed significantly ($p<0.01$) greater inflammatory effects. On the other hand both fractions lacked significant sedative effects.

CONCLUSIONS: Given that fraction 2 had only two constituent compounds (isomasticadienonic and Masticatrienonate), one or both of these compounds should be contributing to the observed analgesic and anti-inflammatory effects.

Key Words:
Schinus molle, Lanosteroid, Isomasticadienonic, Masticatrienonate, Analgesic, Anti-inflammatory, Sedative effects.

Introduction

The treatment of pain remains a challenge to modern medicine as most analgesic drugs, though effective, are limited for use on a chronic basis. Adverse effects of common pain-relieving drugs such as the non-steroidal anti-inflammatory drugs (NSAIDs)[1] are associated with gastric distress and cardiovascular complications[2]. Thus, NSAIDs are restricted to short-term use, although patients with chronic conditions may require daily dosages over prolonged periods or even for life. Consequently, the recent years have seen a return to medicinal plants for treatment of various conditions including pain and inflammation, based on the perception that plant-based medications are more effective, have lower toxicity and can be used for prolonged periods without the health risks associated with pharmaceutical drugs[3,4]. More so, pharmaceutical companies are in search of plants, which may provide potential seed molecules for the development of novel drugs with better efficacy and lower toxicity than current drugs.

Schinus molle is a plant belonging to the family Anacardiaceae. It is native to South America but has been introduced to other tropical and subtropical areas, including parts of South Africa where this species is listed among invasive plants[5]. Traditionally, *Schinus molle* is used to treat wounds and bacterial infections, and al-

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so as an anti-depressant, diuretic and for toothache[5]. Essential oils from *S. molle* have reportedly shown antibacterial[6] and antifungal effects[7]. Other studies have indicated that this plant also has hypotensive[8], anti-inflammatory[9], analgesic[10] and insect repellence[11] effects. A recent article reported that monoterpenes were the predominant constituent of essential oils isolated from the leaves and fruit of *S. molle*; the oils showed anti-oxidant, antibacterial and antifungal properties[12]. The aforementioned studies were carried out with essential oils and hexane extracts of *S. molle* from countries other than South Africa. Environmental influences on plants often result in chemotypes which differ in quantity and quality of constituent compounds in similar species; thus highlighting the importance of investigating the chemical composition of *S. molle* grown in South Africa and to determine some of its biological effects. The aim of this study was therefore to determine the analgesic, anti-inflammatory and sedative effects of the dichloromethane extract fraction of *S. molle* and its constituent fractions.

Materials and Methods

Extract Preparation

Schinus molle seeds were harvested in May 2013 in Mthatha, South Africa. Authentication of the plant was done by Dr Immelman of the Botany Department, Walter Sisulu University, Mthatha and a voucher specimen deposited in the Botany Department Herbarium. The plant material was air-dried and then ground to powder. 445 g of the ground powder was subjected to sequential extraction with hexane, ethyl acetate, dichloromethane and methanol (Figure 1). The dichloromethane extract provided the highest yield, a cream to light yellow extract of 22.50 g. Furthermore, column chromatography of this extract showed distinct spots making it the extract of choice for additional investigation.

The dichloromethane extract fraction was subjected to column chromatography and yielded two fractions (fraction 1 and fraction 2) and was therefore selected for further separation and biological tests. Column chromatography of fraction

