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Analgesic and anti-inflammatory activities of 9-Hexacosene and Stigmasterol isolated from *Mondia whytei*

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Abstract

The aim of the study was to ascertain the analgesic properties of *Mondia whytei* roots and to isolate and characterize the active constituents. Bioactivity guided fractionation of the chloroform root extract yielded stigmasterol and 9-hexacosene. Stigmasterol (15 mg/kg) and 9-hexacosene (30 mg/kg) significantly ($p<0.05$) inhibited chemical nociception induced by intraperitoneal acetic acid. Stigmasterol (7.5, 15, 30 and 100 mg/kg) dose dependently reduced the time spent in pain behavior in both the early and late phases of the formalin test. 9-hexacosene dose dependently caused significant ($p<0.001$) antinociceptive effect on the late phase of the formalin test. Co-administration of naloxone failed to antagonize the analgesic activity of stigmasterol and 9-hexacosene in the formalin test. We concluded that both stigmasterol and 9-hexacosene possess potential analgesic effects which are most likely mediated by their anti-inflammatory activities rather than through opioid receptor system.

Keywords: *Mondia whyte;* Analgesic; Anti-inflammatory

Introduction

Mondi whytei (hook f), belongs to the Asclepiadaceae family. It is a forest floor plant with aromatic rhizomatous roots. It is a climber which grows up to 6 m high, leaves broadly ovate with a base cordate, apex acuminate and corona of 11 – 12 mm long (Kokwaro, 2006). *Mondia whytei* is distributed widely in Africa. In Kenya, it is common in wet and humid areas, particularly common in Western Kenya. (Beentje, 1994). *Mondia whytei* root have traditionally been used to treat various diseases and conditions that include pain, swelling,

post partum haemorrhage, diabetes mellitus among many other claims (Kokwaro, 2006 and Jain *et al.,* 1996).

Previous phytochemical studies have identified major groups that include carotenoids, flavonoids, steroids and tannins from the roots (Msonthi, 1994; Mukonyi and Ndiege, 1999). Parasympathomimetic effects of aqueous root extracts on isolated rabbit heart and jejunum have been reported (Githinji *et al.,* 2007). Significant analgesic activity of the aqueous root extract of *Mondia whytei* is well documented (Githinji *et al.,* 2008).

Based on previous investigations, we studied the analgesic activity of the aqueous, chloroform and n-hexane extract representing extracts containing compounds of varying polarity. Defatted methanol root extract was partitioned with chloroform which was then fractionated with 30% methanol in dichloromethane. The first two fractions to elute had the highest antinociceptive activities in both the acetic acid-induced writhing test and the formalin test. The present study was designed to further examine the analgesic activity and the possible mechanism of the chloroform portion of the defatted methanol root extract as well as elucidating its chemical constituents and providing scientific basis for the clinical use of *Mondia whytei* roots.

Materials and Methods

Harvesting of Mondia whytei roots

Roots of *Mondia whytei* were collected from Kakamega forest, Western province of Kenya during the flowering. The plant material was identified in the Department of Botany, University of Nairobi, Kenya. Voucher Specimen number CG/MW/608 was deposited in the herbarium of Department of Botany, University of Nairobi, Kenya. The skeels of the roots were peeled off when fresh and allowed to dry for three weeks under a shade. One kilogram of the root skeels was pulverized using a Molly grinder and the powder stored in a glass container at room temperature awaiting extraction.

Preparation of the defatted chloroform extract, fractionation and characterization

One hundred grams (100 g) of the root powder were put on a Soxhlet evaporator and extracted at 60 ^{0}C for 48 hours using n-hexane (defatting). The residue marc was dried and re-extracted with methanol to yield defatted methanol extract which was then dissolved in methanol: water (1:3) and successively partitioned with n-hexane and chloroform to yield defatted chloroform, aqueous and n-hexane extracts. Each of the three extracts were evaporated to dryness in a rotary evaporator (Ugo Basile, Italy) at 40 ^{0}C and a pressure of 376 Pascals

Twelve grams of chloroform portion of the defatted *Mondia whytei* methanol root extract were then subjected to column fractionation with 30% methanol in dichloromethane as the eluent. The result was five fractions that were then weighed and placed in an amber coloured sample bottles awaiting nociceptive assays. Yield of fraction I and II (0.3 μg) each were solubilized in dichloromethane and then subjected to Gas chromatography–Mass spectroscopy (GC–MS) analysis.

