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ANTINOCICEPTIVE EFFECTS OF STIGMASTEROL AND 9-HEXACOSENE ISOLATED FROM *MONDIA WHYTEI*(HOOK.F.) ROOT

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ABSTRACT

Mondiawhytei is used for treatment of dysmennorrhea, gastro intestinal colic, post-partum pains among other uses in the practice of traditional medicine in Africa.In the present study, the *in vivo*antinociceptive effects of two compounds,stigmasterol and 9-hexacosene isolated from*Mondiawhytei* roots has been investigated.Bioactivity guided extraction and isolation of stigmasterol and 9-hexacosene was carried out.The formalin test was used inassessing the antinociceptive activity.Stigmasterol reduced the time spent licking, biting and/or lifting the injected paw in both the early and late phases of the formalin test. This reduction was found to be dose dependent and statistically significant ($p<0.001$) at a dose of 30mg/kg body weight.9-hexacosene produced dose-dependent and statistically significant ($p<0.001$) antinociceptive effect on the late phase of the formalin test at a dose of 7.5mg/kg body weight.No motor, neurological or other behavioral deficits were observed.

*Conclusion:*Results of the present study supported the utilization of *Mondiawhytei* in Africa folk medicine and revealed stigmasterol and 9-hexacosene as the major antinociceptive principles in the roots.

Key words*:Mondiawhytei*, Antinociception, Stigmasterol, 9-hexacosene, formalin test.

INTRODUCTION

Mondiawhytei(Hook. f.) is a forest floor plant with aromatic rhizomatous roots that belongs to the A*sclepiadaceous* family. *Asclepiadaceous* has more than 300 genera and 2000 species (Van Heerden and Steyn, 1999). However, there are about 104 species in the family that have been identified in East Africa (Agnew and Agnew, 1994).

The *Asclepiadaceous* family is mostly found in the tropics and subtropical regions. *Mondiawhytei* is distributed widely in Africa. It is found in Guinea in West Africa through Sudan, Uganda, Kenya, Tanzania, Malawi, South Africa and Westwards to Angola (Beentje, 1994).

In Kenya, *Mondiawhytei* is locally known as Mukombela (Luhya), Ogomba (Luo), Olkonkola (Maasai), Mkonkora (Kamba) and Muhukura (Kikuyu). Outside Kenya, the plant is known as Omurondwa (lunyore-Uganda), Ilivi (sudan), Omondi (Zulu) and Mbombogazi (Tanzania) (Kokwaro, 2006).

The roots have been chewed or boiled with porridge to treat post-partum pains, gastro intestinal colic and dysmenorrhea. It is also claimed to have anti-inflammatory, anti-pyretic and antimicrobial activity (Jain *et al.*, 1996). It is also used asan aphrodisiac (Kokwaro, 2006).Parasympathomimetic effects of *Mondiawhytei*aqueous root extract on rabbit heart and

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jejunum is well documented (Githinji*et al.,* 2007) Literature search reveals that there is no systematic study performed in order to authenticate the claims above. The aim of this study was to evaluate the antinociceptive effects of two compounds (stigmasterol and 9-hexacosene) that were isolated from the roots of *Mondiawhytei.*

This paper reports the antinociceptive effects of two compounds, stigmasterol and 9-hexacosene isolated from *Mondiawhytei* roots for the first time.

MATERIALS AND METHODS

Experimental animals

Male Swiss albino mice weighing 20 – 25 g were used taking into account international guiding principles and local regulations concerning the care and use of laboratory animals for biomedical research (University of Nairobi animal welfare committee guidelines).The animals had free access to a standard commercial diet and water*ad libitum* and were kept in rooms maintained at 22 $\pm$ 1 ^{0}C with 12 hours light/dark cycle. All experiments were performed between 8.00a.m and 5.00 p.m.

Collection of plant material and isolation of stigmasterol and 9-hexacosene.

Plant material of *Mondiawhytei* was collected from Kakamega forest, Western province of Kenya in August 2006 and authenticated by Mr. Mutiso (Department of Botany, University of Nairobi, Kenya). A voucher Specimen (CG/MV/608) was deposited in the Department of Botany herbarium, University of Nairobi, Kenya.

The roots were air-dried, milled to moderately coarse powder and defatted with n-hexane (60 – 80 ^{0}C) using Soxhlet extractor. The material was subjected to Soxhlet extraction with methanol. The methanolic extract was exhaustively partitioned with chloroform andthen fractionated using superfrac fractionators eluting with methanol: dichloromethane (3:7). Stigmasterol and 9-hexacosene were isolated as white solid which crystallized in acetone as white and off-white crystals respectively. (Githinji, 2010).

