AN EVALUATION OF THE ANALGESIC ACTION OF AQUEOUS LEAVES EXTRACT OF *HYBANTHUS ENNEASPERMUS* LINN. F. MUELL [VIOLACEAE] IN RODENTS

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Abstract

Hybanthus enneaspermus is a tropical and subtropical shrub used to manage conditions involving inflammation and pain. This study was carried out to evaluate the analgesic activity of aqueous leaves extract of H. enneaspermus (ALHE). The analgesic activity of ALHE (50, 100 and 200 mg/kg) was investigated using acetic acid- and acetylcholine-induced mouse writhing tests, formalin-induced pain and tail clip tests in mice. Naloxone, glibenclamide or pilocarpine was administered to some animals 30 minutes before ALHE prior to induction of pain. Possible contribution of central nervous system (CNS) activity of the extract to its analgesic action was also evaluated using open field and hexobarbitone-induced sleep tests. The extract (50-200 mg/kg) significantly inhibited writhing in the acetic acid- and acetylcholine-induced mouse writhing tests. It was most effective at 100 mg/kg, producing 97.6% and 96.5% inhibition in both tests respectively. Naloxone, glibenclamide and pilocarpine significantly (p<0.001) altered this analgesic effect of the extract. The extract also significantly (p<0.001) increased pain threshold in tail clip test and significantly reduced reaction time in both phases of the formalin-induced pain test. The extract significantly reduced locomotive and exploratory activities of mice in the open field test but did not produce any significant effect in the hexobarbitone-induced sleep test in mice. These findings show that the aqueous leaves extract of Hybanthus enneaspermus possesses analgesic activity, which is mediated by mechanisms likened to those of opioid receptor antagonists, muscarinic receptor antagonists, and K^+ channels opening.

Keywords: *Hybanthus enneaspermus*, Aqueous Extract, Analgesic, Writhing, Acetic Acid, Acetylcholine, Locomotion

Introduction

Pain, which is probably the most important reason for visits to physicians, has been regarded as an enormous health challenge globally. It has been reported that approximately one in five adults experience pain, and one in ten of same suffer chronic pain each year. Although it has been referred to as only a symptom of disease rather than a disease itself, several consequences of mental, physical, social and economic relevance have been attributed to unmanaged pain (Goldberg and McGee, 2011). A number of approaches have been adopted for the management of pain, including the use of drugs such as non-steroidal anti-inflammatory and opioid analgesics. The benefits of these drugs have been however limited by several factors, one of which is adverse drug reactions. Conventional analgesics have been reported as major causes of on-toward adverse drug reactions (Sanchez-Borges *et al.*, 2010). Other draw backs on the therapeutic applications of these drugs are the increasing incidence of tolerance, fear of addiction (for opioid analgesics) and the fact that the drugs are not readily available or affordable, especially as it concerns rural dwellers, who account for up to 53% of the Nigerian population. Indeed over 80% of the population in some Asian and African countries depend on traditional medicine for their health care needs (WHO, 2008). Hence the need for more efficacious and more affordable analgesics of which medicinal plants are very important sources.

Medicinal plants have long been used in the management of pain. Morphine, the opioid analgesic used to manage severe pain as in cancer patients was derived from the latex of immature seed capsules of the poppy flower (*Papaver somniferum*) (Ballantyne and Mao, 2003). Chronic pain is one of the most commonly cited reasons for the use of medical marijuana (Lucas, 2012), derived from *Cannabis sativa*. According to Zogopoulos *et al.* (2013), the evidence of medical marijuana's pain mitigating effects is

generally conclusive. Many other medicinal plants including *Alafia barteri, Mesua ferrea, Amaranthus spinosus, Blumea perrothetiana* and *Amaralia bignoniflora* and *Hybanthus enneaspermus* are used to manage painful conditions. It is advisable not to relent on research efforts to provide evidence based report on the pharmacological basis for the health claims accrued to such plants. Information thus obtained will allow for well-informed application of medicinal plants for pain management as well as encourage cultivation of such for increased benefit to mankind.

