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Phytochemical and Anti-Inflammatory Studies of Ethanol Extract of *Terminalia macroptera* Guill. & Perr. (Combretaceae) Stem Bark in Rats and Mice

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: *Terminalia macroptera* Guill. & Perr. (Combretaceae) is a flowering plant used traditionally for the treatment of various disease conditions including hepatitis, dysentery, piles, edema, fever, gastritis, as aphrodisiac, applied to sprains and as a cleanser for washing sores.

The aim of the work was to evaluate the qualitative and quantitative phytochemical properties and anti-inflammatory activity of the ethanol extract of *T. macroptera* stem bark.

Material and Methods: Preliminary phytochemical screening and quantification was carried out on the extract using standard phytochemical methods. The ethanol stem bark extract was screened using the carrageenan induced rat paw edema model, and xylene induced edema model in mice at doses of 50, 100, 200, and 400 mg/kg doses orally. Lorke's method was used to study the acute oral toxicity effect.

Results: Preliminary phytochemical screening revealed the presence of flavonoids, tannins, terpenoids, saponins, and alkaloids. The total phenolic and flavonoid content of the extract were 164.8±10.37 (GAE/g of dried extract mg/g) and 193.6±6.37 (QE mg/g) respectively. The result showed that the extract produced a significant ($p<0.05$) dose independent inhibition with the maximum inhibition (89.93%) observed at 30 minutes at 50 mg/kg in the carrageenan induced edema and (49.5%) at 400 mg/kg in xylene induced models. No toxic effect was observed at 4.0g/kg of the extract in rats.

Conclusion: The study showed that the ethanol stem bark extract is a good source of various phytochemicals that could be responsible for the observed anti-inflammatory activity of the plant.

Keywords: *Terminalia macroptera*, Anti-inflammatory, Phytochemical, Carrageenan

INTRODUCTION

Inflammation is a dynamic process that is elicited in response to mechanical injuries, burns, microbial infections and other noxious stimuli that may threaten the well-being of the host. It has been considered "King of Human Miseries" (Shah *et al*., 2006). The process involves changes in blood flow, increased vascular permeability, and destruction of tissues via the activation and migration of leucocytes with synthesis of local inflammatory mediators such as nitric oxide, prostaglandins, bradykinins, serotonin, leucotrienes and histamine. It is associated with

characteristic signs such as pain, redness, swelling and loss of function (Shah *et al*., 2008).

Herbal medicine is the use of plants and plant extracts to treat diseases, something mankind has always done. Many modern drugs were originally obtained from plant sources based on their phyto-constituents, even if they are now made synthetically (Amin *et al*., 2004). Currently, much interest is being paid to the search for medicinal plants with anti-inflammatory activity and its constituents, which may lead to discovery of new therapeutic agents that will not only be used in suppressing inflammation but also used in diverse disease conditions where inflammatory response is implicated and are safer compared to allopathic drugs (Shobana and John, 2012).

Terminalia macroptera is widespread in deciduous open woodland and bushy grassland upto 1400m altitude. Its growth is favoured by the tropical climate of Ghana, Mali, Nigeria, Senegal and Uganda (Akpovona *et al*., 2016). Traditionally, the roots of the plant are regarded as efficient anti-microbial remedy; it is also considered a strengthening tonic and a useful treatment in cases of urethral discharge and urinary troubles in women during pregnancy (Silva *et al*., 1997). A decoction of the leaves is used against hepatitis, ringworm and skin diseases (Pham *et al*., 2011). An enema made from a decoction of the bark is used for treating pile, as a wash to cleanse sores and applied topically on wounds. The stem bark and leaves are commonly used in gastritis, edema, cough, tuberculosis, hepatitis and against pain (Pham *et al*., 2011). Various morphological parts of the plant are used in traditional medicine in Nigeria against several ailments (Yakubu *et al*., 2015).

