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Phytochemistry and anti-inflammatory activity of ethanolic root bark extract of *Vepris nobilis* Mziray (Rutaceae family)

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ABSTRACT

Root bark powder of *Vepris nobilis* Mziray (family Rutaceae) collected from Mukura Sub-county, Ngora District, Eastern Uganda, about 300 km from the Capital City Kampala was extracted with 70% ethanol. Phytochemical analyses was carried out as well as in vivo anti inflammatory animal tests. The GC–MS analysis identified 37 compounds in the extract including fatty acids (45.08%), sesquiterpenes (40.38%), furaquinoline alkaloids (10.93%), a phenylpropanoid (2.37%) and a triterpenoid (1.31%). Interestingly, it revealed that at 400 and 600 mg of extract / kg of body weight, formalin induced paw oedema in mice was inhibited much better than by 25 mg of Diclofenac sodium /kg body weight. It was concluded that ethanolic root bark extract of *V. nobilis* has important phytochemicals that need to be evaluated for anti-inflammatory activity as potential lead compounds for anti-inflammatory drug development.

Introduction

The genus *Teclea* Delile subsumed into *Vepris* Mziray constitute plants widely used for medicinal purposes. About 30 species formally in *Teclea* genus are known to exist in Africa, and the most common include *Vepris nobilis, V. trichocarpa, V. natalensis, V.gerrardii, V. occidentalis, V. amaniensis, V. grandifolia, V. hanangensis,* V. simplicifolia, *V. afzelii* and *V. ouabanguiensis* [22].

Vepris nobilis Mziray, formerly *Teclea noblis* Delile (family Rutaceae) is an ever-green plant growing to a height of 3–12 m in forested areas. The leaves are a remedy for managing pain, fever, chronic cough and weight reduction [16]; and the stem bark is used to treat gonorrhoea [20], cough, chest disorders, pneumonia, weight loss, syphilitic and ulcers [3], and dental

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caries [25]. The root bark on the other hand is used to treat rheumatism, arthritis, pneumonia [22], cough, fever, measles, malaria [21], and as anthelmintic [28].

Phytochemical analyses have indicated presence of alkaloid compounds in the leaves [39,5], fruits [17], roots [24], stem bark [11]; sesquiterpenes in the leaves [4]; and essential oils in the roots [25] and leaves [3].

Pharmacological analyses indicated antipyretic, analgesic and anti-inflammatory activities in the leaves [2,3,20]. Antimicrobial activity has also been conducted on the leaves [3,16,27]; stem bark [11] and roots [25,24]. Anti-malarial activity has been reported on the leaves [19] leaves and fruits [17]. Anti-leishmanial and anti-trypanosomal activity was reported on the leaves and fruits [17] while schistosomicide activity was reported on the leaves [23]. This study evaluated the phytochemical and anti-inflammatory activity of the ethanolic extract of *V. nobilis* root bark.

Materials and methods

Collection and identification of plant material

Vepris nobilis root barks (2 kg) were collected from Mukura Sub-county, Ngora District, Eastern Uganda, about 300 km from the Capital City Kampala. The plant was authenticated by a taxonomist at the Department of Botany, Natural Chemotherapeutics Research Institute (NCRI), Ministry of Health, Kampala, Uganda. A voucher specimen (NCRI/O.F./001) was deposited in the herbarium of NCRI.

Preparation of plant material

The collected root bark material was cleaned with tap water to remove soil. The bark was peeled and reduced into small pieces which were then dried in a vacuum oven at a temperature of 40–50 °C for 72 h. The dried root bark pieces were then pulverized into fine powder by using an electric grinder, and stored in an airtight container.

Extraction of plant material

Five hundred grams (500 g) of the root bark powder was extracted by maceration in 70% ethanol (2.5 L) for 72 h with occasional shaking. The resultant solution was then filtered using Whatman filter paper No.1.

