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Analgesic activity and effect of *Maerua angolensis* stem bark extract and fractions on morphine dependence in mice

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Abstract
Pain is associated with most pathological conditions in humans that affects thinking, sleeping, emotion and performance of daily chores, thereby making it an important therapeutic priority for control of pains. In conditions like advanced cancer, the only viable therapeutic option is management of the pain with analgesics, but potent and safe analgesics is limited. Opioids such as morphine are suitable for moderate to severe pain but their frequent use causes physical dependence and tolerance. Since *Maerua angolensis is* a medicinal plant used traditionally in the treatment of pain, the study examined the analgesic effect of the petroleum ether/ethyl acetate extract and fractions prepared from the stem bark of this plant in the mouse tail-flick test using Hargreaves thermal hyperalgesia model. The effect of the extract and fractions on morphine dependence was also assessed in mice. Dependence was induced using subcutaneous injections of morphine at doses of 50, 50 and 75 mg/kg three times daily for 3 days. On day 4, morphine was injected 2 hours prior to the intraperitoneal injection of naloxone. The number of jumps during the 30 minutes period after naloxone injection was taken as a measure of the withdrawal syndrome. The extract and fractions from *Maerua angolensis* (3 – 30 mg/kg, orally) significantly ($P < 0.0001$) and dose-dependently attenuated nociception in the tail-flick test and produced dose-dependent inhibition of the number of jumps comparable to muscimol and baclofen acting on Gamma-Amino Butyric Acid system. Additionally, the inhibition of jumping caused by the extract and fractions was reversed by intraperitoneal treatment of mice with bicuculline (Gamma-Amino Butyric Acid $_A$ receptor antagonist) and aminophylline (a non-selective adenosine receptor antagonist) suggesting stimulation of Gamma-Amino Butyric Acid and adenosine transmission. The antinociceptive effect and the suppression of withdrawal syndrome of morphine dependence established in this study contribute to the analgesic knowledge of this species.

Keywords: Tail-flick test, Morphine dependence, Maerua angolensis

1. Introduction
Pain is associated with most pathological conditions in humans that affects thinking, sleeping, emotion and performance of daily chores, [1, 2] thereby making it an important therapeutic priority for control of pains. In many pathological conditions, particularly HIV/AIDS, diabetes and cancer, the management of pain remains a cause for concern. In conditions like advanced cancer, the only viable therapeutic option is management of the pain with analgesics, but potent and safe analgesics is limited [1]. The non-opioids, paracetamol and acetyl salicylic acid including other non-steroidal anti-inflammatory drugs (NSAIDs), are particularly suitable for mild to moderate pain in musculoskeletal conditions, whereas the opioids such as morphine are more suitable for moderate to severe pain, particularly of visceral origin. However, both opioids and NSAIDs have known toxic and lethal effects which limit their clinical use [3-5]. Gastric irritation is a major side effect of NSAIDs, whereas the frequent use of opioids causes physical dependence and tolerance. A range of agents and systems such as noradrenergic system, adenosine receptor agonists, excitatory amino acid antagonists, protein kinase C inhibitors, glucocorticosteroids, benzodiazepines and arachidonic acid can modulate the morphine withdrawal syndrome [6]. Medicinal plants of recent, especially in developing countries, have been the subject of intense research due to their potential as sources of commercial drugs or as lead compounds in drug development [7]. It is on record that about 80 % of people living in the world, notably those living in the developing world, depend on traditional medicine for their primary health care needs [8]. This is due to the perceived low cost, easy access and the belief that these medicines are devoid of adverse effects as well as Blending readily into the sociocultural life of the people [9].
Maerua angolensis DC (family Capparidaceae) is a medicinal plant used traditionally in the treatment of various painful conditions in Nigeria and some West African countries [10, 11]. It is found in bush and rocky areas of the Savannah in tropical Africa.

Various parts of the plant notably the leaves, roots and stem barks are claimed to relieve pain and also used to manage psychosis, epilepsy, diabetes, peptic ulcer, diarrhoea and arthritis in the traditional medicine [12-15]. Phytochemical study indicated that the methanolic stem bark extract has steroids, saponins, tannins, terpenoids, flavonoids, alkaloids, glycosides, carbohydrates and proteins [12, 13]. Many of these bioactive substances are involved in the modulation of pain sensation. [16] The median lethal dose (LD_{50}) of the stem bark extract of the plant in mice has been reported to be 3,807.9 mg/kg orally and greater than 500 mg/kg intraperitoneally [14] indicating relative safety of the stem bark. The analgesic effect of the stem bark (the most potent plant part) in petroleum ether, ethyl acetate and aqueous ethanol separately were demonstrated in writhing and formalin tests [17]. Since opioids such as morphine are suitable for moderate to severe pain but their frequent use causes physical dependence and tolerance, a medicinal plant such as *Maerua angolensis* known to contain bioactive substances involved in the modulation of pain sensation and used traditionally in the treatment of pain might also have some protective effects on the withdrawal syndrome of morphine dependence in its analgesic activity. This study, therefore, assessed the analgesic effect of the petroleum ether/ethyl acetate extract and fractions prepared from the stem bark of *Maerua angolensis* in the mouse tail-flick test using Hargreaves thermal hyperalgesia model. The effect of the extract and fractions on morphine dependence was also assessed in mice.

