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Full Length Research Article

Toxicity Study and Analgesic Activity of Methanol Extract of Baobab (*Adansonia digitata)* Seed in Mice

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ABSTRACT

The difficulty and complexity associated with the management of pain are related to side effects, efficacy and cost of current agents used in its management. We exploited some natural product resources in Africa in a bid to provide alternatives to the current agents used in pain management. We extracted the seeds of *Adansonia digitata* using methanol. We screened the fractions for phytochemicals, acute toxicity and analgesic properties using the tail-flick and hot plate methods. The phytochemical analysis of the seed powder revealed the presence of alkaloids, flavonoids and steroids. Oral LD_{50} did not reveal any toxicity in mice at doses ranging from 10-5000 mg/kg. Except for urea levels at the highest dose for the oily extract, the methanol extract of *Adansonia digitata* seeds demonstrated relative safety in laboratory mice. The methanol extract of *Adansonia digitata* significantly (p<0.05) prolonged the reaction latency to pain thermally induced in mice by both the tail-flick and hot plate methods. The highest dose of the extract exhibited a mean reaction time range of 12.2-19.9 sec as against 5.2-6.43 sec for the control for the tail-flick method. It also exhibited a mean reaction time range of 15.7-20.0 sec as against 5.48-10.62 sec for the control for the hot plate method. Overall, the extract of *Adansonia digitata* seed did not exhibit any acute toxicity at the experimental doses and demonstrated analgesic activity in mice.

Keywords: Analgesia, *Adansonia digitata,* pain, Hot Plate Method, Tail-Flick Method.

INTRODUCTION

Pain has been defined by the International Association for the Study of Pain as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Raja *et al*., 2020). Drugs used in the clinical management of pain include acetylsalicylic acid, ketoprofen, morphine etc. Challenges encountered with the use of current analgesic agents are that they only provide half of the required pain relief in about one-third of the patients that are administered of the drugs (Finnerup *et al*., 2005). Moreover, the affordability and availability of the potent analgesic agents has been reported to be limited by cost about two decades ago. This is true, especially in developing countries (De Lima *et al*., 2004). Health care providers are still grappling with the side effects of available analgesic agents which include gastro-intestinal disorders, physical dependency and addiction (Hanson *et al*., 2009).

Toxicity profile determination is a sine qua non for development of a potential drug candidate. Acute toxicity provides an insight into a compound's short-term harmful effect when administered within 24 h. It provides information on probable harmful target organs, potentials for toxicity in humans and safe acute doses for further research (Colerangle, 2017). Sub-acute toxicity illustrates a substance's innately harmful effects when administered daily for 1-3 months at a time. Sub-acute toxicity studies how lesions change or advance after a drug candidate is taken on a regular basis. It also covers the area of an acute toxicity investigation (Heimann and Doughty, 2014). Since natural product has been acknowledged as a vital source of active principles in therapeutic agents (Mathur and Hoskins, 2017), and the seeds and other parts of *Adansonia digitata* plants have been documented for use as an analgesic (Kamatou *et al*., 2011), we decided to explore the seeds of the fruit of the plant with a view to ascertain the folkloric claim that the seed of the plant contains analgesic agents and develop a safer and more affordable analgesic agent.

MATERIALS AND METHODS

Materials and chemicals

Agate mortar and pestle (Sigma-Aldrich), Distilled water was prepared using distiller machine manufactured by Laboid international, Solan, Pradesh, India. Hot air oven (Guangzhou Yueshen Medical Equipment Co., Ltd. Guaugzhou, China). Methane, isopropanol and ascorbic acid were purchased from Changzhou Baixing Chemical Co., Ltd., Tianjin, China. All solvents used were of analytical grade and were purchased from Merck (Loba-chemie pvt. Ltd., Mumbai, India). Semi Auto Analyser Microlab 300 (Vital Scientific, US) machine, Diclofenac sodium (BOC Sciences, US).

Plant collection and processing

The fruit of *Adansonia digitata* (Bombacaceae) was obtained from Sokoto Central Market, Sokoto State. They were identified and authenticated at the Department of Pharmacognosy and Ethno-pharmacy, Faculty of Pharmaceutical Sciences, UDUS, Sokoto State, Nigeria where a voucher specimen (No 109806) was deposited. The seed was air dried after separation from the pulp by using mortar and pestle. The sample (400 g) was crushed into powder. The powdered *Adansonia* seed was extracted via Soxhlet extraction using 90%v/v methanol. The

resulting extract was concentrated to dryness under controlled temperature (50-55°C) using an oven and was span to get a clear oil at the top and a mucilaginous residue at the bottom.