Experimental animals

Adult Swiss albino mice aged 5 – 6 weeks and weighing 20 – 25 g were used. The animals were housed in colony cages with free access to food and water and allowed to acclimatize for one week. They were kept in rooms with temperature of 23 $\pm$ 1.0 ^{O}C and relative humidity of 50%. Diurnal rhythms were regulated with a 12-h light: 12-h dark cycle with lights on 6.00 a.m to 6.00 p.m. Each animal was used only once in the experiment. All the experiments were conducted in accordance with the NIH guide on care and use of laboratory animals (NIH Publication No. 80 – 23; revised 1978). More specifically, pain experiments conformed to the guidelines issued by the International Association for the Study of Pain (IASP) for animal pain experimentation (Zimmermann, M., 1983).

Acetic acid-induced writhing test

Eight mice per group were randomly assigned to receive either stigmasterol (7.5, 15, 30 and 100 mg/kg), 9-hexacosene (7.5, 15, 30 and 100 mg/kg) or the vehicle. Acetic acid-induced writhing test was performed as described by Garcia *et al.,* (2004). The animals did not have access to water and food two hours prior to the experiment. With a minimum restraint, 0.1 ml/10 g body weight of 0.7 v/v acetic acid was administered intraperitoneally. Immediately after the injection of the acetic acid, each animal was isolated and placed in a box (30 x 30 x 30 cm) for observation. The numbers of writhes were counted over a period of thirty minutes, recorded and expressed as the percentage protection of writhing using the following ratio:

(Control mean – treated mean) x 100/control mean.

Formalin test

Eight mice per group were randomly assigned to receive either stigmasterol (7.5, 15, 30 and 100 mg/kg), 9-hexacosene (7.5, 15, 30 and 100 mg/kg) or the vehicle. Formalin test was carried out as described by Abbot *et al.,* (1999) and Bannon and Malmberg (2007). Twenty micro liters (20 µl) of 5% formalin was injected intradermally on the plantar surface of the hind paw of each mouse thirty minutes after administration of the test samples/vehicle. The duration of paw licking (s) as an index of painful response was determined at 0 – 5 min (early phase) and 20 – 30 min (late phase) after formalin injection. Morphine (Sigma Aldrich, Switzerland) was used as a positive control drug and was administrated at the dose of 5 mg/kg, subcutaneously, 30 minutes before the test. Naloxone (Roche, Switzerland) was used in order to elucidate the possible mechanism of action of stigmasterol and 9-hexacosene. Naloxone (1 mg/kg) was administered 15 minutes before stigmasterol, 9-hexacosene or morphine administration.

Assay of dimethyl benzene - induced inflammation in mice

The test samples, vehicle or dexamethasone (Rotexmedica, Germany) was administered intraperitoneally one hour before dimethylbenzene (Sigma Aldrich, Switzerland) topical application to the right ear. The edema was measured 1 hour after dimethylbenzene treatment in accordance with the method described by wei *et al.,* (2004). The ear swelling was measur-

ed by subtracting the weight of the left ear from that of the right. The inhibitory ratio (IR) was calculated as follows: IR = (A – B) x 100/A, where A is edema induced by dimethyl benzene alone and B is edema induced by dimethyl benzene plus sample. Each value was the mean of individual determinations in 8 mice.

Sensorimotor activity testing

The pull-up test (Deacon and Gardner, 1984) was performed in order to verify that the presence of any antinociceptive activity in the extract was independent of any confounding factors like muscle relaxant and sensorimotor retardation effects. According to the procedure, the mice were held in a fully extended inverted position one hour after administration of the test samples/control. The end point of the experiment was set when the mouse in an attempt to gain an upright position touched the hand or fingers of the experimenter with both forepaws simultaneously. The latency to the end point was recorded using a stop watch. The cut-off of the experiment was set at fifteen seconds.

