ETHYL ACETATE FRACTION OF *TETRACERA ALNIFOLIA* (WILD) STEM EXTRACT POSSESSES ANTINOCICEPTIVE AND ANTI-INFLAMMATORY ACTIVITIES

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ABSTRACT

The study evaluated the antinociceptive and anti-inflammatory properties of *Tetracera alnifolia* used to treat pains associated with rheumatoid disorders in Southern Nigeria. The anti-inflammatory activity of the alcoholic leaves and stem extracts/fractions of *T. alnifolia* was evaluated using the fresh egg albumin-induced paw edema and xylene-induced ear edema tests in albino rats and mice respectively. The acetic acid-evoked writhing model was used to evaluate the antinociceptive action in albino mice. The extract (150–600 mg/kg, p.o.) produced significant ($p < 0.05$) dose-dependent inhibition of rats paw and mice ear edema elicited by fresh egg albumin and xylene respectively. The extracts (200 – 600 mg/kg) also significantly ($p < 0.05, 0.01$) decreased the nociceptive reaction writhes induced by acetic acid in dose-dependent manner. In respect of analgesic and anti-inflammatory activities of the solvent fractions, the ethyl acetate (EtOAc) fractions were the most potent; the stem fraction (100 mg/kg) eliciting 55.6 % inhibition of paw edema in rats and 72.9 % nociceptive reaction in mice. The EtOAc fraction (100 mg/kg) of the leaves produced 71.8 % inhibition of the writhing movement in mice comparable with the effects of diclofenac. Phytochemical screening of both extracts revealed similar composition; however, saponins and alkaloids were detected only in the stem extract. The extract did not produce any death or visible signs of delayed toxicity when administered orally up to 5000 mg/kg. The results obtained in this study suggest that *T. alnifolia* possesses antinociceptive and anti-inflammatory activities possibly mediated via mechanisms involving inhibition of release and/or actions of pro-inflammatory substances, thus further supporting the use of the plant as analgesic and as an adjuvant in rheumatoid disorders.

KEYWORDS: **Dilleniaceae, Analgesic, Anti-inflammatory, Egg albumin, Xylene, Acetic acid**

INTRODUCTION

A major breakthrough in the chemotherapy of inflammatory and pain disorders was the discovery of non-steroidal anti-inflammatory drugs (NSAIDs), steroidal anti-inflammatory drugs (SAIDs) and disease-modifying anti-rheumatoid drugs (DMARDs) in the past [1,2]. Despite advances in anti-inflammatory and analgesic drug development, their administration may be hampered by some side effects such as renal toxicities, cardiovascular dysfunction and peptic ulcer [3]. These limitations have necessitated strong need, via complementary and alternative medicines, to discover more available, affordable and accessible drugs with lower or least noxious effects. Often, natural products from plants show strong potential for this. Plant-derived constituents have remained indispensable in sustenance of healthcare delivery in many developing countries despite concerns about their safety and standards. One of such plants is *Tetracera alnifolia* (Wild) of the Dilleniaceae family; a common wild medicinal plant in West Africa [4]. Several uses of this local herb in ethnomedicine in Nigerian communities, especially in management of various infectious diseases are well known [5-11] which are attributed mainly to the flavonoids and terpenoids present in the plant [12,13]. The phytochemical research based on ethno-pharmacological information is generally

considered an effective approach in the discovery of novel effective agents from higher plants [14]. In this regard, an extensive ethnopharmacological survey in a riverine community in Nigeria indicated that the cold alcoholic macerate of *T. alnifolia* is used as pain-killer by the agrarian populace; in addition to its anti-inflammatory activity when used as adjuvant in rheumatoid disorders. This corroborated several documentated evidences elsewhere [5], however, previous attempt to validate these claims [15] did not accommodate some of the ethnopharmacological evidences obtained from Nsit Ubium in Nigeria where it is popularly called Ekwong Uruk. This, aside geographical differences and climatic influences when the present study is compared with the previous report [15], can provide relevant phytochemical information and biological activity data necessary for further development. Against these backdrops, we report the phytochemical composition, as well as the analgesic and anti-inflammatory properties of different fractions of alcoholic extract of *T. alnifolia* leaves and stem.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, ethyl acetate, n-hexane, n-butanol and acetic acid were procured from JHD (China) while diclofenac sodium and indomethacin were kind donation by Unicure Pharma, Nigeria and Naxa-25 Pharma, China respectively. All the reagents were of analytical grade and were used without further purification.

