Research Article

Analgesic Activity of Ethanol Extract of *Adenopus breviflorus* (Roberty) Fruit in Mice

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ABSTRACT

Adenopus breviflorus is a perennial climber used locally as an anticonvulsant, sedative and pain-killer in West Africa. Several studies have reported gastrointestinal, reproductive and anti-microbial effects of extracts of *Adenopus breviflorus*, but there is dearth of information on its anticonvulsant effect. This study was therefore designed to investigate anticonvulsant effect of Ethanol Extract of *Adenopus breviflorus* (EEAB) in mice. Three hundred grams of air-dried *Adenopus breviflorus* fruits were cold macerated in 70% ethanol and concentrated using rotary evaporator. The method described by Lorke was used to determine the LD_{50}. The EEAB (250 – 2000 mg/kg, *p.o.*) was studied for its effect on nociceptive responses using hot plate, tail immersion, acetic acid-induced writhing and formalin tests. The mechanism of action was studied using morphine (10 mg/kg, i.p.), naloxone (2 mg/kg, i.p.) and sub-maximal dose (1000 mg/kg) of the extract. Data were analyzed using descriptive statistics and ANOVA at p=0.05. The LD_{50} of the crude extract was found to be 7000 mg/kg *p.o.* All doses of EEAB (250-2000 mg/kg) and morphine (10 mg/kg) significantly ($p<0.05$) induced analgesia in mice by causing an increase in the reaction time to the thermal stimulus of hot plate and hot water relative to their respective controls. All doses of EEAB (250 - 2000 mg/kg) and morphine (10 mg/kg) induced dose-dependent inhibition in paw-licking time in both phases of formalin test relative to their respective controls. Pretreatment with naloxone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) in hot plate, tail immersion and formalin tests. It can be concluded that *Adenopus breviflorus* fruit extract probably possess an analgesic effect which could be mediated via μ-opioid receptor and inhibition of prostaglandins synthesis or release.

Keywords: *Adenopus breviflorus,* Analgesia, Hot plate test, Formalin test, Mice.

INTRODUCTION

Pain is basically a protective mechanism in the human body which occurs as a response to tissue injury or damage and causes the individual to react to remove the painful stimulus, and analgesics have been one of the common therapeutic categories on which research work was done[1].

Plants have been used for therapeutic applications ever since man has been concerned about his health. For centuries, the world has depended on the useful possessions of plants as a source of medicines[2]. Ethnobotanical investigations done in the last few decades had discovered the analgesic properties of plants mentioned in the traditional information. Numerous herbal preparations are being suggested as analgesic in the traditional information. The exploration for new analgesic compounds from the enormous arrays of medicinal plant resources is growing. This is because such information may hold guarantee for the finding of new therapeutic agents capable of inhibiting, decreasing, or relieving pain[3].

Plants characterize a huge natural supply of valuable compounds that might achieve as lead for the expansion of novel drugs[4]. The exploration of the effectiveness of plant-based drugs used in the traditional medicine has been given great considerations because they are cheap and have little side effects, and, according to World Health Association (WHO), about 80% of the world population still relies chiefly on plant-based drugs[5]. One of these medicinal plant species that has a great pharmaceutical value is *Adenopus breviflorus.*

Adenopus breviflorus belongs to the family of Cucurbitaceae. It is commonly called Wild colocynth in English language, "Ogbenwa" in Ibo language and "Tagiri" in Yoruba language[6]. It is a perennial tendril climber. It would usually lie on the ground for want of something to climb and climbs over shrubs and herbs by means of axillary tendrils. The leaves are simple, alternate and palmately veined[7].

Medicinally, the plant is used as a purgative in Tanganyika as well as a vermifuge and cathartic in Nigeria[6]. A decoction from the plant is said to be used in Nigeria for headache[6]. It is used in West Africa for a wide range of gastrointestinal disorders and measles in man. In southern Nigeria its seed-decoction is reportedly given to pregnant women but the purpose is not stated[8]. It is used as an anticonvulsant, sedative and pain killer[9]. It is used with other medicinal plants as concoctions to aid parturition in humans[10]. Livestock farmers employ the fruit extract of the plant for the treatment of Newcastle disease and coccidiosis in animals[10]. The fruit is also used for money-making charms by the Yoruba herbalists of South-West Nigeria because of the cowrie-like inscriptions on its body.