2. Materials and methods

2.1 Plant materials

Fresh stem barks of *Maerua angolensis* were collected from the Samaru campus of Ahmadu Bello University, Zaria, Nigeria and were identified at Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. A voucher specimen (KNUST/FP/12/051) was kept at the herbarium of the Faculty.

2.2 Animals

Male ICR mice (20 – 25 g) were used after approval of protocol for the study by the local ethical committee for animal handling and experimental procedure. All animals were housed in groups of five in stainless steel cages (34 x 47 x 18 cm) with softwood shavings as bedding in the animal facility of the Department, KNUST, with free access to food and water and were maintained under normal laboratory conditions of humidity, temperature (25 ± 1 ^{0}C) and a 12 h/12 h day/night cycle. Each animal was used only once. The investigation conforms to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH No. 85 – 23, revised 1996). In all the experimental studies each group consisted of 5 animals.

2.3 Extraction and fractionation

For the preparation of the extract, 4 kg of dried powdered stem barks was sequentially extracted for 4 days with 10 L of petroleum ether and 10 L of ethyl acetate by cold maceration. The materials were filtered and the filtrates concentrated separately in a rotary evaporator under reduced temperature and pressure to give yellowish syrupy mass. These were dried in the oven (Gallenhamp®, England) at 50 ^{0}C to obtain yields of 2.23 and 3.87 % (w/w) respectively. The two extracts were then combined and subsequently referred to as petroleum ether/ethyl acetate stem bark extract of *Maerua angolensis* (MAE).

The MAE (10 g) was fractionated using the chromatography column technique in which silica gel 60 F_{254} (Merck, 0.20 mm thickness) was used as adsorbent and elution effected using the solvents petroleum ether (100 %), petroleum ether and ethyl acetate (90:10), and petroleum ether and ethyl acetate (50:50) sequentially in order of increasing polarity. A total of 64 fractions were collected in 100 ml aliquots. Continuous elution with 100% petroleum ether yielded 31 fractions (fractions 1 – 31). Further elution with petroleum ether and ethyl acetate (90:10) yielded 24 fractions (fractions 32 – 55) while 9 fractions (fractions 56 – 64) resulted from continuous elution with petroleum ether and ethyl acetate (50:50). Fractions with similar thin layer chromatography (TLC) profiles suggesting similar phytocomponents were combined and concentrated. Fractions 1 -2 were combined giving a yield of 5.5% (w/w) and coded F1 while fractions 32 – 34 were combined giving a yield of 6.7% (w/w) and coded F32. Fractions 3 – 31 and 35 – 64 did not show any spot on TLC and were discarded.

2.4 Assessment of the analgesic effect of *Maerua angolensis* extract and fractions in the tail-flick test using Hargreaves thermal hyperalgesia model

Tail–flick latencies were determined with the IITC Analgesia Meter (model 336, IITC Life Science Inc., Woodland Hills, CA, USA). Mice (male) were individually placed in a transparent plexi glass observation chamber on a clear glass platform for acclimatization period of 15 min to the testing environment. A focused beam of radiant light was delivered to the tail tip until the mouse flicked the tail [18, 19]. Basal reaction times of mice were taken before the administration of the petroleum ether/ethyl acetate extract of *Maerua angolensis* (MAE), the fractions (F1, F32) (3, 10 and 30 mg/kg, p.o.), morphine (0.3, 1 and 3 mg/kg, i.p.) or normal saline (10 ml/kg, i.p.). A timer was set to automatically turn off the light source when the mouse withdrew the tail, and the tail withdrawal latencies (TWLs) measured was defined as the time required for the tail to show an abrupt withdrawal. TWLs were measured again at 1, 2, 3 and 4 h post–drug administration. A cut–off time of 25 s was used in order not to cause any tissue injury to the tail. Mice received two training sessions before the day of testing. Antinociceptive effects exerted by drugs were calculated from the TWLs as a percentage of the maximum possible effect (% MPE) using the formula: [(post drug – pre-drug latencies/ (cut off time – pre-drug latency) X 100].