Phytochemical analyses

Qualitative screening for phytochemicals

Ethanol extract of *V. nobilis* (100 mL) was screened for tannins, reducing compounds, alkaloids, steroid glycosides, anthracenosides, coumarin derivatives, flavonoids, saponins, polyuronides and glucides. Tannins were tested with Ferric chloride test, reducing compounds with Fehling's test, alkaloids with Mayer's reagent test, steroid glycosides with Liebermann-Burchard's test, anthracenocides with Ammonia solution test, courmarin derivatives with Ammonia solution and UV light test, flavonoids with Shibata's reaction test, starch with Iodine solution, glucides with Molisch's reagent test, polyuronides with Ethanol and Methylene blue test and saponins with Frothing test [15]. The level of presence of the phytochemical was indicated by the intensity of colour change i.e. (++) more presence or abundance (+) less presence or less abundant and (-) not present.

Separation and identification of compounds in V. nobilis

Thin layer chromatography (TLC) and GC–MS were used in the separation and identification of compounds in *V. nobilis*. The extract (10 mL) was evaporated to 2 mL, and then 5 mL of 2% HCl was added. The pH of the solution was raised to 9–10 with 25% ammonia solution. The alkaline solution was extracted using a separating funnel with chloroform (3 × 5 mL) and dried with magnesium sulphate [8]. The chloroform layer was concentrated in a rotary evaporator to 1 mL for compound separation and identification using TLC and GC–MS.

TLC separation and identification of compounds

The organic extract was spotted on precoated TLC plates (5 × 10 cm). The plates were developed using the solvent system; n-hexane-ethyl acetate-methanol-25% ammonia solution (3:3:1;0.1). The spots on the plates were visualized under UV light (364 nm) and after spraying with Dragendoff's reagent to confirm presence of alkaloids [15].

GC–MS separation and identification of compounds

The GC–MS analysis was carried out using Shimadzu, model TQ 8040 triple quadruplet equipped with a split injector (Split ratio 1:0) at 250 °C using the method by Erdemoglu et al., [13] with some modifications. In brief, the chloroform layer 1.0 µL aliquots were injected directly into the column -ZB-5SMi, 30 *m* × 0.25 mm × 0.25 µm 50 *m* × 0.32 mm. The column temperature program was employed with an initial temperature of 80 °C, held for 20 min, followed by a temperature increase of 5 °C /min to 180 °C, then held for another 5 min to 250 °C, and 15 min to 310 °C. Helium was employed as the carrier gas at an average linear velocity of 44 0.5 cm/*sec*, prime pressure of 500–900 kPa. The flow control mode had

pressure at 99.8 kPa, total flow of 50 mL/min, column flow of 1.46 mL/min for separation, linear velocity of44.5 cm/*sec*, and purge flow of5.0 mL/min. Detection range *m/z* was 40–600 and mass range (*m/z*) was 20–440. Furthermore, the detector temperatures were 250 °C and 300 °C, respectively. Electron impact (EI) ionisation was carried out at 70 eV and the ion source temperature was 230 °C. Data were processed on GC–MS and compounds were identified based on comparison of mass spectral fragmentation with those in the NIST in the GC–MS library.

Anti-inflammatory activity

Laboratory animals

Male albino mice were obtained from the Natural Chemotherapeutic Research Institute, Kampala, Uganda. Mice were given free access to food and water, and were kept in the laboratory at room temperature with a 12-hour light/dark cycle. Clearance to use laboratory animals for the experiment was obtained from the Institutional Pharmacology Review Board (PRB) at the NCRI.

Preparation of the extract for anti-inflammatory activity analysis

The previously prepared ethanol extract of *V. nobilis* was concentrated using a rotary evaporator at a temperature of 40–50 °C and further dried in the vacuum oven at the same temperature range. The dry crude extract was then reconstituted with distilled water for determination of anti-inflammatory.

Administration of the drug

Paw oedema technique was used to evaluate the anti-inflammatory activity [7]. ·Formalin induced paw oedema was used because it is one of the most suitable test procedures to screen chronic anti-inflammatory agents, since it closely resembles human arthritis [14]. Further, the nociceptive effect of formalin is also biphasic, an early neurogenic component followed by tissue mediated response [38]·

Male albino mice (13–20 g) were randomly selected from their cages and divided into 6 groups (A-F), each consisting of five individuals ($n = 5$). These included A (positive control group), B-E (test groups) and F (negative control group). The positive control group (A) received 25 mg/kg body weight diclofenac sodium, test groups received *V nobilis* extracts at dose of 100 (B), 200 (C), 400 (D) and 600 (E) mg/kg body weight, and the negative control received distilled water 1 mL/kg body weight.

