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ANTINOCICEPTIVE, ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES OF *PENTACLETHRA MACROPHYLLA* AQUEOUS EXTRACTS IN MICE.

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Abstract

The aqueous leaf, stem – bark, seed and fruit pericarp extracts of *Pentaclethra macrophylla* were examined for their cytotoxicity, while only the leaves and seeds were tested for analgesic and anti-inflammatory activities using *in-vivo* and *in-vitro* experimental models. Cytotoxicity haemagglutination assay revealed the following order of toxicity among the plant parts: fruit pericarp > stem - bark > seed > leaf with 71.4, 25.6, 5.3, and 0.5 haemagglutination titre values respectively. The extracts at 30 and 60 mg/kg exhibited analgesic activity and anti-inflammatory property using the flick and hot plate tests, acetic acid induced writhing test; and leucocyte counts, pulmonary oedema and oedema paw of mice in a dose-dependent manner. These findings therefore explain and justify ethnomedical uses of *Pentaclethra macrophylla* in the treatment of itching (inflammatory response) and pain in animals and in man.

Key words: *Pentaclethra macrophylla*, analgesic, anti-inflammatory, toxicity activities.

Introduction

Ethnopharmacological literature has shown some medicinal plants in the diet of local populations of wild animals (gorillas and chimpanzees) in the forest and the same medicinal plants used by local human population for various parasitic infections, inflammations, pain and related illnesses (Cousins and Huffman, 2002). *Pentaclethra macrophylla* is one of the plants in Africa used in traditional herbal practice for the treatment of disorders of both domestic and wild animals and human diseases (Akah, et

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al., 1999) *P. macrophylla* Benth: (Mimosaceae) is also known as the African oil bean tree (Oliver, 1960). The plant is most found in the forests of Eastern, Western and Central Africa (Keay et al., 1969). All the parts of the plant are used for various human ailments. The bark, fruits, seeds and the leaves are used as anthelmintics, for gonorrhea, convulsion and as analgesic (Githens. 1948; Bonquet et al., 1971; Iwu 1993). Whole leaves are always given to domestic and wild animals and ruminants (Rogers et al., 1990), while the aqueous extract of the leaves is administered to man orally (Akah, et al., 1999). Antimicrobial property and the fixed oil extracted from the seeds is used in the preparation of formulation against pruritus, worms and dysentery (Singha, 1963., Gugnani and Ezenwanze 1985., Kamanzi Atindehou et al., 2002). This study, examines the traditionally acclaimed antinociceptive and anti-inflammatory properties of the plant. Since this particular plant is of great economic importance both in domestic animals and in humans, the cytotoxicity effects of all the plant's parts were also determined *in-vitro.*

Plant materials

The leaves, stem - bark, seeds and the fruit pericarp were collected in March 2004 from Umuahia in Abia State and Ile-Ife in Osun State of Nigeria. Identification was made by Mr. A. Daramola and a voucher specimen was deposited at the Department of Botany Herbarium, Obafemi Awolowo University, Ile-Ife.

Extraction procedures

Four aqueous extracts were used. The leaves, stem - bark, seeds and the fruit pericarp extracts were prepared as follows: Seed (200 g) and powdered fruit pericarp (213 g) were boiled separately in 400 ml of distilled water for 30 minutes. The materials were filtered and the filtrates concentrated in a rotary evaporator under reduced temperature and pressure, after which they were lyophilized to obtain the semi-solid aqueous extracts. The stem - bark (225 g) and dried leaves (39 g) were extracted by maceration in 1 L of distilled water for 24 h. The residues were filtered, concentrated and lyophilized to obtain their respective aqueous extracts.

Animals

Swiss albino mice of either sex weighing 20 – 26 g were used. The animals were maintained under normal laboratory conditions of humidity, temperature ($25\pm1^{o}C$) and light (12 h day : 12 h night), and allowed free access to food and water *ad libitum* for at least 5 days, before the commencement of our experiments. The "principle of laboratory animal care" (NIH publication No 85 – 23) guidelines and procedures were followed in this study (NIH publication revised 1985).