Hybanthus enneaspermus of the family Violaceae is a tropical and subtropical shrub distributed in various regions of the world. It has spindle shaped root that is rough and light yellow in colour. The stem is sparingly branched with woody base and spreading erect branches. It also has simple dark green leaves with pink flowers 8 to 10 mm wide and 5 mm wide fruit with seeds. It is commonly called "spade flower" in English and "abíwéré" among the Yorubas in Nigeria. The plant is used as tonics, diuretics, demulcents and the root is diuretic and administered as an infusion in gonorrhea and urinary tract infections (Gill and Akinwunni, 1986). It is used in managing inflammations; problems with sterility, leucorrhea and dysuria, and also as an aphrodisiac (Yoganarasimhan, 2000). Pharmacological investigations have shown that the plant possesses anti-inflammatory, antitussive, antiplasmodial, antimicrobial (Rajakaruna *et al.*, 2000; Weniger *et al.*, 2004), anticonvulsant and free radical scavenging activities (Hemlatha *et al.*, 2003). Phytochemical analysis revealed that the plant contains aurantiamide acetate, isoaborinol, -sitosterol and triterpene (Prakash *et al.*, 1999: Retnam and De Britto, 2003). In Nigeria, it is added to food for pregnant and parturient women as the plant is believed to bring about painless child birth. This study was thus aimed at evaluating the analgesic potential of the aqueous leaves extract of *Hybanthus enneaspermus* (ALHE).

Methods

Plant Material

The fresh portion of the plant, *Hybanthus enneaspermus* was collected from a local market in Mushin, Lagos. It was identified and authenticated by Mr. T.K. Odewo of the Department of Botany and Microbiology of the University of Lagos, Akoka, Lagos. Voucher specimen was deposited in the Lagos University Herbarium with the voucher specimen number LUH 4625 allotted.

Plant Extraction

The fresh leaf (100 g) of *Hybanthus enneaspermus* was washed, blended and boiled in 1 litre of distilled water for 30 minutes. The decoction was left to stand overnight after which it was filtered using cotton wool and then filter paper. The filtrate was then oven-dried at 40 °C to give a greenish brown solid extract. The extract yield was found to be 10%.

Experimental Animals

Albino mice (20-25 g) of either sex were obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Nigeria. The animals were maintained under standard laboratory environmental conditions with access to standard rodent feed and water *ad libitum*. The experimental procedures adopted in this study were in accordance with the 1985 United States National Institute of Health Guidelines for care and use of Laboratory Animals in Biomedical Research.

Phytochemical Analysis

Preliminary phytochemical screening of ALHE was carried out using analytical methods described by Sofowora (1993).

Acute Toxicity Test

Five groups of 5 mice each fasted for 12 hours prior to the experiment were administered ALHE (10 g/kg, p.o.) and observed first for 2 hours post-treatment for immediate signs of toxicity and also observed 24 hours later for signs of morbidity and mortality. A further 14 days observation period was allowed to check for delayed onset of morbidity or mortality (Amida *et al.*, 2007).

Test for Analgesic Activity

The analgesic property of the aqueous leaves extract was investigated using the following animal models.

Acetylcholine-Induced Writhing Test

Albino mice (20-25 g) of either sex in five groups of five animals each were administered distilled water (5 ml/kg, p.o.), ALHE (50-200 mg/kg) or diclofenac (5 mg/kg) one hour before the intraperitoneal administration of 10 mg/kg acetylcholine (Sigma Aldrich, Germany). The number of writhes by the mice in all the groups was then counted for 15 minutes (Saha *et al.*, 2007; Adeyemi *et al.*, 2008). In order to ascertain the mechanism of action of ALHE in this test, 1 mg/kg pilocarpine (Sigma Aldrich, Germany) was administered intraperitoneally 30 minutes before ALHE (100 mg/kg, p.o.) to one group of 5 mice. Percentage inhibition of writhes was determined as follows:

Inhibition (%) = <u>Number of writhes (control) – Number of writhes</u> (treatment) x 100 Number of writhes (control)