Pharmacologically, it has been reported that the methanol extract of its whole fruit has anti-implantation activity[11] and abortifacient activity[12]. The ethanol extract of its whole fruit has been reported to have a broad spectrum antibacterial activity[13] as well as anti-oxidant and anti-ulcerogenic effects[14]. Its ethanol extract has been reported to have a little toxic effect and a lot of beneficial effects on the hematological functions and blood chemistry of male Wistar rats[15].

Since this plant has been reported to be used medicinally as a pain killer[9], this study aims to scientifically authenticate the veracity of this claim.

MATERIALS AND METHODS

Experimental Animals

Adult male mice weighing between 20-25 g bred in the Pre-Clinical Animal House of the College of Medicine, University of Ibadan were used. They were housed under standard laboratory conditions and had free access to feed (Ladokun Feeds Limited, Ibadan, Nigeria) and water; they were acclimatized for two weeks to laboratory conditions before the commencement of the experiments. All experiments were carried out in compliance with the recommendations of Helsinki's declaration on guiding principles on care and use of animals.

Plant Material

Fresh samples of *Adenopus breviflorus* fruit were bought in Bodija Market, Ibadan, and were authenticated in the Taxonomy Unit of the Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan.

Preparation of Crude Ethanol Extract

Large quantity (7.5 kg) of fresh specimens of the whole fruit of *Adenopus breviflorus* were washed free of debris and pulverized using mortar and pestle and air-dried for eight weeks. The resultant dried specimens (300 g) were macerated and extracted with 70 % ethanol for 72 hours at room temperature (26-28 °C). The resulting solution was then filtered using a wire-gauze and a sieve with tiny pores (0.25 mm). The 70 % ethanol was later evaporated using steam bath (40 – 45 °C) to give a percentage yield of 8.6 % of the starting sample. The dried sample was reconstituted in distilled water to make up test solutions of known concentration.

Drugs and Chemicals

The following drugs and chemicals were used: Acetic acid (May & Baker Ltd., England), Aspirin (May & Baker Ltd, England), Formaldehyde (Sigma), Morphine (Martindale Pharma®, U.K), Naloxone (BDH, England).

Toxicity test

The method described by[16] was used to determine the LD_{50}, which is the index of acute toxicity. Male albino mice (20-25 g) were used. This method involved an initial dose finding procedure, in which the animals were divided into three groups of three animals per group. Doses of 10 mg/kg, 100 mg/kg and 1000 mg/kg were administered orally, one doses for each group. The treated animals were monitored for twenty-four hours for mortality and general behaviour. From the results of the above step, seven different doses (2000 mg/kg, 3000 mg/kg, 4000 mg/kg, 5000 mg/kg, 6000 mg/kg, 7000 mg/kg, 8000 mg/kg) where chosen and administered orally to seven groups of animals of one mouse per group respectively. The treated animals were monitored for twenty-four hours. The LD_{50} was then calculated as the geometric mean of the lowest dose showing death and the highest dose showing no death.

Preparation of Stock Solution of EEAB

Ten grams of EEAB were dissolved in 100 mL of distilled water to give a concentration of 0.1 g/mL.

The dosages of EEAB administered in these studies were obtained from the results of the acute toxicity test.

Screening of Analgesic Activity

Hot plate test

The apparatus comprised a water bath filled with hot water containing a metal plate in which each animal was placed for the testing time. The temperature of the water was maintained at 55±0.5°C in order to cause the animal to lick its forelimbs and/or to produce jumping responses. Forty mice were randomly divided into five groups (n=8). Group I was given distilled water (0.2 mL/20 g, *p.o.*), while groups II – V were given EEAB (250–2000 mg/kg, *p.o.*).

Each mouse was dropped gently on the hot plate and the time (in seconds) taken for the mice to lick its paws and/or produce jumping responses was recorded at time 0 minute (before treatment) and at time 30, 60, 90 and 120 minutes after treatment. The cut-off time was set at 15 seconds to avoid tissue damage.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.); the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described[17].

