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Phytochemical Composition and Anti-inflammatory Activity of the Leaf Extract of *Eucalyptus tereticornis*

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Abstract

Eucalyptus tereticornis is a plant well-known for its various medicinal properties. The essential oil produced from *Eucalyptus tereticornis* leaf are found useful in ethnomedicine. This study evaluated the phytochemical composition and *in-vitro* anti-inflammatory activity of the leaf extract of *Eucalyptus tereticornis*. The analyses were done using standard biochemical methods. The anti-inflammatory activity at concentrations of 0.1, 1.0, 5 and 10 mg/ml was measured using three *in-vitro* assays viz; membrane stabilization, inhibition of albumin denaturation and anti-proteinase activity assays. Diclophenac was used as standard drug. The result of phytochemical analysis showed the presence of Tannins (6.02 ± 0.001 mg/g), Alkaloids (2.99 ± 0.00 mg/g), Saponin (7.12 ± 0.004 mg/g), Glycosides (8.68 ± 0.006 mg/g), Terpenoids (1.50 ± 0.002 mg/g), Flavonoids (9.09 ± 0.03 mg/g), Steroids (2.03 ± 0.04 mg/g) and Phenol (5.53 ± 0.01 mg/g). From the anti-inflammatory assay results, the extract and the standard displayed a concentration dependent activities which were significantly different ($p<0.05$). At the highest concentration of 10 mg/ml, the extract displayed 59.60% and 43.75% inhibition of heat and hypotonicity-induced haemolysis respectively while that of the standard were 76.32% and 56.19% inhibition respectively. The extract displayed 53.87% inhibition of albumin denaturation activity while the standard displayed 71.23% activity. The anti-proteinase activity of the extract was 28.60% while that of the standard was 57.65%. This study has shown that the leaf extract of Eucalyptus tereticornis contains bioactive compounds which are important therapeutic agents. The leaf could be used in design of a potent anti-inflammatory drug for management and treatment of diseases associated with inflammatory reactions.

Keywords: *Eucalyptus tereticornis*; Phytochemical; Anti-Inflammatory; Membrane Stabilization; Red Blood Cell; Diclophenac

Abbreviations: NSAIDs: Nonsteroidal Anti-Inflammatory Drugs; DFS: Diclofenac Sodium; ESUT: Enugu State University of Science and Technology; ANOVA: Analysis of Variance.

Introduction

Since ancient times, different parts of plants have been used for medicinal purpose and in recent times, there is increase in the study and search of medicinal herbs by pharmacological researchers. Plants are enriched with different organic compounds, of which many are utilized for medicinal purposes. These plants contain chemical substances called phytochemicals which are known to produce specific physiological actions in the body [1]. These phytochemicals are bioactive chemical compounds found naturally in plants. They are secondary metabolites that contribute to flavor, smell and color of plants. In addition, they form part of a plants natural defense mechanisms against diseases. They have been implicated in therapy and disease control [2]. Most of them such as phenols and flavonoids have

been reported to possess multiple biological properties such as anti-inflammatory, anti-microbial, cytotoxic, anti-tumor and antioxidant activities [3,4].

Inflammation is the reaction elicited by the vascular tissues of the body to injurious stimulant. This can cause pain, a rise in denaturation of protein, permeability of the vascular tissues and alteration of the membrane. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs to reduce pain and inflammation associated with arthritic disorders [5]. NSAIDs are nonspecific COX (cyclooxygenase) inhibitors; COX is responsible for prostaglandin synthesis, which mediates the process of pain and inflammation [5,6]. Diclofenac sodium (DFS) is globally the most widely prescribed NSAID in management of pain and inflammation in various diseases including rheumatoid arthritis and osteoarthritis [6]. However, most of these drugs have some negative side effects such as gastric ulcers leading to bleeding, heart problems and kidney issues. More so, some of these synthetic drugs are costly and may not be affordable for a common man. The high cost coupled with the adverse side effects of synthetic drugs are some of the considerations for an inclination towards medicinal drugs of natural products, which are presumed to be effective in treatment of chronic diseases. Therefore, this necessitates search for more widely available medicinal plants of natural origin with less cost and minimal side effects that will help prevent illnesses associated with inflammatory reactions.

