

Antioxidant Activity of Ethanol Leaf Extract of *Clerodendrum Splendens* G. Don (Verbenaceae)

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Abstract

The aim of the study was to evaluate the antioxidant activity of the ethanol leaf extract of *Clerodendrum splendens*. Three hundred and fifty grams (350 g) of the powdered leaf sample was macerated in 1500 ml of ethanol for 48 hours. The ethanol extract (EE) was partitioned into n-hexane, ethyl acetate and aqueous ethanol using the separating funnel to obtain the respective fractions. Phytochemical screening and acute toxicity studies were conducted for EE. The antioxidant activities of the extracts were analyzed, in comparison with ascorbic acid (AA), using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay for concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml. The percentage inhibition power, IC₅₀ and EC₅₀ values were calculated accordingly. The results of the phytochemical screening showed that alkaloids, tannins, flavonoids, steroids, saponins, glycosides and terpenoids were present in EE. In the acute toxicity study, mice administered a dosage of 5000 mg/kg were without experiencing any fatalities. The DPPH assay indicates that the most active fraction was the aqueous ethanol fraction with the lowest IC₅₀ value of 0.31 mg/ml, while AA had an IC₅₀ value of 0.0285 mg/ml. The FRAP results also indicates that the most active fractions were the ethyl acetate fraction with EC₅₀ values of 0.29 mg/ml, while AA had an EC₅₀ value of 0.057 mg/ml. The antioxidant activity of EE is similar to that of ascorbic acid, and it can be utilized to treat and prevent disorders associated to oxidative stress.

Keywords: *Clerodendrum splendens*, Ascorbic acid, Antioxidants, Oxidative stress

Introduction

The condition known as oxidative stress is brought about by an imbalance between the body's capacity to synthesize and biologically detoxify accumulated reactive products like reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells and tissues. ROS oxidize sensitive biomolecules and stay in the body longer when antioxidant defenses are unable to effectively neutralize them [1]. These reactive species assault essential biological components including DNA, RNA, lipids, proteins, and carbohydrates when their levels are not controlled. This can result in destruction of tissues, cell death, and the eventual emergence of chronic diseases such as diabetes, hypertension, Alzheimer's disease, and cancer [2,3]. One mediator in vascular relaxation that is dependent on the endothelium is the NO⁻ radical. Under oxidative stress circumstances, NO⁻ which is usually synthesized by the enzyme Nitric Oxide Synthase (NOS) reacts with the radical O²⁻ to form ONOO⁻, which damages endothelium [4,5]. However, according to the oxidative stress theory, reactive oxygen species' interactions with the DNA, lipid, and protein constituents of cells result in the build-up of irreversible, oxidation-induced damage that eventually causes ageing. Highly common chronic age-related disorders including diabetes, hypertension, atherosclerosis, and rheumatoid arthritis all exhibit elevated oxidative stress, even if the ageing process itself is determined to be unrelated to oxidative stress [6-9]. Medicinal plants contain various phytoconstituents that have largely been connected to a wide spectrum of pharmacological activities which include antioxidants properties that play a protective role in the body against diseases such as cancer, heart disease, hypertension etc.

Clerodendrum splendens, commonly known as glory tree or flaming glory flower is a member of the Verbenaceae family, and the genus includes over 400 tropical herbs, vines, shrubs, and trees, many of which are cultivated as garden plants [10,11]. It is an evergreen

climber plant that blossoms during the dry season, producing attractive red flowers [12]. The leaves are opposite, simple, 6-12 cm long and 2-6.5 cm broad, with an entire margin, dark green colour from inner side and pale green colored from outside, texture is smooth, leaf is petiolate [13]. Numerous chemical components, including steroids, terpenoids, flavonoids, volatile constituents, cyanogenic glycoside, alkaloids, tannins, saponins, and phenolic compounds, have been isolated from *Clerodendrum splendens* after comprehensive investigation, with some being responsible for the pharmacological activity of the plant [10,14,15].

Traditional medicine uses the leaves of *Clerodendrum splendens* to treat urinary tract infections, and inflammations [10]. In Nigeria, people use a decoction of *Clerodendrum splendens*' roots and leaves to cure inflammation, tumors, malaria, skin conditions, ulcers, and stomachaches [14]. Additionally, rheumatism, asthma, and other inflammatory diseases have been treated with plant extracts from the roots and leaves [10] and according to reports from traditional healers in Southeastern Nigeria, *Clerodendrum splendens* is used to cure syphilis, gonorrhoea, and fibroids [14]. The plant is used as a vermifuge, febrifuge, and to cure beriberi disease in addition to treating cough, scrofulous infection, buboes difficulties, venereal infections, and skin ailments [10,14]. Some members of *Clerodendrum* genera such as *C. serratum*, *C. infortunatum*, *C. inerme* and *C. phlomidis* have all been reported to possess high antioxidant properties [16-19]. The aim of this study was to evaluate the antioxidant activity of ethanol leaf extract of *Clerodendrum splendens*.