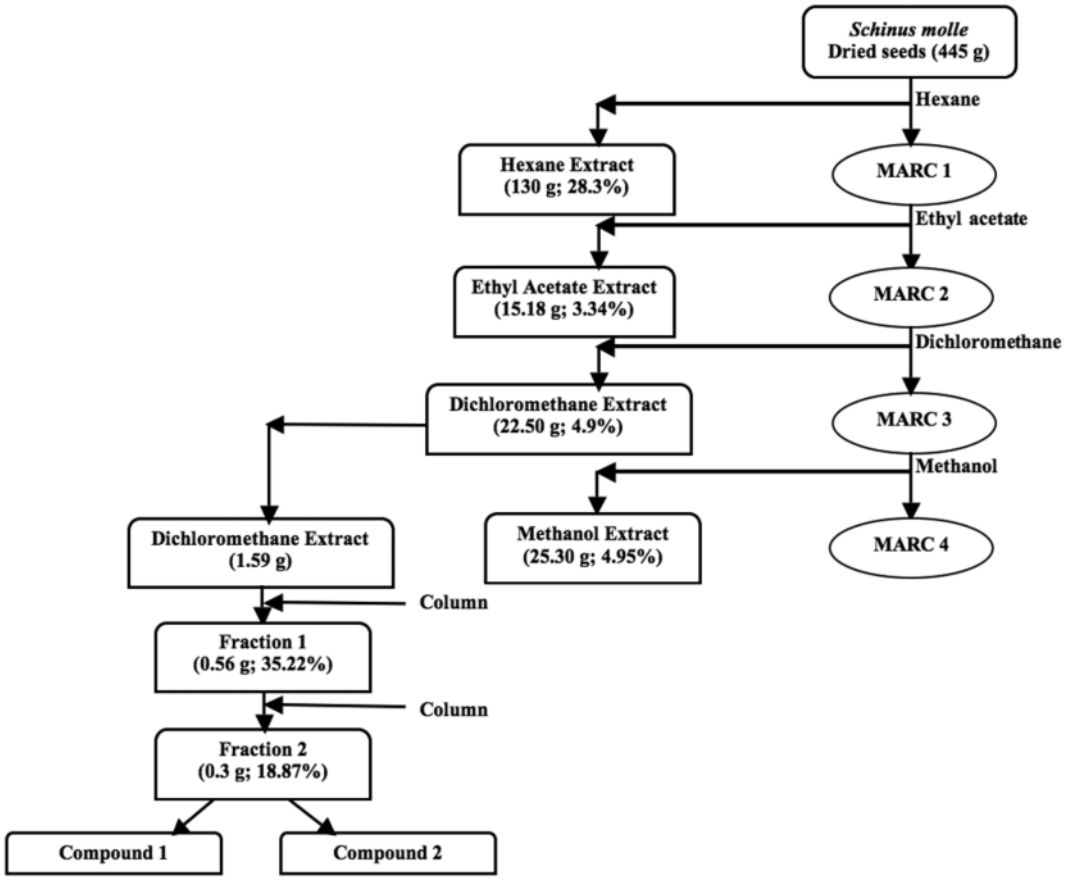

Figure 1. Summary of sequential extraction process of S. molle seeds.

2 led to the isolation of two compounds whose chemical structures were elucidated using 2D NMR techniques.

Fraction 1 and Fraction 2 were tested for analgesic, anti-inflammatory and sedative effects. However because quantities of isolated compounds were too small, their analgesic, anti-inflammatory and sedative effects could not be investigated.

Biological Tests

Experimental Animals

Sprague Dawley rats and Swiss mice were procured from the South African Vaccine Initiative and housed in the animal holding facility in the Faculty of Science. Room temperature was maintained at 24°C while lighting was exclusively by daylight. All animals had free access to food and water except during the experimentation period. Ethical approval for this study was obtained from the Walter Sisulu University Ethics Committee Ref No: Ethics 0009-07 which is in accordance with the guidelines established by the European Union Animal Care (CEE Council 86/609).

Drugs, Reagents and Extracts

Ibuprofen was obtained from Pfizer, South Africa, diazepam from Roche, Basel, Switzerland and ketamine from Rotex-Medica®, Tritau, Germany. All other reagents used were of analytical grade. Fresh egg albumin was prepared from fresh eggs.

Tail Flick Analgesia Test

The analgesic effects of the dichloromethane fractions of *S. molle* were investigated using the tail flick method as described by Nkeh-Chungag et al[13]. Briefly, the animals were lightly restrained and the distal third of the tail placed over the window slit of the Ugo Basile Tail Flick Analgesy-meter (Model: 37450; Varese, Italy) and the IR emission switched on. The animal flicked its tail when it felt pain. The flicking of the tail away from the window slit signalled an automatic cut-off of the timer and latency was displayed in seconds. The maximum time allowed for an animal to respond to the heating of the tail was 20 s. Pre-treatment latencies were determined for all animals before administration of plant extract fractions (fraction 1: 150 mg/kg; fraction 2: 40 mg/kg), ibuprofen (20 mg/kg) or an equivalent volume of physiological saline to groups of five mice each. Tail flick latencies were again determined at hourly intervals after treatment for 5 hours. Results were expressed as the mean difference between post-treatment tail flick latency at different times and pre-treatment of baseline tail flick latency.

