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Evaluation of antibacterial, antioxidant, and anti-inflammatory properties of GC/MS characterized methanol leaf extract of *Terminalia superba* (Combretaceae, Engl. & Diels)

Njideka Ifeoma Ani¹, Kenneth Obinna Okolo^{1*}  and Raymond Ogbonna Offiah²

Abstract

Background: *Terminalia superba* is a well-known medicinal plant used in folk medicine for the management of various diseases and swelling. Validation of its efficacy in standardized scientific models is lacking. This gap needs to be filled as a way of enhancing modern drug discovery. The aim is to evaluate the antibacterial, antioxidant, and anti-inflammatory properties of *T. superba* in known and established models. Also, to establish and possibly correlate the established activity with the phytochemicals identified using GC/MS and qualitative methods.

Results: The result showed a dose-dependent percentage inhibition of DPPH, HO[•], and Fe³⁺ reducing activity. The antibacterial activity showed dose-dependent significant ($p < 0.05$) inhibition against all the organisms used. The anti-inflammatory activity of METS was confirmed in the carrageenan model with significant ($p < 0.05$) inhibition of paw volume when compared to control while significantly decreasing ($p < 0.05$) weight of xylene-induced ear. For instance, after 6 h, there was a reduction of 42%, 33%, and 22% for diclofenac, 200 mg, and 100 mg, respectively, as against 4% in control. The significant ($p < 0.05$) increase in MDA was attenuated by the treatment with METS dose dependently. Phytochemical assay and GC/MS characterization showed that alkaloids, saponins, phenols, quinone, tannins, coumarins, proteins, flavonoids, and amino acids were dominant with fatty acids accounting for 53%. Others are esters (23%), organic compounds (12%), alkanes (9%), and carboxylic acids (3%).

Conclusions: *T. superba* possesses antioxidant, antibacterial, and anti-inflammatory properties which are believed to arise from the secondary metabolites observed in the GC–MS characterization.

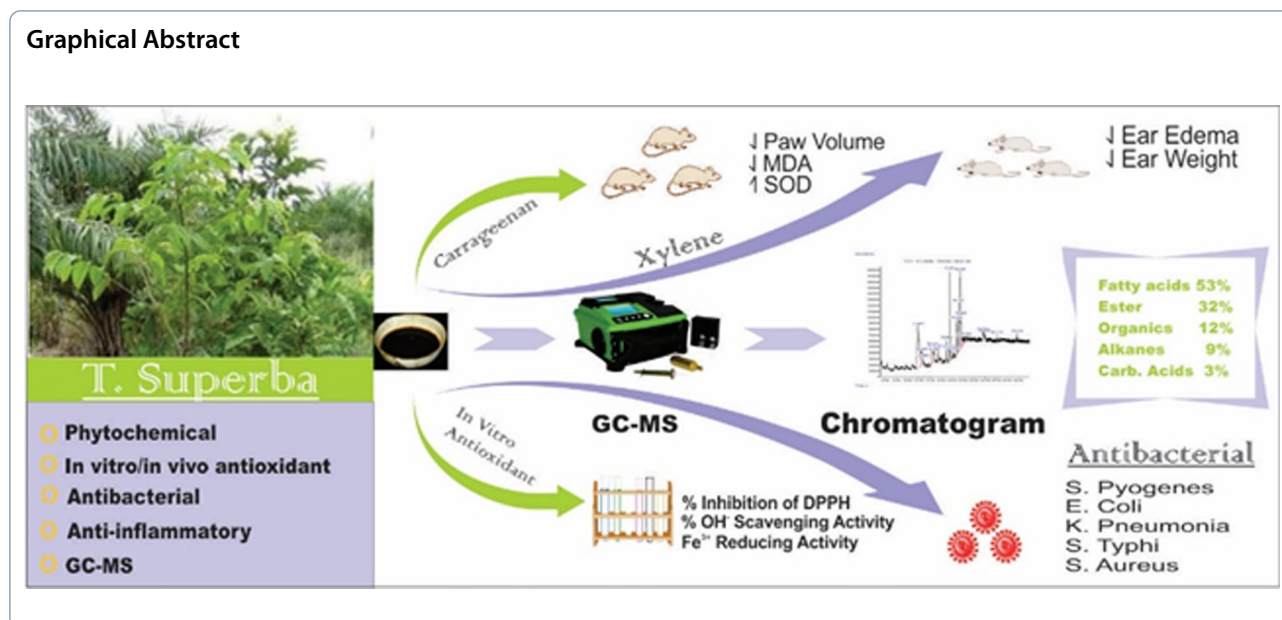
Keywords: *T. superba*, Carrageenan, Xylene, Antimicrobials, Anti-inflammatory, Antioxidant

*Correspondence: kenneth.okolo@esut.edu.ng

¹ Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Agbani, Enugu State, Nigeria

Full list of author information is available at the end of the article

Graphical Abstract



Background

The ethnomedicinal use of diverse and indigenous plants/herbs for the treatment of various disease conditions has become a profitable adventure for humans. Researchers have discovered that the inherent phytochemical constituents found in these traditional plants contribute greatly to their gainful worth in the management of various ailments [79]. *Terminalia superba* (*T. superba*) is one of these ethnobotanical plants whose various parts have folkloric claims and uses for the management, treatment, and prevention of various ill health [6]. It is a deciduous tree which can grow up to 45–50 m in height usually straight with up to 120–150 cm in diameter. *Terminalia superba*, also known as white afara, in Africa, belong to the genus *Terminalia*. The leaf is popular in folkloric medicine for its antimicrobial, laxative, antiulcer, anti-diarrheal, wound healing, and α -glucosidase inhibitory activities [41, 73]. Because of its popularity and medicinal usage, *T. superba* faces the problem of extinction in its natural habit which has implored bio-conservationists to seek its sustainability [9].

In recent times, attention on free radical biology and its untoward effects has been on the increase. The involvement of free radicals (reactive oxygen and nitrogen species) in various hemostatic and pathological states and its consequence in disease development and progression is believed to be at the foundation of many illnesses [15]. Throughout evolution, man is endowed with both internal and external sources of coping with oxidative stress. The internal system involves antioxidant defense systems especially antioxidant defense enzymes like superoxide dismutase (SOD), catalase, glutathione, etc. [54].

The exogenous antioxidant support mainly comes from consuming food that supplies the body with necessary phytochemicals with the ability to terminate free radical reactions [2, 55].