Materials and Methods

Collection of plant materials

Fresh leaves of *Clerodendrum splendens* were collected from Nsukka, Enugu State, Nigeria in June 2023. It was authenticated by Mr. Ozioko of the International Centre for Ethnomedicine and Drug Development (ICEDD). A voucher specimen with the number ESUT/COG/202008 was kept in the herbarium of the Department of Pharmacognosy, Enugu State University of Science and Technology.

Preparation of plant extract

The leaves were shade-dried at room temperature for about 7-10 days and ground with a grinder until a coarse powder is formed. The powdered plant samples were stored in airtight containers. An amount (350 g) of the powdered sample was macerated in ethanol (1500 ml) for 48 hours with the use of mechanical shaker for continuous agitation of the mixture. Muslin was used to filter the extract first, then Whatman (No. 1) filter paper was used for a second filtering step. The dried crude extract was partitioned with n-hexane, ethyl acetate and aqueous ethanol using separating funnel. The individual fractions were concentrated with rotary evaporator set at 40°C and 120 rpm and the resultant extracts were further dried at 40°C in the oven.

Phytochemical screening

The tests for saponins, terpenoids, tannins, flavonoids, steroids, alkaloids and glycosides were conducted following the protocols described [20-22].

Acute toxicity study (LD₅₀)

The protocol according to Lorke's method [23] was used to estimate the oral acute toxicity of the plant extract. Nine mice were used in the first part of Lorke's approach. The mice were split into three groups of three mice each, and they received oral dosages of 10, 100, and 1000 mg/kg body weight of the extract. The mice were then monitored for 24 hours to see whether any of them had died. Following the first phase, in which no mice died, three new mice were divided into three groups of one mouse each, and oral dosages of 1600, 2900, and 5000 mg/kg of the extract were given. The number of fatalities in a 24-hour period was also recorded.

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The procedure outlined by Beatrice *et al.* [24] was followed to carry out the DPPH radical scavenging experiment using 1,1 diphenyl-2-picrylhydrazyl (DPPH). Five different concentrations of the plant extract and fractions (1, 0.5, 0.25, 0.125, and 0.0625 mg/ml) were made using methanol. Standard antioxidant, ascorbic acid was also prepared at the same concentrations and 1 ml of the examined extracts were measured in neat test tubes, mixed in 3 ml of DPPH and made up to 10 ml using methanol. After giving the mixture a good shake, it was kept at room temperature for half an hour in the dark. The same method was done for the standard. The absorbance values were determined with a UV spectrophotometer at 517 nm. The experiment was conducted in triplicates. The DPPH radical scavenging activity was calculated using Equation 1.

$$\text{DPPH scavenging activity (\%)} = \frac{A_b - A_s}{A_b} \times 100 \dots \text{Equation 1}$$

A_b = Absorbance of blank

A_s = Absorbance of sample

A plot of the extract concentrations against the percentage of DPPH free radical inhibitions was used to calculate the half maximum inhibitory concentration (IC₅₀) of the extracts.

Determination of ferric reducing antioxidant power (FRAP)

The ferric reducing power was conducted following the protocols described [24]. Five distinct methanol extract concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/ml were prepared in test tubes and 2.5 ml of 2 M of phosphate buffer was added to each test tube (pH of phosphate buffer was 6.6). The solution was properly mixed and 2.5 ml of potassium ferricyanide was added, shaken slightly and covered with foil and incubated in a water bath for 20 minutes at 50°C. Then, 2.5 ml of 10% trichloroacetic acid was added to the solution and centrifuge at 3000 rpm for 10 minutes. Next, 2.5 ml of the supernatant was taken and mixed with 2.5 ml of deionized water. This was done respectively. The solution was mixed and 0.5 ml of 10% ferric chloride was added. The absorbance was read at 700 nm using a UV spectrophotometer. This was also done for the standard (positive control) that is ascorbic acid. The blank contained water and other reagents with no plant extract. The graph of absorbance at 700 nm against extract concentrations was used to identify the concentrations that might produce an absorbance value of 0.5, which was then regarded as the median effective concentration (EC₅₀). The assay was conducted in triplicates.

