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Original Research Article

PROTEA MADIENSIS OLIVER (PROTEACEAE) ROOT EXTRACT AND FRACTIONS IN INFLAMMATION AND PAIN MANAGEMENT IN MICE

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ABSTRACT

Protea madiensis root, was studied for anti-inflammatory and analgesic properties to verify its local applications. After the collection of whole plants, roots were separated, washed and shade dried. The pulverized root was cold macerated with ethanol (70%) for 72 h, filtered and concentrated *in vacuo* at 40ºC. A portion of the extract was introduced into a separating funnel and successively partitioned with dichloromethane, ethyl acetate and butanol to obtain partitioned fractions (dichloromethane, ethyl acetate and butanol fractions). The remaining ethanol extract was screened for secondary metabolites. Both the ethanol extract and fractions were studied for anti-odematous and analgesic effects in mice using fresh egg albumin-induced paw oedema, xylene- induced oedema, acetic acid-induced writhing, formalin-induced paw licking and thermal-induced pain models. Data obtained revealed ethanol root extract of *P. madiensis* as being able to display dose-dependent anti-inflammatory effect, reduced pains caused by acetic acid, formalin and hot plate effects. All the fractions displayed potent significant ($p \leq 0.05 – 0.001$) analgesic effects that were comparable with the prototype drug, acetyl salicylic acid. This study validates its numerous applications in oedema and pain diseases in folkloric medicine.

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INTRODUCTION

Protea madiensis Oliver, a specie in Proteaceae and found in lowland to upland savannah, reputed for its folkloric application in wounds and inflammatory diseases' management [1], epilepsy, amenorrhea, malaria, headache, fever and dysentery [2]. It is also a remedy for hyperpigmentation and other skin diseases [3]. Biological studies revealed its leaf as potential remedy for wound treatment and for malaria, both as a single remedy and in combination with other plants' extracts [2, 3]. The leaf extract is reported to contain flavonoids, saponins, alkaloids, tannins and glycosides while 2- tridecanone, oleic acid and sitosterol have been isolated from the root extract [1, 3]. The root of *P. madiensis*, according to ethnomedicinal information is a remedy for inflammatory wound management by the Nsukka people of Enugu State. This study became necessary to report the anti-oedematous and analgesic efficacy of the root to further validate its ethnomedicinal uses in pain and inflammatory diseases' care.

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MATERIALS AND METHODS

Preparation of Extract/Fractions

The root of *P. madiensis* was collected in April 2022, at Nsukka Local Government Area in Enugu State and the entire plant was identified by Prof. Margaret Bassey, a Taxonomist in the Department of Botany and Ecological Studies in the University of Uyo, Uyo, Akwa Ibom State, Nigeria. A herbarium specimen [UUPHB(7)i] was deposited in the Department of Pharmacognosy and Natural Medicine Herbarium, Faculty of Pharmacy, University of Uyo. Following identification and authentication, the plant's roots were harvested, detached from the entire plant using a pair of scissor, selected to free it from contaminants, washed with water, chopped into small pieces and dried. Further reduction of the dry materials to powder was achieved by passing through hammer mill followed by cold maceration with 70% ethanol. After 72 h, filtration was achieved with filter paper (Whatman No.1) plugged in glass funnel and the filtrate concentrated using a rotary evaporator (LabTech EV311-V) at 40ºC. Fractionation of the extract was achieved by partitioning 50 g crude extract successively with dichloromethane, ethyl acetate, butanol to obtain the partitioned fractions [dichloromethane (DCM), ethyl acetate (EA), butanol (BUT) and aqueous (AQ)] which were individually concentrated, kept in the refrigerator (4º C) and used from here for the study.

Animal Study

Albino mice (25-30 g) were procured from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Uyo, Akwa Ibom State, Nigeria. They were kept under standard conditions, starved of food 24 h before the experiment and given access to only water. All procedures agreed with the international protocols for care and use of laboratory animals (1996), as approved by the National Institutes of Health and the ethics committee of the Faculty of Pharmacy, University of Uyo, Nigeria [16]

Acute Toxicity Study

OECD/OCDE 423 guideline [4] was adopted for acute toxicity study. The lethal dose (LD_{50}) was achieved by administration of doses of 300 mg/kg and 2000 mg/kg orally to two groups of mice (3 per group) and observation was made for toxicity signs such as abdominal stretching, reduced movement among others, and even death, within 24 h, and LD_{50} extrapolated from OECD/OCDE chart (annex 2d).