Protocol

In both nociceptive tests, conscious (un-anesthetized) mice were used. The doses of the positive drugs were determined on the basis of their pharmacokinetics and clinical use (Ghelardini *et al.,* 1990; McGaraughty *et al.,* 2005; Shimoyama *et al.,* 1998; Nagy, 1983). The test samples were administered intraperitoneally. The doses selection for the test samples was based on the results of preliminary experiments. Control groups were treated with a similar volume of vehicle that had been used to dilute the test samples.

Statistical analysis

All data were expressed as mean $\pm$ standard error of the mean (s.e.m). Data was subjected to analysis of variance (ANOVA) using the GraphPad Prism^{TM} suite of statistical software. When the analysis was restricted to two means, Student's t-test (paired and one tailed) was used. Scheffe' post hoc test was performed for multiple comparisons. The level of significance was set at $p<0.05$.

Results

Chemical analysis

Fraction I and II derived from the chloroform portion of the defatted *Mondia whytei* methanol root extract yielded stigmasterol (steroid) and 9-hexacosene (long chain hydrocarbon), the first time the two compounds were isolated from *Mondia whytei* roots. The structures were elucidated unambiguously by Gas Chromatography hyphenated with Mass Spectroscopy (GC-MS) Fraction I yielded 342 mg of white crystalline compound. This compound was recrystallized in acetone to give white crystals which were non UV active, turned yellow in iodine chamber and black when sprayed with vanillin in concentrated sulfuric acid. It had retention factor (R_f) value of 0.71 in methanol: dichloromethane (3:7) and melting point of 168 ^{0}C (using Gallenkap apparatus).