Experimental animals

A total of eighty-five male and female albino mice weighing between 18-20 g were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria. The animals were acclimatized for two weeks in the experimental room and fed *ad libitum* on commercial mouse chow (Vital Feed, Grand Cereals Ltd) and water throughout the period of the experiment. The University's ethical committee requirements for the use of animals, as well as the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) were followed. The University Research and Ethics Committee approved the research protocol and assigned a reference number UDUS/UREC/2020/010.

Phytochemical screening of the extract

Phytochemical screening of the extracts was performed for the presence of secondary metabolites using standard phytochemical methods as described by Trease and Evans (2002).

Acute toxicity study (LD_{50})

LD_{50} determinations were conducted using the method of Chinedu *et al*. (2013) as detailed in Table 1 below. The LD_{50} value was then determined by calculating the geometric mean of the lowest dose that caused death (a) and the highest dose for which the animal survived (b) after 24 h of administering the compound. LD50= $\sqrt{a \times b}$.

Table 1: Experimental Design of Acute Toxicity Study

PHASE ONE (n=3)		PHASE TWO (n=1)	
Route of administration	**Dose (mg/kg)**	**Route of administration**	**Dose (mg/kg)**
Oral	10	Oral	1,600
Oral	100	Oral	2,900
Oral	1,000	Oral	5,000

Sub-acute toxicity study

Forty two mice were used in the sub-acute toxicity research. All the mice were orally administered the methanol extract. The mice were divided into seven groups, each containing six mice per group. The first group (vehicle control) received the vehicle at a dose of 2 ml/kg body weight, whiles the second, third and fourth groups received 250 mg/kg, 500 mg/kg and 1,000 mg/kg body weight of the extract respectively. The fifth, sixth, and seventh groups were administered 6.25 mg/kg, 12.5 mg/kg and 25 mg/kg of the oil extract respectively. Physiological functions that were monitored including the respiratory rate, occurrence of secretions and excretions, and autonomic activity (such as lacrimation, piloerection, pupil size, and unusual respiratory pattern), changes in skin fur, presence or absence of hair loss (alopecia), eyes and mucous membranes. The mice were weighed weekly to monitor weight changes. On day 29 of administration, the mice were fasted for 12 h and blood samples were collected by cardiac puncture in plain blood sample tubes (Parasuraman *et al,*. 2010). The serum was separated and quantified for glucose, urea, creatinine, total cholesterol, triglyceride, aspartate aminotransferase, total bilirubin, alkaline phosphatase and alanine transaminase using a Semi Auto Analyser Microlab 300 (Vital Scientific, US) machine.

Analgesic study design

Forty two mice were randomly assigned to eight groups of six animals each for the two different experimental models. Group one served as negative control receiving the extract diluent (10 mL/kg). Group two served as positive control and was administered diclofenac sodium intra-peritoneally at a dose of 10 mg/kg. Groups three, four and five were administered 250 mg/kg, 500 mg/kg and 1000 mg/kg of the extract respectively. Groups six, seven and eight were administered 6.25 mg/kg, 12.5 mg/kg and 25 mg/kg of the oil extract respectively. All treatments except those for group two were administered orally.

Tail-flick test.

Analgesic activity of the extract was evaluated using the tail-flick method as described (Fan *et al*., 2014). About 5 cm from the distal end of the tail of each mouse was immersed in warm water maintained at 50ºC. The reaction time (in seconds) was the time taken by the mouse to flick its tail due to pain. The first reading was omitted and reaction time was taken as the average of the next two readings. The reaction time was recorded before (0 min) and at 15, 30, 45, and 60 minutes after the administration of the treatments. The maximum reaction time was fixed at 20 sec to prevent any tail tissue injury. Reading were taken by two independent assessors to avoid bias. Reading that exceeded 20 sec. was considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$MPA = \frac{\text{Reaction time for treatment} - \text{reaction time for saline}}{20\text{sec} - \text{reaction time for saline}} \times 100$$

