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Original article

Antinociceptive and anti-inflammatory activities of ethanolic extract of *Alafia barteri*

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ABSTRACT

This study analyzes the antinociceptive and anti-inflammatory properties of ethanolic leaf extract of *Alafia barteri* Oliv., Apocynaceae, based on its medicinal use in the treatment of toothaches, inflammation and fevers. The antinociceptive effect was assessed in mice using acetic acid-induced writhing, tail clip, tail immersion and formalin assays. Anti-inflammatory activity was evaluated on carrageenan-induced paw oedema in rats, and xylene-induced ear oedema in mice. In acetic acid-induced writhing test, the extract at different doses (50, 100 and 200 mg/kg, *p.o.*) significantly ($p < 0.05$) and dose-dependently reduced pain by 35.04, 56.49 and 84.25%, respectively. The extract also significantly inhibited both the early and late phases of formalin-induced nociception in mice. In the tail immersion test, the extract caused a significant inhibition of pain (34.43% inhibition, after 90 min) at a dose of 200 mg/kg, while the effect of the extract in the tail clip test was only significant at the 100 mg/kg dose. *A. barteri* caused a significant inhibition of paw oedema development in the carrageenan and xylene-induced oedema tests. There was no mortality recorded following treatment with the extract (5 g/kg, *p.o.*). The results support the traditional use of *A. barteri* in the treatment of various diseases associated with pain and inflammation.

Introduction

Non-steroidal anti-inflammatory drugs (NSAID) are used worldwide for the treatment of inflammation, pain and fever, as well as for cardiovascular protection. However, they often produce significant side-effects, which include gastric ulcer, renal damage, bronchospasm and cardiac abnormalities, thus limiting their use (Burke et al., 2006). Drugs of natural origin are an important source for the treatment of many diseases worldwide (Pandima Devi et al., 2003). The research and analysis of plants employed as pain-relievers and anti-inflammatory agents in traditional ethnomedicine is one of the productive and logical strategies in the search for new drugs (Elisabetsky et al., 1995; Vongtau et al., 2004).

Alafia barteri Oliv., Apocynaceae, is a climbing shrub distributed widely in the tropics. It is valued for its efficacy in the traditional

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medicine system in Nigeria and other African countries, as an anti-inflammatory and fever remedy. The infusion of the leaves and twining stem are used for the treatment of inflammation and fever (Burkill, 1985; Iwu, 1993). The decoction of root and leaves of the plant is also taken internally or applied externally to treat rheumatic pain, toothache and eye infections (Odugbemi, 2008). The extracts of the leaves were found to have antibacterial and antifungal activities (Adekunle and Okoli, 2002; Hamid and Aiyelaagbe, 2011). The aqueous leaf extract was reported to display potent antiplasmodial activity (Lasisi et al., 2012). Aside these reports, and to the best of our knowledge, no other pharmacological effects of this plant related to its traditional use for antinociceptive and anti-inflammatory activities have been reported. Therefore, this study aimed to evaluate the antinociceptive and anti-inflammatory properties of the ethanolic extract of *Alafia barteri* leaves.

Materials and methods

Plant material

The leaves of *Alafia barteri* Oliv., Apocynaceae, were collected from Olokemeji forest, Ibadan (7°25' N, 3°31'E), Nigeria, in January, 2012. The plant sample was authenticated by Mr T. K. Odewo of the herbarium unit of the Department of Botany, University of Lagos, and a voucher specimen (LUH 2880) was deposited in the same unit.

Preparation of the plant extract

The leaves were air-dried and coarsely powdered using Christy and Norriis 8' Lab Milling Machine (serial No. 50158). The powdered plant sample (310 g) was extracted with 96% ethanol (1.8 l) for 24 h, at room temperature with constant stirring. This process was repeated twice for complete extraction. The extract was filtered and the filtrate was concentrated at 45°C on a regulated water bath (Julabo TW20GB, USA Inc.). The percentage yield was 2.8% (w/w).