The ubiquity of microorganisms over the ages and their disease-causing ability had been part of human existence [66]. Man had depended on his environment to cope with these diseases causing co-inhabitants of earth. This unique ability to benefit from what is available resulted in the use of plants for the treatment and prevention of diseases resulting from microbes [12]. The effectiveness or otherwise of these plants is believed to stem from its constituents of secondary phytochemicals like tannins, glycosides, flavonoids, terpenes, etc., which the plants rely on for their protection against invaders like insects [30]. In today's modern science, the antimicrobial properties of plants and other natural products could be tested on a range of organisms with a clue on which is susceptible. This practice had yielded several products, giving clues to medicinal leads [58]. A classic example of this opportune is the antimicrobial lipids, fatty acids and monoglycerides. These lipids have increased the chemical space available for identifying new entities with a positive potential to overcome the incidence of resistance [77].

Inflammation is a universally accepted physiological defensive response of the human system to injury and foreign invaders. As a defense system, it mediates mechanisms to protect the mammalian system against harmful stimuli arising from pathogens, irritants, destroyed cells, and thermal or mechanical injury [35, 51]. Kinetically, Kaplan [37] described inflammation as the recruitment of lymphoid and myeloid cells to a site affected by

mechanical stress arising from the injury. These invaders may include biological pathogens (bacteria, viruses, or fungi), while the injury may be a result of physical, chemical, or mechanical damage as well as an autoimmune reaction. Generally, four distinct phases can be recognized, such as inflammatory inducers, inflammatory sensors, inflammatory mediators, and affected tissues [47]. Clinical presentation may include redness due to increased blood flow, swelling because of rapid vascular permeability, pains as a result of nerve fiber sensitization, heat, or loss of function. Once triggered, the initial inflammatory response involves the adhesion of polymorphonuclear leukocytes (neutrophils) to vascular tissues which in turn activates the release of pro-inflammatory cytokines (interleukins, IL-1 β , IL-6, IL-12, IL-18), tissue necrosis factor (TNF), and interferon gamma (IFN γ). Granulocyte-macrophage colony stimulating factor, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) are also activated [35]. Based on the duration of response, two types can be recognized, acute which lasts for a short time as a means of mediating the host defense system against infection. Chronic inflammation is believed to occur following the inability of the body to resolve acute inflammation and usually involves a change in the type of cells present at the inflamed site with tissue damage present. It is believed to be intricately linked to chronic diseases like cancer, rheumatoid arthritis, Alzheimer's, multiple sclerosis, inflammatory bowel disease, psoriasis, immune inflammatory illness, etc. [2, 3, 25]. Human nature is endowed with an active mechanism for the resolution of inflammation. The kinetics of such resolution involves pro-resolving mediators that are highly regulated and intricately linked to trafficking events in inflammation [29]. This process involves switching leukocyte trafficking to inflamed site, reversing vascular permeability and vasodilatation. There is the efficient removal of the pro-inflammatory mediators, cytokines, leukocytes, exudates, and fibrin in a non-phlogistic manner before the inflamed site resolves [23, 40]. The body possesses lipid mediators that have actions on leukocytes, endothelium, epithelial, stromal cells, and tissue necrosis factor alpha (TNF α). These mediators are usually the lipoxins (LXs) especially LXA4, resolvins, protectins, maresins, cyclopentenones, and presqualene [29]. The resolution process leads to the release of mediators to checkmate pro-inflammatory ones released. They include IL-4, IL-10, IL-13, IFN α , and transforming growth factors from macrophages [35]. Once this resolution process fails, chronic inflammation sets in with its attendant consequences of injury [29, 57].

Scientifically, the development of anti-inflammatory drugs relied on animal models of inflammation. Several inflammatory models exist for both acute and chronic

inflammations and reflect possible mechanisms of an inflammatory response [56]. Carrageenan (CGN) and xylene (XLN) models are considered acute inflammatory. CGN, a sulfated polysaccharide, is known to activate an inflammatory cascade by activating toll-like receptors (TLRs), nuclear factor kappa B (NF-Kb), and reactive oxygen species (ROS) leading to the release of IL-8. This lead to edema, infiltration of leukocytes, and an increase in the levels of prostaglandins (PGs) especially PGE $_2$ [8, 50]. Xylene, on the other hand, cause severe vasodilatation and skin edema which histopathologically manifests as an increase in thickness of the ear [65]. Conventional management of inflammation targets blocking mediators and relies on the use of non-steroidal anti-inflammatory drugs and corticosteroids [29, 40]. These conventional drugs possess numerous cardiovascular, renal, gastrointestinal, and endocrine side effects that limit their use, especially in long-term therapy [27]. This has paved the way for the use and development of alternative therapies that are possibly mimetic of inflammatory resolution mediators [29]. Herbs are believed to possess these qualities with fewer side effects when compared to conventional therapies [25]. Secondary metabolites (SMs) in herbs can act on multiple sites in a cell with specific interactions with different biochemical pathways to down-regulate pro-inflammatory signals and enhance inflammatory resolution [35].

This study evaluates the antimicrobial, antioxidant, and anti-inflammatory properties of *Terminalia superba* leaf extract. Also, chemical characterization of the contents of *Terminalia superba* using gas chromatography coupled to mass spectrometry was carried out. The results of this study can serve as basis for the development of new antibacterial regimens especially against drug resistance species and anti-inflammatory therapies that are safer with higher tolerability.

Materials and methods

General experimental procedures

All the chemicals used in this study were of analytical grade and including methanol xylene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and carrageenan was purchased from a local representative of Sigma-Aldrich (Sigma-Aldrich, Germany). Levofloxacin, indomethacin, and Diclofenac sodium were purchased from Pharmacy (Hovid Pharmaceuticals).

Harvesting of the plant leaves

Fresh leaves of *T. superba* were collected from a forest in Enugu state, Nigeria, and authenticated by a botanist at Bioresources Development and Conservation Program (BDCCP) Center, Nsukka, Enugu State, Nigeria, where a voucher specimen was deposited (INTERCEDD/203).

The name was also confirmed with the plant list website. The leaves were hand-picked, free from debris, and other soil remains into a clean container. The fresh leaves were dried under the shade at a temperature of less than 40 °C to minimize the loss of volatile compounds. The dried leaves were pulverized to a coarse powder using a milling machine (Laboratory Mill, Serial No. 4745, Christy and Norris Limited, England). The coarse powder was stored in an air-tight container ready for extraction.

Extraction of plant material

The pulverized leaves of *T. superba* (1100 g) were extracted by cold maceration in 5 L of methanol for 72 h with intermittent vigorous shaking every 2–4 h. The extract was strained with a muslin cloth and filtered with Whatman No. 1 filter paper. The filtrate was concentrated using a rotary evaporator set at 40 °C to reduce the volume to 1/10 of its original volume and dried in a water bath set at 35 °C to obtain the methanol extract (METS). The extract was stored in an airtight amber-colored bottle and stored at 4 °C in a refrigerator until use [49].