Hot plate test

The evaluation of analgesic activity of the extract was also carried out using hot plate method as described (Gunn *et al*., 2011). Each mice were placed on a hot plate maintained at 55ºC within the restrainer. The reaction time (in seconds) or latency period was determined as the time taken for the mice to react to the thermal pain by licking their paws or jumping. The reaction time was recorded before (0min) and at 15, 30, 45, and 60 min after the

administration of the treatments. The maximum reaction time was fixed at 20 sec to prevent any injury to the tissues of the paws. Reading were taken by two independent assessors to avoid bias. Reading that exceeded 20 sec. considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$MPA = \frac{\text{Reaction time for treatment} - \text{reaction time for saline}}{20\text{sec} - \text{reaction time for saline}} \times 100$$

Statistical analysis

Data were presented as mean ± standard error mean (SEM). The results were analyzed using Statistical Package for the Social Sciences (SPSS) version 19. Statistical significance was determined by Student's t-test and one way analysis of variance (as appropriate) and P values less than 0.05 was considered significant.

RESULTS

Yield

The total weight of the extract obtained from the powdered seed of *Adansonia digitata* after methanol extraction using Soxhlet method was 51.48 g. After separation of the extract, about 11.68 g of the extract formed the mucilaginous part while the remaining 39.80 g gave the oily fraction.

Qualitative phytochemical screening revealed the presence of alkaloids, glycosides, saponins and flavonoids. Tannins, amino acids and steroids were absent (Table 2)

Table 2: Phytochemical analysis for the methanol extract of *Adansonia digitata* seed

Phytochemical compound	Presence/absence
Cardiac glycosides	+
Tannins	_
Flavonoids	+
Amino acid	_
Alkaloids	+
Steroids	_
Saponins	+

+ = presence; − = absent.

Acute toxicity

During the first and second phases of the acute toxicity study, oral administration of the oily and mucilaginous forms of the extract (10-5,000 mg/kg) for 24 h did not produce any visible sign of toxicity in the animals. The oral LD_{50} for the methanol extract of *Adansonia digitata* seed in mice was determined to be above 5,000 mg/kg. The highest dose of adopted doses was less 60% of the LD_{50} (Nair and Jacob, 2016).

Sub-acute toxicity study

Throughout the sub-acute toxicity intervention period, there was no difference in the weight of the mice except at the last week where all the groups except group 3 (500 mg/kg mucilaginous extract) demonstrated a significant difference in weight when compared to the vehicle control group (Figure 1).

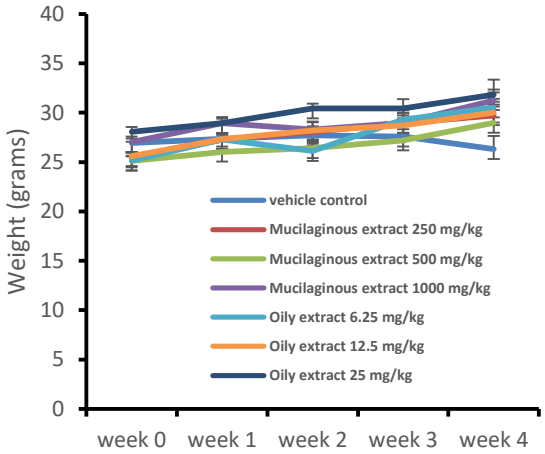

Figure 1: Weekly weight changes in mice following oral administration of graded doses of methanol extract of *Adansonia digitata* seed. Vehicle control=group 1; mucilaginous extract 250 mg/kg = group 2; mucilaginous extract 500 mg/kg = group 3; mucilaginous extract 1000 mg/kg = group 4; oily extract 6.25 mg/kg = group 5; oily extract 12.5 mg/kg = group 6; oily extract 25 mg/kg = group 7.

Irrespective of the dose, the methanol seed extract of *Adansonia digitata* did not affect total cholesterol and triacylglycerol levels. This was also the case for the levels of the three liver enzymes examined after the four weeks intervention with the methanol seed extract. Total bilirubin levels were high for the highest dose of the oily methanol extract. Serum glucose and creatinine levels were all not significantly different among the different groups of mice administered graded doses of both forms of the methanol extract (Table 3). As the dose of the compound increases, also serum urea levels tend to increase.