Spectroscopic methods for quantitative determination of phenolic compounds

Total phenol concentration in the ethanolic extract from leaves of *A. barteri* was determined spectrophotometrically, according to Folin-Ciocalteu colorimetric method (Menichini et al., 2009) and expressed as gallic acid equivalents (mg/g) of dried extract (R^2 = 0.959). Total flavonoid content was measured by the aluminium chloride colorimetric assay (Rajasekaran et al., 2012) and expressed as quercetin equivalents (mg/g) of dried extract (R^2 = 0.961). Proanthocyanidin content was determined by the vanillin-HCl assay as described by Sun et al. (1998). Catechin was used as the standard (R^2 = 0.958) for the calibration curve, and the result was expressed as catechin equivalents (mg/g) of dried extract.

Experimental animals

Swiss albino mice (17-25 g) and Wistar rats (150-200 g) were housed in clean plastic cages and maintained under standard laboratory conditions (temperature 24-28°C and 12:12 light/dark cycles). They were fed with commercial rat food (Livestock Feed PLC, Ikeja, Lagos, Nigeria) and water *ad libitum*. The procedures adopted in this study were in accordance with Guidelines for Care and Use of Laboratory Animals in Biomedical Research of the National Institutes of Health of the United States (NIH, 1985), and approved by the Experimentation Ethics Committee on Animal Use of the College of Medicine, University of Lagos, Lagos, Nigeria (CM/COM/08/VOL.XXV).

Acute toxicity

An acute toxicity assay was performed in accordance to OECD test guidelines 423 (limit test) and as reported by Ong et al. (2011). A single oral dose of *A. barteri* extract (5 g/kg) was administered to a group of seven fasting male mice, while the control group received vehicle (1% Tween 80, 10 ml/kg, *p.o.*). The animals were observed continuously for the first 4 h and then periodically up to 24 h for toxic symptoms and mortality. The mice were further observed for seven days for manifestations of delayed toxicity.

Assessment of antinociceptive effect of the extract

Acetic acid-induced writhing test

The antinociceptive effect was evaluated in mice by acetic acid-induced writhing test (Koster et al., 1959; Singh and Majumdar, 1995). Animals were randomly selected and divided into five groups of six animals each. Group I served as the control and received 1% Tween 80 (10 ml/kg, *p.o.*), groups II, III and IV received plant extract (50, 100 and 200 mg/kg, *p.o.*, respectively) while group V, which served as positive control, received acetylsalicylic acid (ASA) (100 mg/kg, *p.o.*). One hour after the administration of the plant extract or standard drug, the animals received acetic acid (0.6%, 10 ml/kg, *i.p.*). The number of writhes (abdominal contractions and stretches) was counted for 30 min after the administration of acetic acid. The results were evaluated by calculating the mean number of writhes per group and antinociceptive activity was expressed as percentage inhibition of abdominal writhes.

Tail immersion test

The tail immersion test was performed according to the method of Ben-Bassat et al. (1959), as reported by Cha et al. (2011) with minor modifications. The mouse was gently handled and two-thirds of its tail was immersed in a beaker containing water at a temperature of 55 ± 0.5°C. Each animal served as its own control. The reaction time, *i.e.* the amount of time it took the animal to withdraw its tail, was recorded with a stopwatch at 0, 30, 60, 90 and 120 min after the administration of the extract (50, 100 and 200 mg/kg, *p.o.*), vehicle (10 ml/kg, *p.o.*) or morphine (10 mg/kg, *s.c*). To avoid tissue injury, the cut-off time was set at 20 s.