Preparation and administration of test samples

Stigmasterol and 9-hexacosene were dissolved in 5% dimethylsulfoxide (DMSO) and then suspended in normal saline. The control group of animals was treated similar to the test group except that the drug treatment was replaced with appropriate volume of vehicle.

Indomethacin (50 mg/kg), Acetysalicyclic acid (ASA) (400 mg/kg) and dexamehtasone (30 mg/kg) in 5% dimethylsulfoxidewere used as reference drugs.

All injections were given intraperitoneally (i.p) in volumes of 10 ml/kg body weight.

Experimental design

A complete randomized design was adopted in this study. The animals were picked up randomly and assigned to an experimental unit. Similarly the treatment was randomly assigned to an experimental unit.

A matched pair design, where each treated experimental unit had a control unit was adopted during the study. In all experiments, each animal was used only once. Administration of the drugs or vehicles was done blindly.

Formalin test

The procedure as described by Hunskaar and Hole (1987)was used. Itinvolved injection of 20 ul of 2.5% formalin solution (0.92% formaldehyde) in phosphate buffer (pH 7.3) into the plantar surface of the right hind paw of mice. Groups of animals were treated with stigmasterol (7.5, 15, 30 and 100 mg/kg), 9-hexacosene (7.5, 15, 30 and 100 mg/kg), indomethacine 50 mg/kg, acetylsalicylic acid 400 mg/kg and dexamethasone 30 mg/kg body weight. Control animals received a similar volume of vehicle. Animals were placed individually in an observation chambermade of perspex; beneath the floor, a mirror was mounted at an angle of 45^{0}toallow clear observation of the animal's paws. The amount of time the animal spent licking the injected paw, considered as indicative of pain, was recorded for 30 minutes after formalin injection. The initial nociceptive score normally peaked 5 minutes after formalin injection (early phase) and 15 – 60 minutes after formalin injection (late phase), representing neurogenic and inflammatory pain responses respectively. Indomethacine, acetylsalicylic acid and dexamethasone were used as reference drugs.

Statistical analysis of the data

The data was analyzed using analysis of variance (ANOVA). When analysis was restricted to two means, students t-test (paired and one tailed) was used.Scheffe' post hoc test was performed for multiple comparisons. Statistical Package for Social Sciences (SPSS) version 16.0 was used for data analysis.

The level of significance was setat $p<0.05$. Data obtained from experiments were expressed as mean $\pm$ standard error of the mean (s.e.m).

RESULTS

Effects of stigmasterol (C_fMR_dI) and 9-hexacosene (C_fMR_dII) compared with that ofindomethacine, acetylsalicylic acid and dexamethasone in the early and the late phase of the formalin test.

The Formalin Test

Injection of 20 µl of 5% formalin subcutaneously into the plantar surface of the mouse produced distinct behavioural responses, licking, biting and lifting the injected paw. Two distinct periods of pain behaviour were identified: the early phase lasting for the first 5 minutes and the late phase, starting 20 to 60 minutes after the injection of formalin.

Stigmasterol

Intraperitoneal administration of stigmasterol significantly reduced the time spent in pain behaviour at a dose of 30 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.001$) during the early phase. Administration of 7.5 mg/kg and 15 mg/kg caused no significant reduction of the time spent in pain behaviour in the early phase.

Administration of 400 mg/kg acetylsalicylic acid significantly ($p<0.01$) reduced the time spent in pain behaviour in the early phase. Indomethacine (50 mg/kg) and dexamethasone (30 mg/kg) caused no significant reduction of the time spent in pain behaviour in the early phase.

In the late phase, stigmasterol (7.5, 15, 30 and 100 mg/kg) dose dependently reduced the time spent in pain behaviour. The reduction was statistically significant at $p<0.05$. Indomethacine (50 mg/kg), acetylsalicylic acid (400 mg/kg) and dexamethasone (30 mg/kg) significantly ($p<0.01$) reduced the time spent in pain behaviour in the late phase.

There was no significant difference ($p=0.72$) between the effects of acetylsalicylic acid (400 mg/kg) and stigmasterol (100 mg/kg) in the late phase. No overt motor, neurological or behavioural deficits were observed at all the doses tested.

9-hexacosene

Intraperitoneal administration of 7.5, 15, 30 and 100 mg/kg of 9-hexacosene had no significant effect on the time spent in pain behaviour in the early phase. Indomethacine (50 mg/kg) and dexamethasone (30 mg/kg) had no significant effects also. Acetylsalicylic acid (400 mg/kg) significantly ($p<0.001$) reduced the time spent in pain behaviour in the early phase.