Previous studies on the proximate analyses of the plant using spectrophotometry and gravimetry methods showed moisture (5.26±0.11%), ash (2.68±0.06%), fibre (6.67±0.16%), lipid (0.53±0.01%), protein (11.84±0.45%) and carbohydrate (73.01±0.06%) contents (Akpovona *et al*., 2016). Also, various activities of parts of the plant have been investigated, and reported. These include Antimicrobial activity (Silva *et al*., 1997), Anti-Neisseria gonorrhoeae activity (Silva *et al*., 2002), Anti-Helicobacter pylori activity (Silva *et al*., 2012) and obesity management (Akpovona and Onoagbe, 2015).

This study aimed to investigate the phytochemical constituents (quantitative and qualitative) and to scientifically evaluate the anti-inflammatory activity of ethanol extract of *T. macroptera* stem bark *in-vivo* for its folkloric use in Nigeria.

Material and Methods

Collection and identification of plant material

The stem barks of *T. macroptera* were collected along Ilorin-Ekiti road, Kwara State, Nigeria in March 2016. The plant was identified by Mr Bolu-Ajayi and authenticated at the Herbarium unit of the Department of Plant Biology, University of Ilorin, Nigeria where a voucher specimen (UILH/001/1230) was deposited.

Preparation and extraction of plant material

After collection, the stem barks were carefully cleaned to remove dust and sand particles and were chopped into pieces with a clean mortar and pestle. The plant material were air-dried on the bench top, further dried in a hot air oven at 45^{o}C and then ground to powder using a mechanical grinder and thereafter stored in a tightly covered amber coloured glass jar until required for further studies.

The powdered material (800g) was macerated with 96% ethanol for 72 hours at room temperature, with intermittent swirling of the content. The resultant solution was filtered and concentrated *in vacuo* at 30^{o}C using the rotary evaporator to obtain the ethanol extract which was reddish brown in colour. The yield was 22.7 % (w/w).

Phytochemical investigations

Qualitative analysis

Various phytochemical investigations were performed on the ethanol extract of *T. macroptera* following standard procedures (Kokate, 2000). Colour intensity was used to categorize the presence of each phytochemical into copious, moderate or slight (trace).

Test for Alkaloids

Few drops of Mayer's reagent, Hager's reagent and Wagner's reagent were added to the extract. Alkaloids solution produces cream coloured precipitate, prominent yellow precipitate and reddish brown precipitate respectively with these reagents.

Test for Anthraquinones (Borntrager's reaction)

To 2 mL of chloroform extract, dilute (10%) ammonia solutionwas added. A pink-red colour in the ammoniacal (lower) layer indicates the presence of anthraquinones.

Test for Cardiac glycosides (Keller-Killiani test)

To a test tube containing 5 mL of extract was added 2 mL of glacial Acetic acid containing a drop of Ferric chloride ($FeCl_3$) solution. It was then underplayed with 1 mL concentrated Sulphuric acid (H_2SO_4). A

brown ring at the interface indicates a de-oxy sugar characteristic of cardenolides.

Test for Flavonoids
To a test tube containing 1 mL of extract, a few drops of dilute Sodium hydroxide (NaOH) solution was added. An intense yellow colour with NaOH, which becomes colourless on addition of few drops of dilute HCl indicates the presence of flavonoids.

Test for Saponins (Frothing test)
The extract was diluted with 20 mL distilled water and was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam indicates the presence of saponin.

Test for Terpenoids (Salkowski's test)
To a test tube containing 5 mL of extract was mixed with 2 mL Chloroform. 3 mL of concentrated sulphuric acid (H_2SO_4) was then added to form a layer. Formation of a reddish brown precipitate colouration at the interface indicates the presence of terpenoids.

Test for Steroids (Liebermann- burchard test)
The extract (1 mL) was dissolved in 10 mL chloroform and equal volume of concentrated sulphuric acid (H_2SO_4) was added down the side of the test tube. The upper layer turns red and Sulphuric acid layer show yellow with green fluorescent. This indicates the presence of steroids.

Test for Tannins
To a test tube containing the extract, 1 mL of 5% Ferric chloride was added. The presence of tannin is indicated by the formation of bluish black or greenish black precipitate.