Tail clip test

Mice were screened by applying a metal artery clip to the base of the tail to induce pain. The animals that did not attempt to dislodge the clip within 10 s were not used for the experiment. The selected mice were divided into five groups of six mice each. The extract (50, 100 and 200 mg/kg, *p.o.*), morphine (10 mg/kg, *s.c.*) or 1% Tween 80 (vehicle, *p.o.*)

were administered to different groups of prescreened mice. The reaction time of each mouse was then determined 60 minutes post-treatment for oral administration and 30 min post-treatment for subcutaneous administration (Bianchi and Franceschini, 1954; Ishola et al., 2011). A post-treatment cut-off time of 30 s was used. The mean value was evaluated and percentage inhibition calculated as follows: % Inhibition = (post-treatment latency – pre-treatment latency)/(cut-off time – pre-treatment latency) ×100.

Formalin-induced pain test

The formalin test was carried out according to the method of Hunskaar and Hole (1987) as reported by Young et al. (2005) with some modifications. Twenty micro-liter of 1% formalin was injected into the right hind paw of mice, 60 min after administration of the extract (50, 100 and 200 mg/kg, *p.o.*) and Tween 80 (1%), and 30 min after morphine administration (10 mg/kg, *s.c.*). Animals were placed individually in transparent cages and observed for 30 min after the injection of formalin, and the amount of time (in seconds) spent licking and biting the injected hind paw was recorded as an indicator of nociceptive behavior. The initial nociceptive scores normally peaked at 0-5 min (early phase) and 15-30 min (late phase) after formalin injection, representing the neurogenic and inflammatory pain responses, respectively.

Assessment of anti-inflammatory effect of the extract

Carrageenan-induced paw oedema in rats

The anti-inflammatory activity of the extract was evaluated according to the method of Winter et al. (1962), and as reported by Ibrahim et al. (2012) with slight modifications. Five groups of six rats each were treated with the extract (50, 100 and 200 mg/kg, *p.o.*), indomethacin (10 mg/kg, *p.o.*) or 1% Tween 80 (10 ml/kg, *p.o.*). One hour after the administration of the various agents, edema was induced by carrageenan injection (0.1 ml, 1%, w/v in saline) into the subplantar tissue of the right hind paw. The linear paw circumference (cm) was measured using the cotton thread method (Bamgbose and Noamesi, 1981). Measurements were made immediately before injection of the phlogistic agents and afterwards at 1 h intervals, for 5 h.

Xylene-induced ear edema in mice

The xylene-induced ear oedema test was performed as previously described by Tang et al. (1984). Briefly, each mouse was given a single dose of the extract (50-200 mg/kg), dexamethasone (1 mg/kg) or vehicle 1 h before induction of ear oedema by topical application of 0.02 ml xylene on the inner and outer surfaces of the right ear. The left ear was used as a control. Mice were sacrificed by cervical dislocation 1 h after xylene application. Circular sections were excised and weighed. The extent of ear oedema was evaluated by the weight difference between the right and the left ear sections of the same animal.

Statistical analysis

The data are represented as the mean ± SEM. Statistically significant differences between the treatments were tested by One-way ANOVA. Differences were considered significant at $p < 0.05$. All statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA).

Results

Acute toxicity

A single oral administration of the extract (5 g/kg) did not produce any sign of toxicity and no mortality occurred within 24 h after administration and after seven days observation.

Antinociceptive activity

Acetic acid-induced writhing in rats

The extract (50, 100 and 200 mg/kg) produced a significant ($p < 0.001$) and dose-dependent decrease in the number of writhes compared to control (Fig. 1). The inhibition rates of the number of writhing for the extract, at 50, 100 and 200 mg/kg, were 35, 56.5 and 84.3%, respectively. The percentage inhibition of writhes by the extract at the dose of 200 mg/kg was higher than that of acetylsalicylic acid (44.1%).

Tail immersion test

The antinociceptive activity of the extract and morphine in the tail immersion test are shown in Fig. 2. At all doses, the effect of the extract was more pronounced after 90 min. At 90 min, the highest reaction time of 8.8 ± 0.9 s (34.4% inhibition) was observed at the dose of 200 mg/kg. Morphine (10 mg/kg) produced a significant anti-nociceptive effect at all observation times when compared to control, with a maximum increase in tail flick latency at 30 min (91.9%, inhibition), which declined with time.

