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Phytochemical screening and analgesic effect, in mice, of the methanolic leaf extract of *Manniophyton fulvum* Mull.-Arg. (Euphorbiaceae)

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Abstract

Medicinal plants continue to attract attention as an alternative form of therapy. Phytochemical screening, analgesic properties and probable mechanism/s of action of the methanol leaf extract of *Manniophyton fulvum* were carried out following claimed folkloric usefulness in this regard. Standard methods were used to screen for classes of phytochemicals. Acetic acid-induced writhing and hot plate tests in albino mice were used to evaluate the peripheral and central analgesic effects using acetylsalicylic acid and morphine respectively as positive controls, while 50% DMSO served as negative control. Elucidation of the possible mechanism/s of action of the extract was carried using phenoxybenzamine, naloxen, ondansetron, atropine and haloperidol. Alkaloids, saponins, carbohydrates, terpenoids, steroids tannins, and flavonoids were detected in the extract. At 100 mg/kg dose, significant (P<0.01) reduction in pain stimuli, compared to the negative control group in both models was observed. Higher doses of extract (200 and 400 mg/kg) did not produce any significant effect. Observed results suggest that the leaf extract of *M. fulvum* may have an analgesic effect that is centrally and peripherally mediated at a lower dose compared to the high dose. The findings provide possible scientific basis for the ethnomedicinal use of the plant in several pain conditions.

Keywords: Analgesic, *Manniophyton fulvum*, Euphorbiaceae, Hot plate, Acetic acid

INTRODUCTION

Pain is an instinct in higher animals, human inclusive that is manifested as a "distressing sensation and emotional experience that is linked to actual or potential tissue damage and discomfort" [1]. It serves the purpose of notifying the body's defense mechanism to react towards a stimulus to avoid further tissue damage and the cause of discomfort [2]. It is subjective and its perception is influenced by neurological, biological, psychological, hormonal and social - cultural differences among individuals [3]. Although the exact epidemiology of pain is uncertain at this time, it is factored as a leading cause of disability and disease burden worldwide with associated economic loss. Chronic pain impacts negatively on the quality of life and is a marker of life expectancy in the general society [4]. Pain can be classified as norciceptive, neuropathic or inflammatory based on a set of associated symptoms and mechanisms [5]. Present therapy for pain present with several side effects which are

often intolerable. For example, the non-selective, non-steroidal anti-inflammatory (NSAIDs) class of analgesics such as Acetylsalicyclic acid and ibruprofen are known to cause ulceration of the gastrointestinal system, while the selective NSAIDs drugs such as celecoxib and refecoxib are associated with increased risk of cardiovascular adverse effects [6]. Use of opioid analgesics is closely linked to the adverse effects of constipation, respiratory depression, sedation, physical dependence and tolerance among others [7]. Medicinal plants represent an alternative and attractive option in pain management, as they are perceived to be safe, effective, accessible and free from side effects [8].

Manniophyton Fulvum Mull -Arg. is a member of the Euphorbiaceae family, known locally in Bini and Igbo dialect (Southern Nigeria) as "Ebome" and "Egreregre" or "ageregre" respectively [9]. It can grow up to 30 m in height, with stems about 10 cm thick, and is used as a source of food and medicine. Ethnomedicinally, the leaves, stem bark, and root are used in the treatment of diarrhea, stomach-ache, cough, and bronchitis. The red stem-sap is credited with hemostatic properties and is used to aid wound healing. It is equally used to treat dysentery, hemorrhoids, hemoptysis, and dysmenorrhea. Stem decoctions are drunk as a healing portion for gonorrhea, while the leaf sap and dried leaf powder are sprinkled on sores to promote healing. The leaf sap is also used against heart and ear problems, caries, and insanity. Leaf decoctions are used in cases of inflammations, seeds are used against hemorrhoids and the root, stem bark, and leaf are used to treat pain-related complaints [10]. Aphrodisiac, antioxidant, anti-inflammatory, analgesic, and anti-diabetic properties of the root of the plant [11-14] have been reported, while anti-oxidant, anti-inflammatory, anti-diarrheal, and anti-diabetic activities have been determined for the leaves [15-17]. The compounds, 3α,28-dihydroxyfriedelan-1-one, manniotaraxerol A, manniotaraxerol B, 3α-hydroxy-1-oxofriedelane, betulinic acid, friedelin, taraxerol, stigmasterol, β-sitosterol, herranone, docosanoic acid, ursolic acid, nasutin B, bergenin, stigmasterol-3-O-β-D-glucopyranoside, 1,2-di-O-palmitoyl-3-O-(6-sulfo-α-D-quinovopyranosyl)glycerol (S11) and aridanin have been isolated from the twigs of the plant [18]. Previous studies have established the potential of the root of the plant as an analgesic agent [13, 14]. Same have not been documented for the leaves of the plant; hence this study was carried out to evaluate the claimed analgesic potential of the leave of *M. fulvum,* with attention on the methanol extract and profiling of the possible mechanism/s of action in albino mice.