Animals

Albino rats (140 ± 20 g) and mice (30 ± 10 g) of either sex were purchased from the animal house of the Department of Pharmacology and Toxicology, University of Nigeria Nsukka. The animals were placed on standard commercial pelleted feed (Guinea feed, Nigeria) and clean drinking water *ad libitium* throughout the duration of the experiment.

Collection and preparation of plant materials

Based on the folkloric uses, Mr. Okon Abia-Williams collected the fresh samples of the plants (leaves and stems) in January 2017 from Ekpene Ibiok, Nsit Ubium, Nigeria. Mr. Nwafor Felix of the Institute of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka identified and authenticated the plant samples. Thereafter, a voucher specimen (PCG/UNN/0098) was deposited at the herbarium of the Institute. The collected plant materials were cleaned, dried under shade for 2 weeks and pulverized into 1 mm coarse powder.

Extraction of plant materials

A 500 g each of the pulverized leaves and stem was separately macerated in 2 L of methanol (MeOH) for 24 h on magnetic stirrer. The mixtures were filtered and concentrated *in vacuo*. The dried extracts were preserved at 4 ºC prior to further investigation.

Solvent-solvent partitioning of extracts

The dried crude extracts of the leaves (16 g) and stem (4.2 g) was separately dissolved in 500 mL of 10 %v/v MeOH-water and the resulting mixtures successively partitioned with 1.5 L (3 x 500 mL) each of *n*-hexane, EtOAc and *n*-butanol in the increasing order of polarity using separating funnel. All the fractions were also concentrated *in vacuo*.

Phytochemical evaluation

Phytochemical analysis was carried out on the crude extracts and some solvent fractions of the leaves and stem using standard procedure [16] for the presence of alkaloids, tannins, steroids, terpenoids, flavonoids, saponins and glycosides.

Pharmacological evaluation

Acute toxicity test

The LD_{50} of the crude extracts in mice was determined following the two stages according to the method described by Lorke [17]. Doses of 10, 100 and 1000 mg/kg of extracts suspended in 10 %v/v tween 80 were administered in the first stage. Lethality in mice was determined in the second stage at higher doses of 1600, 2900 and 5000 mg/kg of the extracts.

Anti-inflammatory evaluation by rat paw edema model

The albino rats were randomized into groups of 5 animals per group. Experiments were carried out according to procedure described by Winter *et. al.* [18]. The rats, fasted 12 h prior to the experiment, received the extract (150, 300 and 600 mg/kg), fractions (100 mg/kg), diclofenac (5 mg/kg), or vehicle p.o. One hour after treatments, inflammation was induced in rats by injection of fresh egg albumin (0.1 ml, in 10 % tween 80 in distilled water) into the sub-plantar surface of the left hind paw.

The swelling degree of the injected paw was measured by water displacement before and 0.5, 1, 2, 4, and 5 h after the administration of egg albumin. The average edema at every interval was accessed in terms of difference in volume displacement of the injected paw and the anti-inflammatory activity expressed as the percent inhibition of edema.

Anti-inflammatory evaluation by mice ear edema model

The albino mice were randomized into groups of 5 animals per group. Experiments were carried out according to Chen *et. al.* method [19]. Acute inflammation was induced by topical application of 30 µl/ear of xylene on the posterior and anterior surfaces of the right ear one hour post-treatment with the extract (150, 300 and 600 mg/kg), fractions (100 mg/kg), indomethacin (1 mg/kg), or vehicle. One hour later, the animals were sacrificed by cervical dislocation and both ear disc of 6 mm diameter were punched out from each mouse and weighed. The increase in the weight of the right ear disc compared to the left ear indicated the edema.