The paw volume of the mice was measured up to the tibiotarsal junction using a Vernier calliper one hour before subcutaneous injection of formalin in the hind paw using gastric canula. Acute inflammation was induced by injecting 0.2 mL/kg of 2% v/v freshly prepared formalin into the subplantar surface of the right hind paw. Increases in the linear diameter of the right hind paws were taken as an indication of paw oedema, and hind paw size was measured after 30, 60, 120 and 180 min. The average size of paw oedema for each group was obtained. Percentage inhibition of the oedema was calculated using the formula: $(C_o - C_t) / C_o \times 100$, where C_o is the average inflammation (hind paw oedema) of the 'negative control' group A at a given time and C_t is the average inflammation of the test groups and positive control group [26].

Data analysis

The anti-inflammatory tests results were analysed using SPSS software Version 16. The mean values (±SEM) were calculated and differences between the test group and the control were analysed by student's *t*-test to determine significant differences between the control and test groups at significance of $p \leq 0.05$.

Results

Phytochemical analysis

The phytochemicals identified in the ethanol extract of *V. nobilis* were tannins, reducing compounds, alkaloids, steroid glycosides, polyuronides, glucides, starch, coumarin derivatives, and flavonoids (Table 1). Preliminary separation of compounds with TLC analysis technique showed major band spots at R_f values of 0.22, 0.69, 0.78, 0.85 and 0.91 with blue fluorescence visualized under UV light at 254 nm. Except for Rf value =0.22, spots turned yellow in colour after spraying the TLC plate with Dragendoff reagent..

The GC–MS conditions applied provided good resolution for separation and identification of 37 compounds. The major constituents in the extract were fatty acids (45.08%), followed by sesquiterpenes (40.38%), alkaloids (10.93%), phenyl propanoid (2.37%), and triterpenoids (1.31%), respectively. Palmitic fatty acid (18.82%), Germacrene-D-4-ol (7.39%), Neointermedeol (3.90%), Desloratadine (3.55%) were the major fatty acids, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes and alkaloids, respectively. The triterpenoid identified was lupeol (Table 2).

Anti inflammatory activity

The test treatment groups (B-E) that received different concentrations of *V. nobilis* ethanol extract and diclofenac (Group A) showed a marked increase in paw oedema that reached its peak after 30 min from the time formalin was administered,

Table 1
Phytochemical analysis in 70% ethanol root bark extract of *V. nobilis*.

Phytochemical compound	70% Ethanolic root bark extract
Tannins	++
Reducing compounds	+
Alkaloids	++
Alkaloid salts	++
Steroid glycosides	+
Anthracenosides	–
Starch	+
Courmarin derivatives	++
Flavanosides	+
Saponins	–
Polyuronides	++
Glucides	++

Note: + abundant ++, more abundant, - not present.

Table 2
GC–MS analysis of methanol extract of V. nobilis root bark.