EXPERIMENTAL METHODS

Drugs and chemicals. Acetylsalicylic acid (ASA), morphine, ondansetron, phenoxybenzamine, haloperidol, atropine, and naloxone were obtained from Sigma – Aldrich (St Louis, USA), while analytical grade methanol and acetic acid were obtained from Fenlab Chemicals (Edo State, Nigeria).

Plant collection and identification. The whole plant was freshly collected in March 2015 at Iwu village in Ovia North East Local Government Area of Edo state. The plant was authenticated by Dr. Adeyemi Oseyemi of the Forest Herbarium Unit, Forestry Research Institute of Nigeria (FRIN), Ibadan, where a Voucher specimen was deposited and the Voucher Number FH1109931 was issued.

Processing and extraction of plant material. The leaves were separated from the stem, shade dried for 1 week, and milled to powder using an electric grinding machine. The powdered Plant material (620 g) was extracted with 2.5 L of methanol, using the Soxhlet apparatus. The extract (MFE) obtained was filtered through a Whatman® filter paper (No1) and concentrated with a rotary

evaporator and hot water bath maintained at 40°C to obtain a dark sticky mass (57.83 g). This was preserved in the refrigerator at 4°C till needed.

Phytochemical screening. Phytochemical screening was carried out using methods described by Harborne [18] and Sofowora [19] to qualitatively determine the presence of secondary metabolites in the leaves of *M. fulvum.* Metabolites screened for were; alkaloids, saponins, carbohydrates, tannins, flavonoids, terpenoids, reducing sugars, steroids, and glycosides.

Animal handling. Male Albino mice weighing 25-35 g were used for this experiment. They were obtained from and housed in the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. They were exposed to 12 hours of natural light and dark cycles, humidity of 68% ± 3%, and a temperature range of 27 - 31°C. Animals were allowed free access to food and clean water. Ethical approval for the study was obtained from the ethical committee on the use of animals, of the Faculty of Pharmacy, University of Benin.

Test for analgesic activity. (Acetic acid-induced writhing test). This experiment was based on the modification of methods described by Koster et al. [20]. Animals were randomly divided into five groups of five animals each. Group 1 served as the negative control and was given 0.2 ml of 50 % dimethysulfoxide (DMSO). Group 2 animals served as the positive reference and were administered acetylsalicylic acid (ASA) at a dose of 100 mg/kg. Groups 3, 4, and 5 animals were administered MFE at a dose of 100, 200, and 400 mg/kg respectively. All administrations were par oral. One hour after administration of extract/control, acetic acid (AA) (0.6 %w/v) at a dose of 10 mg/ml was administered intraperitoneally to each mouse. The number of writhes produced in each

animal was observed and counted at intervals of 5 minutes for a period of 25 minutes. Percentage inhibition of writhing movement was calculated using the following formula:

% Inhibition of wrt response =
$$\frac{mean\ wrt\ (control) - mean\ wrt\ (test)}{mean\ wrt\ (control)} \times 100$$
………. 1

wrt = writhing response

Hot plate method. This was carried out according to the method described by Eddy and Leimbach, [21]. Animals, grouped as in the AA-induced writhing test (five groups of five animals each) were placed on top of the hot plate (LE 7406; Harvard Bioscience; USA) maintained at 55 ± 1°C. Morphine (10 mg/kg) served as the positive control, while DMSO (0.2 ml) was the negative control. Each mouse was individually placed on the hot plate to determine their reaction to pain induced by heat, which is manifested as lifting, licking of the paws, and subsequent jumping off the top of the hot plate. The time from when animals were placed on the hot plate to manifesting signs of discomfort/pain was recorded as latency time. Latency time was recorded at five minutes before extract/controls treatment and 30, 60, 90, 120, 150 and 180 minutes after administration of extract /controls.

Determination of probable mechanism(s) of action of analgesic activity of *M. fulvum* extract. The AA-induced mouse writhing assay was used in evaluating the possible mechanism of anti-nociception activity of MFE. Six groups of mice of five animals each were pretreated intraperitoneally with ondansetron (1 mg/kg), phenoxybenzamine (0.1 mg/kg), haloperidol (0.1 mg/kg), atropine (1 mg/kg), naloxone (1 mg/kg) and 50% DMSO (0.2 ml) respectively, followed 15 minutes later by oral administration of MFE (100 mg/kg). One hour later, AA (0.6 %v/v; 0.2 ml) was administered to animals in the different groups, and the number of writhes in each animal was observed and recorded. To further understand the possible mechanism of

action of MFE, animals were grouped into four groups of five animals each. Group 1 animals received haloperidol (0.1mg/kg), while groups 2 and 3 animals were treated with MFE (100 mg/kg); haloperidol (0.1 mg/kg) and MFE (100 mg/kg) respectively. Group 4 animals served as negative control and received DMSO (0.2 ml). Animals in each group were exposed to pain by the intraperitoneal administration of AA to induced writhing, one hour after the administration of drug/extract or control and the number of writhes evoked was counted and recorded at interval of 5 minutes for 25 minutes.

Data analysis. Data are presented as mean ± S.E.M and analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test, using the SPSS statistical software package (Version 20.0). Values of $P < 0.05$ were considered statistically significant.

RESULTS
Phytochemical screening. The different classes of secondary metabolites identified qualitatively in the leaves of *M. fulvum* are shown in Table 1. Alkaloid, flavonoids and tannins were abundantly present.
Acetic acid-induced writhing in mice. The extract at a dose of 100 mg/kg significantly ($p < 0.05$) decreased the number of AA-induced writhes in control mice from 89.20±14.75 (not shown) to 34.80±7.03 corresponding to pain inhibition of 60.99%. At higher doses of 200 and 400 mg/kg, the percentage inhibition of writhes in mice was lower signifying reduced

ability to inhibit pain compared to the 100 mg/kg and ASA treated animals (Figure 1).

Hot plate test. The extract at a dose of 100 mg/kg significantly ($p < 0.01$) increased the reaction time of the animals in response to thermal stimuli, from 4.17 ± 0.61seconds in negative control animals to 6.67 ± 0.62 seconds 60 minutes after administration and to 7.73 ± 0.60 seconds, 120 minutes after administration. The same pattern of results was obtained for morphine, the standard drug at a dose of 10 mg/kg with values of 8.55 ± 0.43 seconds and 12.08 ± 0.71 seconds respectively. However, the 200 and 400 mg/kg doses of the extract did not elicit any significant effect compared with the control.

Elucidation of probable mechanism(s) of action of anti-nociceptive action of *Manniophyton fulvum* extract. Pre-treatment of animals with phenoxybenzamine produced 29.65±4.18 number of writhes in mice. This was similar to results observed for naloxon. Ondansetron and atropine pre-treatment evoked a significant increase in writhing and decreased analgesic activity in the animals, but did not completely reverse the anti-nociceptive effect of MFE (100 mg/kg) as shown in Table 1. Co-administration of MFE and haloperidol with AA markedly reduced the number of writhing, necessitating further investigation.