Phytochemical screening

In line with standard protocols, extract of *P. madiensis* (root) was studied for the presence/absence of alkaloids, flavonoids, tannins, saponins, terpenes, cardiac glycosides and anthraquinones. Dragendorff's test for alkaloids; magnesium metal in acid for flavonoids; ferric chloride and bromine water for tannins; froth test for saponins; Salkowski, Keller – killiani and Lieberman's tests for cardiac glycosides while Borntrager's test was used for anthraquinones [5]

Anti-inflammatory Study

Two experimental models were used for this study viz: egg albumin- induced hind paw oedema and xylene-induced ear oedema models [6]. The animals were fasted for 24 h with access to only water, grouped into five per group and administered with extract and fractions (orally) as shown below (Table 1).

Table 1: Extract/fractions/drug treatments

Treatment groups	Doses of extract/fractions/drugs
1	Distilled water 10 mL/kg
2	Ethanol ex. 500 mg/kg
3	Ethanol ex. 1000 mg/kg
4	Ethanol ex. 1500 mg/kg
5	DCM ftn 1000 mg
6	ET ftn 1000 mg
7	BUT ftn 1000 mg
8	AQ ftn 1000 mg
9	Standard drugs (ASA 100 mg/kg/ dexa 4mg/kg)

Where: dexa = dexamethasone: Where ex. = extract, ftn = fraction, ASA= acetyl salicylic acid and dexa = dexamethasone.

For anti-inflammatory study, the initial hind paw weights of the animals were taken, inflamamation was induced with egg albumin (0.1 mL) into the sub planter surface of right hind paw of the mice 1 h sequel to pretreatments with the extract, fractions and ASA (100 mg/kg), the prototype drug. Measurement of paw sizes were done using digital venier caliper. In xylene experiment, 20 µL of xylene was placed on the mice's right ears while the left ear was not treated and taken as control. Difference in weights between these ears were noted and dexamethasone (4 mg/kg) was a standard drug in xylene model [6].

Analgesic Study

In analgesic study, three experimental models were employed viz: acetic acid –induced writhing, formalin – induced paw licking and thermally – induced pain models. Mice were grouped in similar manner as earlier described, pretreated with extract and fractions, thirty minutes before induction of pain intraperitoneally for acetic acid, formalin (2.0 µL formalin (2.5 %) prepared in a solution of phosphate buffer) and hot plate for thermal – induced models, respectively. Number of abdominal stretching movements for acetic acid experiment was recorded for another thirty minutes at five minutes intervals. Reduction in frequency of abdominal stretching between control mice and the pretreated ones, was taken as a measure of analgesia. For formalin model, the number of paw licking was noted at five (5) minutes duration for a total of thirty minutes following treatment with formalin [7, 8]. In the thermal model using hot plate, the time mice were placed on

standardized (T= 50 ± 1°C) hot plate and licking of paws was taken as response to heat latency [6].

Statistical analysis

Data obtained were represented as mean ± SEM, analyzed using analysis of variance and comparison test calculated using version 5.03 GraphPad Prism software. The differences between the means were considered significant at $p \leq 0.05$-0.001.

RESULTS

The LD_{50} of ethanol root extract of *P. madiensis* was extrapolated to be 5000 mg/kg and doses of 500 mg/kg, 1000 mg/kg and 1500 mg/kg used for the study represented the low, median and high doses. The phytochemical constituents (Table 2), anti-inflammatory effect (Tables 3 and 4) and analgesic effect (Table 5-7) of *P. madiensis* root extract and fractions are represented below.

