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ORIGINAL ARTICLE

Analgesic and Antioxidant Activities of Stem Bark Extract and Fractions of *Petersianthus macrocarpus*

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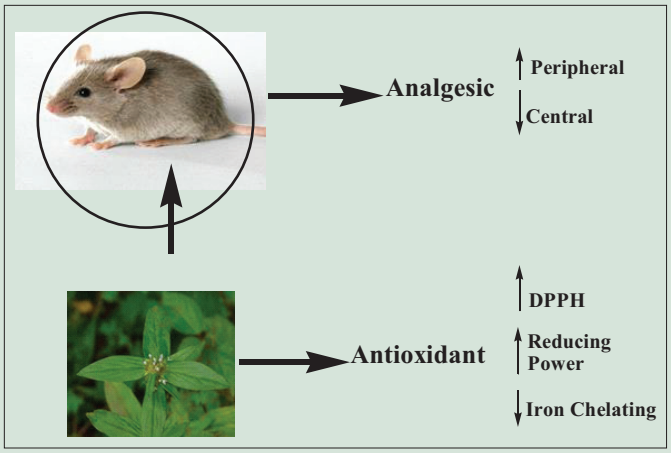
ABSTRACT

Background: *Petersianthus macrocarpus* (Lecythidaceae) is widely used in the folk medicine in Nigeria to relieve pain and fever associated with malaria. This study evaluated the analgesic and antioxidant activities of the methanol extract and fractions of the stem bark of the plant. **Materials and Methods:** The analgesic activity was determined in mice using hotplate and acetic acid-induced writhing models. Morphine sulphate (5 mg/kg, i.p.) and aspirin (100 mg/ml, p.o.) were used as reference analgesic agents. The antioxidant potential was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical; reducing power, iron chelating properties and determination of total phenolic content. **Results:** The extract at 200 and 500 mg/kg, produced an insignificant ($P > 0.05$) increase in pain threshold in hotplate but a significant ($P < 0.05$) increase at 1000 mg/kg. The extract significantly ($P < 0.05$) reduced the writhing induced by acetic acid in mice in a dose dependent manner. Fractionation increased the analgesic activities significantly ($P < 0.05$) in ethyl acetate and aqueous fractions (200 mg/kg). The extract demonstrated strong DPPH radical scavenging activity with IC_{50} 0.05 mg/ml, good reducing power and weak iron chelating activities. The total phenol content was 142.32 mg/gin term of gallic acid. The antioxidant effects were more pronounced in ethyl acetate and aqueous fractions. **Conclusion:** The findings of the study suggested that the extract has strong analgesic and antioxidant activities which reside mainly in the polar fractions thus confirming the traditional use of the plant to alleviate pains.

Key words: Anti-nociceptive, *Lecythidaceae*, pain, radical scavenging, writhing

SUMMARY

- Analgesic and antioxidant activities of extract and solvent fractions of *Petersianthus macrocarpus* investigated indicated that extract has analgesic and antioxidant properties that reside mainly in the polar fractions.



Abbreviations Used: DMSO: Dimethyl sulphoxide, ANOVA: analysis of variance, EDTA: ethylene diamne tetraacetic acid, SDM: standard deviation of mean, PGE: prostaglandins E, PDF: prostaglandins F.

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INTRODUCTION

Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage.[1] The direct and indirect action of chemical mediators, such as arachidonic acid metabolites (prostaglandins and leukotrienes), peptides, serotonin, acetylcholine, cytokines, nitric oxide, among others, which can be produced or released following tissue injury or by exogenous irritants (formalin, acetic acid), are responsible for the multiplicity of events that occur during pain transmission in both the peripheral and central nervous systems.[2-4] Reactive oxygen species (ROS) including free radicals are involved in sensitization of dorsal horn neurons that plays a fundamentally important role in pain. Antioxidant supplements are known to increase the threshold of pain perception.[5,6]

The study of plants used traditionally as analgesics is still a fruitful and logical strategy in the search for new analgesic drugs and pain mechanisms.[7] Drugs such as morphine, steroidal or non steroidal anti-inflammatory agents that are currently being used for the treatment of pain clinically have severe adverse effect thereby necessitating the search for naturally occurring agents with reduced side effects as substitutes.