Administration of haloperidol and MFE to AA-induced animals resulted in a near-complete abolition of writhing in mice (Table 3).

Table 1: Phytochemicals present in the leaves of *M. fulvum*

Phytochemicals	Results
Alkaloids	+++
Carbohydrate	++
Flavonoids	+++
Glycosides	++
Saponins	+
Steroid	++
Tannins	+++
Terpenoids	++

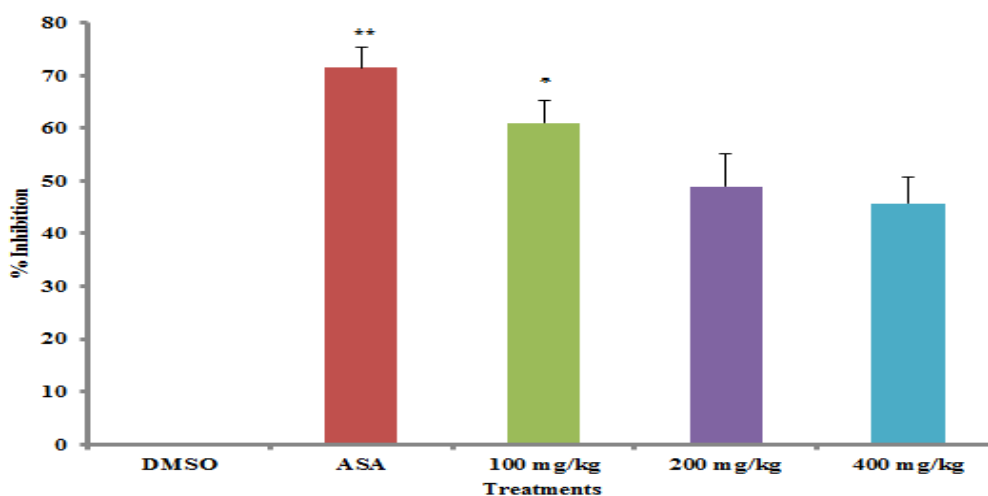**Figure 1**: Effect of MFE on AA-induced writhes in mice. Data are presented as mean ± S.E.M. Statistically significant *p < 0.05; Statistically significant **p < 0.01.
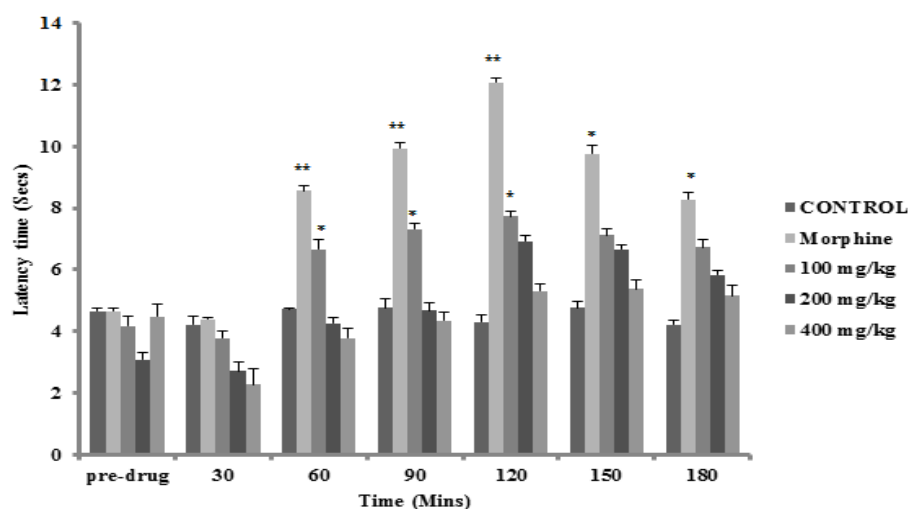

Figure 2: Effect of MFE on reaction time in the hot plate test in mice.