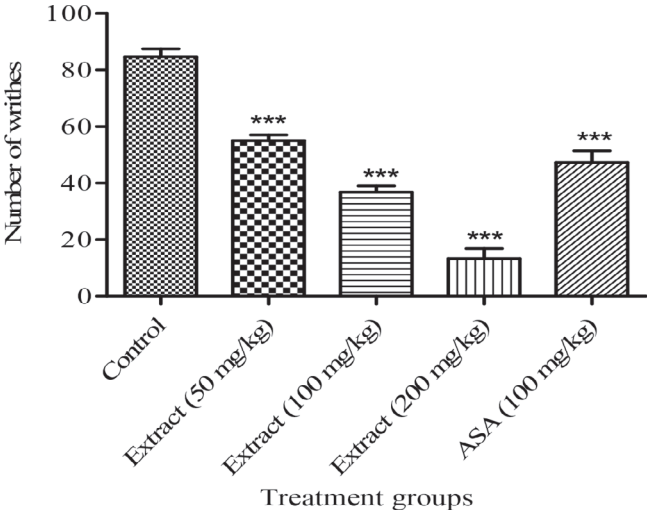

Figure 1 – Effect of *Alafia barteri* on acetic acid-induced writhing in mice. Values are mean ± SEM (n = 6). ***$p < 0.001$ compared to control (One-way analysis of variance followed by Tukey's multiple comparison test).

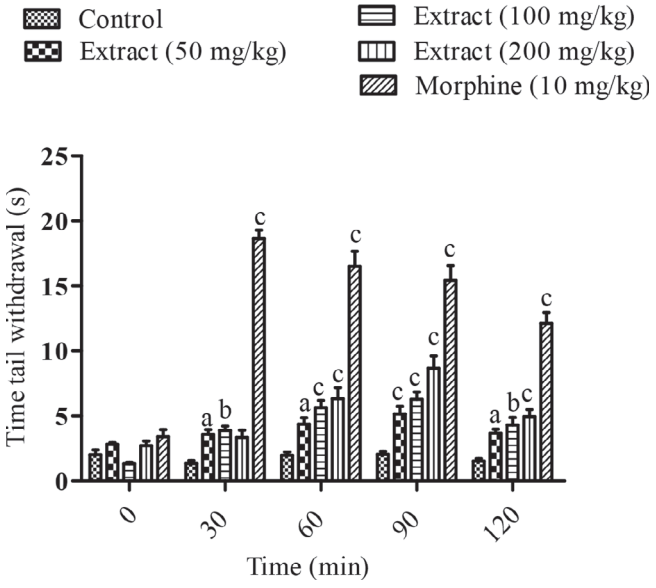

Figure 2 – Effect of *Alafia barteri* on pain induced responses in tail immersion test. $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ indicates significant difference when compare with control using Two-way ANOVA followed by Bonferroni's test.

Tail clip test

The results obtained from the tail clip test are presented in Table 1. The extract exhibited a low and not dose-dependent analgesic effect against tail clip-induced algesic response in mice compared to control. The effect was only significant ($p < 0.05$) at the dose of 100 mg/kg (22.4% inhibition). Morphine (10 mg/kg) exhibited a significant ($p < 0.01$) analgesic activity (55.8% inhibition) in this test.

Formalin test

In the control group the results showed an intensified nociceptive response in early and late phases of formalin test. The mean paw licking response times were 71.3 ± 8.9 s and 91.8 ± 9.8 s respectively (Table 2). The extract at all doses (50, 100 and 200 mg/kg, *p.o.*) significantly ($p < 0.05$; $p < 0.001$) reduced the time spent by the mice licking the injected paws, both in the early and late phases when compared with controls. The effect in the late phase appears to be higher than the earlier phase. The inhibition rates of formalin-induced licking in the late phase compared with the control were 47.4, 55, and 83.5%, respectively. Morphine significantly inhibited pain (100%) over the control, both at early and late phases.