Acetic Acid-Induced Mouse Writhing Test

Albino mice (15-25 g) of either sex allotted into five groups of five mice each were administered distilled water (5 ml/kg, p.o.), ALHE (50-200 mg/kg, p.o.) or diclofenac (5 mg/kg, p.o.) one hour before the intraperitoneal administration of acetic acid (0.6%, 0.2 ml/mouse) obtained from Sigma Aldrich, Germany. The number of writhes by the mice in all the groups was determined for 30 minutes (Adeyemi *et al.*, 2011). In order to determine the possible mechanism of action of ALHE, some animals received naloxone (1 mg/kg, i.p.) or glibenclamide (2 mg/kg, p.o.) 30 minutes before ALHE (100 mg/kg, p.o.) Percentage inhibition of writhes was determined as described above

Formalin-Induced Pain Test

Mice fasted overnight were divided into 5 groups of 5 animals each. The different groups of animals were treated with distilled water (5 ml/kg, p.o.), ALHE (50-200 mg/kg, p.o.) or morphine (2 mg/kg, s.c.). Sixty minutes after administration, 20 μ L of 1 % formalin (Sigma Aldrich, Germany) was injected into the right hind paw of each mouse. The time (in seconds) spent in licking and biting of the injected paw was recorded for each animal. The responses of the mice were observed first at 0-5 minutes (first phase) and then at 15-30 minutes (second phase) after formalin injection (Mbagwu *et al.*, 2007). The percentage analgesic effect was then determined as follows:

Inhibition (%) = <u>Reaction time (control)</u> – <u>Reaction time (treated)</u> x 100 Reaction time (control)

Tail Clip Test

Mice were initially screened by applying a metal artery clip to the root of their tail to mechanically induce pain and animals which failed to attempt dislodging the clip in 10 seconds were screened out. Eligible mice were then divided into 5 groups of 5 mice each. The reaction time of all mice was determined before and after treatment as follows: distilled water (5 ml/kg, p.o.), ALHE (50 - 200 mg/kg, p.o.) or 2 mg/kg of orally administered morphine (Martindale Pharmaceuticals, Ranford, United Kingdom). A cut-off time of 20 seconds was allowed for tail clip test (Adeyemi *et al.*, 2004). Percentage analgesic response was determined using the formula:

% analgesic response = <u>(Post -treatment latency) - (Pre-treatment latency)</u> x 100 (Cut-off time - Pre-treatment latency)

Investigation of Central Nervous System Involvement in the Analgesic Action of ALHE

Open Field Test

An open field made of wood (50 x 50 x 25 cm) was used in this study. The floor of the box was divided into 16 squares (8 x 8 cm). Sixty minutes after oral administration of distilled water (5 ml/kg) or ALHE (50-200 mg/kg, p.o.) to 4 groups of 5 mice each respectively. The mice were placed in turn at the center of the open field and the number of square crossings (with all four paws moved from one square to another), unassisted rearing and assisted rearing were determined for 5 minutes (Mahendra and Bisht, 2011).

Hexobarbitone-Induced Sleep Time Test

Four groups of 5 mice each were given orally, distilled water (5 ml/kg) or ALHE (50 - 200 mg/kg). One hour later, 100 mg/kg of hexobarbitone was administered intraperitoneally to each mouse. The mice were placed in observation chambers and the onset and duration of loss of righting reflex was recorded for each mouse. When there was any doubt, the animal was placed gently on its back again and if it rights itself within one minute, this time was considered the endpoint (Mujumdar et al., 2000).

Statistical Analysis

Results obtained in this study were expressed as mean \pm SEM. Data were analyzed using-one way ANOVA followed by Tukey's multiple comparison post-hoc test using GraphPad[®] Prism 6 Software. Results were considered significant when p <0.05.

Results

Preliminary Phytochemical Screening of ALHE

The observations made in the phytochemical studies are shown in Table 1. The results revealed the presence of detectable amounts of alkaloids, tannins, reducing sugars, anthraquinone and flavonoids, with the absence of saponins, phlobatannins and cardiac glycosides.