DISCUSSION

Phytochemical screening of the leaves of *M. fulvum* showed it contained secondary metabolites of various classes. Flavonoids, which were qualitatively determined to be present in the leaves of *M. fulvum* in this study, have been cited to evoke analgesic activities by blocking cycloxygenase, lipooxygenase and, phospholipase A_2 and C enzyme activities [22]. Furthermore, several flavonoid glycosides have been isolated from the leaves of *M. fulvum* and include; myricetin-3-*O*-β-D-rhamnoside, kaempferol-3-*O*-β-D-rhamnoside, quercetin-3-*O*-β-D-glucoside, quercetin-3-*O*-β-D-rhamnoside, quercetin-3-*O*-β-D-galactoside, rutin and, quercetin [22, 23]. In particular, quercitin and myricetin have been shown to exert analgesic effects by inhibiting inflammatory and neuropathic pain through inhibition of prostaglandin and cytokine production in the AA-induced pain test [24].

Table 2: Effect of pre-treatment with drugs and MFE on acetic acid-induced writhing in mice

Treatment	Mean number of writhes
Ondansetron (1 mg/kg) + Extract	50.00 ± 1.79*
Phenoxybenzamine (0.1 mg/kg) + Extract	29.65 ± 4.18
Haloperidol (0.1 mg/kg) + Extract	2.20 ± 0.33**
Atropine (1mg/kg) + Extract	58.06 ± 15.42*
Naloxone (1mg/kg) + Extract	28.90 ± 5.66
DMSO + Extract (100 mg/kg)	36.56 ± 3.88

Results are presented as means ±SEM; *p<0.05; **p<0.01

Table 3: Effects of co-administration of haloperidol and MFE, haloperidol alone and MFE on acetic induced writhing in mice on writhing

Drug administered	Mean number of writhes
Haloperidol(0.1 mg/kg)	21.50 ± 1.06[*]
Extract (100 mg/kg)	38.50 ± 5.30[*]
Haloperidol (0.1 mg/kg) + Extract (100 mg/kg)	1.00 ± 0.00[**]
Dimethylsulfoxide (0.2 ml/kg)	89.20 ± 14.75

Results are presented as Means ±SEM; *p < 0.05; **p < 0.01

The alkaloid-rich fraction of *Ziziyphus nummularia* showed significant inhibition of pain responses induced in the AA-induce writhing, hot plate and, tail-flick tests, providing evidence for the role of alkaloids in inhibiting pain response induced by various means [25]. This is indicative that flavonoids and alkaloids, present in the leaves of *M. fulvum*, may be partly responsible for the observed analgesic effect of MFE.

The AA-induced mice writhing test as used in this study was to evaluate the peripheral analgesic activities of MFE. The test as a measure of analgesic activity is considered a sensitive assay as it can detect peripheral activities of a drug at doses that may be inactive in other methods such as the tail-flick test [26, 27]. Writhing in mice is characterized by arching of the back, an extension of the hind limb, and contraction of the abdominal muscle as a manifestation of pain and discomfort due to the injection of agents such as acetic acid [28].

Analgesic activity of test compounds/extracts in the AA-induced writhing test is inferred from a decrease in the number/frequency of writhing as previously described [26]. The extract at the lowest administered dose of 100 mg/kg significantly decreased the number of writhing compared to the negative control. This activity was comparable to that produced by ASA, the standard drug. Results presented here are comparable to that obtained from previous studies with extract of the root of *M. fulvum* [14], supporting the claim that the plant may possess potential analgesic properties. However, the analgesic effect obtained from previous studies with the root of the plant was dose-dependent, a pattern of activity which could not be substantiated in this study. The extract at the higher doses of 200 and 400 mg/kg produced less inhibition of writhes compared to the 100 mg/kg dose. Plant extracts owe their biological activities to hundreds of individual constituents which they contain at varying abundance. The mixture of compounds present in an extract at varying concentration can act synergistically, additively or antagonistically [29]. In this study, it can be inferred that the combination of compounds present at higher doses of MFE acted in an antagonistic manner as less inhibition of writhes was observed at higher doses. Acetic acid induces pain through the liberation of endogenous mediators of pain such as serotonin histamine, prostaglandins (PGs), bradykinins and substance P [30].