Quantitative analysis

Determination of Total Phenolic Content
The total phenolic content of the extract was determined by modified Folin-Ciocalteu reagent method (Wolfe *et al*., 2003). Gallic acid was used as a standard at concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL prepared in methanol. 0.5 mL of the extract (0.1 mg/mL) and Gallic acid was mixed with 1 mL Folin Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 0.8 mL (75 g/L) of sodium carbonate. The mixtures were vortexed for 15 seconds and allowed to stand for 30 minutes at room temperature. The absorbance was measured at 765 nm using a UV-VIS spectrophotometer. All determinations were performed in triplicates.

Determination of Total Flavonoid Content
Total flavonoid was estimated using the method of (Miliauskas *et al.,* 2004). 1.5 mL of 2% Aluminium Chloride ($AlCl_3$) in ethanol was added to 2 mL of samples. Concentrations of 1 mg/mL of the extract in methanol was used, while Quercetin concentrations of 0.03, 0.06, 0.09, 0.12 and 0.15 mg/ mL prepared in methanol were used to obtain the calibration curve. The absorbance was measured at 420 nm after 60 mins at room temperature. The estimation of total flavonoids content in the crude extract was carried out in triplicate and the result averaged. The total flavonoid was calculated by the following formula:

$$T_{(QE)} = \frac{(C * V)}{M}$$

Where
T= Total Flavonoid content (Quercetin equivalent), mg/g plant extract
C = Concentration of Quercetin from standard curve mg/ml
V = Volume of extract used during the assay (ml)
M = Mass of extract used during assay (g)

Experimental Animals
Swiss albino mice (17-30 g) and Wistar rats (110-200 g) of both sexes, obtained from the Animal house of the Department of Zoology, University of Ilorin, Nigeria were used. The animals were housed in perforated plastic cages (6 animals per cage) in a well-ventilated room at a controlled temperature and light/dark cycle (25 ± 2^0C, 12 h light/dark cycle) and fed with standard rodent pellets (Livestock Feed plc., Lagos, Nigeria). All animals were allowed to drink water *ad libitum*. The animals were acclimatized to laboratory conditions for 7 days before the start of experiment. However, prior to treatment, the animals were fasted for 12 hours. The animals were treated in accordance with the guideline on the use of animals by University Ethical Review Committee, University of Ilorin, Ilorin, Nigeria (UERC/ASN/2016/338) and in accordance with NIH guidelines for care and use of laboratory animals in Biomedical Research (NIH, 1985).

Acute toxicity test in rats
The acute toxicity study was conducted in accordance with Lorke's method (Lorke, 1983). The study was carried out in two phases using a total of twelve albino male rats. In the first phase, nine rats were divided into three groups of three rats each and received oral doses of 10 mg/kg, 100 mg/kg, and 1000 mg/kg of the extract respectively to possibly establish the range of doses producing any toxic effect. Behavioural parameters and mortality was monitored closely for the first 4 hours and thereafter for 24 hours. No death was recorded. The second

phase was carried out with one animal each receiving doses of 1500 mg/kg, 3000 mg/kg and 4000 mg/kg of the extract to further evaluate toxicity. Behavioural parameters and mortality was monitored closely for the first 1 hour and thereafter over 24 hours for signs of toxicity. They were further observed daily for 7 days for signs of delayed toxicity.

Anti-inflammatory Studies

Xylene induced right ear edema

The method of Junping *et al*., 2005 and Sowemimo *et al*., 2015 were adopted in this experiment. Thirty (30) mice were randomly assigned into six groups of five mice each (n=5). Each group (I, II, III, IV, V and VI) was treated with single oral administration of ethanol extract of *T. macroptera* stem bark (50, 100, 200, 400 mg/kg), normal saline (10 ml/kg), or dexamethasone (1 mg/kg) as positive control respectively. Thirty (30) minutes thereafter, induction of edema by the application of xylene (0.03ml) to the inner surface of the right ear was conducted. The left ear of each animal was used as control. After 15 minutes, the mice were euthanized under ether anaesthesia and both ears were cut off and weighed. The mean of the difference between the right and left ears was determined for each group. The percentage of inflammation inhibited was calculated using the formula;

$$\text{Percentage inhibition} = \frac{(RE_{wt} - LE_{wt})_{saline} - (RE_{wt} - LE_{wt})_{treated}}{(RE_{wt} - LE_{wt})_{saline}} \times 100$$

Where;

