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Anti-inflammatory activities of dichloromethane-methanolic leaf and stem bark extracts of *Ximenia americana* in mice models

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ABSTRACT

Introduction: *Ximenia americana* is a highly branched shrub mainly found in tropics of Asia, Africa, New Zealand, Central and South America among others. In most parts of Africa, *X. americana* is used in folklore to treat various disorders such as oedema, pain, fever, helminthiasis, diarrhea and burns among others. There is no published data on anti-inflammatory activities of organic extracts of *X. americana*. It is against this background that this research was carried out. The study tested for the anti-inflammatory activities of dichloromethane-methanolic (DCM-MeOH) leaf and stem bark extracts of *X. americana* in rats.

Methods: The plant materials were collected from Mbeere North sub-county, Embu county, Kenya. Methanol and dichloromethane in the ratio of 1:1 was used to extract the active compounds. Five to 6 weeks old Swiss Albino mice were employed for the anti-inflammatory studies. Animals were divided into 6 groups of 5 mice each: normal, negative, reference and three experimental groups (50, 100 and 150 mg/kg body weight). Inflammation was induced experimentally using carrageenan. The experimental groups were treated with predetermined dose quantities of prepared extracts. Diclofenac was used as the reference drug. Data was analyzed using one-way analysis of variance (ANOVA).

Results: The extracts from the leaves reduced hind paw circumference by between 0.91% and 16.90% while the stem bark extracts reduced hind paw circumference by between 5.84% and 29.00%. Diclofenac reduced right hind paw circumference by 1.32%-29.60%. Qualitative phytochemical screening showed presence of alkaloids, flavonoids, steroids, saponins, cardiac glycosides, phenolics and terpenoids in the extract.

Conclusion: The study established that the DCM-MeOH leaf and stem bark extracts of *X. americana* is effective in management of inflammation and therefore it can be explored as a possible bio-resource in the development of herbal medicines.

Implication for health policy/practice/research/medical education:
This study confirms the anti-inflammatory potential of the leaf and stem bark DCM-MeOH extracts of *X. americana* in experimental animals. Therefore, the DCM-MeOH leaf and stem bark extracts of *X. americana* might prove useful in managing inflammation and thus serve as an alternative treatment bioresource, which is more effective than the conventional synthetic drugs.

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Introduction

Inflammation (Latin, *inflammatio*), is part of the body response to harmful stimuli. The stimuli can be disease causing organisms, damaged cells, irritants among others (1). Inflammation is meant to be protective and it involves blood vessels, immune cells and various inflammatory mediators (2). There are 5 classical signs of acute inflammation. These five signs are heat, pain, redness, edema and loss of function (3).

Resident cells such as dendritic, Kupffer, mastocytes, histocytes and macrophages, which are in diseased tissue, initiate the process of acute inflammation (4). Pattern recognition receptors (PPRs) are presented on the surface of these cells. These receptors recognize generic molecules

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broadly shared by disease causing organisms that are different from host molecules. They are collectively referred to as pathogen-associated molecular patterns (4). During the onset of disease, the cells are activated; one of their PRRs recognizes pathogen associated molecular pattern (PAMP), causing the release of inflammatory mediators responsible for the cardinal signs of inflammatory process (4). Redness and increased heat on the damaged part is caused by vasodilatation that leads to increased blood flow. Edema is caused by increased permeability of blood vessels which results in leakage of plasma proteins and fluid into tissue. Pain is caused by increased sensitivity as result of released mediators such as bradykinin (4). Loss of function (disturbance of function) is the product of the neurological reflex in response to pain (4).