Fresh egg albumin-induced Anti-inflammatory test

Sprague Dawley rats weighing 200-240 g were randomly assigned to one of the treatment groups of six animals each. Baseline right hind paw volumes were determined plethysmographically using the Ugo Basile plethysmometer (model: 7140; Varese, Italy). Each treatment group received one of four treatments: dichloromethane extract fraction 1 (150 mg/kg), dichloromethane extract fraction 2 (40 mg/kg), ibuprofen (20 mg/kg) or normal saline (0.09% NaCl) orally. Thirty minutes after oral treatments, the right hind paw of rats was injected subplantarly with 100 μl/rat of freshly prepared 50% v/v egg albumin and right hind paw volumes measured hourly for 5 hours. Inflammation was determined by computing the difference between the right hind paw volume at predetermined times after albumin injection – right hind paw at baseline volume.

Sedative test

Hypnosis was induced as described by Mimura et al[14] using ketamine (100 mg/kg, *i.p.*). The mice were randomly divided into 4 groups (n=6). Group 1 received the vehicle (5% DMSO), groups 2 and 3 received fractions 1 (150 mg/kg) and 2 (40 mg/kg) respectively, while group 4 received diazepam (2 mg/kg) and served as the positive control group. All the animals were orally pre-treated for 60 min prior to ketamine injection. The parameters assessed were sleep latency (SL), which is the time from injection to loss of righting reflex; and total sleeping time (time from loss of, to regaining of righting reflex).

Statistical Analysis

GraphPad Instat® (GraphPad Software, Inc., San Diego, CA, USA, www.graphpad.com) was used to perform ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons. The level of significance was set at $p<0.05$. Results were expressed as mean ±SEM.

Results

Elucidation and Identification of Isolates

The 13C NMR showed 30 carbons with 7 CH_3, 9 CH_2, 6 CH, two carbonyl C and 6 qua-

Table I. NMR data for compounds 1 and 2.

Number of carbon	d ^{1}H-NMR	d ^{13}C-NMR		d ^{1}H-NMR	d ^{13}C-NMR
	Compound 1		Ref	Compound 2	
1		35.6	35.51		
2		34.9	34.55		
3	-	216.9	218.32		52.8
4		47.8	47.23		37.3
5		52.3	51.42		
6		24.3	20.22		
7	6.05 *dd*	117.8	27.42	5.95 dt	117.84
8	-	147.08	134.63	-	125.74
9		52.8	132.61		
10		38.5	37.09		43.4
11		21.9	28.01		
12		28.2	30.65		14.13
13		43.5	49.99		129.54
14		51.1	49.99		126.24
15		33.6	29.74		
16		20.5	21.32		
17		48.4	49.99		
18	0.78 (s)	18.2	15.51	0.72 (s)	18.19
19	0.87 (s)	12.7	20.51	0.81 (dd), 1.59 (m)	17.95
20	1.40 (q)	34.0	36.39		
21	0.86 (d)	18.2	18.53	1.68 (2H, m)	14.13
22		35.0	35.80		
23	1.02	27.4	26.83		145.38
24	5.27-5.28 *dd*	145.97	147.35		146.11
25		125.7	125.81	-	170.85
26		172.23	173.40		20.69
27	1.89 (s)	20.5	19.74		21.78
28	1.09 (s)	21.6	21.06		12.95
29	0.98 (s)	26.9	26.65	3.5 (OCH3)	76.26
30	0.98 (s)	24.5	24.15		

ternary carbons for compound 1. The IR spectrum of compound 1 revealed the presence of three major functional groups which were ketone at 1683 cm^{-1}, carboxylic at 1706 cm^{-1} and C=C 1620 cm^{-1}. This information suggests that compound 1 has a lanosteroid skeletal structure (Figure 3). 2D NMR assisted in further elucidating the structure. Compound 1 was then proposed to be an isomasticadienonic acid. The presence of two olefinic protons at d 5.27-5.28 and 6.05 doublet of double bonds confirmed the fact that one of the carbons was next to a CH_2. Comparing the NMR data (Table I) with data from the literature by Paraschos et al[15] we were, therefore, able to establish that the compound was isomasticadienonic acid. Indeed, isomasticadienonic acid has been reported as one of the constituent molecules of *S. molle* seed[9].