RE_{wt}= Right ear weight

LE_{wt}= Left ear weight

Carrageenan- induced rat paw edema

The method of Adeyemi *et al*., 2002 and Sowemimo *et al*., 2015 were employed in this experiment. Thirty (30) albino rats were randomly assigned to six treatment groups of five rats each (n=5). Each group (I, II, III, IV, V and VI) was administered the plant ethanol extract (50, 100, 200, 400 mg/kg p.o), positive control Indomethacin 10 mg/kg, and negative control distilled water 10 ml/kg p.o. respectively. One hour post treatment, edema was induced by injection of carrageenan (0.1ml, 1% w/v in saline) into the sub-plantar tissue of the right hind paw. The linear paw circumference was measured immediately before injection of the phlogistic agent and then, at 30 minutes interval for 3 hours using the plethysmometer. Inflammation was expressed as an increase in paw volume due to carrageenan injection. Hence, analysing the reduction in paw size and calculating percentage inhibition of edema acts as an indicator for anti-inflammatory activity. The percentage inhibition was calculated using the formula:

$$\text{Percentage inhibition} = \frac{(C_t - C_o)_{distilled\ water} - (C_t - C_o)_{treated}}{(C_t - C_o)_{distilled\ water}} \times 100$$

Where;

C_t= Paw circumference volume at time t

C_o= Paw circumference volume before administration of treatment and carrageenan

C_t-C_o= Paw edema

Statistical analysis

The results were expressed as Mean ± Standard Error of Mean (SEM) and Percentages (%). Statistical analysis of the data was done using one-way analysis of variance (ANOVA) and individual comparisons of the group mean values was done using Dunnett's test, with the aid of Graph Pad Prism 6. Results were considered statistically significant at $p<0.05$.

RESULTS

Qualitative analysis

Results of phytochemical screening with the ethanol extract of the plant was observed to test positive to secondary metabolites like alkaloids, flavonoids, terpenoids, tannins, saponins and alkaloids (Table 1).

Quantitative analysis

T. macroptera extract had 164.87±10.37 (GAE/g of dried extract mg/g) and 193.60±6.34 (QE mg/g) total phenolic[1] and flavonoid[2] content, respectively.

[1]: mg/g Gallic Acid Equivalent

[2]: mg/g Quercetin Equivalent

Values were performed in triplicates and represented as mean±SEM

Acute toxicity test

No death was recorded in both the first and second phases of the acute toxicity study. However, there were slight behavioural changes such as increase in paw licking, decreased motor activity and sedation .

Table 1: Preliminary phytochemical screening of *T. macroptera*

Constituents	Test	Observation	Inference
Alkaloids	Mayer's reagent	Creamy white precipitate	+
	Hager's reagent	Yellow precipitate	+
	Wagner's reagent	Reddish brown precipitate	+
Anthraquinones	Borntrager's	No pink colour	-
Cardiac glycosides	Keller-killiani	No reddish brown at interphase	-
Flavonoids	NaOH test	Yellow colouration turns colourless with HCl	++
Saponins	Frothing test	Persistent froth	+
Terpenoids	Salkowski's test	Brown colour at interphase	+
Steroids	Liebermann-Burchard	Greenish colouration	-
Tannins	$FeCl_3$ test	Greenish black precipitate	+++

+++ (copiously present), ++ (moderately present), + (slightly present), - (absent)

Table 2: Effect of *T. macroptera* in acute toxicity study

Phase 1		Phase 2	
Dose (mg/kg)	**Mortality**	**Dose (mg/kg)**	**Mortality**
10	0/3	1500	0/1
100	0/3	3000	0/1
1000	0/3	4000	0/1

Anti-inflammatory activity

Xylene-induced ear edema in mice

The extract significantly ($p<0.0001$) reduced the ear edema induced by xylene and peaked at the dose of 400 mg/Kg (49.41%). The level of inhibition observed at this dose was significantly different from that produced by 1 mg/Kg dexamethasone (59.89%).

