

Full Length Research Paper

Evaluation of the anti-inflammatory and analgesic activities of *Vitex doniana* leaves

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Accepted 15 January, 2022

The anti-inflammatory and analgesic activities of extract of leaves of *Vitex doniana* Sweet (Verbenaceae) were investigated as well as the mechanisms of action. The extract significantly ($P < 0.05$) inhibited the formation of paw edema induced by agar in rats and increased reaction latency to thermal pain in mice in a dose-dependent manner. The extract caused a significant ($P < 0.05$) dose-dependent ulceration of the rat gastric mucosa and concentration-dependent inhibition of hypotonicity-induced haemolysis of red blood cells. Also, the extract significantly ($P < 0.05$) inhibited the activities of phospholipase A₂ and prostaglandin synthase in a concentration-related manner. These suggest that the leaves possess anti-inflammatory and analgesic activities mediated through sequential inhibition of the enzymes responsible for prostaglandin synthesis from arachidonic acid. Phytochemical analysis of the extract revealed the presence of glycosides, tannins, alkaloids, flavonoids and saponins. Acute toxicity studies established an oral LD₅₀ greater than 3 000 mg/kg.

Key words: *Vitex doniana*, anti-inflammatory, analgesic, ulcerogenic activity.

INTRODUCTION

Medicinal plants with anti-inflammatory activity are considerably employed in the traditional treatment of several disorders of inflammation. The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane and Bolting, 1995) which are aimed at host defense and usually activated in most disease conditions. These different reactions in the inflammatory response cascade are therapeutic targets which anti-inflammatory agents including medicinal plants interfere with to suppress exacerbated inflammatory responses usually invoked in such disorders as rheumatoid arthritis, in infection or injury. Inhibition of the synthesis of pro-inflammatory prostaglandins is one of such therapeutic targets to which some of the potent anti-inflammatory agents of clinical relevance (e.g. NSAIDs)

owe their activity (Flower and Vane, 1974; Viana et al., 1997). Several anti-inflammatory medicinal plants have also demonstrated the ability to inhibit the synthesis of prostaglandins (Jager et al., 1996; McGaw et al., 1997).

Vitex doniana (Verbanaceae) is a perennial shrub widely distributed in tropical West Africa, extending eastward to Uganda, Kenya and Tanzania in savanna and high rainfall areas (USDA,). It is commonly known as Mfuru, Mgwoobe (Tanzania), Munyamazi, Muhomozi (Uganda) (USDA,), Dinya, Tinya, Tunci (Fulani) (Atawodi et al., 2003). In ethnomedicine, *V. doniana* is employed in the treatment of a variety of diseases. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea and dysentery (Irvine, 1961; Etta, 1984) indicating that the plant's leaves may possess anti-inflammatory and analgesic properties among others. The roots and leaves are used for nausea, colic and in epilepsy ((Bouquet et al., 1971; Iwu, 1993). In eastern parts of Nigeria, the young leaves are used as vegetable

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for sauces and porridge for meals.

The anti-hypertensive effect of extract of the stem bark of *V. doniana* has been reported (Olusola et al., 1997). The extract exhibited a marked dose-related hypotensive effect in both normotensive and hypertensive rats (Olusola et al., 1997). Extracts of stem bark of *V. doniana* have also demonstrated some level of *in vitro* trypanocidal activity against *Trypanosoma brucei brucei* (Atawodi, 2005). The aqueous methanol extract has also exhibited anti-diarrhea activity (Agunu et al., 2005).

The search for new anti-inflammatory agents from the vast array of medicinal plant sources is intensifying since they may hold promise for the discovery of therapeutic agents with beneficial effect not just in suppressing relevant aspects of the inflammatory cascade but also on diverse disease conditions where the inflammatory response is amplifying the disease process. This present study was carried out to assess the validity of the folkloric uses of this plant in the management of pain and treatment of inflammatory disorders and establish the possible mechanisms of pharmacological action.