In laboratory animals, inflammation can be induced by use of carrageenan. Carrageenan is sulfated polysaccharide mainly used as food additive (5). Carrageenan is readily available in Kenyan market and is a widely studied agent for inducing inflammation in laboratory animals. The inflammatory response induced by carrageenan involves activation of Nuclear factor kappaB (NF-κB) by Toll like receptor (TLR) 4 and B-cell leukemia (BCL) 10 that leads to increased Interleukin (IL)-8 production (5).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac are used in the treatment of inflammation. These drugs confer anti-inflammatory activities through non-selective inhibition of cyclooxygenase enzymes (cyclooxygenase [COX] 1 and 2) which may result in serious side effects such as gastric bleeding (6). Such unwanted effects associated with the use of synthetic drugs can be avoided by alternatively using effective, tolerable and reliable naturally occurring agents (7).

Since time immemorial, plants have been used as source of medicine with many studies being carried out worldwide to confirm their efficacy (8). Traditionally, *Ximenia americana* is used in the treatment of a variety of disorders such as: pain, fever and inflammation (9,10), treatment of helminthiasis (11), diarrhea and wounds (12) and headaches, skin ulcers, kidney and heart problems (13). Mbeere community use *X. americana* to treat a stomach discomforts, fever, oedema, and pain. Despite the broad folklore use of *X. americana*, there is no scientific evaluation of its dichloromethane-methanolic (DCM-MeOH) extracts on inflammation. The current study was designed against this background to specifically bioscreen DCM-MeOH stem bark and leaf extracts of *X. americana* for anti-inflammatory potential.

Materials and Methods

Collection and preparation of plant samples

Leaves and stem barks of *X. americana* were collected with the help of local traditional herbalists from Mbeere-North in Embu county, Kenya. The plant materials were properly sorted, cleaned and packed in polythene bags and transported to biochemistry and biotechnology laboratories of Kenyatta University for further processing. The botanical authentication of the materials was done by a qualified taxonomist and the voucher specimen deposited in Kenyatta University Herbarium. The plant materials were chopped, completely air dried at room temperature followed by grinding into fine homogenous powder with an electric mill and then sieved through mesh sieve.

Extraction

For each sample, 200 g of the powder was soaked in cold 1:1 mixture of methanol and DCM and stirred for 6 hours to extract the active compounds. This was followed by successive filtering of the extracts and the filtrate concentrated under reduced pressure and vacuum using rotary evaporator (Buchii R110). The concentrate was stored in airtight containers at -4°C before use in the bioassay studies (14).

Laboratory animals

Swiss Albino mice of either sex aged 5-6 weeks and weighing between 15-35 g were employed in the study (15). The animals were acquired and bred at the animal breeding and experimentation laboratory in Kenyatta University, Department of Biochemistry and Biotechnology. The animals were kept in standard cages, under the conditions of a standard laboratory of ambient temperature of 25°C and 12-hour light followed by 12-hour dark cycle throughout the experiments. The feeding was on standard pellets for rodents and water was supplied in *ad libitum* (16). Throughout the study, all ethical guidelines and procedures on animal handling were followed (17).

Determination of anti-inflammatory activities

The experimental animals (30 Swiss Albino mice) were divided into 6 groups of 5 mice each and treated as shown in Table 1.

The carrageenan, (0.1 mL and 1% w/v in normal saline), was used to induce inflammation by injecting into the sub-plantar of the right hind paw tissue (18). One hour after induction of inflammation, herbal extracts and DMSO were intraperitoneally administered. The inflammation was measured immediately prior to the injection of carrageenan and the comparison of the paw circumference was done after injection of carrageenan. The measurement of linear paw circumference was done at an interval of 1 hour for 4 hours and comparison was done according to the following formula (19):

$$E_1 = \frac{t_1 - t_0}{t_0} \times 100$$

Where t_0 = initial volume of paw, t_1= final volume of the paw.