Eucalyptus tereticornis is a specie of *Eucalyptus* and belongs to *Myrtacea* family. It originated from Australia but now widely found in other countries [7]. The plant is commonly called Mysore gum, Mysore hybrid or *Eucalyptus* hybrid and mainly used as fiber and timber source. *Eucalyptus tereticornis* grows to a height of 20 to 50 meters with a girth of up to 2 meters. The trunk is straight and is usually un-branched for more than half of the total height of the tree. Thereafter, limbs are unusually steeply inclined for *Eucalyptus* species. The bark is shed in irregular sheets, results in a smooth trunk surface coloured in patches of white, gray and blue, corresponding to areas that shed their bark at different times. It has a narrow, lanceolate green leaves, from 10 to 20 centimeters long, and one to nearly three centimeters wide. Flowers occur in inflorescence of 7 to 11 flowers. The *Eucalyptus tereticonis* is one of the key canopy species of the threatened Cumberland plain wood lands. *Eucalyptus* is well known for the essential oils extracted from the leaves which have been found useful for medicinal purposes as stimulant, anesthetic and antiseptic [8]. There are reports from studies of the anti-hyperglycemic and antimicrobial activities of the leaf extract [9,10]. The leaves are said to possess wound healing abilities when chewed while the decoction is used for relieve of fever and improvement of pulmonary associated difficulties [11].

Materials and Methods

Plant Collection and Identification

Fresh leaves of *Eucalyptus tereticornis* plant were collected from the environment of Enugu State University of Science and Technology (ESUT) Agbani in Enugu State, Nigeria. The plant was identified by a taxonomist in Nnamdi Azikiwe University, Awka, Nigeria and a specimen was deposited in the herbarium with voucher number NAUH169A.

Sample Preparation/Extraction

The leaves were dried at room temperature for two weeks and was ground with electric blender. One hundred grams (100g) of the sample were weighed into a conical flask. The powdered sample was macerated and extracted in 70% ethanol for 72hours. It was sieved and filtered using Whatman no 1 filter paper. The filtrate was evaporated to dryness at 50°C in a water bath.

Phytochemical Analysis

This was done qualitatively and quantitatively.

Qualitative Phytochemical Analysis

The qualitative phytochemical analysis was carried out in order to ascertain the presence of some plant secondary metabolites. The determinations were done by utilizing standard conventional protocols as illustrated by Harborne [12]; Trease, et al. [13]. The preliminary analysis involved testing for the presence of flavonoids, terpenoids, steroids, saponins, alkaloids, tannins, glycosides and phenols.

Quantitative Phytochemical Analysis

The quantitative analysis was also carried out using standard methods. Thus, alkaloid, terpenoid, glycoside and Saponin were determined according to the method of Harborne [12], Tannin was determined according to the method of Van-Burden, et al. [14], flavonoid was determined using the method of Bohn, et al. [15], steroid was determined according to the method of Okeke, et al. [16], total phenol was determined using Folin-Ciocalteu reagent method.

Anti-Inflammatory Assay

The anti-inflammatory activity of the ethanol leaf extract of *Eucalyptus tereticornis* was assayed using three in-vitro anti-inflammatory assays; anti-proteinase activity, inhibition of albumin denaturation and membrane stabilization assays. Diclophenac was used as the reference drug.

Preparation of the Standard Drug

One tablet (60 mg) of diclophenac drug was ground and dissolved in 10 ml of water. The extract was obtained by vortexing the mixture for 10 min and allowed to sediment for 30 min afterwhich the supernatant was filtered. The filtered sample was serially diluted to obtain the various concentrations used.

Anti-Proteinase Activity

Proteinase inhibitory activity of the leaf extract was performed according to the method of saket, et al. [17] and Oyedepo, et al. [18] with some modifications. The reaction mixture [2ml] contained 0.06 mg tripsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (0.1, 1, 5 and10 mg/ml)). The mixture was incubated at 37°C for 5 min and then 1ml of 0.02% (w/v) casein was added. The mixture was incubated for an additional 20 min at 37°C. Perchloric acid (2 ml; 2%) was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The percentage inhibition of proteinase inhibitory activity was calculated thus:

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs Control

Inhibition of Albumin Denaturation

Methods of Mizushima et al. [19] and Saket, et al. [17] followed with minor modification. The reaction mixture which consist of 0.5 ml of test extract and 0.5ml of 1% aqueous solution of bovine serum albumin fraction, pH of the reaction mixture was adjusted to 7.4 using small amount of HCL (1N) at 37°C. However, all the reaction mixtures of the test extracts, standard drug of various concentrations (0.1, 1, 5 and 10 mg/ml) and control (0.5 ml of distilled water and 0.5ml of 1% aqueous solution of bovina albumin fraction to generate 100% albumin denaturation) were incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling the turbidity was measured at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated thus:

Percentage inhibition = (Absorbance of control –Absorbance of Sample) X 100/ Absorbance of control.