Chemicals

All the chemicals used in this study were of analytical grade and including methanol xylene, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and carrageenan was purchased from a local representative of Sigma-Aldrich (Sigma-Aldrich, Germany). Levofloxacin, indomethacin, and Diclofenac sodium were purchased from Pharmacy (Hovid Pharmaceuticals).

Preliminary phytochemical analysis

The extract was subjected to phytochemical analysis using standard procedures [18].

Gas Chromatography–Mass Spectrometry Analysis of methanol crude extract of *T. superba*.

The GC–MS analysis of bioactive compounds from the different extracts was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5MS column (30 m in length × 250 μm in diameter × 0.25 μm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system that utilized high-energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow rate of 1 mL/min. The initial temperature was set at 50–150 °C with an increasing rate of 3 °C/min and a holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in a splitless mode. The relative quantity of the chemical

compounds present in each of the extracts was expressed as a percentage based on the peak area produced in the chromatogram.

Quantitative DPPH radical scavenging assay

The ability of the extract to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was assessed according to the modified method used by Okolo and Orisakwe [53]. The percentage inhibition of DPPH radical scavenging activity was calculated based on the following equation:

$$\% \text{ inhibition} = 100\% \times (A_o - A_s/A_o)$$

where A_o is the absorbance of the control and A_s is the absorbance of the test sample.

DPPH radical scavenging property was quantified using a regression line of best fit where the abscissa represents the concentration, and the ordinate represents the percentage of inhibitory activity for three replicates.

Quantitative hydroxyl ion (OH[•]) scavenging assay

Hydroxyl radical scavenging activity of the extractives was determined by the method of Rahman et al. [60]. The generation of hydroxyl radical was instituted by the Fe³⁺-ascorbate-EDTA-H₂O₂ system (Fenton reaction). The assay principle is based on the quantification of the 2-deoxy-D-ribose degradation product, which forms a pink chromogen upon heating with TBA at low pH. The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mmol/L, pH 7.4), 0.2 mL of extractives/standard at different concentration (12.5–100 μg/mL), 0.2 mL of EDTA (1.04 mmol/L), 0.2 mL of FeCl₃ (1 mmol/L), and 0.2 mL of 2-deoxy-D-ribose (28 mmol/L). The mixtures were maintained at 37 °C in a water bath, and the reaction was started by adding 0.2 mL of ascorbic acid, AA (2 mmol/L), and 0.2 mL of H₂O₂ (10 mmol/L). After incubation for 1 h, 1.5 mL of cold thiobarbituric acid, TBA (10 g/L) was added to the reaction mixture followed by 1.5 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with ice water. The absorbance of the solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition of the percentage of 2-deoxy-D-ribose oxidation on hydroxyl radicals. The percentage of hydroxyl radical scavenging activity was calculated according to the following formula:

$$\% \text{ hydroxyl radical scavenging activity} = [A_0 - (A_1 - A_2)] \times 100/A_0$$

where A_0 is the absorbance of the control without a sample.

A_1 is the absorbance after adding the sample and 2-deoxy-D-ribose.

A_2 is the absorbance of the sample without 2-deoxy-D-ribose.

The percentage inhibition was plotted against concentration, and the experiment was repeated three times at each concentration.

Ferrous reducing antioxidant capacity assay

The ferrous reducing antioxidant capacity (FRAC) of the sample was evaluated by the method of Rahman et al. [60]. The Fe^{2+} is measured by measuring the formation of PerI's Prussian blue at 700 nm. 0.25 mL samples/standard solution at different concentration (12.5–100 $\mu\text{g}/\text{mL}$), 0.625 mL of potassium buffer (0.2 M) and 0.625 mL of 1% potassium ferricyanide, $[\text{K}_3\text{Fe}(\text{CN})_6]$ solution were added into the test tubes. The reaction mixtures were incubated in a water bath for 20 min at 50 °C to complete the reaction. Then, 0.625 mL of 10% trichloroacetic acid (TCA) solution was added to the test tubes. The total mixture was centrifuged at 3000 rpm for 10 min, after which 1.8 mL of supernatant was withdrawn from the test tubes and mixed with 1.8 mL of distilled water and 0.36 mL of 0.1% ferric chloride (FeCl_3) solution. The absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution mixture without plant extracts/standard and was incubated under identical conditions. The absorbance of the blank solution was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing capacity. The experiment was carried out in triplicate.

Animal husbandry

Eight-week-old albino rats and mice of both sexes were obtained from the animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, Enugu State-Nigeria. The rats and mice were of the weight ranging from 150 to 200 g and 17–25 g, respectively. The animals were kept differently in steel cages to acclimatize within the facility and allowed free access to clean water and food ad libitum. They were kept in a well-ventilated room with 12/12-h light/dark conditions and at room temperature. Animal experiments were conducted in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (Pub. No. 85-23, revised 1985), and per the University of Nigeria, Nsukka Ethics Committee established rules on the use of laboratory animals (PHARM/01/072).

Acute toxicity test

The estimation of the mean lethal dose (LD_{50}) of the ME of *T. superba* in mice was done using the modified method described by Lorke [44]. Firstly, nine mice were divided into three groups ($n=3$), received oral administration of 10, 100 and 1000 mg/kg of METS (prepared in 3% tween 80) and were observed for 24 h for a number of deaths. At the end of 24 h, no death was recorded. Consequently, a fresh batch of mice divided into four groups ($n=1$) received 1600, 2900, 3600, and 5000 mg/kg of METS in the second stage of the study and were observed for 24 h for death. Based on the result, it is believed that the extract is safe up to 5000 mg/kg because there was no physical or concealed signs and symptoms of toxic effects or any record of death for all the periods of observation of the animals.

Antibacterial activity test

The effects of METS on microorganisms were evaluated using antimicrobial activity on inflamed wound isolates. The standard bacteria sample were obtained from the pathology laboratory at a medical school in Enugu, Nigeria. The clinical wound isolates were collected in sterile swab sticks from patients before dressing the wounds and characterized based on the method [33]. Patient selection was randomly done with no consideration for gender or age. The swabs were streaked and sub-cultured three times in sterile nutrient agar plates and subsequently maintained on agar slants stored at 4 °C. The isolates were characterized and identified using gram staining, colony characterization, cetrimide agar, gelatin liquefaction, sodium chloride, and Mannitol fermentation tests [52]. An antimicrobial activity test was performed using the agar well diffusion method described by Balouiri et al. [7]. Briefly, sterile Muller Hinton agar plates were flooded with 1×10^6 cfu/ml concentration of microorganisms. Using a sterile cork borer (7 mm diameter), 6 wells were bored on the agar, and three drops of the METS (12.5, 25, 50, 100) mg/ml in 10% dimethyl sulfoxide (DMSO) were placed in the appropriate well. DMSO (10%) was used as control, while levofloxacin served as the standard drug. The plates were allowed 30 min for diffusion and incubated in an inverted form for 24 h at 37 °C.