Qualitative Phytochemical Screening

Qualitative phytochemical screening was done on the extracts to find out whether selected phytochemicals were present (20,21). The secondary metabolites tested for are: alkaloids, tannins, steroids, saponins, cardiac glycosides,

Table 1. Treatment protocol for evaluation of anti-inflammatory activities of DCM-MeOH leaf and stem bark extracts *Ximenia americana* in mice

Group	Status	Treatment
I	Normal control	DMSO[a]
II	Negative control	Carrageenan 100 µg + DMSO (10%)
III	Positive control	Carrageenan 100 µg + 15 mg/kg diclofenac
IV	Experimental group A	Carrageenan 100 µg + 50 mg/kg extract
V	Experimental group B	Carrageenan 100 µg + 100 mg/kg extract
VI	Experimental group C	Carrageenan 100 µg + 150 mg/kg extract

[a] DMSO was used as a vehicle.

phenolics and terpenoids.

Management and statistical analysis of the data

The data on the right hind paw circumference changes was recorded and tabulated in a spread sheet. The data was then imported to Minitab statistical software version 17 for descriptive statistical analysis. The results were expressed as mean ± standard error (SE) of mean for analysis. One-way analysis of variance (ANOVA) was performed to compare the group means followed by Tukey's post hoc test for pair-wise mean separations and comparisons to obtain the specific significant differences among the different groups. Unpaired student *t* test was used to compare mean anti-inflammatory activities between leaf and stem bark extracts of *X. americana*. The statistical significance was considered at $P \leq 0.05$. The data on the percentage change in paw circumference was presented using graphs.

Results

Anti-inflammatory activity of DCM-MeOH leaf extract of *Ximenia americana* in mice

The DCM-MeOH leaf extracts of *X. americana* at the 3 dose levels (50, 100 and 150 mg/kg body weight) reduced right hind paw circumference of carrageenan-induced inflammation in mice (Table 2; Figure 1).

In the first hour, the 3 dose levels (50, 100 and 150 mg/kg body weight) reduced paw circumference by 0.91%, 2.28% and 2.30% respectively (Figure 1). The anti-inflammatory activities of the leaf extract of *X. americana* at the 3 dose levels showed no significant difference from the positive group ($P>0.05$; Table 2). The anti-inflammatory activities at dose levels 100 and 150 mg/kg body weight was significantly different from the normal and negative groups ($P<0.05$; Table 2).

In the second hour, the 3 dose levels of the extract (50, 100 and 150 mg/kg body weight) reduced the paw circumference by 8.47%, 11.62% and 7.85% respectively. There was significant difference between the 3 dose levels of extract from the normal and negative control group at this hour ($P<0.05$; Table 2). The anti-inflammatory effects of the extract at dose level 50 and 150 mg/kg body weight showed no significant difference from the positive group at this hour ($P>0.05$; Table 2).

In the third hour, the 3 dose levels of leaf extract of *X. americana* (50, 100 and 150 mg/kg body weight) reduced the right hind paw circumference of mice by 14.54%, 15.04% and 16.90% respectively (Figure 1). The difference in anti-inflammatory activities of extract was significant from both normal group and negative group ($P<0.05$; Table 2) but there was no significant difference when compared to that of diclofenac ($P>0.05$; Table 2).

In the fourth hour, the 3 dose levels of the leaf extract of *X. americana* (50, 100 and 150 mg/kg body weight) reduced the paw circumference of mice by 2.61%, 15.94% and 9.01% respectively (Figure 1). The anti-inflammatory activity of the extract at this hour for all the dose levels was significantly different from the normal group and negative group ($P<0.05$; Table 2). The anti-inflammatory activity of the leaf extract at the dose level of 100 mg/kg body weight showed no significant difference from the positive control group at this hour ($P>0.05$; Table 2).