In the late phase, 9-hexacosene (7.5, 15, 30 and 100 mg/kg) dose dependently reduced the time spent in pain behaviour. All the doses were statistically significant($p<0.01$). Indomethacine (50 mg/kg), acetylsalicylic acid (400 mg/kg) and dexamethasone (30 mg/kg) caused a statistically significantly ($p<0.01$) reduction in the time spent in pain behaviour.

In the late phase, there was no statistically significant difference in the antinociceptive effects of indomethacine (50 mg/kg) and 9-hexacosene (100 mg/kg) ($p=0.988$), acetylsalicylic acid (400 mg/kg) and 9-hexacosene (30 mg/kg) ($p=0.995$), dexamethasone (30 mg/kg) and 9-hexacosene (30 mg/kg) ($p=0.976$) and dexamethasone (30 mg/kg) and 9-hexacosene (100 mg/kg) ($p=0.962$). No overt motor, neurological or behavioural deficits were observed following injection.

Table 1. Effects of Intraperitoneal Administration of Stigmasterol (7.5, 15, 30 and 100 mg/kg), Indomethacine (50 mg/kg), Acetylsalicylic acid (400 mg/kg) and Dexamethasone (30 mg/kg)

DRUG	DOSE	Time (Seconds) spent in pain behavior
EARLY PHASE		
Vehicle	10 ml/kg	190.76 ± 5.18
Stigmasterol (C_fMR_dI)	7.5 mg/kg	174.39 ± 5.57 n.s
	15 mg/kg	155.81 ± 5.99 n.s
	30 mg/kg	144.78 ± 9.80 *
	100 mg/kg	99.36 ± 2.51 **
Indomethacine	50 mg/kg	186.21 ± 4.52 n.s
Acetylsalicylic Acid	400 mg/kg	65.45 ± 4.65 **
Dexamethasone	30 mg/kg	172.37 ± 9.09 n.s
LATE PHASE		
Vehicle	10 ml/kg	263.22 ± 2.89
Stigmasterol (C_fMR_dI)	7.5 mg/kg	171.16 ± 5.53 *
	15 mg/kg	136.8 ± 7.85 **
	30 mg/kg	103.72 ± 4.4 **
	100 mg/kg	80.62 ± 4.82 **
Indomethacine	50 mg/kg	67.76 ± 8.43 **
Acetylsalicylic Acid	400 mg/kg	85.09 ± 6.8 **
Dexamethasone	30 mg/kg	91.63 ± 6.07 **

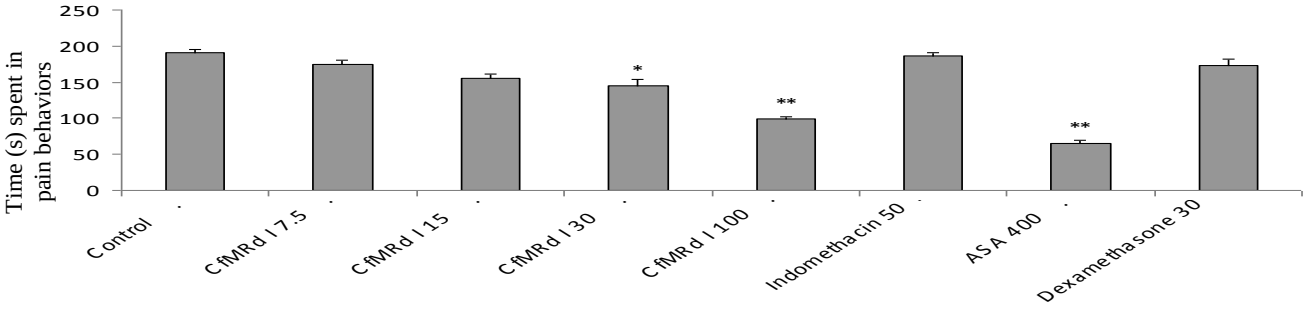
Table 2.Effects ofIntraperitoneal Administration of 9-hexacosene (7.5, 15, 30 and 100 mg/kg), Indomethacine (50 mg/kg), Acetylsalicylic acid (400 mg/kg) and Dexamethasone (30 mg/kg)