Anti-inflammatory studies

Carrageenan-induced oedema

As shown in Table 3, the extract at 50 and 100 mg/kg doses significantly ($p < 0.001$; $p < 0.01$; $p < 0.05$) inhibited the increase of paw oedema from 2 to 5 h. The maximum inhibitory effect of the extract was recorded at the 100 mg/kg dose (78.85%) at 5 h. Oral administration of indomethacin (10 mg/kg) also reduced the oedema with significant inhibition of 44.44% and 46.15% , at 3 and 5 h, respectively.

Table 1
Effect of *Alafia barteri* on tail clip-induced pain in mice.

Treatment	Dose (mg/kg)	Pre-treatment reaction latency (s)	Post-treatment reaction latency (s)	% Inhibition
Control	10 ml/kg	4.3 ± 0.5	2.1 ± 0.2	-
A. barteri	50	2.6 ± 0.5	5.8 ± 0.6	26.2
	100	3.8 ± 0.6	6.3 ± 1.0[a]	22.4
	200	4.2 ± 1.8	4.7 ± 0.3	4.5
Morphine	10	2.6 ± 0.6	9.5 ± 1.7[b]	55.8

Values are mean ± SEM, (n=6). $^{a}p < 0.05$, $^{b}p < 0.001$ compared to control (One-way analysis of variance followed by Tukey's multiple comparison test).

Table 2
Effect of *Alafia barteri* on formalin-induced pain in mice.

Treatment	Dose (mg/kg)	Early phase (0-5 min)	% Inhibition	Late phase (15-30 min)	% Inhibition
Control	10 ml/kg	71.3 ± 8.9	-	91.8 ± 9.8	-
A. barteri	50	43.6 ± 3.9[a]	38.9	48.3 ± 5.0[b]	47.4
	100	46.7 ± 5.0[c]	34.5	41.3 ± 6.5[b]	55.0
	200	24.7 ± 2.9[b]	65.4	15.2 ± 6.5[b]	83.5
Morphine	10	00.0 0.0[b]	100	00.0 ± 0.0[b]	100.0

Values are mean ± SEM, (n=6). $^{a}p < 0.01$, $^{b}p < 0.001$, $^{c}p < 0.05$ compared to control (One-way analysis of variance followed by Tukey's multiple comparison test).

Table 3
Effect of *Alafia barteri* on carrageenan-induced inflammation in rats.

Treatment	Dose (mg/kg)	Increase in paw circumference (cm)				
		1 h	2 h	3 h	4 h	5 h
Control (ml/kg)	10	0.3 ± 0.0	0.4 ± 0.0	0.45 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
Extract	50	0.3 ± 0.0 (24.2)	0.3 ± 0.0 (21.1)	0.4 ± 0.0 (22.2)	0.3 ± 0.1[a] (46.2)	0.2 ± 0.0[b] (55.8)
	100	0.1 ± 0.0[b] (69.7)	0.2 ± 0.0[c] (55.3)	0.2 ± 0.0[b] (66.7)	0.1 ± 0.0[b] (75.0)	0.1 ± 0.0[b] (78.9)
	200	0.3 ± 0.0 (24.2)	0.2 ± 0.1[a] (39.5)	0.2 ± 0.2[a] (48.9)	0.3 ± 0.0[a] (40.4)	0.3 ± 0.0[c] (46.2)
Indomethacin	10	0.3 ± 0.0 (15.2)	0.3 ± 0.0 (26.3)	0.3 ± 0.0[a] (44.4)	0.3 ± 0.0 (36.5)	0.3 ± 0.0[c] (46.2)

Values are mean ± SEM, (n=6). [a]$p < 0.05$, [b]$p < 0.001$, [c]$p < 0.01$ *vs.* control (One-way analysis of variance followed by Tukey's multiple comparison test). Values in parenthesis are % inhibition.