Anti-inflammatory activity of DCM-MeOH stem bark extract of *Ximenia americana* in mice

The DCM-MeOH stem bark extract of *X. americana* showed similar anti-inflammatory activities against carrageenan-induced inflammation in mice (Table 3 and

Table 2. Effects of DCM-MeOH leaf extract of *Ximenia americana* on carrageenan-induced inflammation in mice

Group	Treatment	Percent change in paw circumference after drug administration				
		0 h	1 h	2 h	3 h	4 h
Normal control	DMSO	100 ± 0.00	99.997 ± 0.16^{a}	100.01 ± 0.15^{a}	99.921 ± 0.18^{a}	100.10 ± 0.10^{a}
Negative control	Carrageenan + DMSO	100 ± 0.00	100.46 ± 0.18^{a}	103.46 ± 1.73^{a}	103.74 ± 1.73^{a}	103.79 ± 1.65^{a}
Positive control	Carrageenan + DMSO + diclofenac	100 ± 0.00	98.684 ± 0.40^{ab}	94.986 ± 0.67^{b}	82.34 ± 2.37^{b}	81.40 ± 1.46^{d}
DCM: Methanolic Leaf extract	Carrageenan + 50 mg/kg	100 ± 0.00	99.090 ± 0.22^{ab}	91.526± 0.98^{bc}	85.46 ± 1.71^{b}	97.39 ± 2.43^{ab}
	Carrageenan + 100 mg/kg	100 ± 0.00	97.715 ± 0.58^{b}	88.38 ± 1.80^{c}	84.96 ± 1.85^{b}	84.06 ± 1.91^{cd}
	Carrageenan + 150 mg/kg	100 ± 0.00	97.700 ± 0.87^{b}	92.149 ± 0.36^{bc}	83.10 ± 1.75^{b}	90.99 ± 2.24^{bc}

Values are expressed as Mean ± SEM for 5 animals per group. Statistical comparison were made within a column and values with the same superscript are not significantly different by one-way ANOVA followed by Tukey's post hoc test ($P > 0.05$). Carrageenan =1%; DMSO = 10%; Diclofenac = 15 mg/kg.

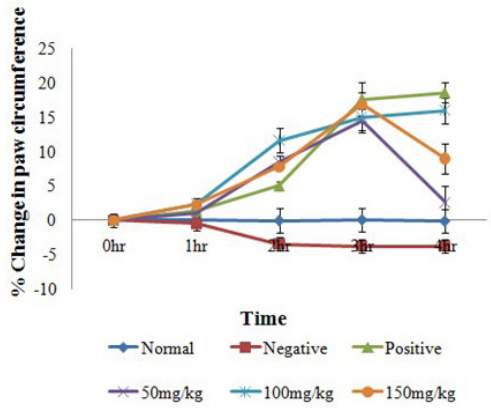

Figure 1. Anti-inflammatory effects of DCM-MeOH leaf extract of *Ximenia americana* on carrageenan-induced inflammation in Swiss albino mice.

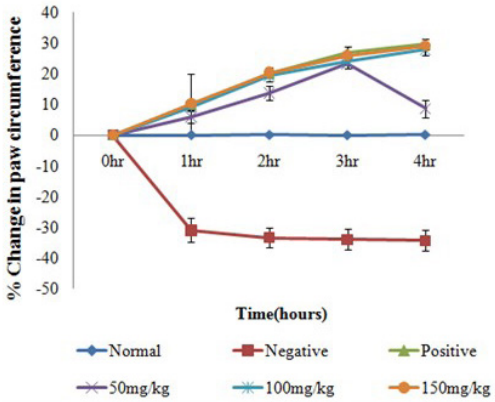

Figure 2. Anti-inflammatory effects of DCM-MeOH stem bark extract of *Ximenia americana* on carrageenan-induced inflammation in mice.

Figure 2). This was indicated by the reduction in the paw circumference.

In the first hour, the 3 dose levels (50, 100 and 150 mg/kg body weight) reduced the right hind paw circumference by 5.848%, 9.174% and 10.27% respectively (Figure 2 and Table 3). In comparison to the diclofenac, the anti-inflammatory activities at the 3 dose levels of the extract did not show significant difference ($P>0.05$; Table 3). The 3 dose levels of the stem bark extract (50, 100 and 150 mg/kg body weight) showed significant difference from the negative control group ($P<0.05$; Table 3).