Microbial sensitivity was determined in triplicate. After incubation, the diameter of the inhibition zone for each well was measured horizontally and vertically, and the mean was obtained. The minimum inhibitory concentration (MIC) was determined as the intercept on the concentration axis of concentration vs. the mean IZD^2 plot.

Induction of carrageenan-induced rat paw edema

Twenty-five albino rats were weighed and randomly divided into five groups ($n=5$) as follows:

Group I: Negative control and received oral administration of distilled water (2 ml/kg).

Group II: Positive control and received the standard drug, Indomethacin (25 mg/kg).

Group III: Treatment group and received 100 mg/kg of ME of *T. superba*.

Group IV: Treatment group and received 300 mg/kg of ME of *T. superba*.

Group V: Treatment group and received 600 mg/kg of ME of *T. superba*.

One hour after the treatments, 0.1 ml of 1% w/v carrageenan (phlogistic agent) in normal saline was injected into the sub-plantar region of the right hind paw of the rats, and the volume of the paw size was measured by water displacement method at times 0, 0.5, 1, 2, 3, 4, and 5 h after carrageenan injection [39].

The percent inhibition of edema was calculated using the following formula:

$$\text{Inhibition of edema (\%)} = \frac{V_c - V_t}{V_c}$$

where V_c is the mean paw edema volume of control at each hour and V_t is the mean paw volume of treated animals at each hour.

After the 5th hour, paw supernatant was collected and used for the quantification of lipid peroxidation.

Quantification of lipid peroxidation and antioxidant enzyme

Paw supernatant lipid peroxidation was quantified as malondialdehyde (MDA) based on the method described by Katerji et al. [38]. The MDA level was calculated according to the method of Todorova et al. [70] and expressed as $\mu\text{g/ml}$. Superoxide dismutase (SOD) was assessed using a commercial kit (Biovision, Mountain View, CA, USA) obtained from a local representative and assayed according to the manufacturer's protocol.

Topical edema of the mouse ear (xylene model)

The effect of the extract on acute topical edema was assessed using xylene-induced ear edema in mice. Mice were divided into four groups ($n=5$). The animals were treated for 4 days. On the 4th day, topical application (5 mg/ear) of METS was applied on the anterior surface of the right ear, while xylene (0.05 ml) was instantly applied on the posterior surface of the same ear. Control animals received an equivalent volume of the vehicle (3%

v/v Tween 80). The left ear was left untreated. Two hours after xylene application, animals were sacrificed and both ears were removed. Circular disks were punched out of the ear lobes using a cork borer (6 mm diameter) and weighed. The difference in the weight of disks from the right treated and left untreated ear was calculated and used as a measure of edema [65]. The level of inhibition (%) of edema was calculated using the relation:

$$\text{Inhibition (\%)} = 100[1 - (R_t - L_t/R_c - L_c)]$$

where R_t is the mean weight of the right earplug of treated animals, L_t is the mean weight of the left ear plug of treated animals, R_c is the mean weight of the right earplug of control animals, and L_c is the mean weight of the left ear plug of control animals.

Evaluation of in vivo antioxidant activity of *T. superba*

Statistical analysis

Data obtained were analyzed by one- or two-way analysis of variance (ANOVA) followed by Turkey multiple comparisons *post hoc* test using Graphpad Prism version 5.0. The values were expressed as mean \pm standard deviation (SD). $p < 0.05$ was considered statistically significant.

Results

Table 1 shows that METS was found to be rich in alkaloids, saponins, phenols, quinone, tannins, coumarins, proteins, flavonoids, and amino acids.

Table 1 Phytochemical constituents present in METS

Constituents	Relative abundance
Alkaloids	+++
Flavonoids	+++
Saponins	+++
Steroids	–
Phytosterols	–
Phenols	+++
Glycosides	–
Quinone	+++
Terpenoids	–
Tannins	+++
Coumarins	+++
Carbohydrates	+
Proteins	+++
Amino acids	+++

(+) slightly present, (++) moderately present, (+++) highly present

Table 2 Phyto-constituents of METS as detected by gas chromatography–mass spectrometry analysis