Retention time	Compound	Nature of compound	Formula	CAS No	Peak area
6.889	δ -Elemene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	20,307–84–0	0.46
7.240	Longicyclene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	1137–12–8	1.15
7.270	α-Cubebene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	17,699–14–8	1.38
7.363	β-Elemene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	515–13–9	0.76
7.566	Calarene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	17,334–55–3	0.74
7.710	Bicyclo[5.2.0]nonane, 2-methylene-4,8	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	NA	1.11
8.085	(+)-Valencene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	10,219–75–7	0.58
8.171	γ-Muurolene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	30,021–74–0	0.57
8.271	Germacrene D	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	37,839–63–7	2.97
8.573	4- epi–cubebol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	38,230–60–3	2.49
8.584	β-Cadinene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	523–47–7	4.86
8.900	α-Elemol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	639–99–6	3.04
9.105	Germacrene B	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	15,423–57–1	0.41
9.267	Germacrene-D-4-ol	Sesquiterpene hydrocarbons	$C_{15}H_{26}O$	74,841–87–5	7.39
9.705	-(-) Cubenol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	19,912–67–5	0.60
9.815	Isospathulenol	Oxygenated sesquiterpenes	$C_{15}H_{24}O$	88,395–46–4	0.65
10.013	*Tau*-Muurolol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	19,912–62–0	2.78
10.166	α-Cadinol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	481–34–5	2.60
10.288	γ-Cadinene	Sesquiterpene hydrocarbons	$C_{15}H_{24}$	1460–97–5	0.74
10.359	Neointermedeol	Oxygenated sesquiterpenes	$C_{15}H_{26}O$	5945–72–2	3.90
10.906	Cadalene	Sesquiterpene hydrocarbons	$C_{15}H_{28}$	483–78–3	0.39
11.191	Oplopanone	Oxygenated sesquiterpenes	$C_{15}H_{26}O_2$	1911–78–0	0.81
13.735	Methyl palmitate	Fatty acid	$C_{17}H_{34}O_2$	112–39–0	0.82
14.313	Palmitic acid	Fatty acid	$C_{16}H_{32}O_2$	57–10–3	18.82
16.611	Methyl oleate	Fatty acid	C19H36O2	112–62–9	0.85
17.148	Linoleic acid	Fatty acid	$C_{18}H_{32}O_2$	60–33–3	4.36
17.265	Oleic acid	Fatty acid	$C_{18}H_{34}O_2$	112–80–1	17.37
17.651	Stearic acid	Fatty acid	$C_{18}H_{36}O_2$	57–11–4	1.27
17.730	*N*-methylflindersine)	Alkaloid	$C_{15}H_{17}NO_2$	91,421–74–8	0.99
18.519	*Teclea*bine	Alkaloid	$C_{18}H_{19}NO_4$	651,737–85–8	1.05
22.230	Phenol,2,4-bis(1-phenylethyl)-	Phenylpropanoid	$C_{22}H_{22}O$	2769–94–0	2.37
22.448	Skimmianine	Alkaloid	$C_{14}H_{13}NO_4$	484–08–2	3.56
22.848	Oleic Acid-13C18 Glycidyl Ester	Fatty acid	$C_{18}H_{34}O_2$	112–62–9	0.61
24.171	Flindersiamine	Alkaloid	$C_{14}H_{11}NO_5$	522–06–5	1.78
26.894	Desloratadine	Alkaloid	$C_{19}H_{19}ClN_2$	100,643–71–8	3.55
26.978	Lupeol	Triterpenoid	$C_{30}H_{50}O$	545–47–1	1.31
29.270	Glyceryl 1-lactate 3-oleate	Fatty acid	$C_{24}H_{44}O_2$	2027–47–6	0.98
	Nature of Compounds				
	Sesquiterpene hydrocarbons				16.87
	Sesquiterpene hydrocarbons				23.51
	Triterpenoid				1.31
	Fatty acids				45.08
	Alkaloids				10.93