MATERIALS AND METHODS

Plant materials

Fresh leaves of *V. doniana* were collected in December 2003, in Nsukka, Enugu State, Nigeria. The plant was identified and authenticated at the International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria, where a voucher specimen (BDCP/InterCEDD 207) was deposited. The fresh leaves were cleaned, dried and pulverized. About 200 g of the powdered leaves was boiled in distilled water for 2 h. The filtrate was partitioned with chloroform-methanol (2:1) to remove traces of fatty constituents from the hot water extraction. The methanol fraction (15.29%) was concentrated and subjected to phytochemical analysis using the general methods of Harbone (1973) and evaluated for biochemical and pharmacological activities.

Animals and tissues

Adult albino rats (150-200 g) and mice (15-35 g) of either sex were obtained from the Laboratory Animal Facility of the Faculty of Biological Sciences, University of Nigeria, Nsukka (UNN). The animals were acclimatized for about 7 days under standard environmental conditions and were maintained on a regular feed (Pfizer Plc, Lagos, Nigeria) and water *ad libitum*. Ox seminal vesicles (OSV) and ox blood were obtained from healthy animals slaughtered in the local abattoir in Nsukka. Strains of *Bacillus pulmilus* used for enzyme assay were obtained from the Department of Microbiology, UNN, Nigeria.

Chemicals

The chemicals used for this study included analytical grades of methanol, hydroquinone, ethylacetate, sucrose, ethylene diamine tetracetate (EDTA), hydrochloric acid, sulphuric acid (BDH chemicals Ltd. Poole England), sodium chloride, chloroform, tri-so-

dium citrate, α -naphthol (May & Baker, England), glutathione, adenosine 5¹- diphosphate (ADP) and hemoglobin (Sigma, U.S.A). Other reagents and solvents were also of analytical grade.

Acute toxicity and lethality (LD₅₀) test

The LD₅₀ of the methanol extract was determined in albino mice using the method of Lorke (1983). The study was conducted in two stages. In stage one, 3 groups of 3 mice each were treated with 10, 100 and 1000 mg/kg of the extract (p.o.) and observed for number of deaths in 24 h. Based on the percentage survival rates, 4 mice were treated with 1500, 2000, 2500 and 3000 mg/kg extract in the second stage and the number of deaths in 24 h recorded. The LD₅₀ was calculated as the geometric mean of the highest non-lethal and the lowest lethal doses.

Anti-inflammatory activity test

Anti-inflammatory activity was assessed in rats using a modification (Ezekwesili and Nwodo, 2000) of the method of Winter et al. (1962). Increase in paw volumes was used to assess inflammation. Four groups of rats (n = 5) were deprived of food but not water for 18 h and then received i.p. injections of the extract (0.5 and 1.0 mg/kg). Thirty minutes later, each animal received subplantar injection of agar (0.1 ml of 2% suspension) in its right hind paw. Paw volume was measured by mercury displacement before and at 1.5 and 5.5 h after agar injection. Control animals received either normal saline (5 ml/kg) or phenylbutazone (150 mg/kg).

Analgesic activity test

Analgesic activity was tested in rats using the hot plate method of Janssen and Jagneau (1957). Twenty five mice of either sex were grouped in five (n = 5 per group). Each group received one dose of the extract (50, 100, 150 mg/kg), normal saline (5 ml/kg) or acetylsalicylic acid (100 mg/kg). At 15 and 60 min after extract administration, animals were lowered onto the surface of a hot plate (50±2°C) enclosed with cylindrical glass and the time for the animal to raise or lick the fore limb was noted as the reaction time (RT). Cut off time in the absence of a response was 90 sec to prevent the animals from being burnt (Sharma et al., 1982).