Analgesic activities by acetic acid induced pain model

The albino mice were randomized into groups of 5 animals per group. Experiments were carried out according to the reported method [20]. The mice received the extract (200, 400 and 600 mg/kg), fractions (200 mg/kg), diclofenac (5 mg/kg), or vehicle p.o. One hour after treatment, 0.6 % acetic acid (10 mL/kg was injected i.p and the number of writhing movement of each mouse was counted for 30 min, commencing 5 minutes after the injection of acetic acid.

Statistical analysis of data

The experimental results were analyzed using the Statistical Package for the Social Sciences (SPSS Inc. Chicago), v. 15.0 software. The paw volumes, the ear weights and the number of writhing were expressed as a mean ± standard error of the mean (SEM) (n=5). One way analysis of variance (ANOVA) was done to test for the significant difference between the means of samples and control at $p < 0.05$ and/or $p < 0.01$ by post-hoc using 2-sided Dunnett's test. In all cases, a $p < 0.05$ or a $p < 0.01$ was considered to be significant.

RESULTS

Phytochemical constituents of stem and leaves of T. alnifolia

The phytochemical evaluation showed the presence of steroids, glycosides, flavonoids, tannins and terpenoids in the leaves extract. In addition to the secondary metabolites present in the leaves, the stem extract contained alkaloids and saponins. Terpenoids and steroids partitioned into the *n*-hexane while glycosides, flavonoids and tannins were detected in the ethyl acetate fraction of the leaves extract. Alkaloids and saponins were abundant in the EtOAc fraction of the stem.

Acute toxicity

On administration of highest dose of 5000 mg/kg, the mice showed no sign of acute toxicity and no deaths were recorded. There were also no significant differences in body weights, behavioural responses or changes in food and water intake compared with the untreated groups. These suggested a high margin of safety in oral administration of *T. alnifolia* alcoholic extracts.

Anti-inflammatory effects of extracts and solvent fractions of stem of T. alnifolia

The anti-inflammatory activities of the crude extract and its fractions on egg albumin-induced paw edema in rats were recorded in Table 1. The stem extracts (300 and 600 mg/kg) significantly inhibited egg albumin-induced paw edema in rats in a dose-dependent manner, $p < 0.05$ as early as 1 h post-inflammation induction. There was significant improvement in the activities of the fractions, with the EtOAc fraction showing 55.6 % inhibition of paw edema compared with 70.8 % in diclofenac-treated 5 h post-treatment. There was no statistical significant difference ($p > 0.05$) in the activities of both *n*-hexane and *n*-butanol fractions when compared with untreated groups within 0 - 2 h post-induction.

Anti-inflammatory effects of extracts of leaves of T. alnifolia

The effects of crude extract of *T. alnifolia* leaves were shown in Table 2. The activities in this case were also dose-dependent. There was statistical significant ($p < 0.05$) difference at higher dose of 600 mg/kg compared with the untreated. However, pair wise comparison 5 h post-induction showed slightly higher paw edema inhibition of stem

compared with the 52. 9 % of the leaves extracts. No significant inhibition of paw edema was obtained in all the fraction-treated groups compared with the untreated.

inhibitory effects, apparently, leaves bulk extracts were slightly more potent than the stem extract at all treated doses, however, fractions of the stem showed relatively higher inhibitory effects.