Petersianthus macrocarpus belongs to the family *Lecythidaceae* and in Nigeria it is used in the treatment of pains, headache, "recurrent" fever and malaria.[8] The aqueous extract of the stem bark is traditionally used in the treatment of constipation, haemorrhoids, veneral diseases and as an abortifacient.[8] The methanolic extract of the stem bark was also reported to produce hypotensive effects.[9,10] In this study, we investigated analgesic and antioxidant potential of methanol extract *P. macrocarpus* stem bark and its fractions.

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MATERIALS AND METHODS

Chemicals and reagents

Deionized water, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Co.), trichloroacetic acid (Sigma-Aldrich Co.), acetic acid, DMSO, anhydrous ferric chloride, potassium ferricyanide, ferrozine, ascorbic acid and other chemicals were all of analytical grade.

Experimental animals

Albino mice (20-22 g) of both sexes were obtained from Laboratory Animal House of College of Medicine, University of Lagos, Idi-Araba, Nigeria. They were kept in cages at room temperature (30 ± 2°C) and food and water were supplied *ad libitum* up to the commencement of the experiment. All protocols were carried out in accordance to internationally accepted principles for laboratory animal use and care.

Plant material

The stem bark of *P. macrocarpus* was collected at Nnewi, Anambra State, Nigeria and identified by Mr. I. K. Odewo, a former curator in the Department of Botany, University of Lagos with voucher specimen number LUH 3153. The barks were dried at 40°C and milled to produce fine powder.

Extraction and fractionation

About 800 g of the powdered stem bark was extracted with methanol (2.5 L) using Soxhlet apparatus for 72 h. The extract was filtered and concentrated to dry powder (7.0% w/w) using rotatory evaporator at 40°C. Extract (30.0 g) was suspended in water then partitioned between n-hexane, chloroform and ethylacetate successively to obtain respective fractions that were concentrated and subjected to analgesic and anti-oxidant investigations.

Preliminary phytochemical screening

Phytochemical screening of the extract was carried out to determine the presence of various secondary metabolites using standard methods.[11]

Acute toxicity studies

Sixty mice were divided into control and test groups containing ten animals each. The control group received 5% DMSO and test groups were treated orally with a solution of the extract (1-5 g//kg) in 5% DMSO). The mice were observed over a period of 48 h and up to 7 days for behavioral changes and mortality.[12]

Analgesic studies

Acetic acid-induced writhing response in rats

The analgesic activity of the plant extract was determined in terms of its ability to inhibit writhing responses of the mice produced by intra peritoneal administration of acetic acid. Different groups of five mice each received orally 5% DMSO as negative control and acetyl salicylic acid (100 mg/kg) or plant extract (200, 400, 1000 mg/kg). Thirty minutes later, 0.7% acetic acid (10 ml/kg) solution was injected intra-peritoneally to all the animals in the different groups. The number of writhes (abdominal constrictions) occurring between 0 and 20 min after acetic acid injection was counted.[13] A reduction of writhes in test animals compared to those in the negative control group was considered as an anti-nociceptive response. The analgesic activities of hexane, chloroform, ethyl acetate and aqueous fractions were tested at 200 mg/kg using similar procedure.