Membrane- Stabilization Assay

Stabilization of erythrocyte membrane was evaluated by exposing RBC to heat-induced and hypotonicity–induced haemolysis.

Preparation of Erythrocyte Suspension

Erythrocyte suspension was prepared according to the method described by Shinde, et al. [20] with some modifications. Fresh whole human blood was collected through the vein using a sterile 10 ml syringe from a healthy human volunteer. The blood was centrifuged at 3000 rpm for 5 min in heparinized centrifuge tubes, and washed three times with equal volume of normal saline (0.9% NaCl). After the centrifugation, the blood volume was measured and reconstituted as a 10% (v/v) suspension with isotonic buffer solution (10 mM Sodium phosphate buffer pH 7.4).

Inhibition of Heat Induced Hemolysis

The analysis was done according to the protocol of Saket, et al. [17] and Shinde, et al. [20] with some modifications. The reaction mixture (2 ml) comprised 1 ml test sample at different concentrations (0.1, 1, 5 and 10 mg/ml) and 10% RBCs suspension (1ml). For control, only saline was added to the test tube. Diclophenac was used as a reference drug. The reaction mixture was incubated in water both for 30 min, at 56°C. The reaction was stopped by cooling the reaction mixture under running tap water. The reaction mixture was then centrifuged at 2500 rpm for 5 min. The supernatant collected was used to measure absorbance at 560 nm. The experiment was performed in triplicate. The percentage inhibition of haemolysis was calculated as follows:

Percentage inhibition = (Absorbance of control –Absorbance of sample) X 100/ Absorbance of control.

Inhibition of Hypotonicity- Induced Haemolysis

Inhibition of hypotonicity-induced hemolysis was done according to the protocol of Azeem et al. [21] with some modifications. Extracts of different concentration (0.0, 1, 5 and 10 mg/ml), reference sample (diclofenac sodium), and control were individually mixed with phosphate buffer pH 7.0 (1 ml), hyposaline (2 ml), and RBC suspension (0.5 ml). The reaction mixtures were incubated at 37°C for 30 min. It was then centrifuged at 3000 rpm. The supernatant was transferred, and absorbance was taken at 560 nm. The percentage haemolysis was calculated by assuming control as 100%

Percentage protection = 100- (Abs sample/Abs Control) x 100

Statistical Analysis

Data was analyzed using SPSS version 23. Statistical significance of the results between samples was determined using one way analysis of variance (ANOVA). Differences between means were considered significant at $P<0.05$.Values

are presented as mean ± standard deviation of triplicate values.

Results

Phytochemical Composition

The result of the qualitative and quantitative phytochemical analysis is presented in Table 1. Flavonoid was the highest in concentration while terpenoid was the least.

Phytochemical	Abundance	Concentration (mg/g)
Tannins	+++	6.02 ± 0.001
Alkaloid	++	2.99 ± 0.00
Saponin	+++	7.12 ± 0.004
Glycosides	+++	8.68 ± 0.006
Terpenoids	+	1.50 ± 0.002
Flavonoids	+++	9.09 ± 0.03
Steroids	++	2.03 ± 0.04
Phenol	+++	5.54 ± 0.01

Table 1: Qualitative and Quantitative Phytochemical Composition of leaf of *Eucalyptus tereticornis*.
Key: +++ (highly present); ++ (moderately present); + (present in trace concentrations).

Anti-inflammatory Activity

Anti-Proteinase Action: Table 2 shows the result of the percentage anti-proteinase activity of the ethanol leaf extract of *Eucalyptus tereticornis* in comparison with the reference drug. The percentage activities of the extract and the reference drug were dose-dependent. Nevertheless, that of the reference drug at all the doses tested were significantly higher than the extract ($p<0.05$).