Table 3: Effect of *T. macroptera* on xylene induced ear edema

Treatment	Dose in mg/kg	Increase in ear weight (mg)	Percentage inhibition (%)
T. macroptera	50	44.27±0.004****	47.74
T. macroptera	100	48.25±0.003****	43.05
T. macroptera	200	43.72±0.003****	48.38
T. macroptera	400	42.78±0.006****	49.41
Dexamethasone	1	33.98±0.005****	59.89
Distilled water	10 mL/kg	84.72±0.005	

Values are expressed as mean±SEM. (n=5)

****p< 0.0001 vs. Control (One way ANOVA, Dunnet's Multiple comparism)

Carragenaan induced rat paw oedema

The stem bark extract of *T. macroptera* (50, 100, 200 and 400 mg/kg) produced a significant ($p<0.001$, 0.01, 0.05) inhibition of edema relative to the control (10 mg/kg distilled water) in a dose independent manner. A peak effect of 89.93% inhibition with 50 mg/kg dose of the extract at 30 minutes was observed (Table 4). The extract compared effectively with the standard drug Indomethacin (10 mg/kg), which produced its peak inhibition of edema (82.02%) at 60 minutes.

DISCUSSION

Medicinal plants are used by 80% of the world population, and it has become imperative to investigate the acclaimed plants especially in treatment of many serious diseases of public health challenges (Adedapo *et al*., 2008). For example, chronic inflammatory diseases remain one of the world's major health problems (Li *et al*., 2003). Due to its implication in virtually all human and animal diseases, inflammation has become the focus of global scientific research, more so, since the currently used anti-inflammatory agents are prone to evoking serious adverse reactions (Park *et al*., 2004). The need for an alternative to the synthetic anti-inflammatory agents from the huge array of medicinal plants resources effective in the treatment of inflammation and devoid of serious side effects is intensifying (Yesilade *et al*., 1997).

The phytochemical analysis shows the presence of secondary metabolites like saponins, flavonoids, terpenoids, tannins and alkaloids while anthraquinones, cardiac glycosides and steroids are absent.

Previous studies have shown that anti-inflammatory and analgesic effects can be as a result of the high polyphenol content of plants especially phenolics and flavonoids (Orhan *et al*., 2007). Plant polyphenols (especially flavonoids) have been reported to possess anti-inflammatory activity (Alcaatraz & Jimenez, 1998) and some of them also act as phospholipase inhibitors (Fowzy *et al*., 1988). Flavonoids are known to prevent the synthesis of prostaglandins by inhibiting the enzyme prostaglandin synthetase, specifically the endoperoxide and have been reported to produce anti-inflammatory effect (Alcaatraz & Jimenez, 1998). The extraction and quantification of

these phenolic compounds in medicinal plants are therefore necessary to allow assessment and eventual value added utilization. *T. macroptera* extract had 164.87±10.37 (GAE/g of dried extract mg/g) and 193.60±6.34 (QE mg/g) total phenolic and flavonoid content respectively. The polyphenolic constituents present in the extract may be responsible for the observed anti-inflammatory activity in this study.

In the investigation, there was no lethality but behavioural changes such as increased paw licking and sedation was observed in the rats. However, no death was recorded within 24 hours of oral administration of the extract. According to Hayes (1989), no dose-related toxicity should be considered above 4000 mg/Kg body weight of herbal preparations. Hence, the results indicate that extract of *T. macroptera* orally administered could be considered as non-toxic.

The xylene induced ear edema has certain advantages in the evaluation of anti-inflammatory steroids (Sowemimo *et al*; 2015). The application of xylene induces neurogenous edema through the release of substance P from sensory neurons which is known to cause severe vasodilation, plasma extravasations and edematous changes of skin which leads to an acute inflammatory response (Junping *et al*., 2005). The increased thickness of ear tissues is caused by these histopathological changes. It is characterized by fluid accumulation and edema. Suppression of this response is taken as an indication of antiphlogistic effect (Atta and Alkofahi, 1998). The extract mildly inhibited the ear edema induced by xylene with peak effect at 400 mg/kg with 49.41% inhibition. According to the results obtained, it is possible to say that *T. macroptera* extract is not a steroidal anti-inflammatory agent since its percentage inhibition is not up to 50 % in comparison to the standard drug (Dexamethasone) which is a known steroidal anti-inflammatory drug with percentage inhibition of 59.89%.