Acute Toxicity Test

The extract produced no mortality at up to 10 g/kg within 24 hours of exposure and no sign of delayed toxicity was observed in the mice within the additional 14 days observation period. **Analgesic Activity**

Acetylcholine-Induced Writhing Test

As shown in table 2, intraperitoneal injection of acetic acid elicited writhing in control mice. ALHE (50–200 mg/kg) produced significant dose-dependent reduction in the number of writhes with peak reduction at 100 mg/kg. At this dose, the extract, in a manner similar to diclofenac (5 mg/kg), produced 96.5% inhibition of the writhing response. This effect of the extract was inhibited by pilocarpine (1 mg/kg) (Table 2).

Acetic Acid-Induced Writhing Test

Intraperitoneal injection of acetic acid also elicited the writhing response in control mice. The extract (50-100 mg/kg) significantly and dose-dependently reduced the number of writhes by the mice with peak effect of 97.6% reduction at 100 mg/kg. Naloxone (1 mg/kg) and glibenclamide (2 mg/kg) significantly inhibited this effect of ALHE by 4.9% and 11.0% respectively (Table 3).

Formalin-Induced Pain Test

Formalin induced the pain responses of paw biting and licking in injected mice. Control mice exhibited this response for a total of 91.00 ± 2.86 and 113.00 ± 3.32 seconds in the first and second phases of the test respectively. In a manner similar to diclofenac (5 mg/kg) and morphine (2 mg/kg), the extract (50-200 mg/kg) significantly reduced the reaction time of mice in both phases of the test. This effect of the extract was greatest at 100 mg/kg with 34.7% and 37.3% reduction of pain response in both phases of the test respectively (Table 4).

Tail Clip Test

Application of the metal artery clip unto the tail of animals in the control group elicited reactions towards clip removal with post treatment latency of 2.08 ± 0.07 seconds. The extract (50-200 mg/kg) caused a significant (p<0.001) increase in reaction latency with the greatest effect (54.4% increased pain threshold) at 100 mg/kg (Table 5).

Open Field Test

The distilled water treated mice made 94.2 ± 1.43 crossings of the squares in the open field and showed 23.2 ± 1.71 assisted and 5.8 ± 1.6 unassisted rearing during the test interval of 5 minutes. ALHE at 50,

100 and 200 mg/kg significantly reduced the number of square crossings, assisted and unassisted rearings compared to the control mice (Table 6).

Hexobarbitone-Induced Sleep Test

The extract did not show any significant alteration in the effect of hexobarbitone on sleep in treated mice. Compared to the control ALHE appeared to reduce the duration of sleep at 50 and 100 mg/kg, while seeming to slightly increase sleep duration at 200 mg/kg (Table 7).

Phytochemical constituent	Inference
Alkaloids	+
Tannins	+
Saponnis	_
Reducing sugars	+
Cardiac glycosides	_
Anthraquinones	+
Flavonoids	+
Phlobatannin	-

ALHE-aqueous leaves extract of Hybanthus enneaspermus

Table 2: Effect of	f ALHE on A	cetylcholine-	Induced Writ	hing Test in Mice
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Treatment	Dose (mg/kg)	No of writhes	% Inhibition
Distilled water (ml/kg)	5	11.40±1.86	-
ALHE	50	3.8±2.2 ^a	66.7
ALHE	100	0.4±0.2°	96.5
ALHE	200	1.6±0.8 ^b	86.0
Pilocarpine + ALHE	1+100	$16.8 \pm 1.9^{\gamma}$	-
Diclofenac	5	0.4 ± 0.4^{c}	96.5

Values are mean±SEM. n=5, $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<001$ vs. control; $^{\gamma}p<0.001$ vs. ALHE at 100 mg/kg (one way ANOVA followed by Tukey's multiple comparison test). ALHE-aqueous leaves extract of *Hybanthus enneaspermus*

Table 3: Effect of ALHE in Acetic Acid-Induced Writhing Test in Mice

Treatment	Dose (mg/kg)	No of writhes	% inhibition
Distilled water (ml/kg)	5	32.8±3.6	-
ALHE	50	48.4 ± 8.4	-47.6
ALHE	100	$0.8\pm0.8^{\circ}$	97.6
ALHE	200	5.8 ± 3.7^{b}	82.3
Naloxone + ALHE	1 + 100	31.2±4.0 ^γ	4.9
Glibenclamide + ALHE	2 + 100	29.2±3.1 ^γ	11.0
Diclofenac	5	0.0±0.0°	100.0