Tail immersion test

This test was performed as described by[18] and as modified by[19]. Forty mice were randomly divided into five groups (n=8). Group I was given distilled water (0.2 mL/20 g, *p.o.*), while groups II – V were given EEAB (250 – 2000 mg/kg, *p.o.*).

The tail of each mouse (up to 5cm) was dipped in a water-bath containing hot water maintained at 55 ± 0.5°C and the time (in seconds) for the mouse to withdraw the tail clearly out of the water was recorded at time 0 minute

(before treatment) and at time 30, 60, 90 and 120 minutes after treatment was taken as the reaction time. The cut-off time was set at 10 seconds to avoid tissue damage.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.); the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described.

Acetic acid-induced writhing test

This model was performed as described by[20]. Forty-eight mice were randomly divided into groups six (n=8). Group I was given distilled water (0.2 mL/20 g, *p.o.*), groups II – V were given EEAB (250 – 2000 mg/kg, *p.o.*), while group VI was given aspirin (150 mg/kg, *p.o.*).

This was followed one hour later by i.p. administration of 10 ml/kg of 0.6% acetic acid. The animals were allowed a 5 minutes observation period in a plexiglass cage (25 cm x 25 cm x 30 cm) before assessment (counting). Nociception was evaluated by counting the number of writhings (abdominal constrictions) displayed by each mouse for 15 minutes. Antinoceptive activity was expressed as the percentage reduction or inhibition of the number abdominal writhes.

The percentage inhibition of writhing was calculated as follows:

$$\%\ inhibition = \frac{Mean\ no\ of\ writhes\ in\ control\ group - Mean\ no\ of\ writhes\ in\ treated\ groups}{Mean\ no\ of\ writhes\ in\ control\ group} \times 100$$

Formalin test

This test was performed as described by[21]. Forty mice were randomly divided into five groups (n=8). Group I was given distilled water (0.2 mL/20 g, *p.o.*), while groups II – V were given EEAB (250–2000 mg/kg, *p.o.*).

This was followed 30 minutes later by the administration of 50 µL (0.05 mL) of 1% formalin into the sub-planter space of the right hind paw. The duration (in seconds) of paw licking was determined 0-5 minutes (1st phase or neurogenic phase) and 20-30 minutes (2nd phase or inflammatory phase) after formalin administration.

In another set of experiments, the animals were divided into three groups (n=8). The first group received morphine (10 mg/kg, i.p.), the second group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with morphine (10 mg/kg, i.p.), the third group was pretreated with naloxone (2 mg/kg, i.p.) 15 minutes prior to treatment with 1000 mg/kg (sub-maximal dose) of the extract. The mice were then tested as earlier described.

Statistical Analysis

The mean and standard error of mean (S.E.M) were calculated for all values. Comparison between the control and experimental groups was done using one–way analysis of variance (ANOVA) with Duncan's Multiple Range Test. Differences were considered statistically significant at $p<0.05$.

RESULTS

The LD_{50} of the crude extract was found to be 7000 mg/kg *p.o.*

The administration of EEAB (250-2000 mg/kg) and morphine (10 mg/kg) to mice significantly ($p<0.05$) reduced the pain response to the thermal stimulus of hot plate as indicated by the increase in reaction time when compared to the control. Pretreatment with naloxone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) (Table 1).

The administration of all the treatment doses of EEAB (250-2000 mg/kg) and morphine (10 mg/kg) to mice significantly ($p<0.05$) induced analgesia by causing an increase in the reaction time to the thermal stimulus of hot water relative to the control. Pretreatment with naxolone (2 mg/kg) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) (Table 2).

Treatment of mice with all the treatment doses of EEAB (250-2000 mg/kg) and aspirin (150 mg/kg) caused a significant ($p<0.05$) reduction in the number of acetic acid-induced abdominal writhes when compare to the control (Table 3).

Treatment of mice with all the treatment doses of EEAB (250-2000 mg/kg) and morphine (10 mg/kg) induced a dose-dependent inhibition in paw-licking time in both phases of the test relative to the control. Pretreatment with naloxone (2 mg/kg BW) reversed the analgesia induced by morphine (10 mg/kg) and the sub-maximal dose of EEAB (1000 mg/kg) in both phases of the test (Table 4).