Gastric ulcerogenic activity test

The ulcerogenic activity of the extract was investigated using the method of Cashin et al. (1979). Rats of either sex were fasted for 18 h with access to water. At the end of the fasting period, the animals received the extract (250 or 500 mg/kg; n = 5) orally. Control animals received indomethacin (30 mg/kg) or normal saline (5 ml/kg). Eight hours after drug administration, animals were sacrificed and the stomachs opened along the greater curvature. The stomach mucosa was examined for ulcer lesions using a hand lens (x20 magnification). The length of lesions on the glandular portion were estimated and summed up to calculate the ulcer index using the method of Aonuma et al. (1980).

Phospholipase A₂ activity test

The preparation of phospholipase A₂ from *B. pulmilus* and assay of the effect of the extract on its activity were performed using the

Table 1. Effect of extract on agar-induced paw edema in rats.

Extract	Dose (mg/kg)	Edema (ml)		Inhibition of edema (%)	
		1.5 h	5.5 h	1.5 h	5.5 h
Control	-	0.48 ± 0.01	0.41 ± 0.01	-	-
Methanol extract	500	0.20 ± 0.01*	0.07 ± 0.01*	58.3	82.9
	1000	0.42 ± 0.01*	0.07 ± 0.01*	12.5	82.9
Phenylbutazone	150	0.30 ± 0.01*	0.20 ± 0.01*	37.5	51.2

* P < 0.05 compared to Control (ANOVA: LSD post hoc test); n = 5.

method of Vane (1971). Aliquots (0.5 ml) of re-suspended erythrocytes were mixed with normal saline containing 2 mM calcium chloride and the enzyme preparation and incubated either in the absence or presence of the extract (0.37, 0.74, 1.10 mg/ml) at 37°C for 1 h. The incubated reaction mixture was centrifuged at 3,000 g for 10 min and the absorption of the supernatant read against the blank at 418 nm. Prednisolone, a known inhibitor of the enzyme was used as control (Morimoto et al., 1979)

Prostaglandin synthase activity test

Prostaglandin synthase was isolated from ox-seminal vesicle using the method of Nugteren et al. (1966). The enzyme activity was assayed using a modification (Nwodo, 1981) of the methods of Yoshimoto et al. (1970) and Flower et al. (1973). The enzyme activity was monitored at 278 nm due to the formation of PGB from PGE₂ by concentrated alkali treatment. The reaction mixture consisted of 1.5 ml cofactor solution (33 mM hydroquinone, 21 mM glutathione and 40 nm haemoglobin, 0.3 ml buffer, 8 mg of enzyme preparation) and 0.5 ml arachidonic acid as substrate. After incubating at 37°C for 2 min, the reaction was stopped by adding 1.5 ml of 0.2 M citric acid, extracted twice with 5 ml ethylacetate and centrifuged at 2,500 g for 10 min. Each time 4 ml aliquots of the top organic layer were pipetted into a clean test tube. The combined ethylacetate extract was evaporated to dryness under a stream of nitrogen gas. The residue was dried overnight in vacuum and then dissolved in 2 ml methanol. A 0.5 ml 3 M KOH solution was added to the solution and allowed to stand for 15 min. The absorbance of tests against blank (the blank contained everything in the reaction mixture but a boiled (denatured) enzyme in place of the active enzyme at 37°C) was read at 278 nm on a Sp 500 (Pye Unicam) spectrophotometer. Triplicate determination was made with the assay mixture containing 0.5, 1 or 5 mg/ml of the extract. Indomethacin (4 mg/ml) was used as control. Enzyme activity was quantified using the method of Nwodo (1981).

Membrane stabilization activity test

The membrane stabilization effect of the extract was evaluated using hypotonicity-induced haemolysis of red blood cells. Briefly, citrated ox- blood samples were centrifuged at 3,000 g for 10 min. The pellets containing the red cells were re-suspended in volumes of saline equal to those of the plasma. An aliquot (0.5 ml) of the red cell suspension was added to 4 ml of water and incubated at 37°C for 1 h. After incubation, the mixture was centrifuged at 3,000 g for 10 min and the absorption of the supernatant measured at 418 nm to assess the extent of lysis of the red cells. The incubation was repeated in the presence of the extract (0.02, 0.04, 0.06 mg/ml). Indomethacin (0.2, 0.4, 0.6 mg/ml) was used as the control (Ghandisan et al., 1991).