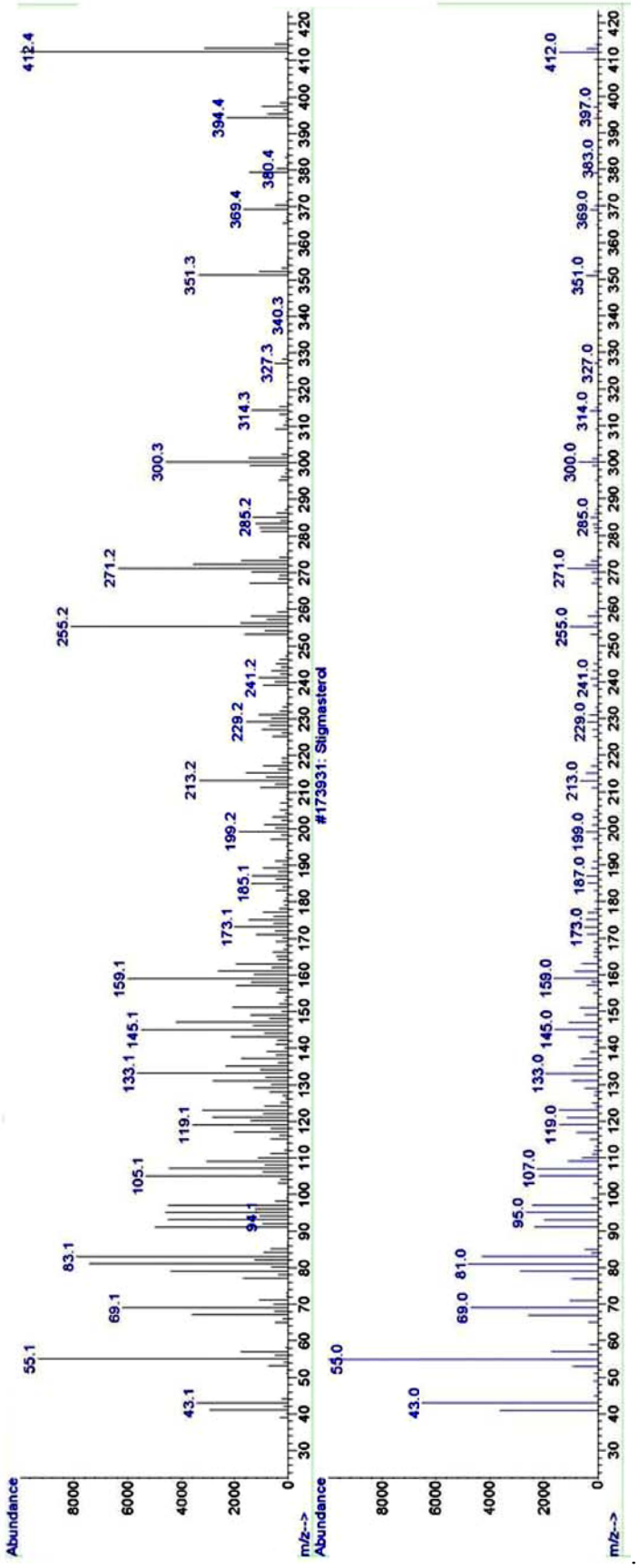

Figure 1. Mass spectrum of C_fMR_d I compared to the mass spectrum of authentic stigmasterol based on NIST05a library data.

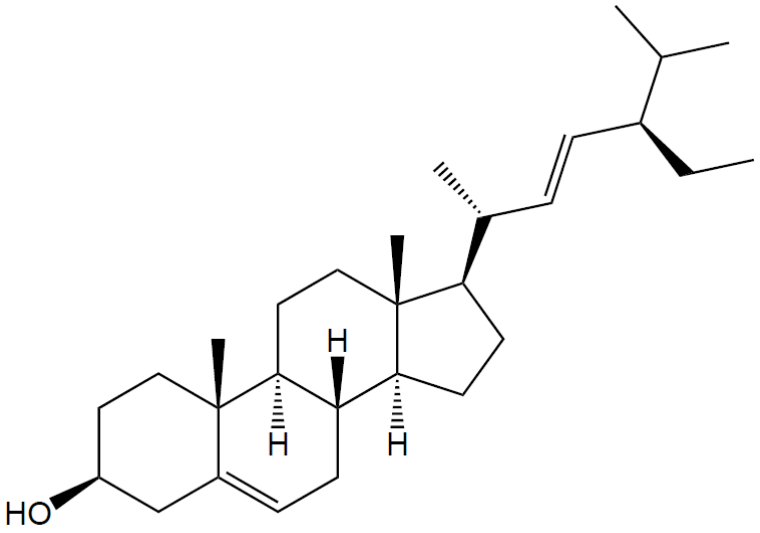

Figure 2. Chemical structure of stigmasterol

Fraction II yielded 82 mg off-white crystals at room temperature, non UV active and turned yellow in iodine chamber and black when sprayed with vanillin in concentrated sulfuric acid. It had an R_f value of 0.48 in methanol: dichloromethane (3:7) and melting point of 79 ^{0}C GC–MS analysis of fraction I yield showed a prominent molecular ion peak at m/z = 412.4, empirical formula, C_{29} H_{48} O, with a fragmentation pattern characteristic for sterols. It had a retention time of 27.624 minutes and purity of 95% in the GC using dichloromethane as the mobile phase (figure 1). It was confirmed by NIST05a spectral library (Agilent, 2005a) as a stigmasterol (figure 2).