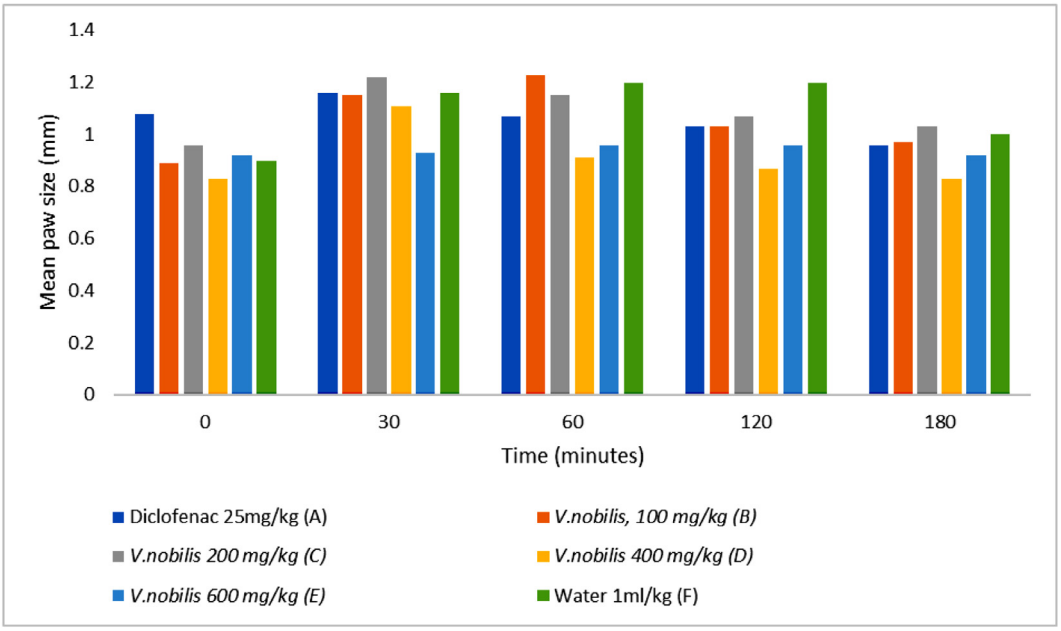


Fig. 1. Effect of ethanolic extract of *V. nobilis* root bark and Diclofenac (25 mg/kg body weight) on induced paw oedema compared to Water (negative control) and Diclofenac (positive control) on albino mice. Values are mean of 5 readings; and 0 min indicates 30 min before inducement of oedema on hind paw.

Table 3
Inhibitory effect of *V. nobilis* methanolic root bark extract and Diclofenac on induced paw oedema.

Treatment	Percentage inhibition 30 min	60 min	120 min	180 min
V. nobilis(100 mg/kg)	16.69 ± 0.72	18.78 ± 0.95	29.26 ± 0.78[b]	37.67 ± 0.71
V. nobilis(200 mg/kg)	11.63 ± 0.74	28.09 ± 1.22	35.40 ± 0.60[b]	39.27 ± 0.94
V. nobilis(400 mg/kg)	39.02 ± 0.67[b]	53.67 ± 1.63[b]	54.77 ± 0.88[b]	57.28 ± 1.40
V. nobilis(600 mg/kg)	22.80 ± 1.10	47.30 ± 1.36[b]	46.30 ± 1.20[b]	48.21 ± 0.99
Diclofenac (25 mg/kg)	17.20 ± 1.08	36.66 ± 1.38[b]	38.61 ± 0.72[a]	43.49 ± 0.67

Values are mean of percentage inhibition and a <0.05, b < 0.0001 are statistically significance different from the positive control group.

and thereafter, experienced a sustained reduction of oedema ($p<$ 0.05–0.001). The extract of 400 mg/kg body weight sustained lowest size of oedema through the 180 min. The mean size of the oedema of the group that received extracts 100 and 200 mg/kg body weight was comparable to Diclofenac sodium 25 mg/kg body weight after 120 min. The negative control group (F) that received distilled water (1 mL/kg body weight) did not experience any reduction in modified paw oedema until after 180 min (Fig 1).

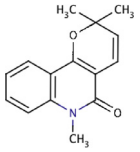
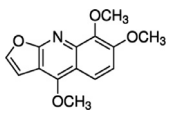
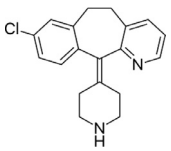
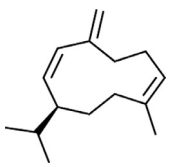
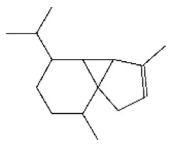
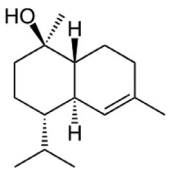
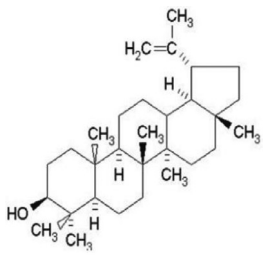
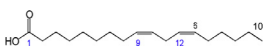
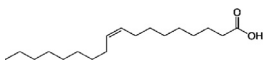
The inhibitory effect of the extract (100 and 200 mg/kg) was not significantly different ($P \leq 0.05$) from that of Diclofenac (25 mg/kg BW) after 30 min. Interestingly, when the plant extract was given at 400 and 600 mg/kg body weight, inhibition of paw oedema had a clearly better effect than Diclofenac (Table 3).