Statistical analysis

Data was analyzed using ANOVA, further subjected to Fischer LSD post hoc test and expressed as Mean ± SEM. Differences between means were regarded significant at P<0.05.

RESULTS

Phytochemical tests

Phytochemical tests on the extract gave positive reactions for glycosides, tannins, alkaloids, flavonoids and saponins.

Acute toxicity

The acute toxicity studies revealed an oral LD₅₀ greater than 3 000 mg/kg.

Effect on agar-induced paw edema formation

The extract significantly (P<0.05) inhibited the formation of paw edema in rats. The magnitude of inhibition increased with time with the effect of the extract comparing well with that of phenylbutazone (Table 1).

Effect on reaction latency to thermal induced pain in mice

The extract caused a dose-dependent increase in reaction latency to thermal pain. At 15 min, the extract (50 mg/kg) significantly (P<0.05) evoked a longer reaction latency than aspirin and also significantly (P<0.05) prolonged the reaction latency at 60 min (Table 2).

Effect on gastric ulcerogenic activity

The extract caused a significant (P<0.05) dose-dependent ulceration of the rat gastric mucosa. The ulcerogenic effect of the higher dose was comparable to indomethacin (Table 3).

Table 2. Effect of extract on latency of pain reaction in mice.

Extract	Dose (mg/kg)	Reaction latency (T) (s)	
		15 min	60 min
Control		6.3 ± 0.6	6.1 ± 0.25
Methanol extract	50	5.1 ± 0.18 [#]	12.3 ± 1.45*
	100	7.0 ± 1.40	13.8 ± 1.83*
	150	8.0 ± 1.00	15.3 ± 0.58*
Acetylsalicylic acid	100	8.3 ± 0.90	12.0 ± 0.60*

[#]**P<0.05 compared to acetylsalicylic acid and control respectively (ANOVA: LSD post hoc) n = 5.

Table 3. Gastric ulcerogenic activity of extract.

Extract	Dose (mg/kg)	Ulcer index (mm)
Control	-	0 ± 0.00
Methanol extract	250	8 ± 1.15*
	500	14 ± 1.73*
Indomethacin	30	15 ± 1.15*

*P<0.05 compared to control (ANOVA; LSD post hoc) n = 5.
Values of ulcer index shown are the Mean ± SEM.

Table 4. Effect of extract on phospholipase A₂ activity.

Extract	Concentration (mg/ml)	Absorbance	Inhibition of enzyme activity (%)
Control	-	1.33 ± 0.06	00.0
Methanol extract	0.37	0.64 ± 0.01*	51.88
	0.74	0.14 ± 0.00*	89.47
	1.10	0.02 ± 0.01*	98.49
Prednisolone	1.00	0.11 ± 0.01*	99.13

Values of absorbance shown are Mean ± SEM of triplicate determination.
*P<0.05 compared to control (ANOVA: LSD post hoc).

Effect on phospholipase A₂ activity

The extract significantly (P<0.05) inhibited phospholipase A₂ activity in a concentration-related manner provoking inhibition comparable to that of prednisolone (Table 4).

Effect on prostaglandin synthase activity

The extract evoked a significant (P<0.05) concentration-related inhibition of prostaglandin synthase activity (Table 5).

Effect on hypotonicity-induced haemolysis

The extract significantly (P<0.05) inhibited hypotonicity-induced haemolysis of red blood cells in a concentration-dependent manner (Table 6).