The NMR spectra for compound 2 indicated the presence of 29 carbon atoms with 6 CH3, 9 CH2, 7 CH and 6 olefinic carbons suggesting that there are three double bonds. Unlike compound 1, the absence of a carbonyl ketone was noted in the FTIR spectrum which was replaced by an ester carbonyl carbon at 1695 cm^{-1}. A lanosteroid skeletal structure fitted perfectly into the above description. The presence of 3 protons at d 5.23 and 5.95 along with 6 carbons at 115.74 ppm, 117.84 ppm. 126.24 ppm, 129.54 ppm, 154.38 ppm and 146.11 ppm confirms the presence of the 3 C=C in the

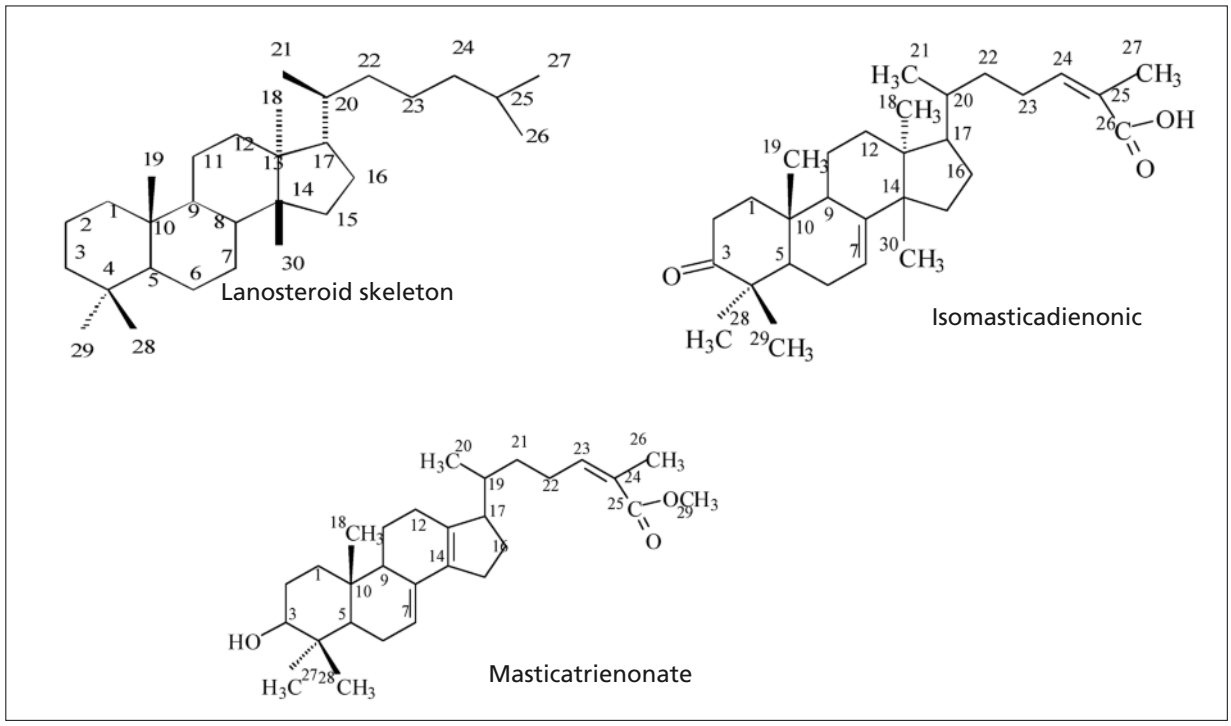

Figure 2. Chemical structures and proposed names of isolated compounds.

compound. The ester carbonyl carbon was 170.85 ppm while the OCH3 of the ester was 76.29 ppm. To the best of our knowledge, this compound has not being reported before. The name masticatrienonate was assigned to compound 2 (Figure 2).