2.5 Assessment of the effect of *Maerua angolensis* extract and fractions on the withdrawal syndrome of morphine dependence

To induce morphine dependence, morphine was injected subcutaneously (s.c.) to mice at doses of 50, 50 and 75 mg/kg three times daily (10.00, 13.00 and 16.00 h, respectively) for 3 days. On day 4, a single dose of morphine (50 mg/kg) was injected [20, 21]. To precipitate morphine withdrawal signs, naloxone was injected (5 mg/kg, s.c.) 2 hours after the last administration of morphine. After the naloxone challenge, mice were immediately placed in a glass cylinder (30 cm high, 20 cm in diameter). The number of jumping episodes (withdrawal symptoms) was recorded for 30 min. Different groups of morphine-dependent mice (as described above) were pre-treated either with MAE, F1, F32 (3 – 30 mg/kg, p.o.), muscimol (0.5, 1 and 2 mg/kg, i.p.), baclofen (0.5, 2 and 3

mg/kg, i.p.) or normal saline (vehicle control, 10 ml/kg, i.p.). An hour after the oral and 30 min after the intraperitoneal administration, the final dose of morphine was administered 2 hours before naloxone and the number of jumps for 30 min recorded.

To investigate the possible mechanisms of MAE and F1 inhibition of withdrawal syndrome of morphine dependence, different groups of morphine dependent mice were pre-treated with MAE or F1 (10 mg/kg, p.o.) plus bicuculline (3 mg/kg, i.p.) or aminophylline (20 mg/kg, i.p.) 1 h before the final dose of morphine. Naloxone (5 mg/kg, s.c.) was administered 2 hours after the last dose of morphine and the number of jumps for 30 min recorded. The results are stated as a change in the number of jumps as compared to the vehicle control or to MAE or F1.

2.6 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) per group. Raw data for the tail-flick test in Hargreaves thermal hyperalgesia model was calculated as the percentage change in maximum possible effect (% MPE). The time-course curves were subjected to two-way (treatment x time) repeated measures analysis of variance (ANOVA) with Bonferroni's post hoc test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). To determine the percentage inhibition for each treatment, the following formula was used:

$$\% \text{ inhibition} = \{AUC_{control} - AUC_{treatment}/AUC_{control}\} \times 100$$

Differences in AUCs were analysed using one-way ANOVA with drug treatment as a between subjects factor. Further comparisons between vehicle- and drug-treated groups were performed using the Newman-Keuls' post hoc test. Doses for 50 % of the maximal effect (ED_{50}) for each drug and 95 % confidence intervals values were determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) using the formula:

$$Y = a + (b - a)/ (1 + 10^{(LogED_{50} - X)}$$

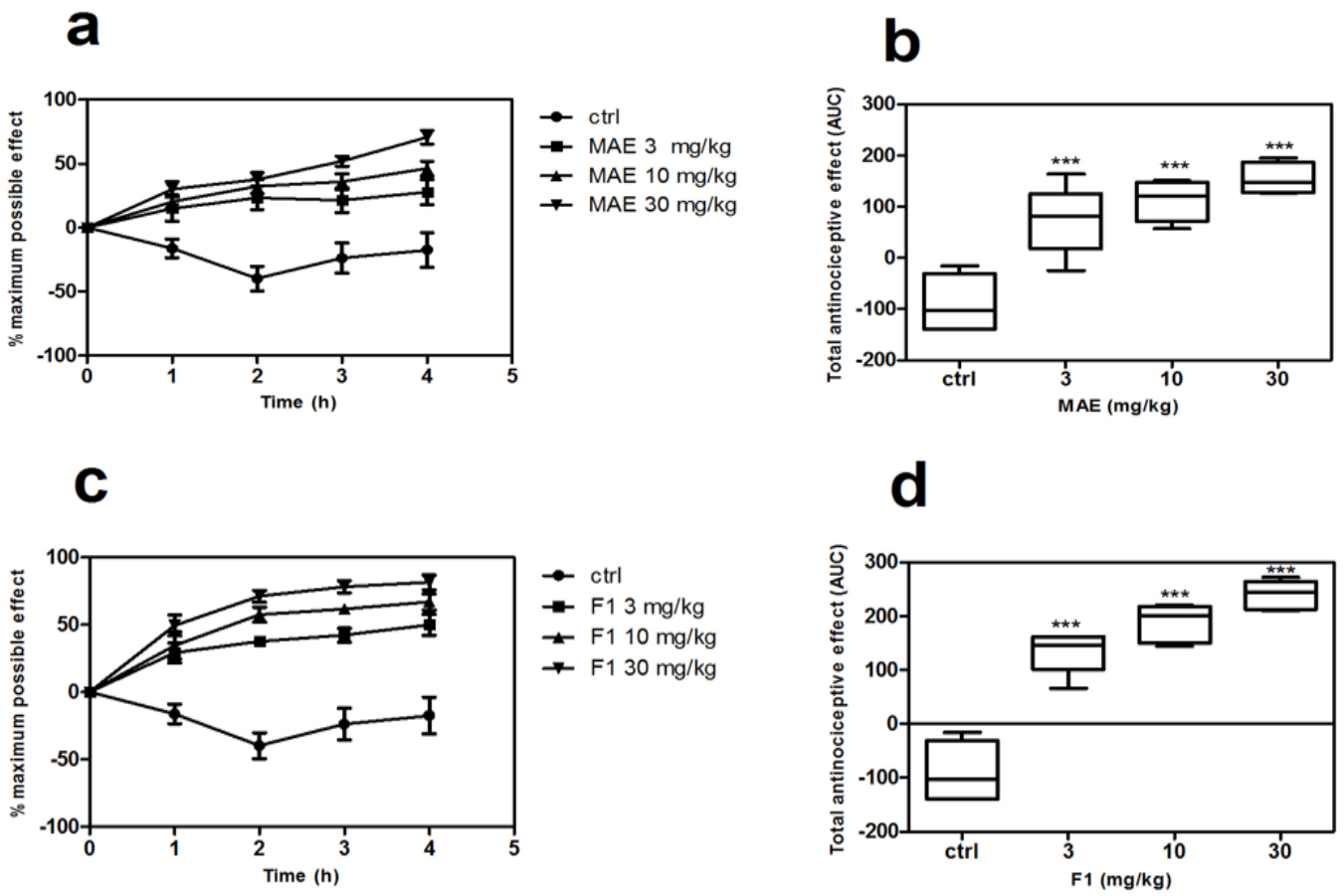
Where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