Concentration (mg/ml)	*Eucalyptus tereticornis* % Inhibition	Diclophenac % Inhibition
0.1	12.51 ± 2.06^a	24.05 ± 2.25^b
1	18.61 ± 0.81^a	43.50 ± 1.25^b
5	22.19 ± 1.75^a	51.59 ± 2.06^b
10	28.60 ± 9.82^a	57.65 ± 5.38^b

Table 2: Anti-proteinase action of ethanol leaf extract of *Eucalyptus tereticornis* in comparison with the reference drug; diclophenac.
Values with different superscript letters on the same row are significantly different ($p<0.05$).

Inhibition of Albumin Denaturation Activity

Table 3 shows the result of the percentage inhibition of albumin denaturation activities of the ethanol leaf extract of *Eucalyptus tereticornis* and that of the reference drug, diclophenac. The activities were also dose-dependent with both samples displaying more than 50% activity at the highest concentration tested. However, the activities of the reference drug were significantly higher than that of the extract.

Concentration (mg/ml)	*Eucalyptus tereticornis* % Inhibition	Diclophenac % Inhibition
0.1	17.05 ± 3.86^a	29.97 ± 5.65^b
1	37.45 ± 1.52^a	42.02 ± 3.50^b
5	49.05 ± 0.72^a	66.16 ± 1.26^b
10	53.87 ± 1.43^a	71.23 ± 0.72^b

Table 3: Inhibition of Albumin denaturation activity of ethanol leaf extract of *Eucalyptus tereticornis* in comparison with the reference drug; diclophenac.
Values with different superscript letters on the same row are significantly different ($p<0.05$).

Membrane Stabilization Activity

Heat-Induced Haemolysis: Table 4 shows the result of percentage inhibition of heat-induced haemolysis of erythrocyte membrane by the extract in comparison with that of the reference drug. The extract activity and that of the reference drug were dose-dependent. The reference drug displayed significant higher activity ($p<0.05$) than the extract.

Concentration (mg/ml)	*Eucalyptus tereticornis* % Inhibition	Diclophenac % Inhibition
0.1	-13.78 ± 1.53^a	39.32 ± 4.38^b
1.0	34.52 ± 0.22^a	49.07 ± 3.72^b
5.0	49.54 ± 5.26^a	71.83 ± 0.00^b
10.0	59.60 ± 1.53^a	76.32 ± 2.41^b

Table 4: Inhibition of Heat-induced haemolysis activity of ethanol leaf extract of *Eucalyptus tereticornis* in comparison with the reference drug; diclophenac.
Values with different superscript letters on the same row are significantly different ($p<0.05$).

Hypotonicity-Induced Haemolysis

Table 5 shows the result of the inhibition of hypotonicity-induced haemolysis of the erythrocyte by the extract and the

reference drug. The activities of both were dose-dependent, however, there was a decline in activity of the reference drug at 10 mg/ml. The activities of the reference drug at all the concentrations tested were significantly higher ($p<0.05$) than that of the extract.

Concentration (mg/ml)	*Eucalyptus tereticornis* % Inhibition	Diclophenac % Inhibition
0.1	10.49 ± 5.89^a	25.31 ± 1.75^b
1.0	20.14 ± 6.44^a	44.83 ± 4.26^b
5.0	42.13 ± 1.53^a	57.72 ± 2.40^b
10.0	43.75 ± 1.42^a	56.10 ± 2.07^b

Table 5: Inhibition of Hypotonicity-induced haemolysis activity of ethanol leaf extract of *Eucalyptus tereticornis* in comparison with the reference drug; diclophenac.
Values with different superscript letters on the same row are significantly different ($p<0.05$).