Table 1: Analgesic activity of EEAB (Hot plate test)

Treatment	Dose (mg/kg)	0 min	30 min	60 min	90 min	120 min
Control	0.2 ml/20g	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
EEAB	250	1.00 ± 0.00	1.00 ± 0.00	1.20 ± 0.20	1.80 ± 0.17	2.20 ± 0.20*
EEAB	500	1.00 ± 0.00	1.40 ± 0.15*	2.00 ± 0.13*	2.80 ± 0.32*	2.80 ± 0.37
EEAB	1000	1.00 ± 0.00	2.00 ± 0.45*	2.00 ± 0.12*	2.80 ± 0.45*	2.80 ± 0.38*
EEAB	2000	1.00 ± 0.00	2.20 ± 0.29*	2.80 ± 0.31*	3.20 ± 0.39*	3.60 ± 0.32
Mor	10	1.00 ± 0.00	2.20 ± 0.26*	2.72 ± 0.32*	3.00 ± 0.36*	3.20 ± 0.26
NAL +	EEAB (1000)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.40	1.00 ± 0.00	1.00 ± 0.00
NAL+	Mor	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.36 ± 0.12	1.62 ± 0.26

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05; Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg).

Table 2: Analgesic activity of EEAB (Tail immersion test)

Treatment	Dose (mg/kg)	0 min	30 min	60 min	90 min	120 min
Control	0.2 ml/20g	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
EEAB	250	1.00 ± 0.00	1.60 ± 0.25	2.00 ± 0.32*	2.00 ± 0.20*	2.80 ± 0.28*
EEAB	500	1.00 ± 0.00	2.00 ± 0.32*	2.40 ± 0.40*	2.80 ± 0.39*	2.80 ± 0.31*
EEAB	1000	1.00 ± 0.00	2.00 ± 0.32*	2.40 ± 0.25*	2.80 ± 0.26*	3.40 ± 0.40*
EEAB	2000	1.00 ± 0.00	2.20 ± 0.41*	5.40 ± 1.54*	10.00 ± 0.58*	10.00 ± 0.10*
Mor	10	1.00 ± 0.00	2.20 ± 0.20*	4.20 ± 0.48*	10.00 ± 0.52*	10.00 ± 0.42*
NAL +	EEAB (1000)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
NAL+	Mor	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.56 ± 0.26	1.60 ± 0.28

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05; Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg).

Table 3: Analgesic activity of EEAB (Acetic acid-induced writhing test)

Treatment	Dose (mg/kg)	No of writhes/ 15 min	% Inhibition
Control	0.2 ml/20g	41.40 ± 1.84	0.0
EEAB	250	33.60 ± 1.06*	18.8
EEAB	500	15.80 ± 1.08*	61.8
EEAB	1000	13.80 ± 0.86*	66.7
EEAB	2000	4.00 ± 0.45*	90.3
Aspirin	150	5.40 ± 0.25*	87.0

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05.

Table 4: Analgesic activity of EEAB and mechanism of action (Formalin test)

Treatment	Licking time (sec)		Percentage inhibition	
	1st Phase	2nd Phase	1st Phase	2nd phase
Control (0.2ml/20g)	76.00 ± 3.73	179.60 ± 3.80	-	-
EEAB (250 mg/kg)	44.00 ± 2.61*	69.00 ± 2.38*	42.1	61.6
EEAB (500 mg/kg)	38.20 ± 2.76*	58.00 ± 2.95*	49.7	67.7
EEAB (1000 mg/kg)	27.60 ± 2.80*	49.40 ± 2.14*	63.7	72.5
EEAB (2000 mg/kg)	4.60 ± 0.55*	1.20 ± 0.34*	93.9	99.3
Mor (10 mg/kg)	36.20 ± 0.48*	17.40 ± 0.75*	52.4	90.3
NAL + EEAB (1000)	73.80 ± 2.40	174.60 ± 3.86	2.9	2.8
NAL + Mor	77.20 ± 2.32	188.40 ± 2.93	-	-

The results are expresses as mean ± S.E.M. (n=8). One way ANOVA revealed significant difference between various treatment groups. * Indicates significant difference from control. *p<0.05; Mor: Morphine (10 mg/kg), NAL: Naloxone (2 mg/kg)

DISCUSSION

Acute toxicity test gives clues on the range of doses that could be toxic to the animal; it could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics[22]. LD_{50} is the dose at which mortality occurs in 50% population of the experimental animals. The higher the value of the LD_{50} for a substance, the relatively safer the substance is assumed to be. The LD_{50} determination for the extract in mice via the oral route was 7000 mg/kg , which was not toxic to the animals, and since the recommended single high dose by OECD guidelines 423[23] for testing acute toxicity is 2000 mg/kg BW; this probably indicates the extract has wide safety margins (low toxicity). Similar result was reportred by[24] in *Eichhornia crassipes* extract treated mice.