Table 2: Phytochemical constituents of *P. madiensis* ethanol root extract

COMPOSITION	TEST	OBSERVATION	OBSERVATION
Alkaloids	Dragendorff's	Brick red precipitate	++
Flavonoids	Magnesium metal in acid	Red precipitate	+++
Tannins	Ferric Chloride	Blue-black precipitate	++
	Bromine Water	Decolourization	++
Saponins	Frothing	Froth after minutes	++
Terpenes	Chloroform & sulphuric acid	No pink colour at interphase	++
Cardiac glycosides	Salkowski	Reddish brown colouration at the interphase	-
	Keller -Killiani	Interphase had brown ring	+
	Lieberman	Interphase had pink colour	+
Anthraquinones	Borntrager's test	Colour observed at lower phase was not pink	+

Key: - absent, + sparingly present, ++ moderately present and +++ highly present

Table 3: Anti-inflammatory effect of ethanol root extract/fractions of *P. madiensis* on egg albumin-induced hind paw oedema in mice

Treatments	Paw	Mean	Diameter (mm)		
	1.0 h	2.0 h	3.0 h	4.0 h	5.0 h
Distilled Water 10 mL/kg	0.76 ± 0.10	0.80 ± 0.09	0.54 ± 0.07	0.62± 0.08	0.60± 0.07
Ethanol extract 500 mg/kg	0.73 ± 0.06	0.64 ± 0.05	0.54± 0.06	0.47± 0.10	0.38± 0.09
Ethanol extract 1000 mg/kg	0.64 ± 0.05	0.54 ± 0.07*	0.46± 0.06*	0.82± 0.03	0.41± 0.04
Ethanol extract 1500 mg/kg	0.48 ± 0.08*	0.40 ± 0.06*	0.28± 0.07**	0.30± 0.06**	0.24± 0.07**
DCM 1000 mg/kg	0.50 ± 0.08*	0.50 ± 0.02**	0.31± 0.03**	0.28± 0.02**	0.23±0.03**
ET 1000 mg/kg	0.55 ± 0.03	0.48 ± 0.06**	0.36± 0.06**	0.25± 0.03**	0.19±0.03***
BUT 1000 mg/kg	0.73 ± 0.06	0.61 ± 0.09	0.54± 0.09	0.46± 0.09	0.42± 0.09
AQ 1000 mg/kg	0.68 ± 0.02	0.61 ± 0.04	0.48± 0.03*	0.38± 0.02*	0.40± 0.02
ASA 100 mg/kg	0.50 ± 0.02**	0.45 ± 0.02**	0.34± 0.04**	0.24± 0.04**	0.16±0.05**

SEM= Standard Error of Mean, ASA= Acetyl Salicylic Acid, *= p≤ 0.05, **= p≤ 0.01, *** p≤ 0.001,
DCM= dichloromethane , ET = ethyl acetate, BUT= butanol, AQ=Aqueous and n=5

Table 4: Effect of ethanol root extract and fractions of *P. madiensis* on xylene – induced ear oedema

Treatment/Dose	Mean Ear Weight difference [g] ± SEM
Distilled Water [10 mL/kg]	0.056 ± 0. 002
Ethanol extract 500 mg/kg	0.032 ± 0.005**
Ethanol extract 1000 mg/kg	0.028 ± 0.004***
Ethanol extract 1500 mg/kg	0.022 ± 0.004***
DCM 1000 mg/kg	0.042 ± 0.002
ET 1000 mg/kg	0.034 ± 0.006**
BUT 1000 mg/kg	0.042 ± 0.002
AQ 1000 mg/kg	0.044 ± 0.002
ASA 100 mg/kg	0.022 ± 0.002***

SEM= Standard Error of Mean, ASA= Acetyl Salicylic Acid, *= p≤ 0.05,**= p≤ 0.01, *** p≤ 0.001 and n=5

Table 5: Analgesic effect of ethanol extract/fractions of *P. madiensis* on acetic acid – induced writhing