Hot plate method

The analgesic activity of the extract and its fractions were assessed using hot-plate method.[14] About 150 Swiss albino mice of either sex were screened and those that did not respond within 15 s were left out of the experiment. Thirty (30) selected mice were divided into six groups of five animals each and received orally 5% DMSO solution, extract (200-1000 mg/kg in 5% DMSO) and morphine 5 mg/kg intra-peritoneally respectively 30 min prior to the test. Each mouse was then placed on a hot plate at 50°C and the time taken to lick the hind paw or to jump was recorded. The response times of these mice were measured 30 min prior and after the treatment. Analgesic activity was expressed as the increase in response time with respect to control. The analgesic activities of the hexane, chloroform, ethyl acetate and aqueous fractions were tested at 200 mg/kg using similar procedure. The mean reaction time for each treated group was determined and compared with the result for each group before treatment. Percentage increase in response time (I %) was derived using the formula:

$I\% = \{(I_t - I_o)/I_o\} \times 100$,

Where I_t = reaction time at time, t,

I_o = reaction time at time zero.

Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu reagent and was measured as gallic acid equivalent.[15] The extract (100 mg/ml, 1.0 ml) was mixed thoroughly with 5 ml Folin-Ciocalteu reagent (diluted ten-fold) and after 5 min, 4.0 ml of sodium carbonate (0.7 M) was added and the mixture was allowed to stand for 1 h with intermittent shaking. The absorbance was measured at 765 nm in a spectrophotometer. All determinations were carried out in triplicate.

Antioxidant activities

Evaluation of free radical scavenging effect

The determination of the free radical scavenging activity of the extract was carried out using the DPPH (1,1-diphenyl-2- picrylhydrazyl) assay.[16] Various concentrations of extract (0.05-1.00 mg/ml) in methanol were prepared. To 1 ml of each solution, 3 ml of methanol was added and then 1 ml of a 1mM DPPH in methanol was added to make up to 5 ml. The mixture was shaken and allowed to stand at room temperature in a dark chamber for 30 min. The change in color from deep violet to light yellow was then measured at 517 nm. The experiment was repeated with fractions (0.1 mg/ml) and ascorbic acid (0.005-0.200 mg/ml) which served as positive control. All determinations were carried out in triplicates. The same procedure was repeated without the extract for blank experiment. The decrease in absorbance was then converted to percentage scavenging activity (% SA) using the formula:

% Radical scavenging activities (% SA) = $[(A_b - A_s)/A_b]/x100$

Where: A_b = Absorbance of the blank solution;

A_s = Absorbance of the test (extract) solution or the standard (ascorbic acid).

Reducing power assay

The reducing power was determined using Yen and Chen method[17] with little modifications, the aliquot of various concentrations of the standard and test extract (0.01- 2 mg/ml) in 1.0 ml of methanol were mixed with 2 ml of phosphate buffer (pH 7.4) and 2 ml of (1%) potassium ferricyanide. The mixture was incubated at 50°C for 20 min. after cooling, aliquots of 2 ml of 10% trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 20 min. The upper layer of solution (2 ml) was taken and diluted with 2ml of deionized water and a freshly prepared 0.5 ml of (0.1%) ferric chloride solution. The absorbance was measured at 700 nm in UV-visible spectrometer (Systronic double beam-UV- 2201). A blank was prepared without the extract. The experiment was repeated with fractions (0.1 mg/ml). Ascorbic acid was used as the standard and the experiment was carried out in triplicate.

Metal chelating activity assay

The chelating of ferrous ions by extract was estimated using Ebrahimzadeh *et al.* method[18] with modifications. Various concentrations of extract (0.1-2.0 mg/ml) in methanol were prepared. To 0.5 ml of each solution, 0.1 ml, 1 mM $FeCl_2$ was added and mixture was allowed to stand for 5 min. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine, the mixture was made up 4 ml with ethanol and shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. Increase in absorbance, indicates poor Fe^{2+} chelating activity. Ethylene diamine tetra-acetic acid (EDTA) was used as positive control. Blank containing no extract was used as the negative control. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated as:

$[(A_0 - A_s)/A_0] \times 100$.

Where: A_0 = Absorbance of the control/blank;

As = the absorbance of the extract or EDTA (positive control).

Statistical analysis

All data were expressed as mean ± standard deviation of mean. Analysis of variance was performed by ANOVA procedures and $P < 0.05$ was considered significant.