Peak	R. time	% Area	Molecular weight (g/mol)	Molecular formula	Compound name
1	11.877	19.87	144.21	C ₈ H ₁₆ O ₂	R*,R*-5-Hydroxy-4-methyl-3-heptanone 1-Penten-3-ol oxalic acid, monoamide, n-propyl, pentadecyl ester
2	12.460	6.45	172.26	C ₁₀ H ₂₀ O ₂	Decanoic acid, 3-methyl-5-hydroxy-2,2-dimethylhexan-3-one
3	12.699	1.92	132.16	C ₆ H ₁₂ O ₃	Butoxyacetic acid Decane, 1-fluoro-
4	14.435	7.30	156.26	C ₁₀ H ₂₀ O	Ether, 6-methylheptyl vinyl Cyclopropane, 1-(2-methylbutyl)-1-(1-methylpropyl)-4-Amino-N-(methylimino)-1,2,5-oxadiazol-3-imine oxide
5	14.527	1.60	438.8	C ₃₀ H ₆₂ O	Docosyl octyl ether, Chloro-methyl-methoxy-amine
6	14.703	2.59	162.14	C ₆ H ₁₀ O ₅	3-Deoxy-d-mannonic lactone 7-Oxabicyclo [4.1.0] heptane, 1-methyl-
7	15.474	2.69	181.27	C ₁₁ H ₁₉ NO	Oxazole, 5-hexyl-2,4-dimethyl-2-tert-Butyl-pyrroline
8	16.982	2.84	268.4	C ₁₇ H ₃₂ O ₂	Hexadecanoic acid, methyl ester Hexadecanoic acid,
9	17.486	1.37	334.4	C ₂₀ H ₃₀ O ₄	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester Dibutyl phthalate
10	17.621	12.02	256.42	C ₁₆ H ₃₂ O ₂	n-Hexadecanoic acid
11	18.208	0.76	210.27	C ₁₂ H ₁₈ O ₃	1-Octenylsuccinic anhydride,1-Heptadec-1-ynyl-cyclohexanol
12	18.800	5.31	296.5	C ₁₉ H ₃₆ O ₂	cis-13-Octadecenoic acid, methyl ester, 11-Octadecenoic acid, methyl ester
13	19.102	6.08	206.37	C ₁₅ H ₂₆	1H-Cycloprop[e]azulene, decahydro- 1,1,4,7-tetramethyl-, [1aR-(1a.alpha ha.,4. beta.,4a. beta.,7. beta., 7a.eta.,7b. alpha.)]- Bicyclo[3.1.0]hexane, 6-methylene-
14	19.425	12.60	282.5	C ₁₈ H ₃₄ O ₂	cis-Vaccenic acid, Oleic Acid
15	19.604	5.69	282.5	C ₁₈ H ₃₄ O ₂	OleicAcid, Octadecanoic acid, 9-Octadecenoic acid, (E)-
16	19.915	1.66	207.31	C ₁₃ H ₂₁ NO	Oleic Acid, 4,7,7-Trimethylbicyclo [2.2.1] hepta n-2-one O-allyloxime
17	20.268	2.33	208.38	C ₁₅ H ₂₈	7-Pentadecyne, 9,12-Octadecadienoic acid, (Z, Z)- 9,17-Octadecadienal, (Z)-
18	20.564	0.75	264.4	C ₁₇ H ₂₈ O ₂	2-Propenoic acid, 2-methyl-, 3-[tris(2-methoxyethoxy) silyl] propyl ester, 3,7,11-Tri-decatrienoic acid, 4,8,12-trimethyl-, methyl ester, (Z, E)- 2,4-Dimethylaniline, N N-di(trifluoroacetyl)-
19	23.602	2.60	282.5	C ₁₈ H ₃₄ O ₂	6-Octadecenoic acid, 9-Octadecenoic acid, (E)-Oleic Acid
20	23.931	1.54	264.4	C ₁₂ H ₁₂ N ₂ OS ₂	4-Thiazolidinone, 3-ethyl-5-[(phenylamino)methylene]-2-thioxo-Oleic Acid, Acetic acid, 4-(7 methylidenebicyclo[3.3.1] non-2-en-3-yloxy)butyl ester
21	26.110	1.24	340.17	C ₁₀ H ₁₀ F ₆ O ₆	Glyceric acid (ISP-TFA),2-Trifluoroacetoxypentadecane (1R,4R)-1-methyl-4-(6-Methylhept-5-en-2-yl) cyclohex-2-enol
22	29.714	0.95	577.2	C ₁₆ H ₄₈ O ₇ Si ₈	Octasiloxane, 1,1,3,3,5,5,7,7,9,9, 11,11,13,13,15,15-hexadecamethyl-Heptasiloxane, 1,1,3,3,5,5,7,7,9,9

Gas chromatography–mass spectrometry of detected compounds

The GC–MS of METS showed the following class of compounds: fatty acids (53%), ester (23%), organic compounds (12%), alkanes (9%), and carboxylic acids (3%).

Table 2 shows the phyto-constituents of METS as detected by gas chromatography–mass spectrometry analysis.

GC/MS chromatogram of METS

Effect of methanol extract of METS on carrageenan-induced paw edema

There was statistically significant inhibition of the inflammatory activity of the administered carrageenan. The highest anti-inflammatory activity of the standard, indomethacin, and 100 mg/kg dose of the extract was seen at the 4th hour; the 300 mg/kg dose showed

Table 3 Phytoconstituents of METS with known pharmacological activity

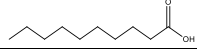
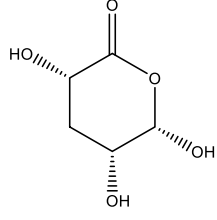
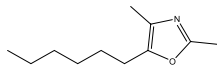
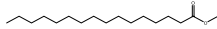
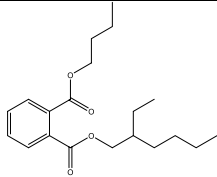
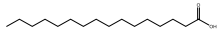
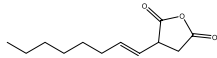
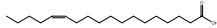
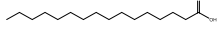
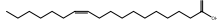
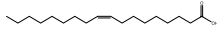


S. no.	Compound name	Structure of compound	Pharmacological activity(s)
1	Decanoic acid (Capric acid)		Anti-inflammatory, antibacterial [74]
2	3-Deoxy-d-mannonic lactone		Antibacterial, antioxidant [21]
3	Oxazole, 5-hexyl-2,4-dimethyl-		Antioxidant [42]
4	Hexadecanoic acid, methyl ester		Antioxidant, anti-inflammatory, and antiarthritic [5, 46]
5	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester		Antimicrobial [19]
6	N-hexadecanoic acid		Anti-inflammatory, Antioxidant [1]
7	1-Octenylsuccinic anhydride,		Antioxidant [72]
8	(Z)-octadec-13-enoic acid		Antibacterial [59]
9	Hexadecanoic acid		Antibacterial [63]
10	cis-Vaccenic acid		Antibacterial activity, and anti-inflammatory [34, 62]
11	Oleic acid		Antioxidant, anti-inflammatory, antimicrobial activity [10, 20]
12	7-Pentadecyne		Anti-inflammatory [17]
13	6-Octadecenoic acid		Anti-inflammatory, antioxidant, and antibacterial [26, 45]

Table 4 Effect of METS on In vitro antioxidant activities: DPPH scavenging assay (%), percentage hydroxyl ion (OH⁻) scavenging assay, and ferric reducing antioxidant power (FRAP)

Concentration µg/ml	% Inhibition of DPPH		% OH ⁻ scavenging activity		Fe ³⁺ reducing activity (absorbance)	
	METS	Ascorbic acid*	METS	Ascorbic acid*	METS	Ascorbic acid*
25	37.00 ± 6.17	54.90 ± 8.50	55.00 ± 7.19	48.22 ± 10.60	0.33 ± 0.02	0.44 ± 0.02
50	48.21 ± 7.60	58.89 ± 9.80	52.83 ± 6.91	62.58 ± 9.88	0.35 ± 0.02	0.45 ± 0.01
100	60.05 ± 2.68	67.51 ± 10.45	43.57 ± 10.60	63.01 ± 11.25	0.40 ± 0.01	0.46 ± 0.02

Data represented as mean ± SD (N=3)

*Standard antioxidant

Table 5 Inhibitory properties (inhibition zone diameter in mm) of METS on different pathogenic bacteria

Types of bacteria	Inhibition zone diameter in mm (Mean ± SD)				
	Gram negative		Gram positive		
Microorganism	<i>E. coli</i>	<i>Salmonella typhi</i>	<i>Streptococcus pyogenes</i>	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumonia</i>
12.5 mg/ml METS	0.9 ± 0.01	0.9 ± 0.01	0.7 ± 0.02	0.9 ± 0.01	0.9 ± 0.02
25 mg/ml METS	1.0 ± 0.01	1.2 ± 0.01	1.1 ± 0.02	1.1 ± 0.03	1.1 ± 0.01
50 mg/ml METS	1.3 ± 0.02	1.3 ± 0.02	1.4 ± 0.03	1.5 ± 0.03	1.3 ± 0.02
100 mg/ml METS	1.6 ± 0.03	1.6 ± 0.02	1.7 ± 0.03	1.8 ± 0.03	1.7 ± 0.02
10 µg/ml Levofloxacin	2.1 ± 0.05	3.1 ± 0.02	1.9 ± 0.05	3.5 ± 0.06	3.1 ± 0.04

Inhibition zone diameter (mm). Volume of crude extract used (10 µl). Inhibition Zone diameter excludes the diameter of the well (7 mm)

its highest activity at the 3rd hour, while that of the 600 mg/kg dose was at the 5th hour. There was no significant reduction in inflammation for all the treatment groups at 0.5 h.