Statistical analysis

GraphPad software was used to statistically analyze the data using One-way Analysis of Variance (ANOVA). The Tukey comparison test was then used to determine significance. Significance level of P<0.05 was applied and Microsoft Excel 2013 was used to create the graphic illustrations.

Results and Discussions

Numerous chronic conditions and prevalent disease conditions have been linked to oxidative stress that eventually causes the oxidation of susceptible biomolecules, resulting in those diseases [1]. Twenty-four hours (24 h) after the extract was administered, *Clerodendrum splendens* did not produce any noticeable symptoms of toxicity up to a level of 5000 mg/kg. The results of the first and second phases of acute toxicity study, as presented in [Table 1](#) validated that there had been no discernible alterations in the mice's motor activity, awareness and no paralysis, sluggishness, tachycardia, restlessness, diarrhea, convulsions, or coma were recorded.

Table 1: Results of acute toxicity studies of the crude extract.

Phases	Treatments	Numbers of death/replicate
I	Group 1 (10 mg / kg)	0/3
	Group 2 (100 mg / kg)	0/3
	Group 3 (1000 mg / kg)	0/3
	Group 4 (1600 mg / kg)	0/3
II	Group 5 (2900 mg / kg)	0/3
	Group 6 (5000 mg / kg)	0/3

The percentage yield result showed that 13.16% of the powered *Clerodendrum splendens* plant sample's components were fully extracted using ethanol. The proportion of 13.16% indicates that the ethanol solvent is appropriate for extracting a respectable amount of the plant sample's bioactive components. The percentage yield of the crude extract for the fractions were 1.76, 4.37 and 14.15% for the n-hexane, ethyl acetate and aqueous ethanol fractions respectively.

The result of the phytochemical analysis, as shown in [Table 2](#), shows that the crude extract of *Clerodendrum splendens* contains secondary metabolites such as tannins, glycosides, flavonoids, saponins, terpenoids, and steroids. The presence of these phytochemicals in these plant materials could be said to be responsible for their physiological and pharmacological activities. Polyphenolics, like tannins and flavonoids which are present in both plants, display excellent antioxidant activity due to the presence of hydroxyl groups that act as positive moieties for antioxidant activity [25].

Table 2: Results of Phytochemical screening

S/n	Secondary metabolites	Observations
1.	Saponins	+
2.	Terpenoids	+
3.	Tannins	+
4.	Flavonoids	+
5.	Steroids	+
6.	Alkaloids	+
7.	Glycosides	+

Key: + = Present - = Absent

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical is a quick, easy, and affordable way to assess a compound's antioxidant potential. It is frequently used to assess a compound's capacity to function as a hydrogen provider and free radical scavenger. The DPPH test depends on the stabilized free radical DPPH being eliminated. The DPPH radical has a dark purple hue in solution, but when reduced as well as transformed into DPPH-H (gain of hydrogen), it turns colorless or light yellow [26,27].

Based on Fidrianny *et al.* [28] criterion for assessing the DPPH free radical scavenging effects, the plant under study exhibited significant antioxidant properties, as evidenced by IC₅₀ values of its crude extracts and fractions being less than 50 mg/ml. The % inhibition power results obtained from the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, which is based on the gain of hydrogen, exhibited a concentration-dependent effect for the standard drug (Ascorbic acid), crude and fractions of the leaf extract of *Clerodendrum splendens* as depicted in Table 3. The percentage inhibition power increased as the concentration increased as shown in Table 3. The half maximal inhibitory concentration (IC₅₀), which is the concentration of the drug or extract at which 50% inhibition power can be obtained, is the determinant of antioxidant activity for the assay [24]. The lower the IC₅₀ value, the higher the antioxidant activity. The most active fraction for the leaf extract of *Clerodendrum splendens*, as also shown in Table 3, is the aqueous ethanol fraction with the lowest IC₅₀ value of 0.031 mg/ml which is comparable to the IC₅₀ value of 0.028 mg/ml for the standard drug. The n-hexane fraction had the least activity with the highest IC₅₀ values of 0.0645 mg/ml.