In the second hour, the 3 dose levels of the extract reduced the right hind paw circumference by 13.72% and 19.17% and 20.33% respectively as shown (Figure 2 and Table 3). The anti-inflammatory activities of the stem bark extract of *X. americana* at the 3 dose levels did not show significant difference from diclofenac ($P>0.05$; Table 3). The 3 dose levels showed significant difference from the normal and negative control groups ($P<0.05$; Table 3).

The 3 dose levels of the stem bark extract of *X. americana* (50, 100 and 150 mg/kg body weight) reduced the paw circumference of mice by 23.18%, 23.91% and 25.97% respectively in the third hour (Figure 2). There was no significant difference of the anti-inflammatory activities at all the 3 dose levels from the positive control ($P>0.05$; Table 3). This was as opposed to the anti-inflammatory activity of the 3 dose levels of the extract which was significantly different from the normal group and negative control group ($P<0.05$; Table 3)

In the fourth hour, the 3 dose levels of the extract (50, 100 and 150 mg/kg body weight) and diclofenac reduced inflamed hind paw circumference by 8.62%, 27.73%, 29.00% and 29.60% respectively (Figure 2 and Table 3). The dose levels of 100 and 150 mg/kg body weight showed no significant difference from the positive control group ($P>0.05$; Table 3). The dose level of 50 mg/kg body weight showed significant difference from the positive control group ($P<0.05$; Table 3). The highest anti-inflammatory activity was associated with the dose level of 150 mg/kg body weight whereby circumference of inflamed paw was decreased by 29%. However, the anti-inflammatory activity at the dose level of 50 mg/kg body weight of the extract was significantly different from 100 and 150 mg/kg body weight ($P<0.05$; Table 3) but there was no significant difference from the normal control group ($P>0.05$; Table 3).

Comparison between the anti-inflammatory activities of leaf and stem bark extract of *Ximenia americana*

In comparison, at the dose level of 50 mg/kg, there was significant difference in the anti-inflammatory activities of DCM-MeOH leaf and stem bark extracts of *X. americana* in the first and third hour of the test period ($P=0.05$ and 0.006 respectively). However, there was no significant

Table 3. Effects of DCM-MeOH stem bark extract of *Ximenia americana* on carrageenan-induced inflammation in mice

Group	Treatment	Percent change in paw circumference after drug administration				
		0 h	1 h	2h	3h	4h
Normal control	DMSO	100 ± 0.00	100.00 ± 0.21^{b}	99.967 ± 0.06^{b}	100.06 ± 0.07^{b}	99.939 ± 0.06^{b}
Negative control	Carrageenan + DMSO	100 ± 0.00	130.97 ± 3.85^{a}	133.45 ± 3.32^{a}	133.97 ± 3.44^{a}	134.34 ± 3.40^{a}
Positive control	Carrageenan + DMSO + diclofenac	100 ± 0.00	90.020 ± 0.43^{c}	79.915 ± 0.66^{c}	73.06 ± 1.97^{c}	70.40 ± 1.70^{c}
DCM: Methanolic stem bark extracts	Carrageenan + 50 mg/kg	100 ± 0.00	94.160 ± 1.82^{bc}	86.28± 2.29^{c}	76.82 ± 1.40^{c}	91.38 ± 2.91^{b}
	Carrageenan + 100 mg/kg	100 ± 0.00	90.826 ± 0.90^{c}	80.83± 1.83^{c}	76.09 ± 1.19^{c}	72.27 ± 1.80^{c}
	Carrageenan + 150 mg/kg	100 ± 0.00	89.731 ± 0.68^{c}	79.67 ±1.60^{c}	74.03 ± 1.55^{c}	71.004 ± 0.94^{c}

Values are expressed as Mean ± SEM for 5 animals per group. Statistical comparison were made within a column and values with the same superscript are not significantly different by one-way ANOVA followed by Tukey's post hoc test ($P>0.05$). Carrageenan =1%; DMSO = 10%; Diclofenac = 15 mg/kg.