Treatments	Number of Writhing as Mean ± SEM					
	5 min	10 min	15 min	20 min	25 min	30 min
Distilled Water 10 mL/kg]	9.80 ± 3.20	9.80 ± 3.20	26.40 ± 4.50	26.80 ±2.82	37.80± 3.50	39.80± 4.66
Ethanol extract 500 mg/kg	9.80 ± 3.43	9.80 ± 3.43	21.00 ± 7.89	26.20 ±2.45	30.20±1.56	36.00± 4.23
Ethanol extract 1000 mg/kg	3.80 ± 1.49*	3.80 ± 1.49*	3.80 ± 2.13***	12.00 ±2.68**	25.80±2.58*	34.80± 1.36
Ethanol extract 1500 mg/kg	1.20 ±0.74**	0.40 ± 0.25***	6.40 ±2.75***	6.60±1.81***	4.00±1.64***	3.00± 1.64***
DCM 1000 mg/kg	0.00 ± 0.00***	5.00 ± 3.13***	13.80 ± 3.65**	16.80±3.86**	12.20±284***	8.80± 3.48***
ET 1000 mg/kg	0.00 ± 0.00**	6.20 ± 2.38***	13.20 ± 4.18**	16.00±1.52**	14.40±2.75***	12.20±1.16***
BUT mg/kg	1.20 ± 0.74**	3.20 ± 1.36***	11.20 ± 1.72***	4.40± 3.43***	8.20± 2.69***	4.00± 0.55***
AQ 1000 mg/kg	0.00 ± 0.00***	5.20 ± 2.80***	15.00 ± 2.45*	19.80±1.32**	13.60± 0.40***	11.60±2.75***
ASA 100 mg/kg	0.40 ± 0.25**	1.80 ± 0.86***	7.60 ± 2.29***	8.00±2.68***	6.80± 2.69***	6.40±2.34***

SEM= Standard Error of Mean, ASA= Acetyl Salicylic Acid, *= p≤ 0.05, **= p≤ 0.01, *** p≤ 0.001 and n=5

Ta**ble 6:** Analgesic effect of ethanol extract/fractions of *P. madiensis* on formalin – induced paw licking

Treatments	Number of Paw Licking (Mean ± SEM)					
	5 min	10 min	15 min	20 min	25 min	30 min
Distilled Water 10 mL/kg	26.4 ± 5.60	13. 00 ±1.05	11.80 ±2.46	16.80 ± 3.38	14.80± 3.95	19.60± 4.89
Ethanol extract 500 mg/kg	22.40 ±4.26	9.20 ± 1.28	6.20 ± 2.78**	15.00 ± 3.29	12.40±3.03	13.20± 2.69*
Ethanol extract 1000 mg/kg	17.20 ±0.80	1.00 ±0.45***	3.20 ± 1.59***	3.40 ±1.22***	3.60±1.22***	8.60± 1.47**
Ethanol extract 1500 mg/kg	16.20 ±1.59	4.20 ± 1.20**	2.00 ±0.84***	3.60±1.57***	4.20±0.49**	5.40± 0.69***
DCM fraction 1000 mg/kg	13.00 ±2.68*	5.80 ± 0.37*	4.20 ± 0.92**	5.80±1.32***	4. 00±1.00**	2.80±0.20***
ET ftn 1000 mg/kg	16.40 ± 1.12	3. 00 ± 1.23***	1.20 ±0.73***	1.60±0.98***	1.20±0.49***	1.60 ±0.98***
BUT ftn 1000	12.40 ±1.22*	2.40 ±0.98***	2.40 ± 0.60***	4.80±0.49***	4.00± 1.67**	3.60±1.80***

mg/kg							
AQ ftn mg/kg	1000	16.40 ±2.46	2.60 ±0.40***	5.20	±2.40 **	5.20±1.75***	5.20± 2.25***	5.80± 0.37**
ASA mg/kg	100	14.60 ±1.60*	2.80 ± 1.39***	2.60 ± 0.68***		3.20±0.74***	2.80±1.11***	3.40±1.50***

SEM= Standard Error of Mean, ASA= Acetyl Salicylic Acid, *= p≤ 0.05, **= p≤ 0.01, *** p≤ 0.001 and n=5

Table 7: Analgesic effect of ethanol extract/fractions of *P. madiensis* on thermally-induced pain

Treatment/Dose	Mean Weight [g] ± SEM
Distilled Water 10 mL/kg	11.00 ± 0.84
Ethanol extract 500 mg/kg	16.20 ± 0.92
Ethanol extract 1000 mg/kg	23.20 ± 0.37**
Ethanol extract 1500 mg/kg	25.60± 2.99**
DCM 1000 mg/kg	26.60 ± 2.99**
ET 1000 mg/kg	31.60 ± 4.39***
BUT 1000 mg/kg	39.60 ± 0.40***
AQ 1000 mg/kg	25.60 ± 2.99**
ASA 100 mg/kg	35.00 ± 3.69***