Effect of extracts and their fractions on acute topical edema of the mouse ear

To further confirm the acute anti-inflammatory activities observed, xylene-induced ear edema model was adopted. The results were shown in Figure 1. Both extracts showed dose-dependent

Table 1: Anti-inflammatory effect of stem extract and fractions on egg albumin induced rat paw edema

Treatment/ Dose, mg/kg	Mean paw volume ± SEM, ml (percentage inhibition)				
	0.5 h	1 h	2 h	4 h	5 h
Control	0.80±0.18	0.83±0.12	0.73±0.08	0.74±0.09	0.72±0.05
Extract, 150	0.77±0.06 (3.8)	0.74±0.11 (10.8)	0.64±0.09 (12.3)	0.58±0.09 (21.6)	0.45±0.04* (37.5)
Extract, 300	0.57±0.05 (28.8)	0.53±0.05* (36.1)	0.47±0.04* (35.6)	0.48±0.07* (35.1)	0.49±0.01* (31.9)
Extract, 600	0.49±0.03 (38.8)	0.51±0.07* (38.6)	0.44±0.06* (39.7)	0.38±0.03* (48.6)	0.39±0.03* (45.8)
***n*-Hexane fraction, 100**	0.76±0.09 (5.0)	0.75±0.11 (9.6)	0.62±0.03 (15.1)	0.54±0.03* (27.0)	0.57±0.05* (20.8)
EtOAc fraction, 100	0.78±0.01 (2.5)	0.69±0.05 (16.9)	0.57±0.04 (21.9)	0.49±0.02* (33.8)	0.32±0.02* (55.6)
***n*-Butanol fraction, 100**	0.79±0.07 (1.3)	0.72±0.07 (13.2)	0.60±0.06 (17.8)	0.60±0.01 (18.9)	0.56±0.04* (22.2)
Diclofenac, 5.0	0.54±0.08* (32.5)	0.44±0.12* (47.0)	0.40±0.08* (45.2)	0.35±0.09* (52.7)	0.21±0.05* (70.8)

*Data expressed as mean ± SEM (n = 5); *p < 0.05* are significant compared to negative control group

Table 2: Anti-inflammatory effect of leaves extract on egg albumin induced rat paw edema

Treatment/ Dose, mg/kg	Mean paw volume ± SEM, ml (percentage inhibition)				
	0.5 h	1 h	2 h	4 h	5 h
Control	0.82±0.04	0.79±0.06	0.70±0.04	0.72±0.02	0.70±0.05
Extract, 150	0.77±0.07 (6.1)	0.78±0.03 (1.3)	0.70±0.03 (-)	0.62±0.02 (13.8)	0.53±0.04 (24.3)
Extract, 300	0.68±0.04 (17.1)	0.71±0.06 (10.1)	0.51±0.03 (27.1)	0.55±0.04 (23.6)	0.50±0.02 (28.6)
Extract, 600	0.57±0.04 (30.5)	0.57±0.04 (27.8)	0.50±0.03* (28.6)	0.40±0.02* (44.4)	0.33±0.04* (52.9)
Diclofenac, 5.0	0.52±0.05* (36.7)	0.46±0.04* (41.8)	0.40±0.05* (42.9)	0.32±0.06* (55.6)	0.20±0.03* (71.4)