DRUG	DOSE	Time (seconds) spent in pain behavior
EARLY PHASE		
Vehicle	10 ml/kg	193.35 ± 5.50
9-hexacosene (C_fMR_dII)	7.5 mg/kg	184.74 ± 2.69 n.s
	15 mg/kg	176.40 ± 5.80 n.s
	30 mg/kg	165.22 ± 5.86 n.s
	100 mg/kg	164.12 ± 9.76 n.s
Indomethacine	50 mg/kg	191.89 ± 6.19 n.s
Acetylsalicylic Acid	400 mg/kg	62.86 ± 3.52 **
Dexamethasone	30 mg/kg	169.53 ± 6.53 n.s
LATE PHASE		
Vehicle	10 ml/kg	258.67 ± 6.06
9-hexacosene (C_fMR_dII)	7.5 mg/kg	476.93 ± 6.67 *
	15 mg/kg	126.50 ± 6.81 **
	30 mg/kg	109.69 ± 2.91 **
	100 mg/kg	71.31 ± 3.00 **
Indomethacine	50 mg/kg	78.42 ± 2.04 **
Acetylsalicylic Acid	400 mg/kg	132.65 ± 2.70 **
Dexamethasone	30 mg/kg	118.46 ± 2.42 **

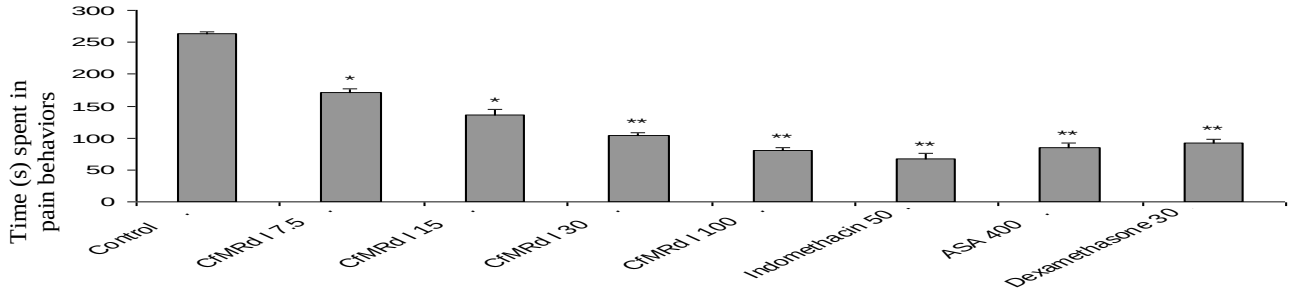
* p< 0.05, **p< 0.01 and n.s = not significant

Figure 1. Effects of administration of stigmasterol (7.5, 15, 30 and 100 mg/kg), indomethacin (50 mg/kg), acetylsalicylic acid (ASA) (400 mg/kg) and dexamethasone (30 mg/kg) on time (s) spent in pain behaviors in the formalin test