RESULTS

Phytochemical screening

The preliminary phytochemical screening of the extract revealed the presence of secondary metabolites such as saponins, phenolic compounds and steroidal nucleus while anthraquinones and alkaloids were not detected.

Acute toxicity study

In this study, oral administration of graded doses of the extract to the mice did not produce any significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects. No mortality or any toxic reaction like convulsion, ataxia, diarrhea or increased dieresis was recorded in any group after observation for 72 h after administering the extract to the mice. The extract was safe at the tested doses.

Analgesic activity

Effect of extract on acetic acid-induced writhing

The results of the acetic acid writhing test in mice are shown in Table 1. At all tested doses, the extract inhibited the writhing responses of the mice caused by the intra-peritoneal administration of acetic acid in dose dependent fashion. The percentage inhibition of the writhing reflex also increased from zero in the negative control group to 70.53% in the highest dose of the extract (1000 mg/kg) treated group which was higher than the 62.63% observed in the group that received acetyl salicylic acid (100 mg/kg). The inhibitory effects of aqueous, ethyl acetate, chloroform and hexane fractions (200 mg/kg) were 77.52, 72.93, 3.87 and 41.86%, respectively.

Effect of extract in hot-plate test

The results of analgesic activity of the extract assessed using hot plate test are shown in Table 2. The extract at 200 and 500 mg/kg produced slight increase in mean post reaction time when compared to the group that received 7% DMSO (negative control group). However at 1000 mg/kg, there was a significant ($P < 0.05$) increase in the latency for jumping or licking when compared to DMSO group but less than morphine (10 mg/kg) used as positive control [Table 2]. The aqueous, ethyl acetate, chloroform, and hexane fractions (200 mg/kg) had 100.41, 86.35, 56.40 and 56.98% elongation of reaction time to thermal stimulus induced pain.

Table 1: Effect of methanol extract of *Petersianthus macrocarpus* stem bark on acetic acid induced writhing in mice

Sample	Dose (mg/kg)	Number of writhes in 20 min	Percentage of inhibition
5% DMSO		47.5±0.18	-
Aspirin	100	17.75±0.21	62.63
Extract	200	27.01±0.95	43.16
	500	20.25±1.02	57.37
	1000	14.00±0.47	70.53

DMSO: Dimethyl sulfoxide; Values are expressed as mean ± SDM; *n*=5

Table 2: Effect of methanol extract of *Petersianthus macrocarpus* stem bark on thermal stimulus induced pain (hot test) in mice

Sample	Dose mg/kg	Reaction time in sec (mean±SDM)		
		Basal (s)	30 min after	Percentage of elongation
5% DMSO		5.0±1.42	5.48±1.31	9.6
Morphine	10	5.75±1.54	11.83±2.08	105.74
Extract	200	5.79±1.31	6.35±1.09	9.67
	500	7.41±0.98	8.35±1.11	12.69
	1000	7.29±0.46	13.45±1.61	84.5

DMSO: Dimethyl sulfoxide; SEM: Standard deviation of mean; Values are expressed as mean ± SDM; *n*=5

DPPH radical scavenging activity

The free radical scavenging ability of the extract was tested by reduction of stable radical DPPH to the yellow colored diphenyl picrylhydrazine. The *in-vitro* investigations revealed that with increase in concentration, the radical scavenging ability of the extract increased by preventing the formation of the DPPH radical [Figure 1]. The activity was significantly less ($P < 0.05$) than that of ascorbic acid used as positive control. The IC50 values were 0.05 and 0.02 mg/ml for extract and ascorbic acid respectively. The ethyl acetate and aqueous fractions (0.1 mg/mL) showed the greatest activity 93.42 and 93.72% respectively while n-hexane and chloroform had lower activity 28.98 and 40.55% respectively.