All the doses administered for group 2 (diclofenac) and the extracts (oily and mucilaginous) demonstrated significant difference in mean reaction time when compared to their respective vehicle controls (group 1) for both the tail flick and the hot plate methods (Tables 4 and 5). Using the tail flick method, the mucilaginous extract showed a gradual decrease in mean reaction time (although not significant at $p<0.05$) as its dose increased. The oily extract demonstrated a direct relationship with the mean reaction time as its dose increased (Table 4).

Table 3: Serum biochemical parameters in mice following oral administrattion of graded doses of methanol extract of *Adansonia digitata* seed

Groups	***1***	***2***	***3***	***4***	***5***	***6***	***7***
Biochemical parameter	***2 ml/kg***	***250 mg/kg***	***500 mg/kg***	***1,000 mg/kg***	***6.25 mg/kg***	***12.5 mg/kg***	***25 mg/kg***
Total cholesterol (mg/dL)	97.6 ± 11.7^{a}	101.2 ± 9.4^{a}	89.2 ± 7.8^{a}	97.3 ± 8.6^{a}	100.2 ± 5.6^{a}	98.7 ± 4.7^{a}	101.3 ± 5.6^{a}
Triacyl glycerol (mg/dL)	132.0 ± 21.6^{b}	125.0 ± 10.5^{b}	128.0 ± 27.7^{b}	131.2 ± 27.1^{b}	129.4 ± 13^{b}	131.6 ± 12^{b}	130.1 ± 12^{b}
Total bilirubin (mg/dL)	1.3 ± 0.2^{c}	2.0 ± 0.3^{c}	1.3 ± 0.4^{c}	1.2 ± 0.4^{c}	1.2 ± 0.2^{c}	1.4 ± 0.2^{c}	3.7 ± 0.3^{d}
Alanine aminotransferase (u/L)	113.2 ± 8.7^{e}	119.3 ± 19.0^{e}	114.6 ± 24.9^{e}	119.2 ± 29.2^{e}	117.3 ± 16.3^{e}	116.4 ± 19.2^{e}	117.4 ± 18.5^{e}
Aspartate transaminase (u/L)	64.4 ± 13.2^{f}	74.0 ± 13.8^{f}	54.6 ± 19.0^{f}	57.6 ± 15.3^{f}	60.2 ± 16.4^{f}	55.0 ± 15.6^{f}	56.6 ± 11.37^{f}
Alkaline phosphatase (u/L)	60.2 ± 16.4^{g}	55.0 ± 25.6^{g}	35.6 ± 21.37^{g}	49.0 ± 14.8^{g}	74.0 ± 13.8^{g}	54.6 ± 19.0^{g}	57.6 ± 15.3^{g}
Glucose (mmol/L)	4.9 ± 2.2^{h}	5.7 ± 2.4^{h}	7.9 ± 2.1^{h}	6.5 ± 1.2^{h}	6.7 ± 1.^{jh}	6.6 ± 1.1^{h}	8.7 ± 0.6^{h}
Urea (mmol/L)	6.7 ± 1.0^{j}	6.8 ± 1.1^{j}	8.2 ± 0.6^{j}	7.3 ± 0.9^{j}	6.7 ± 2.4^{j}	7.9 ± 2.1^{j}	13.5 ± 1.2^{k}
Creatinine (mg/dL)	1.1 ± 0.3^{l}	0.9 ± 0.3^{l}	1.5 ± 0.4^{l}	1.1 ± 0.3^{l}	1.1 ± 0.1^{l}	1.07 ± 0.2^{l}	1.02 ± 0.2^{l}

Data represent mean ± SD (n=6). Rows superscripted by the same letter are not significantly different at $p < 0.05$ in Tukey's multiple comparison test.

Table 4: Mean reaction time and maximum possible analgesia of methanol seeds extract of *Adansonia digitata* in mice using tail flick method