Drugs and Chemicals

The following drugs were used: [Disprin® (acetylsalicyclic acid – ASA)]-(Reckiti

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Benckiser); Carrageenan (Sigma); and indomethacin (KGN Pharmaceuticals). Glacial acetic acid (BDH), All the chemicals are of Analar grade. Formaldehyde (Sigma), ethanol (BDH), methanol (BDH), sodium tri citrate (BDH), DMSO - Dimethyl sulfoxide (Sigma), methyl paraben (Sigma), sodium chloride (BDH), disodium hypophosphate (BDH) and sodium dihydrogen phosphate (BDH) .

Analgesic Activity

Evaluation of the analgesic properties of the leaf, seed, stem-bark and fruit pericarp extracts of the plant was carried out by using three different models of noxious stimuli; namely, chemical, mechanical and thermal stimuli.

Tail immersion test method

Control group of mice (n = 5) received normal saline (0.3ml/kg i.p.) only, and the mean reaction time (in seconds) was determined. Test groups of mice (5 mice per extract dose- or reference drug-dose) were treated with the aqueous extracts of the leaf and seed (30 and 60 mg/kg i.p.) or ASA (100 mg/kg i.p.) respectively. 1 hour following the drug- or reference drug (ASA) administration, the tail (up to 5 cm) of each mouse was immersed in hot water maintained at 50 ± 1^0C (in a 1-L water bath). For both the control and test animals, the reaction time (in seconds) was taken as the time when the animals withdrew their tails completely from the hot water in the bath (Parimaladevi *et al*., 2003). The test mean reaction time (in seconds) was calculated for each plant extract dose, ASA and the control.

Hot Plate test method

Control group of mice (n = 5) received normal saline (0.3ml/kg i.p.) only. The control mean reaction time (in seconds) was determined and recorded. The test group mice (5 mice per extract dose- or reference drug-dose) were treated with different doses of the leaf and seed (30 and 60 mg/kg i.p.) or ASA (100 mg/kg i.p.) respectively. One hour following the extract or ASA-administration, the mice were separately placed on a hot plate (Thermajust, Model 475,TechniLab Instruments, N.J, 07440) maintained at 55 ± 1^0C. For both the control and test animals, the reaction time (in seconds) was taken as the time when each of the mice jumped out of the beaker on the hot plate. The test mean reaction time (in seconds) was also determined for each plant extract dose and ASA.

Acetic acid-induced writhing method

Control group of mice (n = 5) received normal saline (0.3ml/kg i.p.). Mice in the test groups received different doses of the leaf and seed extracts (30 and 60 mg/kg i.p) or ASA (100 mg/kg i.p.) respectively. One hour following the extract, ASA- or normal saline administration, 0.1 ml of a 3% acetic acid solution was injected to each of the test mice intraperitoneally (Koster *et al.*, 1959). The number of abdominal

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contractions that occurred within the next 20 minutes following acetic acid administration were counted and recorded. A significant reduction in the number of acetic acid-induced abdominal contractions of the treated mice, compared to the contractions in the untreated control mice, was taken as an indication of analgesic activity.

Anti-inflammatory Activity
Carrageenan-induced pleurisy in mice

The method used in these experiments was modified from that described in detail by Vinegar *et al.* (1982) – cited by Badilla *et al;* (2003). 5 groups of 5 mice each, were treated with different doses of the leaf and seed extracts (30 and 60 mg/kg i.p), IND (10 mg/kg i.p.) and normal saline [control] (0.3 ml/kg i.p) respectively. One hour after treatment, all the animals received an intrapleural injection of 0.1 ml carrageenan on the right side of the thorax. 2 hours later, the mice were anaesthetized with chloroform, and the pleural cavity was washed with.0.1 ml of distilled water. The number of leucocytes in the pleural cavity was determined and recorded.