Oxidative stress and SOD activity

Administration of CGN to paw led to an increase in oxidative stress and a decrease in antioxidant enzymes as evidenced by an increase in Malondialdehyde and SOD, respectively. However, administration of METS significantly ($p < 0.05$) decreased this rise in malondialdehyde and subsequently increased the level of SOD.

Xylene-induced ear edema test

The effects of METS on acute inflammation induced by xylene in mice are shown in Table 6. Compared with the control, there is significant inhibition in 200 mg/kg METS group (21.78%, $p < 0.01$) and in 200 mg/kg group (32.93%, $p < 0.01$). Also, there is a significant inhibition in Diclofenac-treatment group (42.29%) of ear edema ($p < 0.01$) when compared to negative control.

The values are presented as mean ± standard deviation ($n = 5$). The asterisk denotes the significance levels in comparison with the negative control: *, ** ($p < 0.05$ and 0.01), respectively.

Discussion

The use of herbs in the management of diseases and ailments seems to be on the increase. This is believed to be due to the safety conception attached to plants, the high cost, numerous side effects, and unavailability of orthodox drugs [75]. A growing number of studies point to the benefits inherent in secondary metabolites produced by these plants to protect themselves from outside invaders [78]. *T. superba*, a perennial tree, is believed to belong to this group [6].

Qualitative phytochemical analysis of METS (Table 1) revealed its richness in alkaloids, phenols, tannins, flavonoids, saponins, coumarins, quinones, proteins, and amino acids. Tannins, phenols, and flavonoids are well-known for their antioxidant properties which may combat oxidative stress that had been implicated in inflammation and the mechanism of action of a phlogistic agent used in this work, CGN [35, 67].

Gas chromatography coupled with mass spectrometry analysis provided further insight into the chemical makeup of *T. superba* that almost half of the secondary molecules detected are fatty acids (Tables 2 and 3). Fatty acids are particularly known for their antibacterial properties which researchers believe are devoid of resistance [13, 77] (Table 4).