Table 3: Results of the % inhibition power and IC₅₀ values of the Standard, crude extract and fractions

Sample	0.0625 mg/ml	0.125 mg/ml	0.25 mg/ml	0.5 mg/ml	1 mg/ml	IC ₅₀ (mg/ml)
Standard (Ascorbic acid)	97.76±0.046	97.84±0.046	97.86±0.046	97.94±0.046	98.56±0.375	0.0285
Crude extract	77.59±0.052*	86.86±0.080*	90.84±0.354*	91.38±0.172*	92.13±0.165*	0.036
n-hexane	48.96±1.497*	58.34±0.286*	65.64±0.677*	68.86±0.565*	78.19±1.950*	0.0645
Ethyl acetate	80.97±0.244*	90.19±0.240*	93.54±0.203*	95.35±0.037*	95.51±0.037*	0.0345
Aqueous ethanol	89.00±0.167*	91.57±0.085*	92.05±0.160*	93.84±0.080*	94.59±0.206*	0.031

Values are presented as Mean±SD. There is a significant difference across all groups when compared to the standard at $p<0.05$ using one-way ANOVA. (*) represents group with significant difference when compared with the standard drug of the same concentration.

In contrast to other assays that evaluate the inhibition of free radicals, the Ferric Reducing Antioxidant Power (FRAP) test is the only one that analyses antioxidants in a sample directly [29]. When ferric iron (Fe²⁺) is reduced to ferrous ion (Fe²⁺), a blue-colored result, the absorbance values from the FRAP assay indicate the equivalent quantity of antioxidants that donate electrons [29]. Increase in absorbance at the wavelength used for the assay (700 nm) indicates an increase in reducing power [30].

The results of the FRAP protocol conducted on the standard drug (ascorbic acid), the plant extract and fractions also exhibited a concentration-dependent effect as depicted in Table 4. The absorbance values increased with increase in concentration as shown in Table 4. The antioxidant activity of the plant samples used in the experiment was determined by the median effective concentration (EC₅₀), which is the concentration of an antioxidant that will result in an absorbance value of 0.5 [24]. The absorbance value of 0.5 indicates that 50% of the ferric ions in the test sample have been reduced to ferrous ions. The most active fraction of the plant extract was the ethyl acetate fraction with the lowest EC₅₀ value of 0.29 mg/ml while the standard has an EC₅₀ value of 0.057 mg/ml. The least active fraction remains the n-hexane fraction with EC₅₀ values of 1.09 mg/ml. Thus, in descending order of their activity in the FRAP assay as shown in Table 4.

Table 4: Result of the absorbance values and EC₅₀ values of the Standard, crude extract and fractions of the ethanol leaf extract of *Clerodendrum splendens*

Sample	0.0625 mg/ml	0.125 mg/ml	0.25 mg/ml	0.50 mg/ml	1.00 mg/ml	EC ₅₀ mg/ml
Standard (Ascorbic acid)	0.553±0.012	0.672±0.025	3.00±00	3.00±00	3.00±00	0.057
Crude extract	0.215±0.006*	0.301±0.007*	0.476±0.012*	0.581±0.011*	0.993±0.062*	0.37
n-hexane	0.113±0.003*	0.214±0.002*	0.291±0.005*	0.365±0.016*	0.442±0.010*	1.09
Ethyl acetate	0.254±0.020*	0.363±0.010*	0.495±0.008*	0.839±0.002*	0.856±0.004*	0.29
Aqueous ethanol	0.192±0.012*	0.229±0.024*	0.26±0.018*	0.387±0.032*	0.814±0.02*	0.57

Values are presented as Mean±SD. There are significant changes across all groups when compared to the standard at $p<0.05$ using one-way ANOVA. (*) represents group with significant difference when compared with the standard drug of the same concentration

The antioxidant activity of a plant can be determined by the amount of radical scavenging compounds, such as polyphenolics and carotenoids, present in the plant and the ability of the solvent, being used for extraction, to extract the phytochemicals responsible for the antioxidant activity of the plant [31]. This could account for the high antioxidant activities of the most active fractions (Aqueous ethanol and ethyl acetate) and ethanol crude extracts of the two plants, indicating their ability extract high levels of radical scavenging compounds and the poor activity of the n-hexane fraction due to its inability to properly extract the phytochemicals responsible for the

radical scavenging activity. Generally, the plant has proven to have good antioxidant property, comparable to the standard and can be effective in the treatment of oxidative stress related diseases.

Conclusion

In conclusion, the study indicates that the leaf extract of *Clerodendrum splendens* has a high radical scavenging potential which is dose dependent. Thus, the extract can be used as a natural source of antioxidants for the treatment and prevention of oxidative stress related disease conditions.

Conflict of interest

The authors have declared no conflict of interest.

Authors' contributions

Conceptualization: PEO; Supervision: COE; CEOU; Plant sample collection, extraction and partition: ACE, PEO; DPPH and FRAP assays: PEO, ACE; Acute toxicity study: ROO, PEO; Phytochemical screening: JIA, PEO; Review & Editing: COE, CEOU; Data Curation & Software: ACE, SOO; Data analysis: SOO, CEO.

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