Fraction II had a prominent molecular ion peak at m/z = 364.0, empirical formula, C_{26} H_{52}. It had a retention time of 22.412 minutes and purity of 62.4% in the GC using dichloro-methane as the mobile phase (figure 4). It was confirmed as 9-hexacosene (figure 3)

Acetic acid-induced writhing test

The results of the acetic acid-induced writhing responses in mice, which indicate the analgesic activity of the test samples, are presented in Table 1. Stigmasterol produced inhibition of writhing in a dose-dependent manner at the tested doses of 7.5, 15, 30 and l00 mg/kg, with writhing inhibitions of 16.67%, 29.10%, 39.46% and 55.61% respectively. Stigmasterol at a dose of 15 mg/kg body weight and above caused a significant ($p<0.05$) inhibition of the writhing responses induced by acetic acid as compared to the control. The

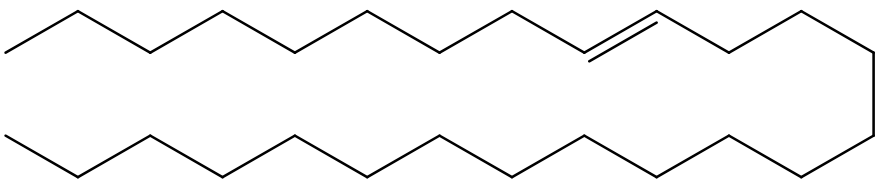

Figure 3. Chemical structure of 9-hexacosene.