DISCUSSION

Extract of leaves of *V. doniana* has demonstrated anti-inflammatory and analgesic activities by suppressing paw edema induced by agar in rats and prolonging reaction latency to thermally induced pain in mice. Agar causes inflammation of the rat paw similar to carrageenan and the extract inhibited the development of paw edema in the treated animals at 1.5 and 5.5 h post injection of irritant corresponding to the two phases of the inflammatory response. The extract also prolonged the reaction latency to pain thermally-induced in mice by the hot plate. These suggest that the extract may possess both anti-inflammatory and analgesic activities probably mediated through a common mechanism. Anti-inflammatory and analgesic activities are commonly possessed by the non-steroidal anti-inflammatory drugs (NSAIDs). These NSAIDs exert anti-inflammatory effect principally by inhibiting the synthesis of prostaglandin (Vane, 1971) an eicosanoid mediator of the inflammatory response (Foegh and

Table 5. Effect of extract on prostaglandin synthase activity.

Extract	Concentration mg/ml	Absorbance	Enzyme activity	Inhibition of enzyme activity (%)
Control	-	1.570 ± 0.040	7.15	0.00
Methanol extract	0.1	0.110 ± 0.005*	5.54	22.52
	0.5	0.055 ± 0.003*	3.07	57.06
	1.0	0.004 ± 0.001*	1.08	84.89
	5.0	0.020 ± 0.006*	0.54	92.45
Indomethacin	4.0	0.030 ± 0.003*	2.00	72.02

Values of absorbance shown are Mean ± SEM of triplicate determination.

*P<0.05 compared to the control (ANOVA; LSD post hoc). Percent inhibition of enzyme activity was calculated relative to the control.

Table 6. Effect of extract on hypotonicity-induced haemolysis.

Extract	Concentration (mg/ml)	Absorbance	Inhibition of haemolysis (%)
Control	-	0.46 ± 0.02	0.00
Methanol extract	0.1	0.21 ± 0.01*	54.35
	0.2	0.20 ± 0.01*	56.52
	0.4	0.15 ± 0.00*	67.39
Indomethacin	0.2	0.15 ± 0.01*	67.39
	0.4	0.13 ± 0.01*	71.74
	0.6	0.16 ± 0.01*	65.22
Distilled Water		0.65 ± 0.28	-

Values of absorbance shown are Mean ± SEM of triplicate determination.

*P<0.001 compared to control (Normal saline) and distilled water (ANOVA; LSD post hoc). Percent inhibition of haemolysis was calculated relative to control.

Ramwell, 2001). In addition to their involvement in the inflammatory response, prostaglandins cause pain (Roberts and Morrow, 2001) and sensitize the skin to painful stimuli (Dray, 1995) probably because they sensitize pain receptors to mechanical and chemical stimulation (Roberts and Morrow, 2001) such as the pain-producing effect of mediators (e.g. histamine, kinins e.t.c) which are released in tissue injury and inflammation. Inhibition of prostaglandin synthesis may account for the analgesic activity of the leaf extract as it contributes in large part to the analgesic activity of the NSAIDs.

The possible involvement of prostaglandin inhibition in the anti-inflammatory and analgesic activities of the extract prompted the evaluation of its irritant effect on the rat gastric mucosa. The extract caused overt ulceration of the rat gastric mucosa typical of classical inhibitors of prostaglandins which cause similar gastric lesions by depriving the gastric mucosa of cytoprotective prostaglandin (Oren and Ligumsky, 1994). This suggests that the extract may actually inhibit the synthesis of prostaglandins.