SEM= Standard Error of Mean, ASA= Acetyl Salicylic Acid, **= p≤ 0.01, *** p≤ 0.001 and n=5

DISCUSSION

Phytochemical profile of the root extract of *P. madiensis* revealed the presence of alkaloids, saponins, tannins, flavonoids and cardiac glycosides (Table 2). Roles of flavonoids and saponins in ameliorating oxidative stress and reducing inflammation/pain is well established [9]. The intrinsic ability of these extract and fractions in reducing oedema and pain may be related to the presence of these phytochemicals.

The acute toxicity result extrapolated from OECD chart revealed an LD_{50} of 5000 mg/kg. Following the LD_{50} value of 5000 mg/kg, the various doses used in this study were 500 mg/kg, 1000 mg/kg and 1500 mg/kg representing low, median and high doses of ethanol extract of *P. madiensis* administered to the mice while the fractions were administered with 1000 mg/kg, representing the median dose. Lethal dose of 5000 mg/kg and above are considered practically non-toxic during acute study and therefore, the ethanol extract of *P. madiensis* may be considered safe on short periods of administration and when given orally [10].

In the anti-inflammatory study, the result revealed that, ethanol extract of *P. madiensis* (Tables 3 and 4) reduced inflammation induced by egg albumin and xylene dose-dependently, and these reductions were significant statistically (p≤ 0. 05 – 0.001) in comparison to distilled water and in same way like ASA and dexamethasone, which are standard anti-inflammatory agents. In the egg albumin model, dichloromethane (DCM) and ethyl acetate (ET) fractions demonstrated marked anti-inflammatory effect almost throughout the duration of study while in xylene model, ET was most potent. The intrinsic property of extract and partitioned fractions in reducing oedema caused by egg albumin is related to their ability to block histamine and serotonin, mediators released when inflammation is studied with egg albumin as a phlogistic agent [11]. Phospholipase A_2 is a major mediator in the pathophysiology of xylene –induced pain model. The capacity of these extracts in reducing topical inflammation of the xylene type, may be linked to their ability to inhibit the actions of Phospholipase A_2 [12].

The responses of ethanol extract/fractions to pain experimental models (Tables 5-7) revealed their ability to reduce the number of abdominal stretchings in comparison to distilled water in a way that was like the action of ASA, a standard drug. All the partitioned fractions demonstrated good pain moderation in acetic acid experiment. Likewise, in formalin pain model, both the ethanol extract/partitioned fractions demonstrated marked reductions in the number of times mice licked their paws. Against thermal-induced pain, the ethanol extract and fractions also displayed marked activity comparable to distilled water and similar to the standard drug, ASA. Acetic acid induces pain by inflammation thus causing permeability of capillaries and peritoneal fluid concentration of prostaglandins. This model normally simulates visceral pain, hence a major distinguishing evidence between central and peripheral pains. The fact that these extracts moderated the frequency of writhing movement caused by *P. madiensis*, suggests that, the produced effect may be through peripheral mechanism [13, 14]. Formalin pain type is biphasic in nature suggesting the ability of both the extract and fractions in reducing the frequency of paw lickings through both central and peripheral mechanisms [14]. Hot plate experiment normally indicates narcotic involvement in pain study. This model implicated the extract and fractions of *P. madiensis* as acting centrally in increasing the time mice spent on hot plate before response to heat [15].

CONCLUSION

The data obtained in the study therefore, adds scientific credence to the applications of *P. madiensis* (root) in cultural based treatment of diseases of pain and inflammation.

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AUTHORS' CONTRIBUTION
Uwemedimo Francis Umoh, conceptualized, supervised the study and edited the manuscript; Paul Sunday Thomas supervised animal study and edited the original manuscript; Chidiebere Ariasike, collected and prepared the plant material; Olubenga Anthony Ojo, wrote the draft of the manuscript; Sifon Ephraim Akpan and Ememobong Iniunam Iniunam carried out the study.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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