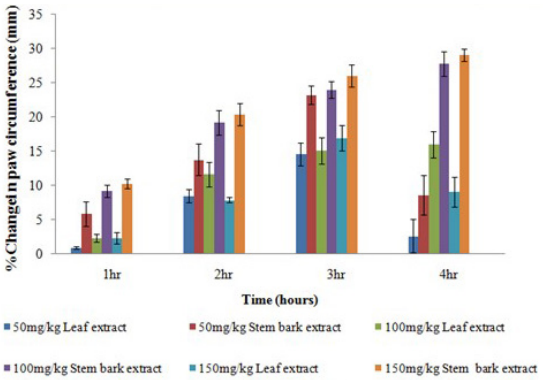

Figure 3. Comparison of percentage reduction in paw circumference of DCM-MeOH leaf and stem bark extract of *Ximenia americana* on carrageenan-induced inflammation in mice.

Table 4. Qualitative phytochemical composition of DCM-MeOH leaf and stem bark extracts of *Ximenia americana*

Phytochemicals	*Ximenia americana* leaf extract	*Ximenia americana* stem bark extract
Alkaloids	-	+
Flavonoids	(+)	(+)
Steroids	-	-
Saponins	(+)	(+)
Cardiac glycosides	+	+
Phenolics	(+)	(+)
Terpenoids	-	+

Present phytochemical are denoted by (+) sign, absent phytochemical are denoted by (-) sign while + (trace) denotes slightly present phytochemical.

difference in the second and fourth hour at this dose level (P=0.08 and 0.157 respectively) (Figure 3).

At the dose level of 100 mg/kg, there was significant difference in the anti-inflammatory activities of the DCM-MeOH leaf and stem bark extracts of *X. americana* for the 4 hours of the test period (P=0.001, 0.022, 0.007 and 0.003 respectively) (Figure 3).

At the dose level of 150 mg/kg, there was significant difference in the anti-inflammatory activities of the DCM-MeOH leaf and stem bark extracts of *X. americana* for the 4 hours of the test period (P=0.000, 0.002, 0.006 and 0.000 respectively) (Figure 3).

Qualitative phytochemical screening

The qualitative phytochemical screening of DCM-MeOH leaf and stem bark extracts of *X. americana* showed the presence of alkaloids, cardiac glycosides, flavonoids, phenolics, saponins and terpenoids. However, alkaloids and terpenoids were absent in the leaf extract of the *X. americana* while the steroids were absent in both leaf and stem bark extracts (Table 4).

Discussion

The present study was designed to evaluate the anti-inflammatory activities of DCM-MeOH leaf and stem bark extracts of *X. americana* in rat models.

The DCM-MeOH leaf and stem bark extracts of *X. americana* showed a significant anti-inflammatory activity on carrageenan-induced paw edema in mice (Tables 2 and 3). The standard screening models for inflammation in experimental animals comprise of carrageenan-induced hind paw edema, cotton pellet induced granuloma and Freund's adjuvant (22).

Carrageenan is a natural carbohydrate obtained from edible seaweeds and is generic name for a family of gel-forming polysaccharides seaweeds (23). There is a very wide use of carrageenan as an agent for inducing inflammation (24). Carrageenan solution 1% in normal saline for intraplantar injection at the dosage of 50-150 µL is readily used (25). Carrageenan induces inflammation in two phases; early phase (90-180 minutes) characterized by the release of histamines and serotonins while the late phase (270-360 minutes) characterized by release of prostaglandins, lysosomes and proteases (26,27). Thus, carrageenan was used as the phlogistic agent for the present study.