The fitted mid-points (ED_{50}s) of the curves were compared statistically using F test. [22] GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was taken to be statistically significant.

3. Results

3.1 Analgesic effect of *Maerua angolensis* extract and fractions in the tail–flick test using Hargreaves thermal hyperalgesia model

An increase in tail-flick latency calculated as % MPE was observed in all mice pre-treated with test drugs [(MAE: $F_{3, 80} = 57.71$, $P < 0.0001$; F1: $F_{3, 80} = 119.9$, $P < 0.0001$; F32: $F_{3, 80} = 135.4$, $P < 0.0001$; morphine: $F_{3, 80} = 136.8$, $P < 0.0001$) two-way ANOVA (treatment x time) (Figure 1a, c, e and g)]. The petroleum ether/ethyl acetate stem bark extract, the fractions of *Maerua angolensis* (3 – 30 mg/kg, p.o.) and morphine used as reference analgesic (0.3 – 3 mg/kg, i.p.) caused dose-dependent antinociceptive effect which were significant [(MAE: $F_{3, 16} = 22.38$, $P < 0.0001$; F1: $F_{3, 16} = 63.36$, $P < 0.0001$; F32: $F_{3, 16} = 59.73$, $P < 0.0001$; morphine: $F_{3, 16} = 75.56$, $P < 0.0001$)] (Figure 1b, d, f and h). The ED_{50} values showed antinociception of morphine (0.2092 ± 0.1063 mg/kg) was more potent than F1 (1.656 ± 0.1224 mg/kg) more potent than F32 (1.680 ± 0.1403 mg/kg) more potent than MAE (1.760 ± 0.2235 mg/kg).