Equally, increased levels of PGE2, PGF2α and lipoxygenase products in peritoneal fluids as well as increased sensitivity of local peritoneal receptors are involved [31, 32]. The inhibition of the AA-induced writhing in mice in this study, suggests that the analgesic activity of MFE may be peripherally mediated, possibly through the inhibition of the activity and/or production of one or more of the aforementioned mediators.

The hot plate model was used to evaluate the centrally mediated analgesic activities of MFE. The hot-plate model measures the behavioral activities of rats when placed on a hot plate maintained at a temperature which is not damaging to the skin of rats (55ºC), but able to elicit reaction such as hind paw lifting, hind paw licking, or jumping off the hot plate. The standard class of drug used in this model is an opioid analgesic. This is because opioids act centrally to limit the input of nociceptive information into the central nervous system thus reducing central hypersensitivity [33]. The hot plate model is suitable for measuring the analgesic activities of opioids but not sensitive in measuring the analgesic effects of other analgesics such as non-steroidal anti-inflammatory drugs [34]. From the result of the hot plate test, the extract at the lowest dose of 100 mg/kg demonstrated significant analgesic activity which was comparable to that obtained with morphine, the standard drug. This suggests that MFE may possess analgesic activity that possibly involves the central mechanism. This model uses morphine as the reference drug and morphine demonstrated a potent analgesic effect in this model indicating the sensitivity of this test [35].

Generally, analgesics are classified into peripherally acting and centrally acting analgesics. Peripherally acting analgesics act by blocking the generation of impulses at the chemoreceptor site of pain, while the centrally acting analgesics increase the threshold of pain, alter the physiological response to pain

and also, suppress the patient's anxiety and apprehension due to pain [36]. To further gain insight into the possible mechanism of analgesic effect of the MFE, the AA-induced mouse writhing test was used. The result obtained provides evidence suggesting a possible involvement of the serotonergic and/or muscarinic receptors in the anti-nociceptive effect of MFE. Pre-treatment with ondansetron, a serotonin $5HT_3$ receptor antagonist, and atropine a competitive reversible antagonist of the muscarinic acetylcholine receptors, significantly reversed the analgesic effect of the extract. These receptors are situated in the central nervous system and peripheral nervous system respectively. The reversal of the analgesic effects of MFE upon pretreatment with atropine and ondansetron, suggests the non-involvement of the cholinergic (albeit the muscarinic) and serotonergic receptor system or possibly an antagonistic interaction. Studies have suggested nociceptive effects for serotonin [37] and that the anti-nociceptive activities of various analgesics depend on the integrity of descending serotonergic system [38].

The anti-nociceptive effect of the extract was not antagonized by pretreatment with phenoxybenzamine and naloxone indicating that the adrenergic and opioidergic systems may not be involved in the anti-nociceptive effect of the extract. However, pretreatment with haloperidol a dopamine D_2 receptor antagonist returned results that showed a high reduction in writhing, suggesting a possible involvement of the dopaminergic system in anti-nociception. Further tests conducted with haloperidol alone revealed a drastic reduction in pain responses. Since a combination of haloperidol and extract produced a marked inhibition of pain, it is possible to infer that haloperidol and the extract act synergistically to inhibit pain caused by acetic acid. Dopamine D_2 receptors are present in the superficial dorsal horn [39], where dopamine is involved

in the inhibition of the responses to harmful stimuli of the dorsal horn neurons [40].

Conclusion. This study has shown that the methanol leaf extract of *M. fulvum* possesses significant analgesic properties in mice which may be mediated through peripheral and central mechanisms. It also revealed that the possible mechanism of detected analgesic activity may be through involvement of the dopaminergic system. Equally, the extract was observed to more active at lower doses compared to higher ones. Some secondary metabolites were, also identified in the extract.

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