Discussion

In recent times, there has been an increase in search of natural products with biologically active constituents and biological activities. The leaf extract of *Eucalyptus tereticornis* was subjected to phytochemical screening and anti-inflammatory activity. This study revealed the presence of phytochemicals which are active medicinal chemical constituents such as saponins, tannins, glycoside, flavonoids, phenols, alkaloids, terpernoids, and steroids as shown in Table 1. Flavonoid was the highest in concentration (9.09 ± 0.03 mg/g). Glycosides (8.68 ± 0.006 mg/g), saponin (7.12 ± 0.004 mg/g), tannins (6.02 ± 0.001 mg/g) and phenol (5.54 ± 0.01 mg/g) were also highly present while alkaloids (2.99 ± 0.00 mg/g) and steroids (2.03 ± 0.04 mg/g) were slightly present. Terpenoid was least in concentration (1.50 ± 0.002 mg/g). These phytochemicals have been reported to possess medicinal properties. Flavonoids and phenols possess antioxidant activities and play vital roles in scavenging of free radicals [22]. They are reportedly involved in many physiological activities which include anti-inflammatory, anti-oxidant, anti-allergic [23], anti-microbial, anti-diarrheal and anti-cancer [24]. Plant extracts that contain glycosides are said to be useful as flavouring agents in pharmaceutical preparations [25]. This shows that leaf extracts of *Eucalyptus tereticornis* could be used as flavouring agents. This confirms the usage of its essential oil in perfumery and fragrance industries. Saponins are also glycosides which are amphipathic in nature and are used as adjuvants in the treatment of cancer [26]. They lower the amount of circulating cholesterol and prevent its uptake by forming complexes with dietary cholesterol in the intestinal walls [27]. Tannin possess wound healing properties as well as anti-inflammatory, antimicrobial and analgesic properties [28]. Alkaloids have been reported to possess anti-inflammatory effects [29]. Plant steroids are used in therapy as arrow poisons or cardiac drugs [30]. The amount of steroid present in the leaf extract of *Eucalyptus tereticornis* is relatively enough and could be used in promoting nitrogen retention in osteoporosis and in animals with wasting illness [31,32]. Terpenoids possess medicinal activities such as anti-carcinogenic, antimalarial, anti-ulcer, antimicrobial and diuretic activity [33].

The results of the anti-inflammatory activity show that ethanol leaf extract of *Eucalyptus tereticornis* possess anti-inflammatory properties. This was demonstrated using three in-vitro anti-inflammatory assays, viz; anti-proteinase, inhibition of albumin denaturation and membrane stabilization assays. Proteinases cause tissue damages during inflammatory reactions such as in arthritis. Serine proteinases reside in the lysosomal granules of neutrophils [34]. Proteinase inhibitors protect against the action of these proteinases. From this study, the leaf extract of *Eucalyptus tereticornis* displayed a dose-dependent inhibitory action against the activity of proteinase.

The extract and reference drug inhibited albumin denaturation. The activities were dose-dependent but the reference drug displayed significantly ($p<0.05$) higher activity than the extract. However, the extract displayed more than 50% activity (53.87 ± 1.43 %) while the reference drug gave 71.23 ± 0.71% activity at the highest concentration tested (10 mg/ml). From studies, it has been reported that protein denaturation causes inflammation leading to inflammatory diseases such as rheumatoid arthritis [35,36]. Therefore, the leaf extract of Eucalyptus tereticornis could serve as an anti- rheumatoid arthritis agent as there has been reports on the ability of compounds capable of inhibiting thermally-induced protein denaturation having potential therapeutic value and could be used as anti-inflammatory agent [37,38].

The result of the erythrocyte membrane stabilization assay showed that the extract and the reference drug stabilized the human erythrocyte against heat and hypotonic-induced haemolysis. The activity was dose-dependent producing more than 50% activity at the highest concentration tested (10 mg/ml). The erythrocyte membrane components have been demonstrated to be similar to that of lysosomal membrane, hence, agents with the ability to stabilize human erythrocyte from heat and hypotonic-induced haemolysis could be a potential source of anti-inflammation [39].Therefore, the leaf extract of *Eucalyptus tereticornis* could be considered as a potential source of human erythrocyte stabilizer.

Although the reference drug; diclophenac displayed significantly higher ($p<0.05$) activities, the result showed

that the extract possess anti-inflammatory activity. These anti-inflammatory activities could be linked to the presence of the polyphenolic compounds such as flavonoids, phenols, alkaloids, tannins and steroids as shown in this study. These compounds have been shown to possess anti-inflammatory properties [40].

Conclusion

This study has shown that the leaf extract of *Eucalyptus tereticornis* contains bioactive compounds which are important therapeutic agents. From the anti-inflammatory activity displayed, the leaf could be used in design of a potent anti-inflammatory drug for management and treatment of diseases associated with inflammatory reactions.

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