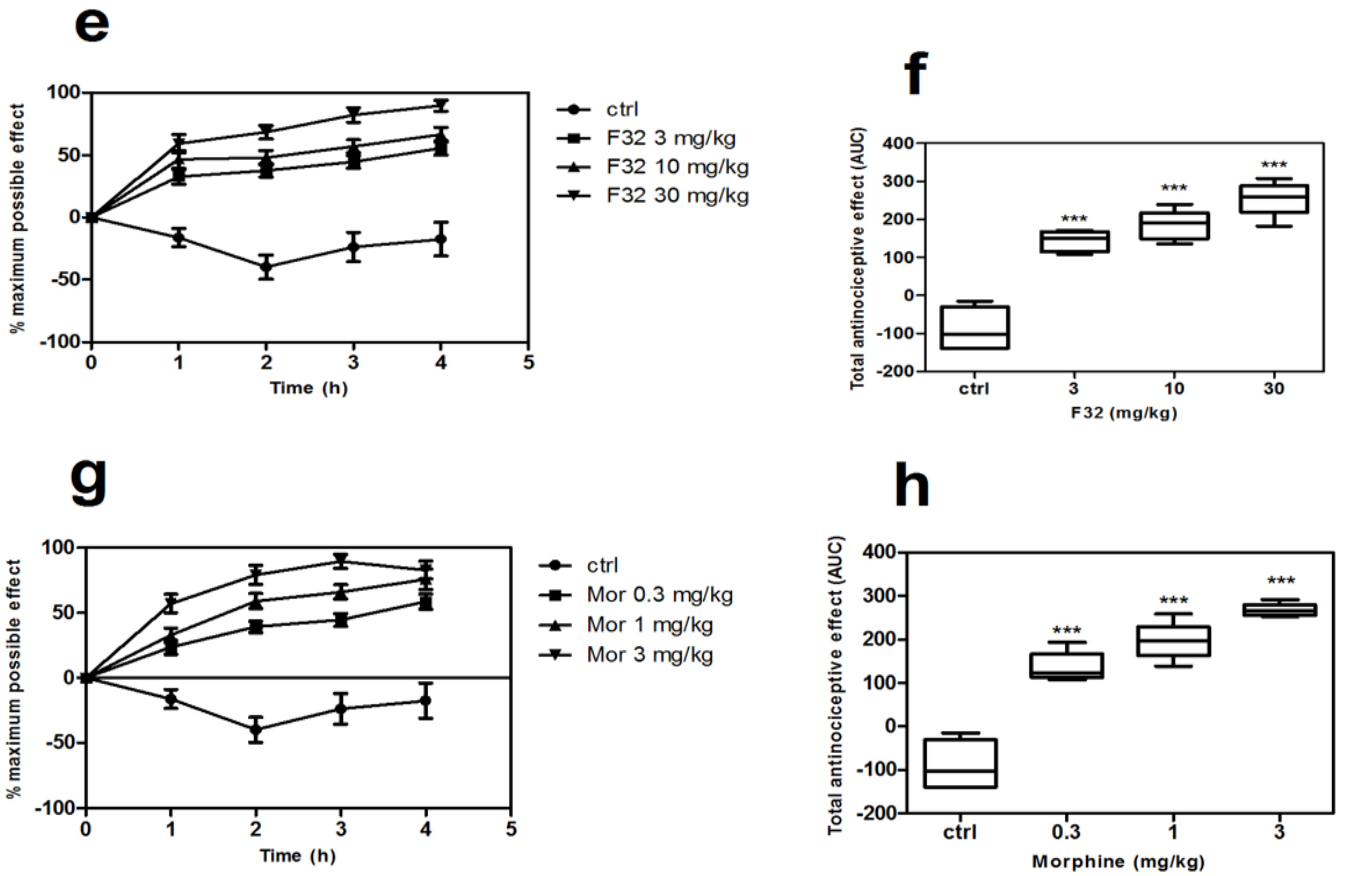
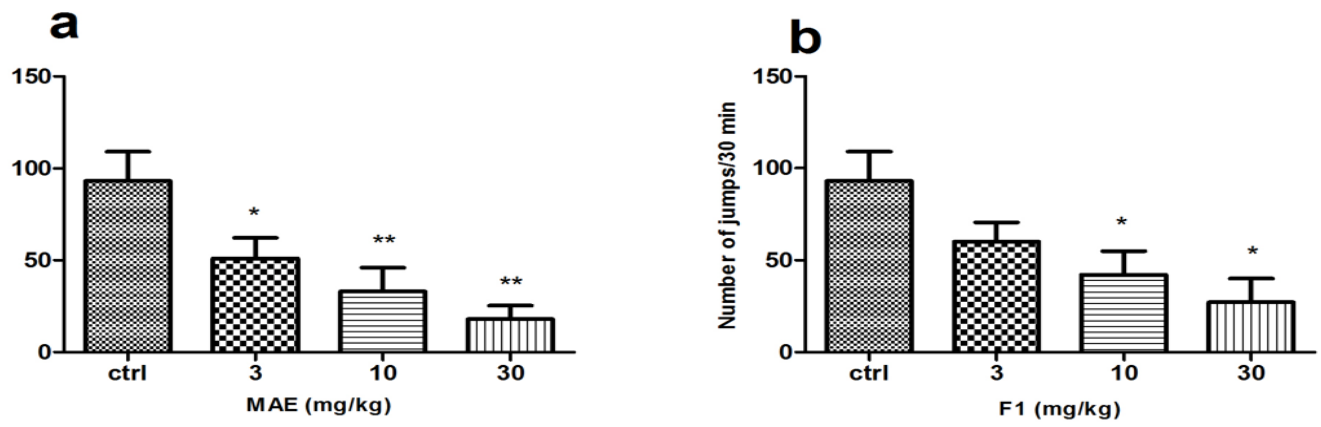


Fig 1. Effect of MAE, F1, F32 (3 – 30 mg/kg, p.o.) and morphine (0.1 – 3 mg/kg, i.p.) on the time course curve of tail-flick in Hargreaves thermal hyperalgesia model (a, c, e and g) and the total nociceptive score (calculated as AUC) (b, d, f and h) in the mice. Data are expressed as mean ± SEM (n = 5). The lower and upper margins of the boxes (b, d, f and h) represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles, respectively. The median is shown as the horizontal line within the box. ***P<0.001 compared to vehicle-treated group (one-way ANOVA followed by Newman-Keuls post hoc test). MAE, Maerua angolensis extract; F1, Maerua angolensis fraction 1; F32, Maerua angolensis fraction 32; ctrl, control.

3.2 Effect of *Maerua angolensis* extract and fractions on the withdrawal syndrome of morphine dependence

Administration of MAE; F1 and F32 1 h before the last dose of morphine in the presence of naloxone significantly ($F_{3, 16}$ = 6.980, P = 0.0032; $F_{3, 16}$ = 4.598, P = 0.0167 and $F_{3, 16}$ = 3.152, P = 0.0539) and dose dependently supressed the jumping behaviour in mice. MAE, F1 and F32 at the highest doses used blocked the morphine-dependent withdrawal effect by 80.7 ± 7.4, 71 ± 12.9 and 67.7 ± 10.6 %, respectively (Figure 2a, b and c). The intraperitoneal administration of the gamma-amino butyric acid (GABA) $_A$ and GABA $_B$ receptor agonists, muscimol and baclofen (30 min before the final dose of morphine), also significantly ($F_{3, 16}$ = 4.519, P = 0.0177 and $F_{3, 16}$ = 14.38, P < 0.0001 respectively) and dose-dependently reduced the jumping reaction (Figure 2d and e).