*Data expressed as mean ± SEM (n = 5); *p < 0.05* are significant compared to negative control group
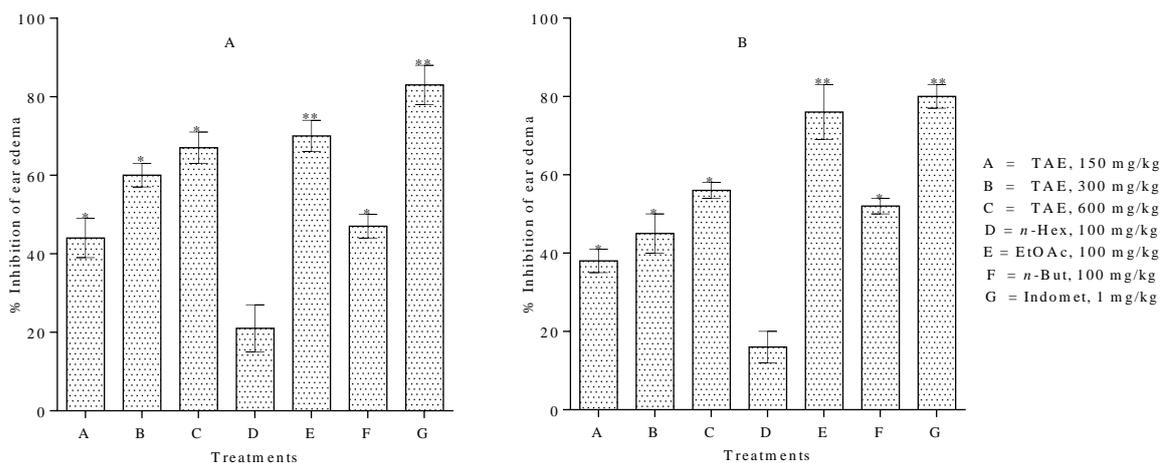
Figure 1: Effect of leaves (A) and stem (B) extracts/fractions on xylene-induced ear edema in mice. *Mean ± SEM (n = 5); *p < 0.05, **p < 0.01* compared with negative control group, TAE = *T. alnifolia* extract.

Effect of T. alnifolia leaves and stem extracts/fractions on acetic acid induced writhing in mice.

In addition to the anti-inflammatory activity, the analgesic effect of the leaves and stem on acetic acid-induced writhing was evaluated. The results were shown in Figure 2. It was observed that all administered doses significantly ($p < 0.05$ or $p < 0.01$) decreased the number of writhing in mice compared with the negative control. The maximum inhibition was 87.9 % and 72.9 % at dose of 600 and 100 mg/kg of stem extract and its EtOAc fraction respectively. The leaves extract and its EtOAc fraction produced significant ($p < 0.01$) but lower inhibition of 85.2 and 71.8 % respectively.
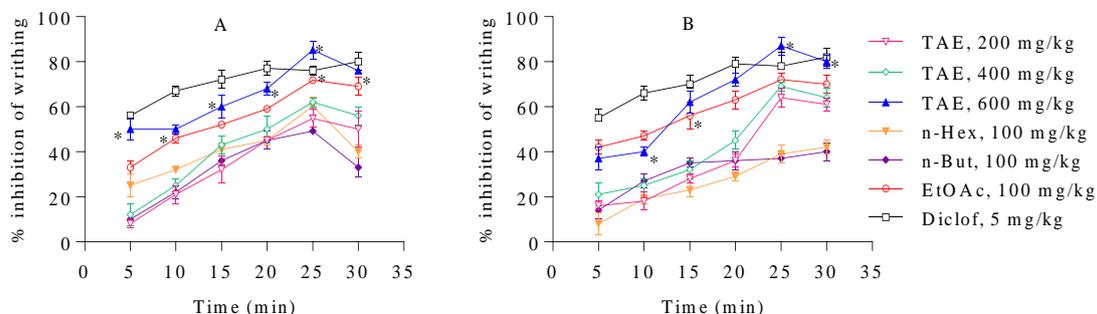

Figure 2: Effect of leaves (A) and stem (B) extracts/fractions on acetic acid-induced writhing in mice, Mean ± SEM, n = 5, p < 0.01 compared with untreated groups, TAE = *T. alnifolia* extract.

DISCUSSION

Traditionally, several plants in different forms are used against inflammatory diseases [21] and several mechanisms of action have been proposed to explain the anti-inflammatory actions of phytochemicals, such as anti-oxidative and radical scavenging activities, modulation of cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes, and neutrophils) and modulation of pro-inflammatory enzyme activities [22]. This study identified the critical potentials of *T. alnifolia* extracts prepared in line with the ethnopharmacological information in management of pain and rheumatoid disorders; and further confirmed by different animal model tests that leaves and stem extracts as used by traditional healers possess analgesic and anti-inflammatory properties thus complementing its anti-malarial, antidiarrheal, antitussive and antimicrobial activities [5-11]. The safety of these ethnomedicinal uses were also supported by this study as the LD_{50} of both extract in mice was greater than 5000 mg/kg.