Pulmonary oedema

The lungs of the animals sacrificed in the Section " Carrageenan – induced pleurisy in mice" above were dissected free from the trachea and weighed. Significant changes in the test 'wet-lung weight' compared to the distilled water-treated controls, was considered to reflect pulmonary oedema (Staub, 1974). Pulmonary oedema was calculated from the formula:

$$\text{Pulmonary oedema} = \frac{\text{Lungs wet weight}}{\text{Body weight}} \times \frac{10{,}000}{1}$$

Acute inflammation

Carrageenan-induced paw oedema in mice was used as a model of acute inflammation. 0.1 ml of a 1% carrageenan solution was injected into the plantar surface of the right hind paws of the mice. Control group mice (n = 5) received normal saline (0.3 ml/kg i.p) treatment only, while animals in the test groups were treated with the leaf and seed extracts (30 and 60 mg/kg i.p.), or IND (10 mg/kg i.p.) one hour before carrageenan injection. Two hours after carrageenan injection, the mice were anaesthetized by dropping them in a jar containing cotton wool soaked with chloroform, and both the right and left hind limbs were cut identically at the ankle joint and weighed. The differences in weight gave the amount of oedema developed in the right hind limbs (Subramoniam et al., 2001).

Cytotoxicity assay

The cytotoxicity of the leaf, seed, bark and fruit pericarp extracts of *P. macrophylla* were monitored by haemagglutination activity, using formaldehyde fixed

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bovine erythrocytes as described by Peumans et al. (1982), Sadique et al. (1989) and Wang et al. (1995).

Preparation and Fixation of bovine erythrocyte

Bovine (*Bos taurus*) erythrocytes fixed with formalin were prepared according to the modified procedures of Sadique et al (1989). Bovine fresh blood collected from N'Dama, a representative of *B. taurus* breed into sterile conical flask containing 3.8% trisodium citrate solution. 20 ml of thoroughly mixed blood was centrifuged at 4000 rpm for 10 min. on a Gallenkamp centrifuge. The packed red blood cells were washed with 10 mM phosphate buffer saline (PBS) pH 7.2 until a clear supernatant was obtained. The washed packed RBC were suspended in (5%v/v) formaldehyde-phosphate buffer saline (1:12.3 v/v) solution. The mixture was left at room temperature for 24 h. The final fixed RBC were washed and centrifuged with PBS 3 times, and preserved with 0.1% methyl parabene to prevent microbial growth and stored at 4^{o} C.

Heamagglutination assay

100 µl of PBS was aliquoted into 96 well microtitre plates. The first row was used as control without the extracts. The extracts (100 µl) were added into the first well of the second row, and a 2-fold serial dilution was made until the last well in row three. Then 50 µl of bovine RBC was added to all the wells. They were incubated at room temperature for 1 h. The presence of buttons in the centre of the well indicates no agglutination and the heamagglutination titre value of the extracts were read as the reciprocal of the last dilution showing agglutination

Results

A quick in-vitro bioassay to investigate the cytotoxicity of an extract/compound is employed by using the heamagglutination assay method in fixed bovine erythrocytes. According to Table 1, the result showed that out of the four extracts tested, the fruit pericarp was the most toxic. The order of toxicity was: Fruit pericarp > Stem-bark > Seeds > Leaves. The leaf is not toxic. Their heamagglutination (HA) titre values were 71.4; 25.6; 5.3 and 0.5 respectively.

The result of the analgesic evaluation of the leaf and seeds are shown in Table 2. The leaf and seed extracts exhibited analgesic activity in the three tests of acetic acid-induced abdominal contractions, hot-plate and flick-tail tests at 30 and 60 mg/kg i.p. in mice. In the acetic acid-induced writhing, the leaf and seeds extracts produced the following % inhibitions of induced abdominal contractions: 87.1, 88.4% and 39.8, 59.7% respectively. Using the hot-plate model, the leaf and seeds extracts produced percentage increase in pain threshold as 46.0, 54.1% and 31.1, 113.5% respectively. Likewise, in flick test, the same trend of analgesic activity was observed, the leaf and seeds extracts exhibited

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16.7, 72.2% and 16.7, 538% increase in pain threshold. The standard drug, acetylsalicylic acid produced 71.3%, as % inhibition of induced abdominal contractions in acetic acid

Table 1: Cytotoxicity activities of the fruit pericarp, stem-bark, leaf and seed extracts of *Pentaclethra macrophylla* and acetylsalicylic acid (ASA) on formaldehyde fixed bovine erythrocytes. Values represent Means ± SEM of triplicate tests.