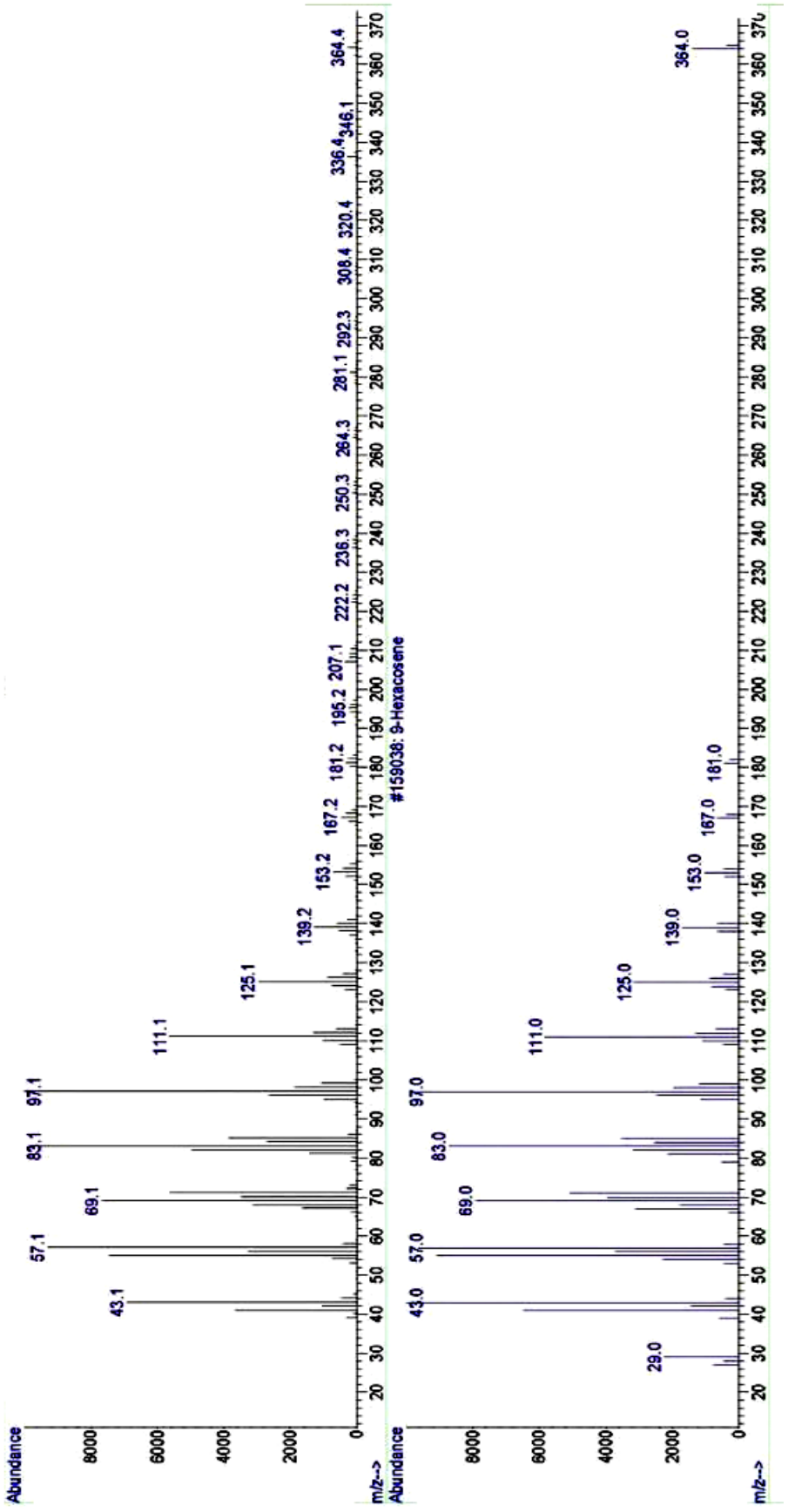

Figure 4. Mass spectrum of C_fMR_d II compound to the mass spectrum of authentic 9-Hexacosene Based on NIST05a Library Data.

Table 1: Effects of stigmasterol and 9-hexacosene on acetic acid-induced writhing responses in mice

Treatment	Dose (mg/kg)	Number of writhings (Mean ± S.E.M.)	Inhibition %
Control	-	38.52 ± 3.37	-
Stigmasterol	7.5	32.11 ± 2.79	16.67
	15	27.31 ± 1.84*	29.10
	30	23.32 ± 1.92**	39.46
	100	17.10 ± 2.91**	55.61
9-hexacosene	7.5	35.13 ± 3.14	8.80
	15	30.11 ± 2.89	21.83
	30	27.22 ± 2.18*	29.34
	100	21.75 ± 2.44**	43.54
Indomethacine	10	16.23 ± 2.26**	58.86

* = $p<0.05$, ** = $p<0.01$ compared to the control, n = 8

effects of stigmasterol (100 mg/kg) were comparable to that of indomethacine (10 mg/kg) with values of 55.61% and 58.86% protection respectively.

On the other hand, 9-hexacosene showed dose dependent inhibition of writhing achieving significant ($p<0.05$) inhibition of writhing at a dose of 30 mg/kg. These results taken together indicate that stigmasterol possesses stronger analgesic activity as compared to 9-hexacosene. The present data give pharmacological support to the validity of the analgesic effect of *Mondia whytei* roots.

Formalin test

The results of the formalin test are presented in Table 2. Stigmasterol (7.5, 15, 30 and 100 mg/kg) dose dependently reduced the time spent in pain behavior. The reduction was

Table 2: Effects of stigmasterol, 9-hexacosene, indomethacine, morphine and naloxone following intraperitoneal injection.

Group	Dose (mg/kg)	Licking Time (s)	
		Early phase	Late phase
Control	-	190.76±5.18	263.22±2.89
Stigmasterol	7.5 mg/kg	174.39±5.57 n.s	171.16±5.53 *
	15 mg/kg	155.81±5.99 n.s	136.8±7.85 **
	30 mg/kg	144.78±9.80 *	103.72±4.40 **
	100 mg/kg	99.36±2.51 **	80.62±4.82 **
9-hexacosene	7.5 mg/kg	184.74±2.69 n.s	176.93±6.67 *
	15 mg/kg	176.40±5.80 n.s	126.50±6.81 **
	30 mg/kg	165.22±5.86 n.s	109.69±2.91 **
	100 mg/kg	164.12±9.76 n.s	71.31±3.00 **
Indomethacine	50 mg/kg	186.21±4.52 n.s	78.42±2.43 **
Morphine	5 mg/kg	94.21±1.52 **	73.61±3.82 **
Naloxone + Morphine (5 mg/kg)	1 mg/kg	162.39±3.27 n.s	224.37±4.67 n.s
Naloxone + Stigmasterol (100 mg/kg)	1 mg/kg	109.26±2.64 **	92.86±2.81 **
Naloxone + 9-hexacosene (100 mg/kg)	1 mg/kg	-	76.36±2.82**