Carrageenan-induced hind paw oedema is a standard experimental model of acute inflammation, thus employed in this study. It has been reported that the carrageenan- induced rat paw model is a suitable *in vivo* model to study anti-inflammatory effects of natural products since it involves several mediators (Woldesellassie *et al*., 2011). It is also used to study non-steroidal anti-inflammatory drugs (Mazzanti and Braghiroli, 1994; Sowemimo *et al*., 2015). It is the phlogistic agent of choice for testing anti-inflammatory drugs, as it is not known to be antigenic and is devoid of apparent systemic effects (Woldesellassie *et al*., 2011). Moreso, the experimental model exhibits a high degree of reproducibility. Carrageenan- induced rat paw edema is believed to be a triphasic response. The first phase begins immediately after injection of carrageenan and diminishes in 1.5 hours and is mediated through the release of cytoplasmic enzymes, histamine, and serotonin from the mast cells. The second phase (1.5-2.5 hours) is maintained by kinin-like substances and the last phase (2.5-6 hours) is related to the release of prostaglandin, protease, lysosome and slow reacting substances (Suba *et al*., 2005). The knowledge of these mediators involved in different phases is important for interpreting mode of drug action.

In this study, sub-plantar injection of carrageenan produced edema development which increased progressively with time in the control group. The result from this study indicates that the extract showed significant inhibitory effect on rat paw edema development in the first phase since the extract showed peak inhibition at 30 minutes post induction of carrageenan which is suggestive that its action is within the first hour and can be said to be possible inhibitor of serotonin and histamine since they are the mediators released within the first hour of carrageenan administration.

Table 4: Effect of *T. macroptera* on Carragenan induced rat paw edema

Increase in paw volume								
Treatment	Dose (mg/kg)	T_0	T_{30}	T_{60}	T_{90}	T_{120}	T_{150}	T_{180}
T. macroptera	50	1.48±0.18	1.53±0.18* (89.94)	1.71±0.14** (86.06)	2.49±0.20 (53.45)	2.99±0.35 (0.50)	3.36±0.14** (-80.41)	3.27±0.23* (-90.59)
T. macroptera	100	1.46±0.15	1.69±0.31 (81.00)	1.96±0.30 (77.06)	2.75±0.29 (44.66)	3.87±0.30* (-50.88)	3.16±0.10 (-56.56)	3.31±0.22* (-89.37)
T. macroptera	200	1.18±0.05	1.33±0.24*** (87.66)	1.88±0.33 (67.89)	2.69±0.17 (35.17)	2.52±0.29** (16.17)	3.13±0.22 (-80.04)	1.73±0.187 (44.17)
T. macroptera	400	1.01±0.09	1.32±0.16* (74.84)	1.69±0.13* (68.89)	2.34±0.05 (42.5)	2.69±0.15 (-5.14)	1.73±0.04 (33.27)	1.59±0.14* (40.49)
Indomethacin	10	1.81±0.04	2.08±0.25 (78.08)	2.20±0.29 (82.02)	2.37±0.38* (75.52)	2.39±0.37 (63.53)	2.27±0.31 (57.30)	2.34±0.08 (45.00)
Distilled water	10 mL/kg	1.28±0.23	2.51±0.21	3.46±0.23	3.60±0.56	2.88±0.28	2.36±0.15	2.26±0.12

Values are expressed as mean±SEM. (n=5). Figure in parenthesis indicate percentage inhibition of edema development ***p<0.001 vs. Control (one way ANOVA, Dunnet's Multiple Comparism); **p<0.01 vs. Control (one way ANOVA, Dunnet's Multiple Comparism); *p<0.05 vs. Control (one way ANOVA, Dunnet's Multiple Comparism).

CONCLUSION

This study showed that the ethanol extract of *T. macroptera* stem bark is a good source of various phytochemicals that could be responsible for the observed anti-inflammatory activity which is possibly mediated by inhibition of the synthesis and/or action of serotonin and histamine. Thus, justify the use of *T. macroptera* stem-bark in the preparation of ethno-medicine used in the treatment of ailments associated with inflammation. Isolation of active principles responsible for the anti-inflammatory activity of the plant and investigating its mechanism of action are subjects for further studies.

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