Groups	Treatments (oral)	Reaction time in seconds (mean ± standard error of mean)			
		15 min	30 min	45 min	60 min
1	Vehicle control	6.43 ± 1.60	5.20 ± 0.87	5.48 ± 1.00	5.86 ± 0.60
2	Diclofenac 10 mg/kg	14.9 ± 2.60*	13.1 ± 2.30*	17.6 ± 2.22*	16.5 ± 2.19*
3	Mucilaginous extract 250 mg/kg	17.6 ± 2.38*	17.9 ± 2.03*	16.6 ± 2.23*	19.9 ± 0.08*
4	Mucilaginous Extract 500 mg/kg	16.1 ± 1.62*	16.8 ± 1.89*	17.9 ± 1.65*	18.0 ± 1.17*
5	Mucilaginous extract 1000 mg/kg	14.6 ± 0.70*	15.9 ± 1.52*	15.4 ± 2.68*	16.1 ± 2.28*
6	Oily extract 6.25 mg/kg	12.2 ± 2.02*	13.4 ± 2.52*	16.6 ± 2.05*	13.6 ± 2.19*
7	Oily extract 12.5 mg/kg	13.8 ± 1.74*	14.6 ± 1.81*	17.3 ± 1.63*	18.0 ± 1.71*^{b}
8	Oily extract 25 mg/kg	18.5 ± 1.24*	19.7 ± 0.28*	17.1 ± 2.42*	14.6 ± 3.14*^{b}

Values are expressed as mean ± standard error of mean of four replicates. Figure in parentheses represents maximum possible analgesia. *P < 0.05 versus control, ^{b}P < 0.05 treatment versus baseline (control) of the respective treatment.

Table 5:: Mean reaction time and maximum possible analgesia of methanol seeds extract of Adansonia digitata in mice using hot plate method

Groups	Treatment (oral)	Reaction time in seconds (mean ± standard error of mean)			
		15 min	30 min	45 min	60 min
1	Normal saline	6.30 ± 0.47	6.98 ± 0.94	5.48 ± 0.55	10.62 ± 0.79^{b}
2	Diclofenac 10 mg/kg	19.8 ± 0.23*	19.9 ± 0.1*	19.9 ± 0.03*	16.9 ± 1.73*
3	Mucilaginous extract 250 mg/kg	19.7 ± 0.21*	16.6 ± 1.36*	15.7 ± 1.51*	16.9 ± 1.85*
4	Mucilaginous Extract 500 mg/kg	19.3 ± 0.75*	19.6 ± 0.31*	18.1 ± 0.82*	16.7 ± 1.44*
5	Mucilaginous extract 1000 mg/kg	20.0 ± 0.00*	20.0 ± 0.00*	20.0 ± 0.00*	20.0 ± 0.00*
6	Oily extract 6.25 mg/kg	20.0 ± 0.00*	20.0 ± 0.00*	20.0 ± 0.00*	20.0 ± 0.00*
7	Oily extract 12.5 mg/kg	20.0 ± 0.00*	20.0 ± 0.00*	20.0 ±0 .00*	19.3 ± 0.45*
8	Oily extract 25 mg/kg	19.0 ± 0.87*	19.1 ± 0.51*	18.5 ± 0.98*	17.8 ± 0.78*

Values are expressed as mean ± standard error of mean of four replicates. Figure in parentheses represents maximum possible analgesia . *P < 0.05 versus control,

DISCUSSION

In this study, we verified the acute and sub-acute toxicity of the methanol extract of *Adansonia digitata* seeds. We also employed pain-state models to screen for analgesic activity in mice using thermal stimuli, such as tail-flick and hot plate methods.

During drug development process, determination of the toxicity profile of a promising therapeutic agent is prerequisite to establishment of efficacy. Acute toxicity study is usually the first type of toxicity study investigated *in vivo* during preclinical drug development. It is aims at provide a guide for the dose range of a novel therapeutic agent and provide preliminary information on the mode of toxic action of the agent in question (Mohs and Greig, 2017). The methanol extract (oily and mucilaginous) of *Adansonia digitata* seeds exhibited an oral LD_{50} of over 5,000 mg/kg body weight. This provides a spectrum for dosage selection and optimization for further therapeutic and toxicity studies. The methanol extract of *Adansonia digitata* seeds (oily and mucilaginous) did not alter serum glucose, liver enzymes and serum lipids investigated. The serum urea level is the only serum biochemical parameter that differed among the groups. This could be due to a building acute renal failure as this was only found in mice administered the highest dose of the oily methanol extract after 28 days sub-acute toxicity study.

The tail-flick and hot plate methods can be used to illustrate centrally mediated anti-nociceptive responses,

which generally focus on alterations above the spinal cord level (Bravo *et al.,* 2019) The hot plate method is considered a supraspinally structured response, whereas the tail-flick approach mediates a spinal reflex to a nociceptive input (Heinricher *et al*., 2009).