Consistent with this finding is the inhibitory effect of the extract on prostaglandin synthase isolated from ox seminal vesicles and phospholipase A₂ an acyl-hydro-lase. These enzymes sequentially mediate the synthesis of prostaglandins. Prostaglandins are synthesized *de*

novo by the action of prostaglandin synthase from the free fatty acid precursor, arachidonic acid which is released or mobilized from membrane phospholipids by the action of phospholipase A₂ (Foegh and Ramwell, 2001). Phospholipase A₂ cleaves free fatty acids from membrane phospholipids; in this case from erythrocyte phospholipids. The enzyme activity of phospholipase A₂ was assayed using its action on erythrocyte membrane on which it creates leakage thus causing hemoglobin to flow out into the medium. The enzyme activity is thus directly related to the amount of hemoglobin in the medium hence the increase in absorbance since hemoglobin absorbs maximally at 418 nm. Inhibition of phospholipase A₂ suggests that the extract may suppress the synthesis of free fatty acids from membrane phospholipids and consequently deprive prostaglandin synthase of precursors or substrates for the production of prostaglandins. Anti-inflammatory and immunosuppressive steroids inhibit arachidonic acid and its metabolites (prostaglandins) by induction of lipocortin which inhibits phospholipase A₂ (Schimmer and Parker, 2001). The sequential inhibition of these two enzymes leads to potent suppression of prostaglandins synthesis and possible amplification of the anti-inflammatory activity of the extract. Thus the extract may exert anti-inflammatory effect by the sequential inhibition of these enzymes while

the analgesic effect may additionally derive from a possible interaction with central nociceptors. Pain induced by thermal stimulus of the hot plate is specific for centrally mediated nociception (Parkhouse and Pleuvry, 1979) and the analgesic effect of the NSAIDs has also been attributed to effects at peripheral or central neurons (Gebhart and McCormack, 1994; Konttinen et al., 1994).

The extract also exhibited membrane stabilization effect by inhibiting hypotonicity-induced lyses of erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane (Gandhisan et al., 1991) and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membranes is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Chou, 1997). Some NSAIDs like indomethacin and acetylsalicylic acid are known to possess membrane stabilization properties (Murugesu et al., 1981; Furst and Munster, 2001) which may contribute to the potency of their anti-inflammatory effect. Though the mechanism of the membrane stabilization by the extract is not known yet, hypotonicity-induced haemolysis may arise from shrinkage of the cells due to osmotic loss of intracellular electrolyte and fluid components. The extract may inhibit processes which stimulate or enhance the efflux of these intracellular components.

The ulcerogenic potential of extract of leaves of this plant appears to contradict the use as food. However, the ulcerogenic effect may not be obtainable when the leaves are used as food given the small quantity used, the possible effect of chemical components of other condiments and seasonings on the ulcerogenic constituents, and the presence of food items traditionally consumed with it. Although the gastric ulceration and hemorrhage associated with inhibitors of prostaglandin synthesis may impair the therapeutic usefulness of *V. doniana* leaf extracts, the discovery of the isoforms of the cyclooxygenase enzyme (Witzgall et al., 1982) and differences in the ulcerogenic capabilities of their specific inhibitors imply the possibility of circumventing this side effect while retaining high potency of analgesic and anti-inflammatory effects. The result of acute toxicity test of the extract also suggests a remote risk of acute intoxication. This implied high degree of relative safety of the plant leaves is emphasized by the use as vegetable in eastern Nigeria and consumption by primates (Rogers et al., 1990; Nishihara, 1995; Cousins and Huffman, 2002).

Phytochemical analysis of the extract has revealed the presence of constituents known to be responsible for the anti-inflammatory and analgesic activities of some plants. The isolation of phytochemical constituents of *V. doniana* is yet to be documented. The plant may contain novel

anti-inflammatory and analgesic phytochemical constituents with therapeutically exploitable mechanism of prostaglandin synthesis inhibition.

In conclusion, the results of this study have shown that the leaves of *V. doniana* possess anti-inflammatory and analgesic properties mediated by prostaglandin synthesis inhibition. Membrane stabilization may contribute to the anti-inflammatory effect. The study also provides empirical evidence for the use of the leaves of *V. doniana* in folkloric treatment of inflammatory disorders and pain.

ACKNOWLEDGEMENT

The authors are grateful to Dr. C.U. Iroegbu of Department of Microbiology, University of Nigeria, Nsukka, for the generous gift of the *Bacillus pulmillus* strains used in this work.

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