* $p< 0.05$ and **$p< 0.01$; n.s = not significant; n = 6

Table 3: Effects of stigmasterol, 9-hexacosene and dexamethasone on ear edema induced by dimethyl benzene in mice

Treatment	Dose (mg/kg)	Number of writhings (Mean ± SEM.)	Inhibition %
Control	10 ml/kg	2.98 ± 0.28	-
Stigmasterol	7.5 mg/kg	2.48 ± 0.22	16.78
	15 mg/kg	1.98 ± 0.21*	33.56
	30 mg/kg	1.48 ± 0.18**	50.34
	100 mg/kg	1.06 ± 0.09**	64.42
9-hexacosene	7.5 mg/kg	2.56 ± 0.30	14.09
	15 mg/kg	2.04 ± 0.20*	31.54
	30 mg/kg	1.81 ± 0.28**	39.26
	100 mg/kg	1.61 ± 0.23**	45.97
Dexamethasone	10 mg/kg	1.42 ± 0.23**	52.35

* p< 0.05 and **p< 0.01; n.s = not significant; n = 6

significant at 30 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.001$) during the early phase. Naloxone (1 mg/kg) did not abolish the analgesic effect of stigmasterol (100 mg/kg) whereas it abolished the analgesic effect of morphine (5 mg/kg). 9-hexacosene (7.5, 15, 30 and 100 mg/kg) and indomethacine (50 mg/kg) caused no significant reduction of the time spent in pain behavior in the early phase.

In the late phase, stigmasterol and 9-hexacosene dose dependently and significantly ($p<0.05$) reduced the time spent in pain behavior. Naloxone (1 mg/kg) did not abolish the analgesic effect of stigmasterol (100 mg/kg) or 9-hexacosene (100 mg/kg) whereas it abolished the analgesic effect of morphine (5 mg/kg). Indomethacine (50 mg/kg) significantly ($p<0.01$) reduced the time spent in pain behavior in the late phase. No overt motor, neurological or behavioural deficits were observed at all the doses tested.

Dimethyl benzene-induced ear edema test

Stigmasterol and 9-hexacosene dose dependently inhibited ear edema. However, 9-hexacosene was less potent in inhibiting ear edema at all the doses tested compared to stigmasterol. At 30 mg/kg dose, the anti-inflammatory activity of stigmasterol was comparable to that of dexamethasone at a dose of 10 mg/kg (50.34% vs. 52.35%) and were both significant ($p<0.01$). The results demonstrate the anti-inflammatory properties of stigmasterol and 9-hexacosene and may justify the use of this plant for the treatment of inflammatory diseases in African traditional herbal medicine.

Discussion

Plants exhibiting analgesic properties are of great interest for a variety of reasons. *Mondia whytei* roots are rich source of several biological molecules, such as organic molecules, produced via the secondary metabolism, including flavonoids, saponins, steroids, carotenoids and glycosides (Mukonyi and Ndiege, 1999). Antinociceptive effects of *Mondia whytei* aqueous root extract have been reported (Githinji *et al.,* 2008). In the present work, the root powder of *Mondia whytei* was fractionated and the main analgesic

fraction determined. From this effective fraction, a steroid (stigmasterol) and a long chain carbon compound (9-hexacosene) was obtained using GC-MS spectral means. NIST05a spectral library was used to confirm the identity of the two compounds. Both compounds exhibited significant anti-nociceptive activity in acetic acid- induced writhing test and the formalin test.