Reducing power assay

The reducing power of the extract was determined using ascorbic acid as the positive control. The reducing ability increased with concentration [Figure 2]. The reducing power showed good linear relationship in the extract (R^2 =0.7616) as well as ascorbic acid (R^2 =0.9133). The maximum absorbance for crude extract was 2.890 at 1.0 mg/mL compared to 3.228 of ascorbic acid used as positive control. The reducing ability of fractions however, increased from hexane < chloroform < aqueous < ethylacetate with absorbance 0.577, 0.759, 2.409 and 2.921 respectively.

Iron chelating power

Ferrous ion chelating ability of the extract was shown in Figure 3. The extract showed 43.49% iron chelating ability at 0.2 mg/ml whereas EDTA used as positive control showed 99.36% at the same concentration. The chelating ability of the extract was significantly lower ($P < 0.05$) than EDTA a known metal chelator. The IC50 of extract and EDTA were 0.31 and 0.02 mg/ml respectively.

DISCUSSION

There is an increasing interest in the development of analgesics from natural products due to side effects of commercially available drugs (opioids and non steroidal anti-inflammatory drugs).[19,20] In this study, stem bark extract and fractions of *P. macrocarpus* stem bark were investigated for central and peripheral analgesic activities. Acetic acid induced nociception is highly sensitive and commonly used in the evaluation of mild peripheral

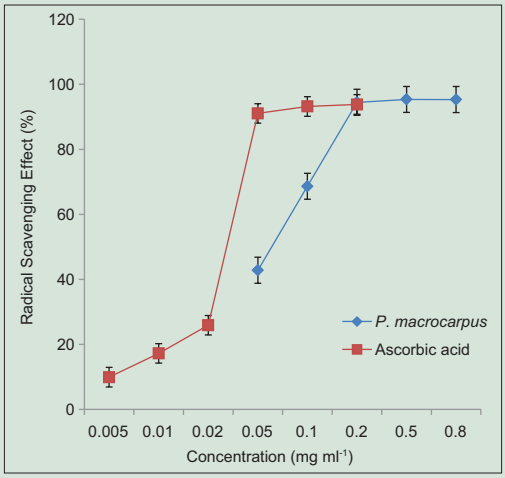

Figure 1: Radical scavenging effects of *Petersianthus macrocarpus* and ascorbic acid on DPPH radical

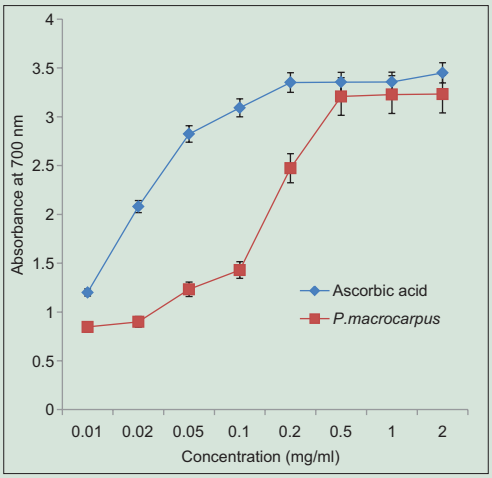

Figure 2: Reducing power of *Petersianthus macrocarpus* and ascorbic acid

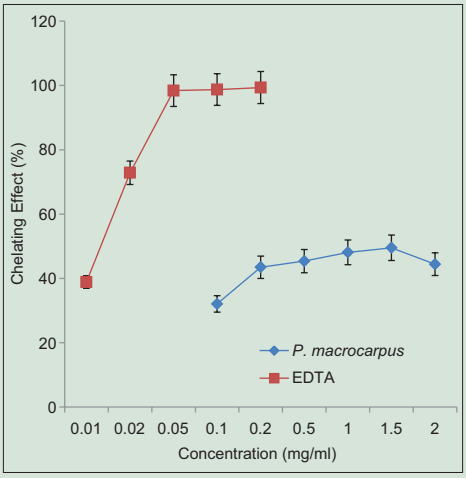

Figure 3: Chelating effect of *Petersianthus macrocarpus* and EDTA

analgesic compounds.[21] The results of the study indicated that extract demonstrated analgesic action in mice by inhibiting acetic acid induced writhing. The inhibitory effect of fractions increases in the order hexane < chloroform < ethylacetate < aqueous. This indicates that the activity resides mainly in ethyl acetate and aqueous fractions.