Pain as a real complaint in clinical training, has different causing factors. Although there are many analgesic drugs for prescription, but because of many complexities including broad side effects, different origins of pain and weak potency of many conventional drugs[25], medicinal plant substitution has been recommended for this purpose[26].

The hot plate method has been found to be very effective for evaluating drugs possessing analgesic property which act centrally[27]. The extract caused increase in reaction time in the hot plate test which probably indicates its central analgesic effect. Similar result was reported by[28] in *Phyllanthus lawii* extract treated mice.

The tail immersion and hot plate models have been used to study centrally acting analgesics[29]. In these models, sensory nerves sensitise the nociceptors and the involvement of endogenous substances such as prostaglandins are minimized[30]; the extract induced increase in reaction time in the tail immersion test which suggests its centrally mediated antinociceptive activity. Similar result was reported by[31] in *Trapa natans* root extract treated mice.

Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting in release of free arachidonic acid from tissue phospholipid[32] via cyclooxygenase and prostaglandin biosynthesis[33]. In other words, the acetic acid induced writhing test has been associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products[34]. The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability[35]. The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglanding synthesis, a peripheral mechanism of pain inhibition[36]. The extract inhibited writhing responses induced by acetic acid which suggests that its antinociceptive effect could be peripherally mediated via inhibition of synthesis and/or release of prostaglandins. Similar result was reported by[37] in *Alstonia boonei* extract treated mice.

The formalin–induced pain model is very useful for elucidating the mechanism of pain and analgesia[38]. Drugs that act mainly centrally, such as narcotics, inhibit both phases of the formalin–induced pain, while peripherally acting drugs such as NSAIDs only inhibit the late (second) phase[39]. This biphasic model represented neurogenic and inflammatory pain respectively[21]. The extract inhibited both phases of formalin–induced pain which suggests that it has both central and peripheral antinociceptive actions. This probably implies that the extract can be used to manage acute and chronic pain. The mechanism by which formalin triggers C – fiber activation remained unknown for a relatively long time. Recently, however,[40] demonstrated that formalin activates primary afferent neurons through a specific and direct action of TRPAI, a member of the transient receptor potential family of cation channels, expressed by a subset of C – fiber nociceptors and this effect is accompanied by increased influx of Ca^{2+} ions. TRPAI channels at primary sensory terminals were also reported to mediate noxious mechanical stimuli[41]. These experiments suggest that Ca^{2+} mobilization through TRPAI cation channels is concomitant with noxious chemicals and mechanical stimuli as they produce their analgesic action. Hence, the antinociceptive action of the extract could be due to the inhibition of influx of intracellular Ca^{2+} ions through TRPAI cation channels. Similar result was reported by[42] in *Teucrium chamaedrys* extract treated rats.

The mechanism of action of three analgesic models used revealed that naloxone, a potent opoid antagonist, reversed the antinociceptive effect of the extract, which suggests that the analgesic effect of the extract could be mediated via interaction with the opioid system. Similar results were reported in *Hofmeisteria schaffneria, Vitis vinifera* and *Murraya koeniggi* extracts treated mice[43-45].

It can be concluded that *Adenopus breviflorus* fruit may possess an analgesic effect which provides scientific basis to the folkloric claim of the plant in the management of pain, and its analgesic activity could be mediated via μ - opioid receptor and inhibition of prostaglandins synthesis or release.

Recommendations

The forkloric claim of *Adenopus breviflorus* as a pain killer has been explored scientifically in animal models in this study. Hence, it is recommended that people suffering from acute and chronic pains may use the extract of *Adenopus breviflorus* fruit in the nearest future after isolation and characterization of the active component(s) and clinical trials.

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