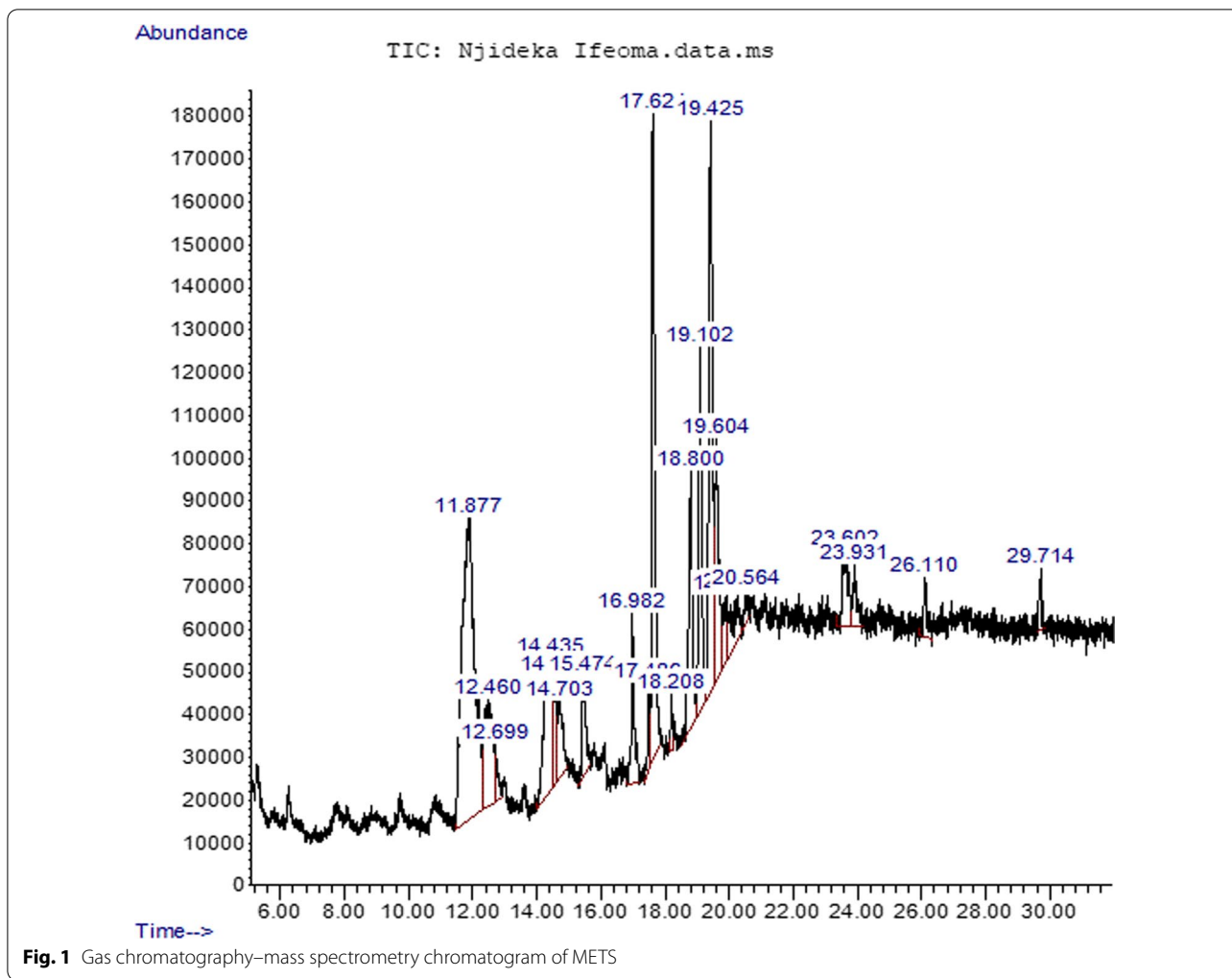


Fig. 1 Gas chromatography–mass spectrometry chromatogram of METS

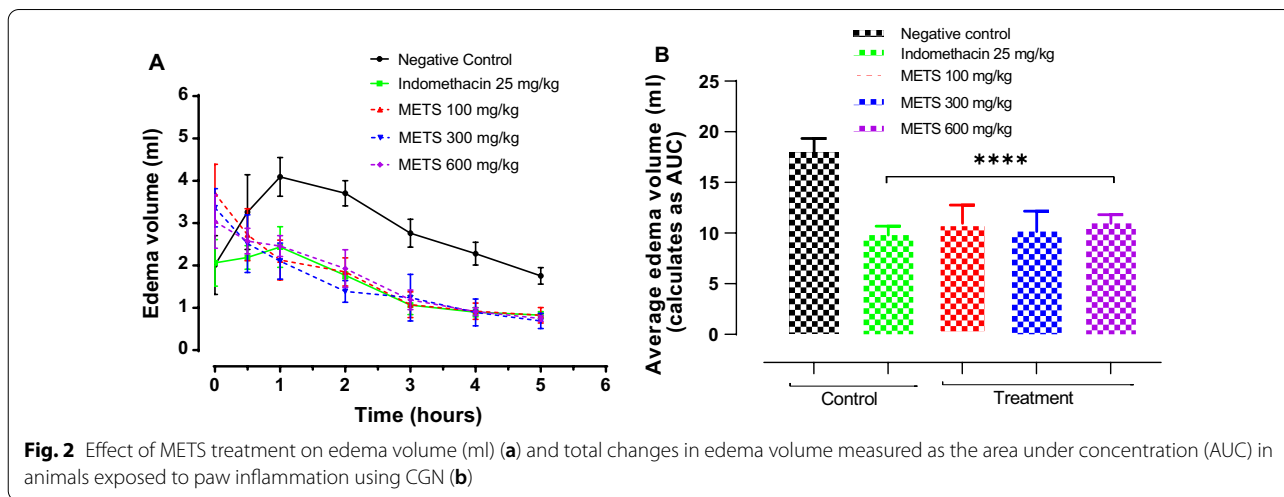
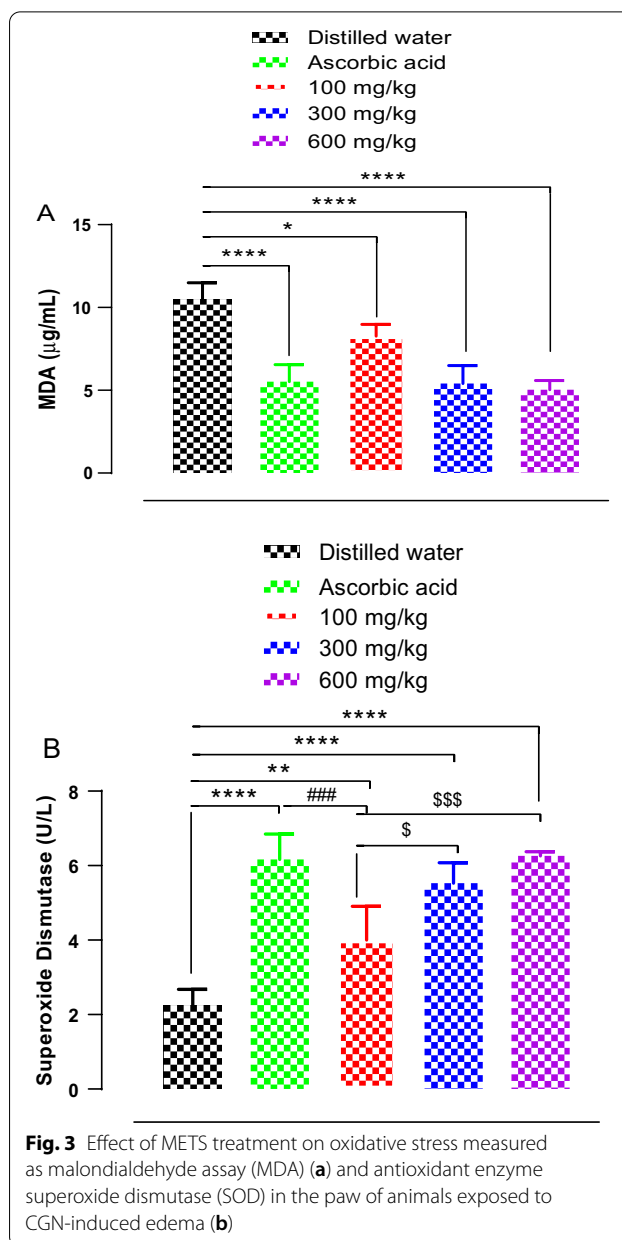


Fig. 2 Effect of METS treatment on edema volume (a) and total changes in edema volume measured as the area under concentration (AUC) in animals exposed to paw inflammation using CGN (b)

The antibacterial properties of METS (Table 5), which are believed to be attributable to the fatty acids component of *T. superba*, arise from the chemical behavior of fatty acids as single-chain amphiphiles with bacterial cell wall destabilizing properties [77]. This activity against both gram-positive and negative bacteria is known to agree with the work of Kabara [36]. Though could be bacteriostatic or bactericidal depending on concentration, species, or environmental factors, the mechanism is based on its amphipathic properties [14]. They alter the electron transport chain, membrane enzymes, and oxidative phosphorylation which controls bacteria energy production. This will lead to compromise in membrane integrity resulting in cell permeability, inhibition of growth, lysis, and ultimately death [61]. Decanoic acid (capric acid) is known for its excellent activity against resistant *Brachyspira hyodysenteriae*, *Salmonella spp*, *E. coli*, and *Campylobacter jejuni* [24, 69]. Mohamed [48] showed that 1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester isolated from hemolymph of scarab beetle *Scarabaeus sacer* (Coleoptera Scarabaeidae) had good antibacterial activity against *Enterobacter cloacae*, *Escherichia coli* (both gram negative), *Bacillus subtilis*, and *Staphylococcus aureus* (gram positives) which is in agreement with this study. Also the antibacterial properties of hexadecanoic acid, vaccenic acid, and oleic acid against resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *K. pneumoniae*, *B. subtilis*, and *S. pyogenes* are well-documented in the literature [62, 63]. El Shoubaky and Salem [16] showed in his work on brown algae with high content of fatty acids had good activity against *Padina pavonica* and *Hormophysa triquetra*. It is then scientifically reasonable to believe that the effects of *T. superba* against the aforementioned organism could at least partly be attributed to the fatty acid content [11] (Fig. 1).