Xylene-induced oedema

The oedema inhibitory value of the extract at the doses of 50, 100 and 200 mg/kg were 46.15, 53.85 and 78.85%, respectively (Fig. 3). A statistically significant peak effect (78.85% inhibition) was observed at the dose of 200 mg/kg, compared to control. Dexamethasone (1 mg/kg, *p.o.*) exhibited a significant inhibitory effect on xylene-induced ear oedema with inhibition value of 100%.

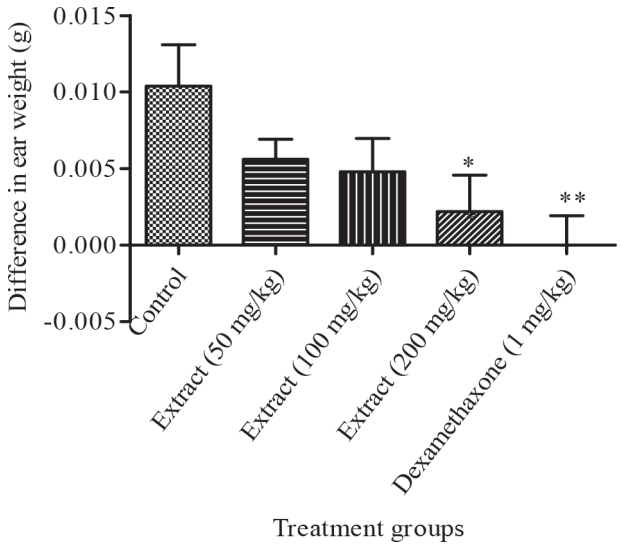

Fig. 3 – Effect of *Alafia barteri* extract on xylene-induced mouse ear oedema test. Values are mean ± SEM, (n = 5). *$p < 0.05$ vs. control (One-way analysis of variance followed by Dunnett's post test).

Polyphenol content

The total phenolic content expressed as gallic acid equivalents for *A. barteri* extract was 541.40 mg GAE/g, while the flavonoid and proanthocyanidin contents were 310.66 mg quercetin equivalent/g and 529.39 mg catechin equivalent/g of dried extract, respectively.

Discussion

The study investigated the antinociceptive and anti-inflammatory effects of *Alafia barteri* Oliv., Apocynaceae leaves ethanolic extract. The antinociceptive activity was carried out in a broad panel of rodent models of acute pain; such as, the acetic acid-induced writhing, tail clip, tail immersion and formalin tests. These methods are classical nociception models used to screen prospective antinociceptive compounds or plant extracts.

Antinociceptive assessment using the acetic acid-induced writhing test showed that oral administration of *A. barteri* produced a statistically significant inhibition of writhes compared to the control. This is an indication of the peripheral analgesic activity of the extract, since any agent that lowers the writhing number demonstrates analgesia by inhibiting prostaglandin synthesis, a peripheral mechanism of pain inhibition (Alam et al., 2009; Loganayaki et al., 2012). Although this method proves to have great sensitivity to central and peripheral analgesic drugs, the abdominal writhing response may be suppressed by muscle relaxants as well as other drugs, leaving scope for the misinterpretation of results (Le Bars et al., 2001).

The tail clip and tail immersion methods were used to evaluate the central mechanism of analgesic activity of the extract. The extract exhibited low activity in these two models of nociception. It is known that centrally acting analgesic drugs elevate the pain threshold of mice related to pressure and heat (Singh and Majumdar, 1995). The tail clip test is useful in elucidating centrally mediated antinociceptive responses, which focuses mainly on changes above the spinal cord level (Vongtau et al., 2004; Özkay and Can, 2013) while the tail immersion, a thermal-induced nociception, indicates narcotic involvement (Besra et al., 1996). Thermal nociceptive tests are more sensitive to opioid-μ receptors, and non-thermal tests are more sensitive to opioid-k receptors (Haider et al., 2012). The results obtained from these two models suggest that the antinociceptive activity of the extract may not significantly involve central mechanism of action.