The pain sensation in acetic acid induced writhing method is elicited by triggering of localized inflammatory response resulting in release of free arachidonic acid from tissue phospholipid[22] via cyclooxygenase (COX) and prostaglandin biosynthesis.[23] The acetic acid induced writhing is therefore associated with increased level of PGE-2 and PGF-2 in peritoneal fluids as well as lipoxygenase products.[24] The analgesic activity of the extract and its fractions might be due the presence of some phyto-constituents which inhibit prostaglandin synthesis or block pain sensation.

Thermal painful stimuli are commonly used for assessing central anti-nociceptive response. Hot plate test determines possible central action where analgesic effects *via* supra spinal and spinal receptors are mediated through opioid agents.[25] In this study, the extract and its fractions exhibited weak anti-nociceptive action in mice compared to the morphine. The effect of the fractions increases in the order hexane < chloroform < ethylacetate < aqueous. This suggests that the activity resides mainly in ethyl acetate and aqueous fractions. The increase in stress tolerance ability of the mice indicates that higher center may be involved its analgesic activity.

We also evaluated the antioxidant activities of the extract and its fractions. Reactive oxygen species produced by living organisms has been implicated in various diseases such as cancer, ageing, diabetes, cardiovascular diseases and inflammation.[26] Free radicals are also known as contributing factor in modulation of pain and tissue injury.[27] Therefore, antioxidants from medicinal plants that may reduce the risk of various chronic diseases are beneficial. DPPH is a stable free radical scavenger which converts unpaired electrons to paired ones through proton donation. The results also showed that extract and its fractions exhibited strong radical scavenging power suggesting that their constituents are capable of donating protons thus quenching radicals. The total phenolic content of the extract measured using Folin-Ciocalteu reagent was calculated as 142 mg/g in terms of gallic acid. Reports have implicated phenolic compounds in antioxidant and analgesic activities due their interactions with prostaglandins and superoxides.[28]

The conversion of Fe^{3+} to Fe^{2+} in the presence of extract and fractions was measured to determine the reducing power. The reducing properties are generally associated with the presence of reductones (anti-oxidants), which have been shown to exert anti-oxidant action by breaking the free radical chain.[29] The anti-oxidant constituents of the extract or fractions caused reduction of ferric-cyanide complex to the ferrous form due to hydrogen donation from phenolic compounds.[30]

It has been reported that chelating agents are effective secondary anti-oxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion.[31] The metal chelating power of the extract and fractions were measured by ferrous ion ferrozzine complex. Ferrozzine combines with ferrous ion to form a red coloured complex that absorbs at 562 nm. In the presence of chelating agent, the ferrozine complex formation is disrupted leading to reduction in red color of the complex.[32] The decrease in absorbance of this complex is an indication of increased activity. The result of this study indicated that the extract has poor capacity for iron binding suggesting that iron chelation has little or no role in its anti-oxidant activity.

Preliminary phytochemical screening showed the presence of polyphenolic compounds and saponins which were reported to be triterpenoid saponins, petersaponins III and IV.[33] These are potential free radical scavengers and their activity against DPPH radical is related to their chemical structures.[34] The anti-oxidant activities of the extract and the fractions might not be unconnected with the activities of these compounds.

CONCLUSION

In conclusion, the present study demonstrated that extract of *P. macrocarpus* stem bark possesses strong analgesic and antioxidant activities which reside mainly in ethylacetate and aqueous fractions thus justifies its use in folklore medicine in treating pain related diseases. However, there is need to isolate compound (s) responsible for the observed pharmacological activities which may lead to development of novel compound for drug discovery.

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Conflicts of interest

There are no conflicts of interest.

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