The acetic acid-induced writhing test was used to evaluate the peripheral anti-nociceptive activity. The test is able to determine the anti-nociceptive effect of compounds at dose levels that might appear inactive in other methods like tail-flick test (Bentley et al., 1983). However, acetic acid-induced writhing test is considered to be non-selective anti-nociceptive model since many drugs without analgesic effects in man can effectively inhibit writhing responses (Sanchez- Mateo *et al.,* 2006). Our results indicated that stigmasterol and 9-hexacosene could significantly reduce the number of writhing, showing powerful anti-nociceptive effects. However, the results of writhing test alone could not ascertain whether the anti-nociceptive effect was centrally or peripherally mediated. In order to confirm it, the formalin test was carried out. The advantage of the formalin model of nociception is that it could discriminate pain in its central and/or peripheral components. The test consists of two different phases that can be separated in time: the first one (early phase) is generated in the periphery through the activation of nociceptive neurons by the direct action of formalin and the second phase (late phase) occurs through the activation of the ventral horn neurons at the spinal cord level (Tjolsen *et al.,* 1992). Central analgesic drugs, such as narcotics, inhibited equally both phases, while peripherally acting drugs, such as steroids (hydrocortisone and dexamethasone) and NSAIDs (indomethacine), suppresses mainly the late phase (Trongsakul et al., 2003).

Stigmasterol though a steroid, showed significant ($p<0.05$) antinociceptive effects in early phase of the formalin test. It was predicted that stigmasterol acted as a neurosteroid. Neurosteroids are those steroids which are synthesized *de novo* in the brain and directly activate $GABA_A$ receptor. The $GABA_A$ receptor is a pentameric protein (Schofield *et al.,* 1987) whose activation by agonists opens an associated chloride ion channel, leading to an increase in chloride ion influx that results in membrane hyperpolarization (Majewska, 1987). Steroids like stigmasterol therefore exerts their antinociceptive effects in the early phase of formalin test by altering the neuronal excitability. Both the potentiation and activation appear to be mediated through a site(s) distinct from the well known barbiturate and benzodiazepine allosteric sites of the $GABA_A$ receptor. This potentiation is stereoselective and mediated by a steroid-induced prolongation of the burst duration of the $GABA_A$–activated chloride ion channel opening. The possession of a hydroxyl group in α–configuration at C-3 of the steroid ring A is an important determinant of potency (Harrison. *et al.,* 1987). Stigmasterol unlike dexamethasone possesses the hydroxyl group in α – configuration at C-3 of the ring A.

On the other hand, 9-hexacosene had antinociceptive effects only in the second phase of the formalin test. It produced graded, dose-dependent and statistically significant ($p<0.05$) antinociceptive effects. 9-hexacosene is an unsaturated hydrocarbon with a double bond at C-9 with structural resemblances to endoperoxides. It was therefore suggested that structure activity relationship existed between 9-hexacosene and endoperoxides. This provides a plausible hypothesis into the mechanism of action of 9-hexacosene. Considering the spatial

arrangement of endoperoxides inside the cyclo-oxygenase enzyme (a 534 amino acid molecule), it is possible that 9-hexacosene enters into the cyclo-oxygenase enzyme tunnel and because of the slight differences with the normal substrate (endoperoxides), 9-hexacosene is unable to form linkages at appropriate sites within the cyclo-oxygenase enzyme and thereofre cannot be hydrolyzed to form pro- inflammatory mediators. 9-hexacosene was thought to acts as a false substrate. To verify possible anti-nociceptive mechanisms, we examined the effect of naloxone, a non- selective opioid receptor antagonist, on the anti-nociceptive activity of both stigmast-erol and 9-hexacosene. Since naloxone failed to antagonize the antinociception produced by neither stigmasterol nor 9-hexacosene, it was thought that the observed analgesic effect is not mediated through opioid receptors.

Dimethyl benzene-induced ear edema test was further employed to evaluate the anti-inflammatory activity of both stigmasterol and 9-hexacosene. Both compounds showed significant anti-inflammatory activity, consistent with the indications revealed in the second phase (inflammatory) of the formalin test, implying that the analgesic activity of stigmasterol and 9-hexacosene may be partly mediated by its anti-inflammatory action.

In conclusion, the study demonstrated the analgesic activity of stigmasterol and 9-hexacosene in the test models of nociception induced by chemical stimuli. It is suggested that the observed anti- nociceptive activity of stigmasterol might be mediated both peripherally and centrally. The antinociceptive activity of 9-hexacosene could be related to its anti-inflammatory activity. These observations merits further studies regarding the precise site and the mechanism of action.

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Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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