In order to further evaluate and clarify the possible mechanism of the antinociceptive effect of the extract, the formalin test was carried out. In the formalin test, two distinct phases of intensive licking and biting activities have been identified. The early phase (first 5 min) is thought to be caused predominantly by C-fibre activation due to the peripheral stimulus caused by formalin. A second burst of licking behaviour occurs after 15 to 30 min and seems to be related to the inflammatory response elicited by formalin. This phase is termed inflammatory (Hunskaar and Hole, 1987; Milano et al., 2008). The extract demonstrated antinociceptive activity in blocking both phases of the formalin response. However, the effect of the extract was more pronounced in the late phase. Manifestation in the late phase is due to inflammation causing a release of serotonin, histamine, bradykinin and prostaglandins, which at least to some degree can cause the sensitization of the central nociceptive neurons (Verma et al., 2005). It then implies that the antinociceptive activity of the extract possibly resulted from its peripheral action, which might also suggest an anti-inflammatory action.

The *in vivo* anti-inflammatory activity of the extract was investigated using two common inflammatory animal models, *i.e.* carrageenan-induced paw oedema in rats, and xylene-induced ear oedema in mice. Carrageenan-induced inflammation is useful in detecting orally active anti-inflammatory agents; therefore, it has a significant predictive value for anti-inflammatory agents acting through mediators of acute inflammation (Badole et al., 2012). The development of oedema induced by carrageenan injection produces a biphasic event. In the first phase, during the first hour, histamine, serotonin and bradykinin are the mediators involved, while prostaglandins are implicated in the second phase (3-5 h) (Marrassini et al., 2010). The extract exhibited a moderate inhibitory effect at the early phase but significantly inhibited the paw oedema in late phase of inflammation. The effect of the extract in this model may be attributed to the inhibition of the release of pro-inflammatory mediators of acute inflammation, especially prostaglandins.

Xylene can induce mouse ear oedema, which may involve inflammatory mediators such as histamine, kinin and fibrinolysin. These mediators can promote vasodilation and increase vascular permeability (Li et al., 2011). Xylene ear oedema model also allows the evaluation of anti-inflammatory steroids which inihibit phospholipase A_2 (PLA_2) enzymes (Ndebia et al., 2011). The ethanolic extract of *A. barteri* was able to reduce inflammation in this model. This finding suggests that the extract may interfere with the actions of inflammatory mediators and enzymes (PLA_2) to produce the anti-inflammatory effect in this model.

Phenols are the most wide spread secondary metabolite in plant kingdom. There is abundant literature regarding medicinal plants establishing relations between anti-inflammatory, analgesic and phenol/flavonoid content (Calixto et al., 2000; Deliorman et al., 2007; Saeed et al., 2010; Deng et al., 2011). Although no record of chemical constituents isolated and characterized from this plant species was found, and the methods used for the identification of phytochemical constituents are preliminary in nature, it could be suggested that the antinociceptive and anti-inflammatory effects recorded for *A. barteri* in this study, may be caused by the total polyphenolic constituents present in the plant.

In summary, comparing the results obtained in the antinociceptive activity models; the ethanolic extract of *A. barteri* was relatively potent in acetic acid writhing test, thus, indicating peripheral antinociception. Similarly, the highly significant inhibitory effect of the extract on the nociceptive response in the late phase of the formalin test suggests that the extract could suppress inflammatory nociception. Furthermore, in agreement with the results from the antinociceptive tests, the extract also elicited anti-inflammatory effects. The results obtained in this study established the antinociceptive and anti-inflammatory actions for the extract. However, the mechanism of these actions is uncertain, and the active chemical compounds responsible of the antinociceptive and anti-inflammatory activities of the extract remains to be elucidated.

Authors' contributions

EI and CE contributed in collecting plant samples and identification, running the laboratory work and analysis of the data. FRA contributed to biological studies and analysis of the data. AJK contributed to critical reading of the manuscript. MOS designed the study, supervised the laboratory work and wrote manuscript. All the authors have read the final manuscript and approved submission.

Conflicts of interest

The authors declare no conflicts of interest.

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