In the hot plate model, it was found that the *Adansonia digitata* extract exhibited significant ($p<0.05$) analgesic activity by increasing the reaction time of the mice compared to vehicle control (saline treated mice) at all-time points and especially doses at 1000 mg/kg, 6.25 mg/kg and 12.5 mg/kg shows equivalent analgesic property compared with standard diclofenac drug. With reference to the maximum possible analgesia (MPA) value, the analgesic effects of the oily and mucilaginous forms of the extract and diclofenac sodium were evident within 15 min following administration. We can also suggest that both forms of the extract have longer duration of action than the diclofenac drug as indicated by high latency time even at 60 min time interval. The oily fraction of the extract demonstrated significant potent analgesic property in comparison with the mucilaginous fraction and the standard drug. It reveals longer reaction time and high duration of action. It may be due to the hydrophobic nature of the oily fraction of the extract which gives it better pharmacokinetic advantages. This could also be due to the presence of higher concentration of alkaloids. This is to say that the hot-plate latency of the extract at all-time points was cumulatively higher than that of the reference drug (diclofenac sodium), which is a mild to moderate analgesia with long duration of action, thereby the extract exhibited a very good significant trend of higher reaction time compared to diclofenac sodium in mice.

In tail-flick model, the methanol extract from the pulverized seeds of *Adansonia digitata* exhibited significant analgesic activity by increasing the reaction time of the mice compared to control (saline treated mice) at all-time points. Diclofenac sodium was used as reference drugs, which are considered mild to moderate analgesic. In comparison with control, the extract (especially at 250mg/kg and 500mg/kg) and the oil fraction at (especially at 12.5 mg/kg and 25 mg/kg) produced the most significant anti-nociception effect during all observation times, followed by the diclofenac treated group. The tail-flick method is based on the observation that analgesic-like compounds are selectively able to prolong the reaction time of typical tail-withdrawal effect in mice. This method is also useful in differentiating central opioid-like analgesics from peripheral analgesics. Analgesic drugs usually act by elevating pain threshold of animals towards heat and pressure (Subedi *et al*., 2016). Therefore, the analgesic effect of the extract (mucilaginous and oily) on this pain-state model indicates that it might have potent analgesic effects. With reference to the MPA value, the analgesic effects of both forms of the extract and diclofenac sodium were evident within 15min of administration. However, the extract (especially 250mg/kg and 25 mg/kg oil) showed long-lived analgesia as the MPA gradually increased down the time interval as compared to diclofenac sodium. The tail-flick latency of the extract at all-time points was cumulatively higher than that of the reference drug, which is a mild to moderate analgesia with long duration of action, thereby the extract exhibited a good significant trend of higher reaction time compared to diclofenac sodium. Both treatments (oil and extract) produced comparable reaction times, suggesting that the pulverized seed of *Adansonia digitata* could be a better natural alternative for mild pain relief.

Many of the alkaloids, flavanoids, steroids, and tannin isolated from medicinal plants have been reported to possess significant analgesic activity (Sengupta *et al,* 2012). In addition, the presence of cardenolides and saponins in plant products with analgesic and anti-inflammatory properties have also been established (Hassan *et al.,* 2012). The presence of flavonoids in the leaves and whole plant of *Adansonia digitata* has been reported likewise (Khan *et al*., 2006). These active ingredients may be responsible for the analgesic and anti-inflammatory properties of *Adansonia digitata.*

Since nonsteroidal analgesic and anti-inflammatory drugs such as diclofenac produce their therapeutic effects through the inhibition of prostaglandin synthesis (Saleh and Hassan, 2019) and the extracts demonstrated analgesic effects similar to diclofenac, therefore by correlation *Adansonia digitata* may inhibit prostaglandin synthesis by inhibiting cyclo-oxygenase 2 (COX-2) as one of its possible mechanism of action.

CONCLUSION

This study has demonstrated that fractions of extract from the seed extract of *Adansonia digitata* exhibited relative safety and analgesic activity in mice. These findings further support the folkloric use of the plant to treat pain and inflammatory conditions, and shows that it may contains active analgesic agents. In view of these findings, efforts could be made to further evaluate the anti-inflammatory property and characterize the active components of this plant. Further work should be done to elucidate the mechanisms of action of its components as analgesic.

Declaration of competing interest

The authors declare that they have no known competing financial or personal interest.

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