Values are mean \pm SEM. n=5, ^bp<0.01, ^cp<0.001 vs. control; ^{γ}p<0.001 vs. ALHE (100 mg/kg) (one way ANOVA followed by Tukey's multiple comparison test). ALHE-aqueous leaves extract of *Hybanthus enneaspermus*

Table 4: Effect of ALHE in Formalin-Induced Pain in Mice

Treatment	Dose (mg/kg)	Response duration (0-5 minutes) (s)	% Inhibition	Response duration (15- 30 minutes) (s)	% Inhibition
Distilled water (ml/kg)	5	91.00±2.86	-	113.00±3.32	-
ALHE	50	68.80±0.86°	24.4	79.60±3.11 ^d	29.6

ALHE	100	59.40±1.29 ^d	34.7	70.80±2.60 ^d	37.3
ALHE	200	68.20±1.91°	25.1	77.00 ± 1.14^{d}	31.9
Diclofenac	5	57.20±2.42 ^d	37.1	54.00 ± 1.92^{d}	52.2
Morphine	2	50.60±5.55 ^d	44.4	22.00±3.65 ^d	80.5

Values are mean \pm SEM. n=5, ^cp<0.001, ^dp<0.0001 compared to control (one way ANOVA followed by Tukey's multiple comparison test). ALHE-aqueous leaves extract of *Hybanthus enneaspermus*

Treatment	Dose (mg/kg)	Pre-treatment reaction latency (s)	Post treatment reaction latency (s)	% Inhibition
Distilled water (ml/kg)	5	0.89	2.08±0.07	-
ALHE	50	1.35	7.24±0.28°	31.6
ALHE	100	1.13	11.39±0.21 ^d	54.4
ALHE	200	1.44	8.70 ± 0.28^d	39.1
Morphine	2	0.80	14.51±1.55 ^d	71.4

Values are mean \pm SEM, n=5, °p<0.001, ^dp<0.0001 vs. control (one way ANOVA followed by Tukey's multiple comparison test). ALHE-aqueous leaves extract of *Hybanthus enneaspermus*

Treatment	Dose (mg/kg)	No. of square Crossings	No. of assisted rearings	No. of unassisted rearings
Distilled water (ml/kg)	5	94.2±1.4	23.2±1.7	5.8±1.2
ALHE	50	75.6±1.9°	16.6±1.7 ^a	2.8±0.7ª
ALHE	100	63.6±3.5 ^d	$10.4{\pm}1.0^{d}$	0.2±0.2°
ALHE	200	66.2±2.9 ^d	11.0±1.3°	0.0±0.0°

Values are mean \pm SEM. n=5. ^ap<0.05, ^cp<0.001 and ^dp<0.0001 vs control (one way ANOVA followed by Tukey's multiple comparison test). ALHE-aqueous leaves extract of *Hybanthus enneaspermus*

Table 7: Effect of ALHE on Hexobarbitone-Induc	ed Sleep Test
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Treatment	Dose (mg/kg)	Duration of sleep (minutes)
Distilled water (ml/kg)	5	34.2±9.3
ALHE	50	28.0±3.0

ALHE	100	32.0±6.5
ALHE	200	39.6±10.1

Values are mean ± S.E.M, n=5. ALHE-aqueous leaves extract of Hybanthus enneaspermus

Discussion

The use of *Hybanthus enneaspermus* in folkloric medicine for conditions of pain including painless child birth formed a rationale for this study. The pain due to uterine contraction experienced in labour during child birth is reported by local users to be ameliorated by the plant. Acetylcholine is one of several mediators implicated in such pain (Samuels, 2009). Intraperitoneal injection of acetylcholine and acetic acid elicited writhing (a pain response characterized by a wave of abdominal musculature contraction followed by extension of the hind limbs) in mice. This induction of writhing by chemical substances injected intraperitoneally results from the sensitization of nociceptors by pain mediators in the peritoneal cavity.