Early Phase



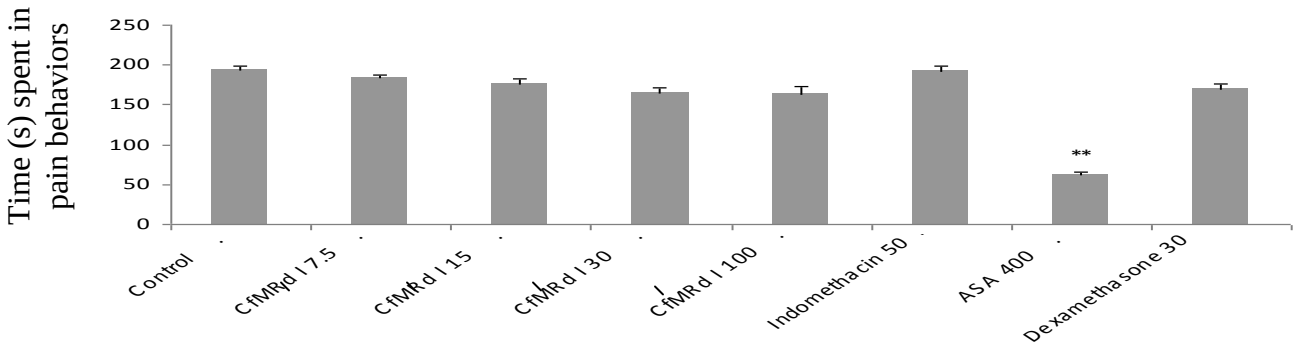
Late Phase



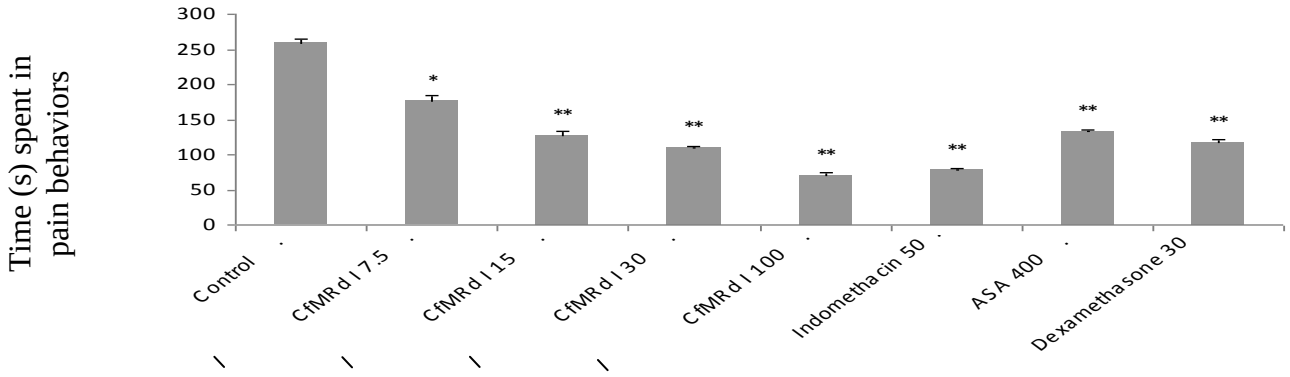
* p< 0.05 and **p< 0.01

Figure 2. Effects of intraperitoneally administered 9-hexacosene (7.5, 15, 30 and 100 mg/kg), indomethacin (50 mg/kg), acetylsalicylic acid (ASA) (400 mg/kg) and dexamethasone (30 mg/kg) on time (s) spent in pain behaviors in formalin test

Early Phase



Late Phase



* p< 0.05 and **p< 0.01

DISCUSSION

Stigmasterol dose dependently and significantly (p<0.05) reduced the amount of time the animal spent in pain behavior in both the early phase and the late phase of the formalin test at a dose of 30 mg/kg and above. These results suggested that the antinociceptive effects of stigmasterol is both non-genomic characterized by its fast action in exerting antinociceptive effect in the first phase and also genomic as characterized by delayed action in exerting antinociceptive effects in the second phase of formalin test.

Stigmasterol like dexamethasone is a steroid and was expected to exert antinociceptive effects on the late phase of the formalin test only. This is because steroids are known to inhibit phospholipase A_2 (Vane and Botting,1987) thereby blocking generation of pro-inflammatory mediators.

The finding that stigmasterol exerted antinociceptive effects in the first phase of formalin test was unpredicted and therefore aroused interest in understanding the mechanisms underlying the basis of steroidal activity. Stigmasterol was thought to act like a neurosteroid. Neurosteroids are those steroids which are synthesized in the brain in contrast to the exogenous steroids. Pregnane steroids directly activate $GABA_A$ receptor. 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.

γ-aminobutyric acid (GABA) the principal inhibitory neurotransimitter in the CNS, activates two types of receptor, the ionotropic GABAA receptor and the metabotropic (G protein-coupled) GABAB receptor. The GABAA 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. Stigmasterol at a dose of 100 mg/kg was equipotent to acetylsalicylic acid at a dose of 400 mg/kg. Acetylsalicylic acid at a dose of 400 mg/kg is known to exert antinociceptive effects in the early phase and is equipotent to morphine 5 mg/kg body weight (Hunskaar, 1987a).

On the other hand, 9-hexacosene had an antinociceptive effects only in the second phase of the formalin test. It produced graded, dose-dependent and statistically significant ($p<0.05$) antinociceptive effects (Table 2). 9-hexacosene at a dose of 30 mg/kgwas equipotent to 400 mg/kg body weight acetylsalicylic acid (Figure 2). The same dose of 9-hexacosene had no statistically significant antinociceptive effects during the early phase whereas 400 mg/kg acetylsalicylic acid significantly reduced the amount of time the animal spent in pain behavior in the early phase ($p<0.05$). 9-hexacosene and indomethacine were found to be equipotent at doses of 100 mg/kg and 50 mg/kg body weight respectively in the late phase.

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 therefore cannot be hydrolyzed to form pro-inflammatory mediators.

9-hexacosene therefore acts as a false substrate.

CONCLUSION

Stimasterol and 9-hexacosene were isolated from the roots of Mondiawhyteifor the first time. Stigmasterol and 9-hexacosene were shown to be strongly responsible for the antinociceptive activities of Mondiawhytei roots. This authenticates the use of Mondiawhytei(Hook. f.) root in pain management.

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