Treatments (Extracts)	Concentrations where agglutination occurs (mg/ml)	Heamagglutination Titre values
Leaves	2.0 ± 0.00	0.5
Seeds	0.187 ± 0.04	5.35
Stem-bark	0.039 ± 0.00	25.64
Fruit pericarp	0.014 ± 0.002	71.43
Concanavalin A (Cytotoxic reference drug).	0.15 ± 0.03	6.40
Acetylsalicylic acid [ASA]	1.25 ± 0.34	0.80

model while 122.3% and 450.0% as percentage increase in pain threshold in hot plates and flick test models respectively.

Using three different models of determining the anti-inflammatory property of the leaf and the seeds extracts, the activity shown in both mice right hind paw and pulmonary oedema formation were equipotent compared to that of the standard drug indomethacin (Table 2). Effects were also demonstrated against the leucocytes, but the activity was less compared to that of indomethacin. In oedema formation of mice right hind paw, both extracts were twice as potent as standard drug indomethacin , by demonstrating between 16.7 – 66.7% inhibition of oedema formation, while indomethacin gave 25.0%. In pulmonary oedema and leucocyte counts, the extracts were less potent. However, both extracts exhibited significant effects where anti-inflammatory property was well demonstrated.

Discussion

Ethnomedical and ethnoveterinary uses of plant secondary metabolites as remedies and medication have now taken a new dimension. It is becoming increasingly apparent that there are similarities in the plants used ethnomedicinally by humans and those exploited by domestic animals for self-medication (Huffman, et. al., 1996, Lans, et. al., 2001). We studied different parts of *Pentaclethra macrophylla* which are used as diet by wild animals (gorillas and chimpanzees) and the local human population in Africa for various parasitic infections, inflammations, pain and related disorders (Akah, et al., 1999*;* Cousins and Huffman 2002).

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The two extracts of the leaves and seeds were active and possess analgesic and anti-inflammatory properties. According to these findings, it was detected that the seed extract evaluated were less potent than ASA while the leaves extract exhibited greater activity than that of ASA in acetic-acid induced writhing model. This activity therefore explains the leaves and the seeds analgesic effect experienced in both ethnoveternary medicine and ethnomedicines. The effect of the extract on the oedema induced by carrageenan in mice hind paw also showed a significant ($P < 0.01$) anti-inflammatory activity (Table 2). Percentage inhibition of oedema produced by *P. macrophylla* seed and leaf aqueous extracts were found to be 66.7% and 50.0% at 60mg/kg respectively. IND (10mg/kg) inhibited the oedema volume by 25.0%. It is known that mast cells and leucocytes are groups of cells widely distributed within the connective tissues and small blood vessels. They play a central role in inflammation and allergic reactions, and produce elevated serine proteinase in the airways of asthmatic patients (Hill, et. al., 1999, Krishnaswamy et. al., 2001). The present results indicate that the leaf and seed extracts of *P. macrophylla* have anti-inflammatory activity against acute – inflammation in carrageenan oedema paw weight, induction of pulmonary oedema and in reducing the numbers of leucocytes in the inflamed pleurisy cavity. It is evident that the extract from the seed is more active than the leaves in mice oedema paw weight model. The effects of both extracts, however, at 60 mg/kg was shown to be as twice as the activity of the standard drug indomethacin (10 mg/kg) in mice right hind paw oedema formation. This result suggests that the constituents in these two extracts could inhibit chemical mediators responsible for inflammation and that the inhibitory role in the migration of leucocytes to the site of inflammation is a strong indication in this study. This could therefore support the anti-inflammatory (anti-allergy / anti-itching) property of this plant (Singha, 1963., Akah, et. al., 1999).