Discussion

The search for anti inflammatory drugs from plants for treatment of pain and inflammation has intensified in recent years.The root bark extract of *V. nobilis* exhibited in this study a strong anti-inflammatory activity at dose levels of at least 400 mg/kg BW after 120 min (two hours) that was higher than that of diclofenac drug (25 mg/kg). It is important to note that during induction of inflammation, the first one-two hours usually involves body release of histamine and serotonine followed by kinins in the third hour, and prostaglandins in the fourth to fifth hour. There is already a strong paw oedema inhibition after 30 min for 400 mg of extract / kg body weight, which was higher than diclofenac. Perhaps, it can be inferred in this study that 400 mg/kg body weight of *V. nobilis* root bark extract inhibited release of histamine and serotonine in the second hour before inhibiting the activity of membrane-associated cyclooxygenase (COX 1) enzyme. Most nonsteroidal anti-inflammatory drugs (NSAIDs) work by inhibiting the activity of COX [33].

Since phytochemicals responsible for anti-inflammatory effect was not known, we conducted phytochemical screening of the extract of *V. nobilis*. Some of the phytochemicals identified in the extract like tannins, coumarins, flavonoids, triterpenoids, glycosides and alkaloids have been recognized for their anti-inflammatory activity [26,30,35]. The phytochemicals identified in this study were similar to those reported for the methanol/chloroform root bark extract of *V. nobilis* [24].

Table 4
Mode of action for anti-inflammatory activity of identified compounds in the root bark extract of *V. nobilis*.

Nature of Compounds	Compounds	Chemical structure	Mode of action	References
Alkaloid	*N*-methylflindersine		Has an inhibition effect against *N*-formylmethionylleucylphenylalanine (f-Met-Leu-Phe) induced superoxide production that is associated with inhibitory effects of prostaglandins.	[9,10]
	Skimmianine		Suppresses pro-inflammatory cytokine production and their upstream events, and inhibits neutrophil infiltration.	[31]
	Desloratadine		Inhibits stimulated superoxide generation.	[1]
Sesquiterpene	Germacrene-D		Exert effect at early stages of inflammation through the central nervous system.	[6]
	α-Cubebene		Attenuates tumour necrosis factor-alpha (TNF-α-), stimulated endothelial adhesion to monocytes by inhibiting intracellular reactive oxygen species (ROS) production, the activation of redox-sensitive nuclear factor kappa beta (NF-κB) transcription factor and expression of VCAM-1 and E-selectin	[12]
	α-Cardinol		Suppresses nitric oxide production by LPS-stimulated macrophages.	[34]
Triterpenoid	Lupeol		Modulates immune system and generation of inflammatory factors,reduces eosinophils infiltration and in Th2 associated cytokines (IL-4, IL-5, IL-13) levels that trigger the immune response in asthmatic condition. Reduces myeloperoxidase activity suggesting mechanism to be related to the neutrophil migration.	[32] [18] [36]
Fatty acid	Linoleic Acid		Inhibits NF-κB activation via activation of peroxisome proliferator-activated receptors (PPARγ), molecular targets of drugs.	[40]
	Oleic Acid		Activates different pathways of immune competent cells and inhibits secretion of phospholipases A1 and A2 (PLAs)	[9]

Anti-inflammatory activity of flavonoids is by inhibition of key enzymes responsible for synthesizing prostaglandins; triterpenoids by reductions of the cells expressing inducible nitric acidsynthase (iNOS) and alkaloids by inhibition of vascular permeability induced by histamine [29]. Alkaloids also inhibit inflammatory signal activation and pro-inflammatory mediator production [37].

Further separation of these phytochemicals using GC–MS confirmed the presence of nine compounds with anti-inflammatory activity (Table 4). Although most of the compounds identified by GC–MS had previously been reported in the aerial parts of *V. nobilis* [5,17,11], anti-inflammatory compounds that had not been reported were desloratadine in alkaloids, α-Cubebene and α-Cardinol in sesquiterpenes as well as oleic and linoleic in fatty acids. This suggests that the root bark extract of *V. nobilis* has anti-inflammatory activity like the aerial parts, though the former registered the effect at higher concentration (400 mg/kg) compared to that of the latter (200–300 mg/kg) as reported by Mascolo et al., [20].

Based on this finding, it is possible to suggest that ethanol extract of *V. nobilis* root bark has anti-inflammatory effects like its aerial parts, thus justifying its use as a traditional medicine remedy for the management inflammatory conditions. However, there is a need to evaluate the anti-inflammatory activity of each identified compound by GC–MS from the root bark as potential lead compounds for anti-inflammatory drug development.

Declaration of Competing Interest

None.

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