Effect of METS on CGN-induced paw edema showed a significant improvement in inflammation in comparison with control (Fig. 2). Inflammation was induced using a known, acceptable and versatile model of acute inflammation, CGN [25]. CGN is known to activate ROS, TLR, and NF- κ B leading to an increase in IL-8, IL-1, IL-6, PGE₂, and infiltration of neutrophils with the attendant result of vasodilatation, fluid leakage, and edema [8, 50]. Following inflammatory induction, the mammalian system is endowed with resolving systems like antioxidants, lipoxins, resolvins, protectins, cyclopentenones, etc., which terminates the inflammatory process [29, 76]. If this resolving system fails, chronic inflammation sets in with its attendant damages [2]. To cope with diseases and ailments involving inflammation, mammals relied on the environment especially herbs to solve such problems [79]. The reduction in the inflammatory response to



CGN (Table 6) is in agreement with the works of [25, 31]. Herbs, by their nature of being endowed with secondary metabolites, are believed to have effects on multiple targets in a cell with specific interactions in its biochemical pathways leading to resolution of inflammatory process [4, 35, 40]. Flavonoids, phenols, and tannins are known to regulate pro-inflammatory responses by up-regulating Heme oxygenase-1 (HO-1) through activation of MAPK which is in agreement with this work [67]. Capric acid (Tables 2 and 3 is known to have attenuated inflammatory response by inhibiting cytokines; TNF α and IL-6. IL-6 is one of the cytokines activated by CGN and *T. superba*

Table 6 Effect of METS on xylene-induced edema

Treatments	Number of mice used	Mean right ear weight (g) ± SD	Mean left ear weight (g) ± SD	% inhibition of treated ear
Negative control	5	0.09 ± 0.01	0.14 ± 0.02	4.30
Positive control (diclofenac 0.1%)	5	0.12 ± 0.01	0.13 ± 0.02	42.29**
100 mg (METS)	5	0.08 ± 0.02	0.13 ± 0.02	21.78**
200 mg (METS)	5	0.09 ± 0.01	0.11 ± 0.02	32.93*

* and **: significantly different from the negative control at $p < 0.05$ and 0.01 respectively

is richly endowed with capric acid. It is scientifically reasonable to posit that the inhibitory effect of METS in CGN could be attributed to IL-6 inhibition. Also, capric acid by its effect on ROS could attenuate the inflammatory response following CGN because of its antioxidant properties. Generally, fatty acids are known to have anti-inflammatory properties [64], and *T. superba* has 53% of its identified secondary metabolites as fatty acids (Table 2). All these fatty acids could have similar mechanism as the capric acid [22].

Also, inflammatory pathway is known to involve oxidative stress [50]. Supernatant obtained from the paw indicate alteration in the high level of MDA and lower level of SOD as a result of CGN administration (Fig. 3). These alterations which confirmed increase in oxidative stress were attenuated by the administration of METS. In vitro antioxidant assay of METS (Table 4) had shown a dose-dependent inhibition of DPPH, hydroxyl ions, and iron-reducing properties of *T. superba* which is found in other works [53].

Cell generation of reactive oxygen species serve both physiological and pathophysiological functions [32]. Following an injurious challenge like CGN, inflammatory processes ensues resulting in the activation of many cascade of pathways involving cytokines, acute phase proteins, and chemokines [32]. Activation of cytokines and chemokines like NF- κ B and TNF α lead to lipid peroxidation and inflammation [68, 71]. Antioxidants like the phenolics, flavonoids, etc., as found in *T. superba* has the ability to donate electrons and neutralize free radicals thereby preventing inflammation and its associated injuries [28]. We believe then that part of the anti-inflammatory property of *T. superba* could be accounted via its rich antioxidant property.

Application of xylene to the animal ear induced topical inflammatory process as evidenced by increase in the ear size (Table 6). The groups that received METS (100 mg and 200 mg) and standard (diclofenac) showed percentage inhibition of inflammation up to 22, 33, and 42, respectively. Xylene causes vasodilatation and edematous stress to the skin when applied. Suppression of these features signifies antiphlogistic effect. This increase in

histopathological changes in the ear was attenuated by METS in a dose-dependent manner which is in agreement with the works of [43, 65]. In the work of Li et al. [43], it is believed that these prevention of pathological alterations occurred because of decrease in ROS and attenuation of pro-inflammatory cytokines. The secondary metabolites observed in METS (Tables 2 and 3) is known to possess these properties.

Gas chromatography–mass spectrometry of detected compounds

The GC–MS of METS showed the following class of compounds: fatty acids (53%), ester (23%), organic compounds (12%), alkanes (9%), and carboxylic acids (3%).

Conclusion

T. superba possesses antioxidant, antibacterial, and anti-inflammatory properties which are believed to arise from the secondary metabolites observed in the GC–MS characterization. These observations can serve as the foundation for development of new therapies to overcome antibiotic resistance and manage inflammation related diseases.

Abbreviations

AA: Arachidonic acid; BDCP: Bioresources Development and Conservation Programme; CGN: Carrageenan; COX-2: Cyclo-oxygenase 2; DMSO: Dimethyl sulfoxide; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; EDTA: Ethylenediaminetetraacetic acid; Fe³⁺: Iron III; FeCl₃: Ferric chloride; FRAC: Ferrous reducing antioxidant capacity; GC/MS: Gas Chromatography–Mass Spectrometry; PGs: Prostaglandins; HCl: Hydrochloric acid; H₂O₂: Hydrogen peroxide; IFN: Interferon; IL: Interleukins; iNOS: Inducible nitric oxide synthase; IZD: Inhibition zone diameter; LXs: Lipoxins; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; METS: Methanolic extract of *Terminalia superba*; MIC: Minimum inhibitory concentration; NF- κ B: Nuclear factor kappa B; OH: Hydroxyl ion; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TBA: Thiobarbituric acid; TCA: Trichloroacetic acid; TLR: Toll-like receptors; TNF: Tissue Necrosis Factor; XLN: Xylene.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by NIA, KOO, and ROO. The first draft of the manuscript was written by NIA, and all authors commented

on previous versions of the manuscript. Conceptualization was done by NIA; methodology was done by KOO; formal analysis and investigation were carried out by NIA and KOO; writing—original draft preparation were done by ROO; writing—review and editing were done by KOO and NIA. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This work was approved by the botanical office/animal and welfare committee of Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, with approval Number UNN/PHARM/01/072.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Agbani, Enugu State, Nigeria. ²Department of Pharmacology and Therapeutics, College of Medicine, Enugu State University of Science and Technology, Enugu, Enugu State, Nigeria.

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