The DCM-MeOH leaf and stem bark extracts of *X. americana* produced significant anti-inflammatory activities on carrageenan-induced paw edema in Swiss albino mice (Tables 2 and 3). These findings relate with the activity of various herbal plants tested in animal models. The aqueous ethanol root bark extract of the *X. americana* extract (10 and 100 mg/kg body weight) showed a significant inhibition of carrageenan-induced mice paw inflammation in which the anti-inflammatory effects were proportional to the dose concentration (28). Similar work on aqueous extract of *Bauhinia purpurea* leaves in animal models demonstrated significant anti-inflammatory activity on carrageenan-induced inflammation (29). Similarly, there was effective anti-inflammatory effect of the ethanolic and aqueous extracts of *Kalanchoe pinnata* on carrageenan induced edema in rats (30).

Nonsteroidal drugs such as diclofenac, ibuprofen, indomethacin among others are routinely prescribed in the management of inflammation (31). These drugs work by inhibiting prostaglandin biosynthesis by blocking the enzyme cyclooxygenases (COX) (32). Therefore, the anti-inflammatory activities of the leaf and stem bark extracts of *X. americana* to manage inflammation may be attributed to the inhibition of prostaglandin synthesis. The anti-inflammatory effect of the DCM-MeOH extracts of *X. americana* at the dose levels of (50, 100 and 150 mg/kg body weight), over a period of 3 hours in carrageenan-induced inflammation was comparable to that exhibited by the positive control group. Previous studies with some other plants such as *Maytenus obscura*, *Caesalpinia volkensii* (33) and *Solanum trilobatum* (34) also showed a similar effect in this model. The findings demonstrate that the extracts act in a dose dependent manner in the late phase which might be involving arachidonic acid metabolites as they are associated with edema reliant on mobilization of neutrophils. This dose dependent trend might be attributed to the passive diffusion of bioactive

molecules through plasma membrane from peritoneal cavity.

In determination of anti-inflammatory activities of the extract in mice, 3 dose levels (50,100 and 150 mg/kg body weight) were used. Similar studies carried out in evaluating anti-inflammatory activities of medicinal plants in animal models employed similar dose ranges (33,35,36).

The anti-inflammatory effect of both extracts was comparable to the group treated with diclofenac. The leaf extract of *X. americana* reduced the paw edema by between 0.91%-16.90% while the stem bark extract reduced it by between 5.84% and 28.99%. Diclofenac reduced the paw edema by between 1.32% and 29.6%. Thus, the extracts possess anti-inflammatory potential comparable to the reference drug (diclofenac) and can be considered as suitable anti-inflammatory agent.

The anti-inflammatory activity of the DCM-MeOH leaf and stem bark extracts of *X. americana* may be due to the presence of phytochemical constituents. Qualitative phytochemical screening of the leaf extract confirmed presence of saponins, flavonoids, cardiac glycosides and phenolics while the stem bark extract shoed presence of alkaloids, flavonoids, saponins, cardiac glycosides, phenolics and terpenoids (Table 4). The presence of these phytochemicals has been associated with the anti-inflammatory activities in the animal models (37).

Conclusion

In conclusion, the present study confirms the anti-inflammatory potential of the leaf and stem bark DCM-MeOH extracts of *X. americana* in experimental animals. The significant reduction in right hind paw circumference which was comparable to the reference drug shows that these extracts are endowed with significant anti-inflammatory activities.

Therefore, the DCM-MeOH leaf and stem bark extracts of *X. americana* might prove useful in managing inflammation and thus serve as an alternative treatment bioresource, which is more effective than the conventional synthetic drugs.

The present study showed that DCM-MeOH leaf and stem bark extracts of *X. americana* contain a class of phytochemicals attributable to anti-inflammatory properties. Therefore, the study scientifically confirms and supports the use of *X. americana* in traditional management of inflammation.

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Authors' contributions

GDM prepared the manuscript and was involved in execution of the work; KDM took care of the experimental animals and followed ethical issues; NMP conducted experimental design development and literature review; MND performed data analysis and discussion. All read and confirmed the publication of the manuscript.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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