In the study, the extract significantly inhibited the writhing induced by acetylcholine in mice. This inhibition was prevented by pilocarpine, a muscarinic agonist, suggesting that one mechanism involved in the analgesic effect of ALHE is by its blockade of muscarinic receptors.

Mediators such as prostaglandins, substance P as well as peritoneal mast cells and acid sensing ion channel pathways have been reported to play a role in acetic acid-induced writhing response (Ribeiro *et al.*, 2000). The extract in this study significantly inhibited this response in treated mice, indicating that the extract has significant antinociceptive action, which may be mediated by inhibition of the release or action of any of the implicated mediators. More specifically, inhibition of the effect of ALHE by naloxone demonstrates that the extract possibly interacts with opioid receptors to mediate its analgesic action. In addition, glibenclamide, an adenosine triphosphate dependent potassium (K_{ATP}) channel blocker, also significantly reversed the antinociceptive activity of ALHE, suggesting that the analgesic effect of ALHE is also mediated via an influence on the opening of K_{ATP} channels. It is reported that the opening of this channel, which allows for the efflux of K⁺ leads to membrane hyperpolarization state, resulting in the reduction of membrane excitability associated with nociception (Lawson, 1996).

Formalin induced pain test in mice consists of two different phases of pain perception (demonstrated by paw licking and biting); an early phase due to direct stimulation of sensory nerve fibres by the direct action of formalin, and a late phase that is due to the activities of inflammatory mediators, such as histamine, prostaglandins, serotonin and bradykinins. It is reported that centrally acting analgesics like narcotics inhibit both phases equally, while peripherally acting drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) (for example diclofenac), suppress mainly the late phase (Kumar *et al.*, 2010). The significant reduction in the duration of pain response by mice in both phases reveals the potential of the extract to relieve both centrally and peripherally mediated pain.

Pain is centrally modulated via a number of complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems (Pasero *et al.*, 1999). The tail-clip test is used for elucidation of the action of analgesics against mechanically induced pain, which is centrally mediated (Paschapur *et al.*, 2009). The significant increase in pain threshold by ALHE (50-200 mg/kg) in this model again suggests the involvement of ALHE in the relief of centrally mediated pain, which may be attributed to its influence on any of the systems involved in such pain.

Pharmacological models such as open field and hexobarbitone-induced sleep tests that directly measure CNS activity were performed to determine possible involvement of ALHE in the CNS. In the open field test, the influence of ALHE on locomotive activity of treated mice was investigated. Increased locomotor activity is associated with increase in alertness, while decreased locomotor activity is indicative of sedative or CNS inhibitory effect. In this study, the extract significantly decreased

locomotion (reducing number of square crossings) as well as exploratory behaviour (reducing number of assisted and unassisted rearing) by the mice, thus indicating CNS depressant activity. This finding demonstrates that ALHE is capable of influencing the CNS. It is possible that the analgesic effect of ALHE may be partly accounted for by its CNS depressant activity. However ALHE did not significantly influence hexobarbitone-induced sleep. Hexobarbitone belongs to the barbiturate class of anaesthetic agents, used to induce hypnosis and sedation through its modulation of gamma aminobutyric acid (GABA) receptors. The inability of the extract to potentiate the sleep action of hexobarbitone indicates that ALHE possesses mechanism(s) apparently non-GABA related for its CNS depressant effect.

Preliminary phytochemical screening revealed the presence of phytochemicals such as tannins, alkaloids, reducing sugars, anthraquinone and flavonoids, some of which could have been responsible for the analgesic effect of ALHE observed in this study. Alkaloids and flavonoids have been shown to possess analgesic activity (Sawant *et al.*, 2004; Davis *et al.*, 2009). The sedative actions of alkaloids have also been reported (Xu *et al.*, 2007).

Conclusion

The findings in this study suggest that the aqueous leaves extract of *Hybanthus enneaspermus* possesses analgesic activity possibly mediated through its interaction with opioid and muscarinic receptors as well as its influence on K_{ATP} channel. An additional activity that may contribute to the analgesia by the extract is its CNS depressant effect. The results obtained demonstrate a pharmacological basis for the use of the plant in traditional medicine for the management of painful conditions.

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