The anti-inflammatory property of *T. alnifolia* was evaluated by the egg albumin-induced rat paw edema and acute topical edema of the mouse ear tests. Egg albumin-induced inflammation results from the release of histamine and serotonin at early phase of mast cell degranulation; release of bradykinin and pain at second stage (1 – 2 h) and release of eicosanoids at late stage (3 – 4 h) [23]. *T. alnifolia* extracts (300 and 600 mg/kg) and EtOAc fraction (100 mg/kg) suppress the development of paw edema in the egg albumin test. It is plausible; therefore, that *T. alnofolia* reduces the release of these vasoactive substances involved in egg albumin challenge. Our findings supported previous work showing that aqueous extract of this and other species of *Tetracera* produced anti-inflammatory effects [15]. The anti-inflammatory effect of *T. alnifolia* in the egg albumin model led us to evaluation of the effect of the extracts and fractions in a neurogenic inflammatory test (xylene-evoked ear edema). During the neurogenic phase, bradykinin and substance P are released [24]. In this study, *T. alnifolia* extracts (150 – 600 mg/kg) and fractions (EtOAC and *n*-butanol) significantly suppressed xylene-induced ear edema. Xylene-induced ear edema is known to cause severe vasodilation and increases vascular permeability associated with a neurotransmitter (substance P) spread all over CNS. Substance P elicits neurogenic inflammation when peripherally stimulated causing swelling and heaviness of the ear [25]. The two models taken together, the results suggest that *T. alnifolia* apparently inhibits neurogenic inflammation by suppressing the release and/or action of bradykinin, serotonin, histamine or substance P.

Furthermore, the antinociceptive property of *T. alnifolia* was evaluated by the mouse writhing test. Here, the i.p injection of acetic acid evoked writhing, a syndrome characterized by abdominal musculature contraction, followed by extension of the hind limbs in mouse. The induction of writhing by acetic acid injected intraperitoneally results from the sensitization of nociceptors by prostaglandins [26]. The dose-dependent inhibition of writhing induced by acetic acid in this study by *T. alnifolia* extracts (200 – 600 mg/kg) and fractions (EtOAc and *n*-butanol, 100 mg/kg) suggests significant peripherally mediated antinociceptive activity based on the association of the model with stimulation of peripheral receptors, especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity [27]. Our finding in this study supported previous work on the aqueous extract of this plant [15]; however, the significantly higher inhibition of

mice writhing obtained here (> 60 %) compared with 40 % at administered dose of 400 mg/kg suggest phytochemical differences based on the optimized extracting solvent as advised by the herbal traditional users.

In this study, therefore, it must be noted that the chemical composition or the concentration of the active principle(s) was unknown. However, phytochemical constituents of plants have been implicated in several biological activities of plants, including *T. alnifolia* [15]. The major significant phytochemical differences observed in this case were in the alkaloid and saponin compositions, which have also been implicated elsewhere in antinociceptive or anti-inflammatory efficacy of plants [28,29]. Interestingly, the consistently higher activities of stem extract and its EtOAc fraction, in addition to the loss of anti-inflammatory activity of the leaves extract on fractionation suggest that alkaloids and/or saponin content of *T. alnifolia* could be the major active phytoconstituents. To confirm the connection of the present study with these findings, however, would require (i) isolation of the active compound(s) from the EtOAc fractions; which is currently in progress and (ii) quantification of the compound responsible for these activities.

CONCLUSION

This study has demonstrated that *T alnifolia* extracts and their EtOAc fractions possess analgesic and anti-inflammatory properties which are possibly mediated through mechanisms involving inhibition of action/release of pro-inflammatory substances. The experimental findings also revealed that stem extracts were more potent than leaves extracts and these further confirmed the folkloric uses of *T. alnifolia* in Nigeria.

CONFLICT OF INTEREST

The authors alone are responsible for the content of this research and report no conflict of interest

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