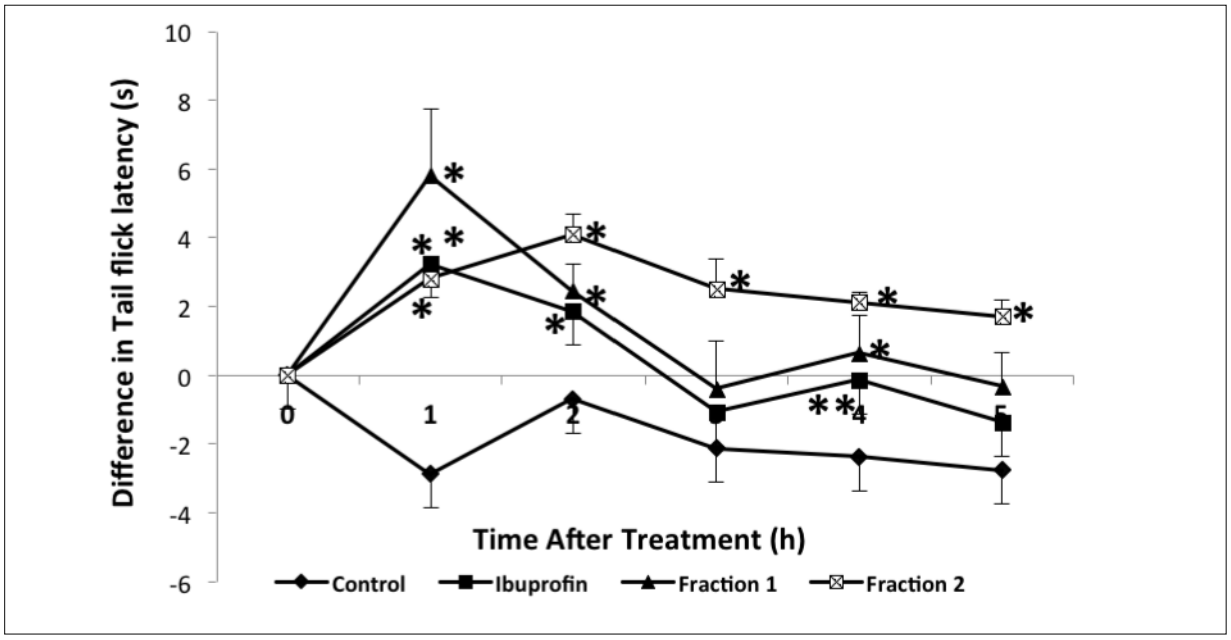

Figure 3. Analgesic effects of Fraction 1 and Fraction 2 of the dichloromethane extract of S. molle using the tail flick test. Points represent mean data from five mice while vertical bars represent the standard error of the mean. Where Δ is difference in tail flick latencies. $^{*}p<0.05$, $p<0.01$ compared to the control values.

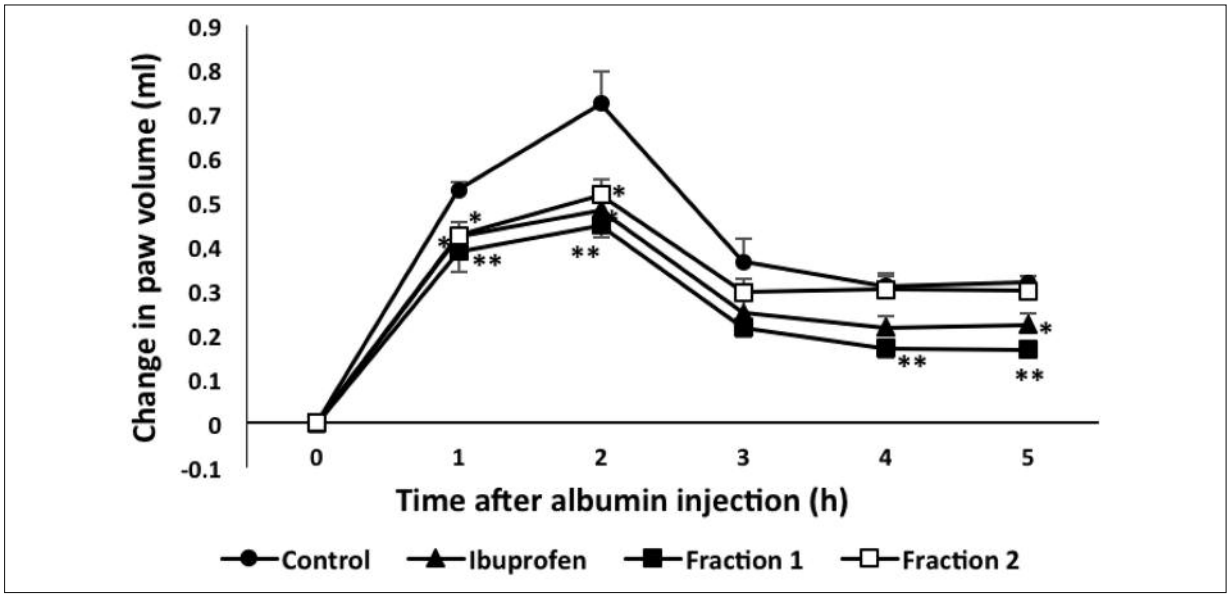

Figure 4. Effect of Fractions 1 and 2 on egg albumin-induced rat paw oedema. $^{*}p<0.05$, $^{**}p<0.01$, statistically different from control.