Cytotoxicity assay was used to assess the effect of the extracts, concanavalin A (cytotoxic reference drug) and ASA on haemagglutination ability on bovine RBC. This is to explore the abilities of agents to maintain the integrity (or preventing the lysing) of the cell membranes skeletal protein. It is one of the mechanisms used to explain their anti-inflammatory property of pharmaceuticals in RBCs and WBCs (Barnhill, et al., 1984, Anderson, et al., 1996). NSAID has been shown to stabilize the cell membrane protein (Bowman and Rand, 1980) in a way to preserve the intergrity of RBC. Therefore, an agent that stabilizes cell membrane is capable of preventing cell damage. The results in this study indicated that ASA did not lyse the RBC with its very low haemagglutination titre value hence they are capable of preventing membrane damage caused by injury during inflammation.

The study also revealed that the leaves extract was not toxic, its haemagglutination value was even less than that of ASA. This could be the reason for the use of this plant for animals and humans as antihelminthics, analgesic, anti-diarrhoeal, antispasmodic and antimicrobial agents (Akah, et al., 1999., Cousins and Huffman 2002). The cytotoxic haemagglutination titre value of the seed is less compared to the standard cytotoxic reference drug Concanavalin A. The seeds are used to poison fish and arrows and eaten to induce abortion, and are edible as food snacks called “ugba” after careful processing by fermentation (Kingsley, 1995; Isu and Ofuya, 2000). However, both the fruit pericarp and the stem bark were so toxic compared to the reference drug.

Table 2: Analgesic activities of the leaf and seed extracts of *Pentaclethra macrophylla* and acetylsalicylic acid (ASA). on acetic acid-induced abdominal contractions, hot-plate and flick-tail tests; and antiinflammatory activity of the leaf and seed extracts and indomethacin (IND) on oedema formation, leucocyte counts and pulmonary oedema. Values represent the Means ± SEM of 5 mice. Significant-t-test level *P< 0.05, **P< 0.01

Treatment (mg/kg)	Flick test (sec)	% increase in Pain Threshold	Hot-plate test (sec)	% increase in Pain Threshold	Acetic-acid induced writhings (No/20 mins)	% inhibition	Mice right hind paw (Oedema formation)	% inhibition	Pulmonary oedema	Leucocyte counts
Control (0.3ml normal saline)	3.6 ± 0.6	0	7.4 ± 0.7	0	63.5 ± 2.4	0	0.06 ± 0.01	0	90.7 ± 2.3	121 ± 18.1
Seeds 30	4.2 ± 0.6	16.7	9.7 ± 0.6*	31.1	38.2 ± 9.8**	39.8	0.05 ± 0.02	16.7	64.8 ± 28.9	63.7 ± 3.7
Seeds 60	23.0 ± 2.9	538	15.8 ± 1.9**	113.5	25.6 ± 7.1**	59.7	0.02 ± 0.0002*	66.7	62.0 ± 3.7	49 5 ± 3.6
Leaf 30	4.2 ± 0.5	16.7	10.8 ± 0.6*	46.0	8.2 ± 1.0**	87.1	0.06 ± 0.0004	0.0	66.1 ± 8.2	79.5 ± 5.3
Leaf 60	6.2 ± 0.5**	72.2	11.4 ± 0.7**	54.1	9.6 ± 5.0**	88.4	0.03± 0.001*	50.0	70.1 ± 3.1	80.5 ± 8
Indomethacin (IND) 10	-	-	-	-	-	-	0.045 ± 0.001	25 0	73.1 ± 3.5	39 8 ± 3 8**
Acetylsalicylic acid (ASA) 100	19.8 ±1.95**	450	16.45 ±2.0**	122.3	18.2 ± 2.85**	71.3	-	-	-	-

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In conclusion, the results obtained in this study provide strong evidence that the aqueous extracts from the fruit pericarp and stem bark of *P. macrophylla* are highly toxic and those of leaves and seeds are not. The leaves and seeds extracts demonstrated analgesic and anti-inflammatory properties to justify their usefulness in itching, pain and inflammatory reactions in ethnoveternary and ethnomedical practice.

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