The mechanism involved against morphine withdrawal symptoms was tackled using antagonist of GABA $_A$ receptors (bicuculline) and the non-selective adenosine receptors antagonist (aminophylline). The inhibitory effect of MAE was antagonized in the presence of bicuculline and aminophylline (Figure 3a and b), whereas the effect of F1 was found to be suppressed in the presence of aminophylline but not in the presence of bicuculline (Figure 3c and d).



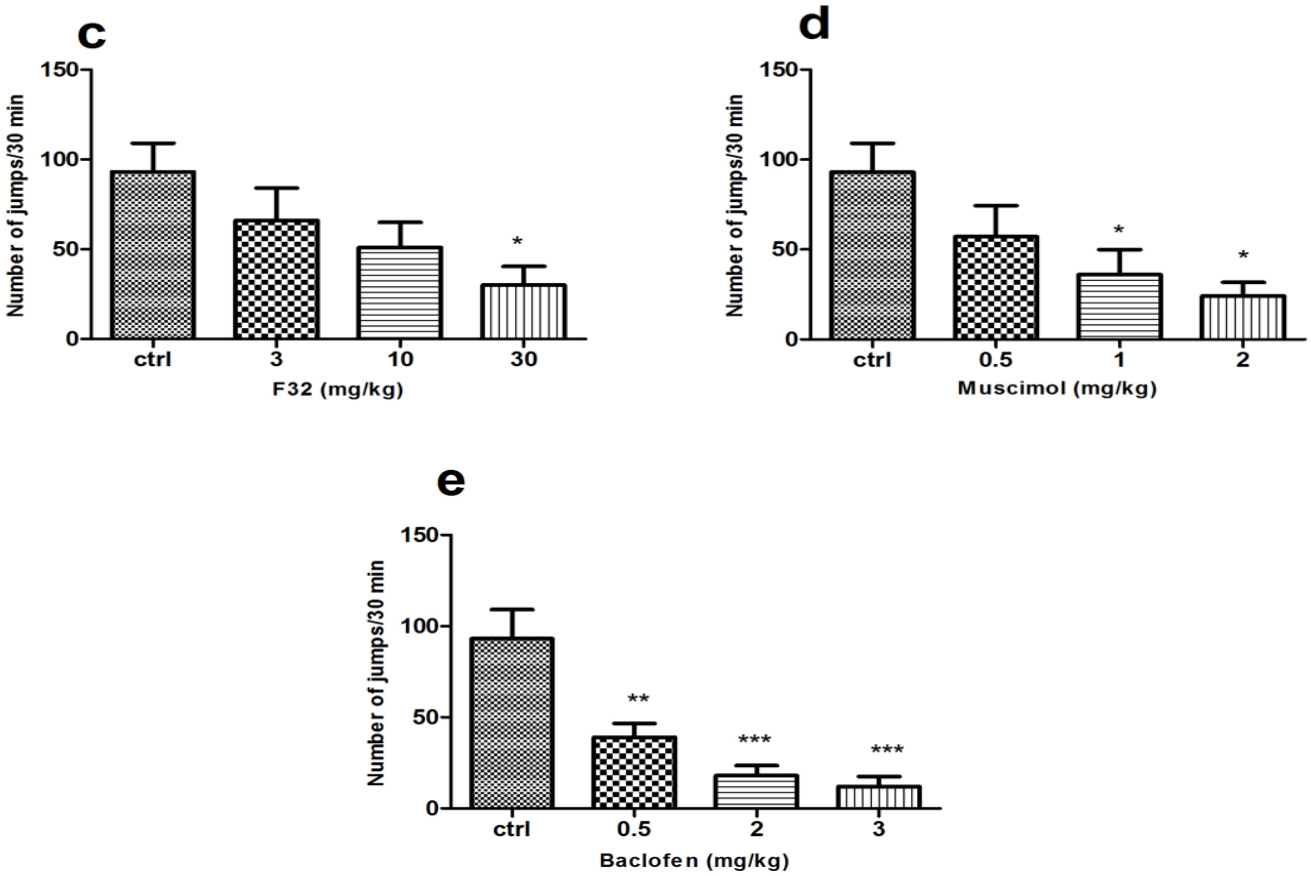


Fig 2: Effect of MAE (a), F1 (b), F32 (c) (3 – 30 mg/kg, p.o.), muscimol (d) (0.5 – 2 mg/kg, i.p.) and baclofen (e) (0.5 – 3 mg/kg, i.p.) on the withdrawal syndrome of morphine dependence in mice. Each column represents the mean of 5 mice, and the error bar indicates the SEM. Asterisks denote the significance levels compared with control groups (one-way ANOVA followed by Newman Keuls post hoc test): $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. MAE, Maerua angolensis extract; F1, Maerua angolensis fraction1; F32, Maerua angolensis fraction 32; ctrl, control.