Analgesic Effects of the Extract Fractions

The analgesic effects of ibuprofen and fraction 1 were highest at 1 hour after treatment. For Ibuprofen, fractions 1 and 2 of the dichloromethane extract showed significant analgesic effects during 1-2, 4 hours after treatment after which there was a rapid decline in the pain-relieving effects of both ibuprofen and fraction 1. On the other hand, fraction 2, which is prepared from fraction 1, showed sustained analgesic effects from 1-5 hours after treatment showing a progressive decrease in activity after peaking at 2 hours post-treatment (Figure 3).

Anti-inflammatory Properties

Paw inflammation peaked at 2 hours after albumin injection and then resolved itself progressively. Ibuprofen and the two extract fractions significantly ($p<0.05$, $p<0.01$) reduced albumin-induced inflammation during 1 and 2 hours after albumin injection. During the third hour, however, treatment did not seem to confer significant protection against the effects of the phlogistic agent. Nevertheless, during the 4 and 5 hours, fraction 2 again showed significant ($p<0.01$) anti-inflammatory effects compared to the control group. The anti-inflammatory effects of ibuprofen were better than those of fraction 1 but weaker than the effects of fraction 2. Indeed, fraction 2 showed significantly ($p<0.05$ and $p<0.01$) better anti-inflammatory effects than fraction 1 during 4 and 5 hours after the albumin injection respectively (Figure 4).

Sedative Effect of Fractions 1 and 2

Fraction 1 and 2 did not alter sleep latency and total sleeping time induced by ketamine compared to the negative group. However, diazepam (2 mg/kg) caused a non-significant decrease in sleep latency but induced significant ($p<0.05$) prolongation of total sleeping time on ketamine-induced hypnosis (Figure 5).

Discussion

We evaluated the analgesic, anti-inflammatory and sedative effects of two fractions from the dichloromethane extract of *S. molle* and showed that fraction 2 was responsible for the observed effects of the extract.

Fraction 2 contained two compounds which were identified as isomasticadienonic acid and masticatrienonate. The former compound has been isolated and characterised from *chios mastic* gum[15,16]. Indeed, it was isolated from *S. molle* as early as 1978[17] though its biological effects were not evaluated. Compound 2, on the other hand, has not yet been described in the literature and is therefore considered a novel compound. However, due to the low yield of the constituent compounds of fraction 2, they were not used for biological tests.

Our results showed that both fractions 1 and 2 of the dichloromethane extract of *S. molle* induced a higher tail-flick latency in mice compared to both ibuprofen and saline treated animals. The tail-flick method relies on an automated recording system of tail withdrawal latencies in response to a thermal stimulus. This model is sensitive to central acting drugs such as the opioid analgesic[18] and only poorly sensitive to the non-steroidal anti-inflammatory drugs (NSAIDs)[19]. Indeed our study showed that ibuprofen, a NSAID, conferred minimal protection against heat-induced pain. Thermal stimuli are generally transmitted from the periphery via C fibres to the spinal cord for integration. Opioid analgesics act by preventing the relay of pain stimuli to upper centres and thus prevent pain sensation[20,21] while NSAIDs prevent pain by reducing the sensitivity of peripheral nociceptors to prostaglandins and nerve sensitisation[22]. Fraction 2 probably has a different mechanism of action compared to that of ibuprofen. Indeed, the chemical structure of the constituent compounds of fraction 2 shows that it contains a steroidal compound and may have anti-inflammatory effects similar to those of steroidal compounds.