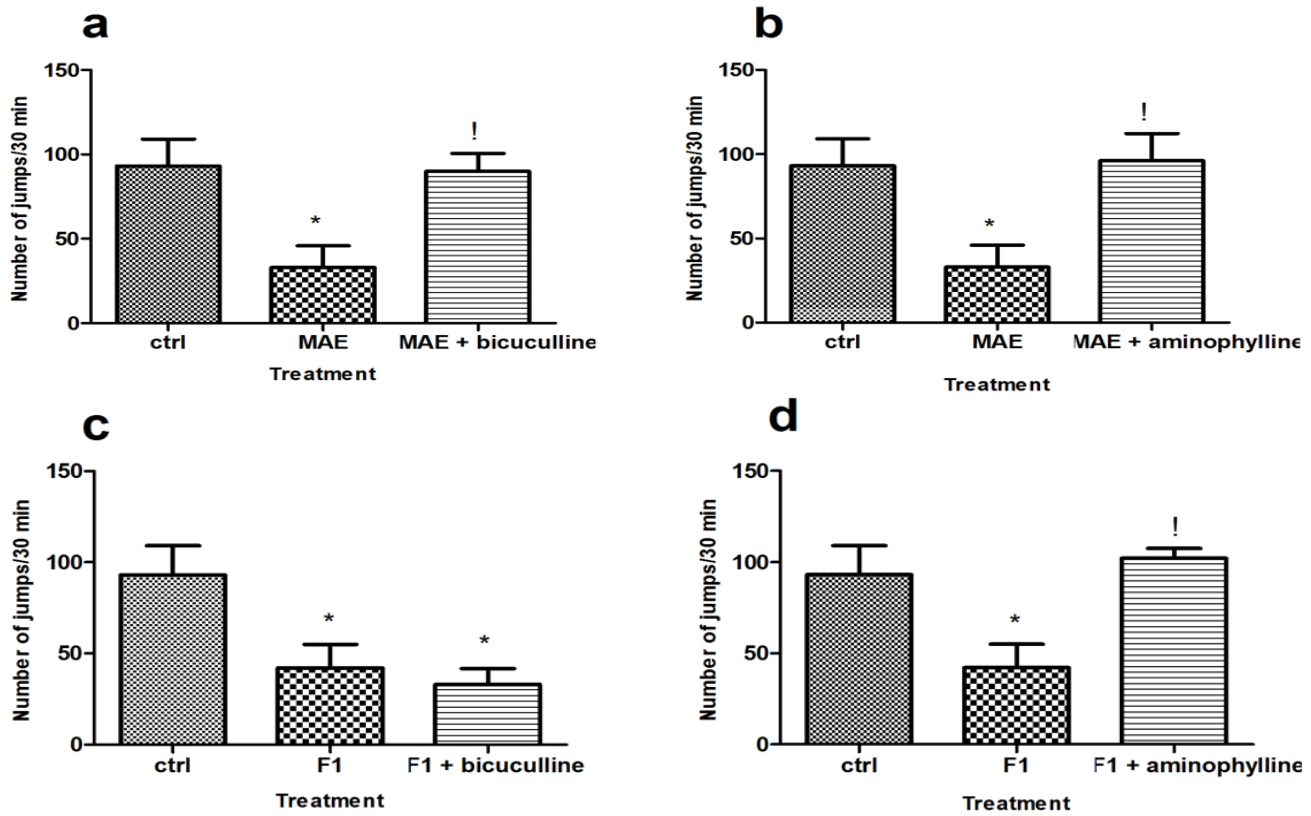


Fig 3. Effect of bicuculline (3 mg/kg, i.p.) and aminophylline (20 mg/kg, i.p) on the inhibitory effect of MAE (a, and b) and F1 (c and d) against morphine dependence withdrawal symptoms. Each column represents the mean of 5 mice, and the error bar indicates the SEM $^{*}P<0.05$ compared to control, $^{!}P<0.05$ compared to MAE or F1 (one-way ANOVA followed by Newman-Keuls post hoc test). MAE, Maerua angolensis extract; F1, Maerua angolensis fraction 1; ctrl, control.