Albumin-induced inflammation like carrageenan-induced inflammation occurs in two phases. The first phase may last from 1 to 2 hours while the second phase may last for up to 6 hours after injection of the phlogistic agent. The first phase is caused by the release of histamine, serotonin and bradykinin from mast cells and resident macrophages. These chemicals increase vascular permeability and leukocyte migration thus facilitating movement of fluids into interstitial spaces. The second phase however, involves the synthesis of prostaglandins from arachidonic acid with the involvement of the enzyme cyclooxygenase 2 (COX-2), IL 1β, TNF-α among others[23,24]. These proteins tend to increase the inflammatory response. NSAIDs treat inflammation by inhibiting COX-2 thus reducing the amount of prostaglandins synthesised. These drugs also inhibit leukocyte adhesion and migration and decrease cytokine production[25,26]. Ibuprofen and both extract fractions showed significant anti-inflammatory effects 1 hours after albumin injection. However, fraction 2 alone was able to provide sustained anti-inflammatory effects beyond the first hour indicating that it was able to inhibit both phases of the albumin-induced inflammation. These findings indicate that either one or both constituents of fraction 2 could be responsible for the observed analgesic and anti-inflammatory effects of the extract.

Fractions 1 and 2 of the dichloromethanol extract failed to influence sleep latency and total sleeping time. Decrease in sleep latency and prolongation of total sleep time on ketamine-induced hypnosis indicate sedative effect[27]. Hence, it can be suggested that fractions 1 and 2 tested in this study lack sedative activity. Many standard analgesic and anti-inflammatory agents such as paracetamol, diclofenac and aspirin do not normally possess sedative activities but they are nonetheless very potent and effective in managing pains and related conditions. It is also possible for plant extract to possess analgesic and anti-inflammatory activity but lack sedative activity as reported for the mushroom *Pleurotus florida*[28]. Analgesics devoid of sedative effect could be of benefit as its use during the day would not interfere negatively with normal working activities.

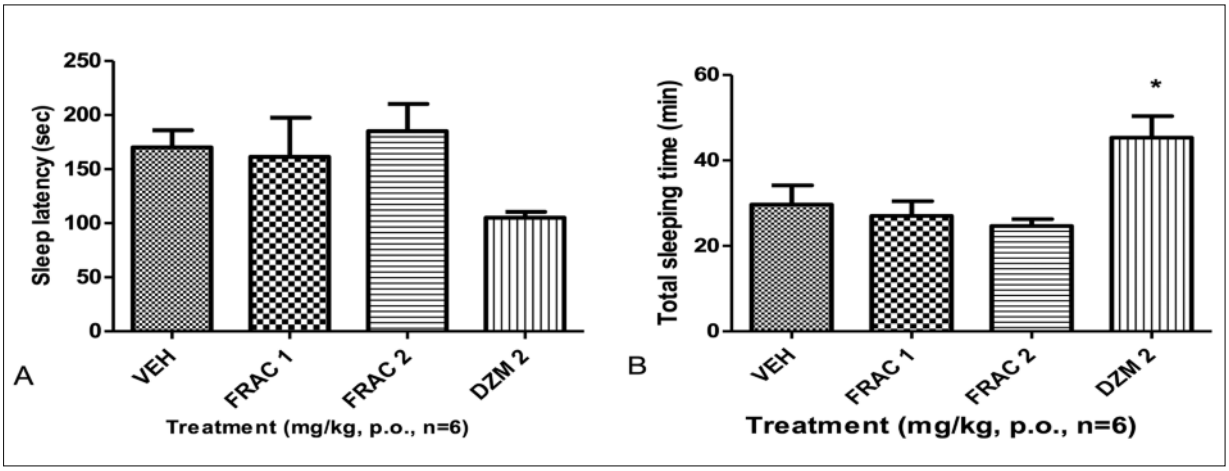

Figure 5. Effect of fraction 1 and 2 on ketamine-induced hypnosis. ***A***, sleep latency; ***B***, total sleep time. Each bar represents mean±SEM. VEH: vehicle (5% DMSO); FRAC: fraction and DZM: diazepam. $^{*}p<0.05$.

Conclusions

We isolated and characterized the constituent compounds of the dichloromethane extract of *Schinus molle*. These compounds were identified as isomasticadienonic and masticatrienonate. The extract fraction containing these compounds showed potent analgesic and anti-inflammatory effects in rat models. Either or both of these compounds may contribute to the purported analgesic and anti-inflammatory properties of *Schinus molle*. Future work will involve isolation of these compounds in sufficient amounts to evaluate their analgesic and anti-inflammatory effects.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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