4. Discussion

Results of the present study show that oral administration of the petroleum ether/ethyl acetate extract and fractions prepared from stem barks of *Maerua angolensis* produced significant and dose–dependent antinociceptive effect when evaluated in thermal model of nociception in rodents. The antinociceptive effects of the petroleum ether and ethyl acetate extracts in the second phase of formalin test has already been reported [17]. The tail-flick test is specific and popular in detecting centrally acting analgesics and is selective for opioid-derived analgesics [23, 24]. The primary heat hyperalgesia generated by a strong thermal stimulation in the stimulated skin area is mainly caused by sensitization of primary afferent nociceptors. [25] It has been demonstrated that the excitation of transient receptor potential vanilloid 1 ($TRPV_1$) receptors-bearing primary afferents is essential for the induction of states of central sensitization in humans [26, 27]. Specific agonists such as capsaicin and noxious heat selectively excites these afferents. The extract and fractions then probably interacts with opioid receptors similar to morphine (reference analgesic) to produce their antinociceptive effect but this needs further investigation. Activation of mu-opioid receptor inhibits the activity of $TRPV_1$ via $G_{o/i}$ proteins and the cyclic adenosine monophosphate pathway (cAMP) [28]. The presence of flavonoids and tannins among other constituents in the stem bark as reported in literature [12, 13] might be responsible for the analgesic activity. This result give further credence to the traditional use of the plant in the treatment of pain.

The present results furthermore show that MAE and fractions reduced the morphine withdrawal signs. Withdrawal from acute morphine dependence is accompanied by centrally mediated side effects, such as physical dependence [21, 29]. Physical dependence is distinguished by excessively definite behavioural abstinence signs such as hyperirritability, anxiety and restlessness after withdrawal of morphine or administration of opioids antagonists [6, 29]. In morphine dependent mice abstinence sign such as jumping is produced by naloxone. In this study, the extract and fractions showed inhibitory outcome against withdrawal syndrome of morphine dependence which was comparable in extent to drugs acting on GABA systems, such as muscimol and baclofen. Some neurotransmitters, including GABA, dopamine, noradrenaline, serotonin, adenosine and glutamate have been associated in the expression of opioid withdrawal [21]. It has earlier been reported that sensitization to opioids seems to be linked with increased dopaminergic transmission in nucleus accumbens which was established to be related with accelerated locomotor, and behavioural response [30]. It is well known that morphine also produces an increase in whole brain GABA concentration [31]. GABA is an inhibitory neurotransmitter which acts on $GABA_A$ and $GABA_B$ receptors, localized on dopaminergic and glutamatergic neurons, and regulate the release of dopamine and its afferent inputs in nucleus accumbens and ventral tegmental area. [30] However in intermittence or withdrawal of morphine GABA discharge is consequently decreased which result in up-regulation of dopaminergic system leading to abstinence behaviour [31]. Thus, GABA-related compound modify the behavioural sensitization to opiates. The results in this study show that MAE and fractions similar to muscimol and baclofen ($GABA_A$ and $GABA_B$ receptor agonists, respectively), blocked naloxone-induced jumping behaviour in morphine dependent mice in a dose-related way, suggesting involvement of GABA system in their antinociception. The inhibitory effect of MAE against abstinence behaviour was significantly antagonized in the presence of bicuculline ($GABA_A$ receptors antagonist), implying that effect of MAE is mediated through $GABA_A$ receptors and this is consistent with some studies [6, 20, 21, 30, 32, 33]. Therefore, MAE may modulate withdrawal syndrome via potentiating the GABA system. Similarly, non-selective adenosine receptors antagonist aminophylline suppressed the inhibitory effect produced by MAE and F1 on naloxone-induced withdrawal syndrome in mice signifying their mechanism, at least in part, through adenosinergic system. The role of cAMP and adenosine in acute opioid withdrawal has since been proposed at behavioural level. Chronic morphine treatment up-regulates the adenylyl cyclase that leads to an increase in extracellular cAMP and adenosine modulating GABA release. Though in withdrawal, there is an adenosine-dependent inhibition of GABA release so, adenosine analogues or an increase in endogenous adenosine neutralizes sign of morphine withdrawal. It is on record that inhibition of A_1 adenosine receptor or adenosine tone inhibits GABA release by inhibition of phosphodiesterase activity [21]. Hence, MAE and F1 effect on GABA release through adenosine system is confirmatory to their inhibitory responses against morphine withdrawal. Treatment of morphine dependence and withdrawal syndrome is limited to opiate replacement therapy and symptomatic treatment of withdrawal signs, [31] but from this study and several similar studies, [31, 34] herbal treatment may be a rational option for the treatment of morphine dependence and withdrawal. In summary, results imply that stimulation of GABA transmission may be a possible way for the antinociception of MAE and F1. This outlook may be valuable to minimize the adverse effects associated with opioid analgesics. Additionally MAE and F1 with possibility of being GABA receptor agonist can be effective in neuralgia and chronic pain associated with spasticity.

5. Conclusion

With the findings of the present study taken together, it is concluded that the extract and fractions obtained from *Maerua angolensis* stem bark possesses central analgesic effect and suppressed morphine withdrawal syndrome via stimulation of GABA and adenosinergic transmission, and so can be exploited for development in therapy. The antinociceptive effect and the suppression of withdrawal syndrome of morphine dependence established in this study contribute to the analgesic knowledge of this species.

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