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ASSESSMENT OF CYTOTOXICITY, ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF THE ETHYLACETATE EXTRACT OF *CALLIANDRA PORTORICENSIS* ROOT BARK

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ABSTRACT: *Calliandra portoricensis* a medicinal plant, is widely use in the treatment/management of various diseases in Nigeria. The ethyl acetate extract of the *Calliandra portoricensis* root bark were tested to evaluate the cytotoxicity, antioxidant (*in vitro*) and free radical scavenging activity. The different assay methods, including total antioxidant activity, free radical (nitric oxide, hydroxyl radicals and lipid peroxidation) scavenging assays were used to evaluate the antioxidant potential of the ethylacetate extract. While cytotoxic activities were evaluated using brine shrimps lethality assays. The methanol extract showed total antioxidant capacity of 2.362µg/mg of plant extract expressed as ascorbic acid equivalents (AAE) compared to 1µg/mg ascorbic acid; the extract exhibited two-fold scavenging activity with IC_{50} of 364.175 and 313.52µg/ml for nitric oxide and hydroxyl radicals compared to IC_{50} of 231.31µg/ml and 228.78µg/ml of ascorbic acid as standard respectively. The extract conferred 50% protection at the concentration of 51.92µg/ml on lipid peroxidation induced by $FeSO_4$ in liver mitochondria. The Brine shrimp lethality bioassay of the extract showed cytotoxic activity of (LC_{50} = 0µg/ml) which falls within 0-100µg/ml considered to be lethal. In conclusion, the study clearly indicated that the ethyl acetate extract of *Calliandra portoricensis* root bark possesses potent bioactive compounds, good antioxidant and free radical scavenging activity along with moderate toxicity which can be harness and purified into useful therapeutic drugs.

INTRODUCTION: Free radicals including superoxide anions, hydroxyl radicals, hydrogen peroxide and nitric oxide are byproducts of biological reactions [1] which are germane to physiological processes such as energy generation, phagocytosis, regulation of cell growth and intracellular signaling [2]. Albeit, free radicals has implicative deleterious effects in the depletion of immune system antioxidants, change in gene expression and induce abnormal proteins and contribute to more than one arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS as a result of damage to biomolecules (DNA, membrane lipids, proteins) [3-4]. A potent safe scavenger of these radicals may serve as a possible preventive intervention for free radical-induced diseases [5]. Recent studies showed that plants produces a wide array of bioactive principles or compounds including polyphenolic compounds (e.g., flavonoids, tannins)

which exerts antioxidant activities either independently or synergistically with *in vivo* antioxidants [6-11]. Plants constitute a rich source of medicine and have replaced synthetic compounds/drugs because of their probable carcinogenic, mutagenic activities against vital organs (lungs, liver etc) [12].

Calliandra portoricensis, is a perennial shrubby plant with slender branches, cream colored flowers and flat fruits. It is widely distributed in West Africa especially in Togo (Misahohe), Gold Coast (Odumase, Aburi), Southern Nigeria (Bonny, Oban, Aguku and Lagos); West Indies and the Atlantic Coast of America. Its leaves are used to treat tonsillitis, spasmodic, diarrheal, malarial, stomach ulcer and other gastrointestinal disorders [13-16]. Its roots have been reported to possess anti-inflammatory, antifungal and antibacterial activities [17-18]. In South eastern Nigeria, traditional herbalists have effectively used extracts of *Calliandra portoricensis* to treat the lethal envenomation of carpet viper (*Echis ocellatus*). [19]. In light of this, this work is therefore designed to evaluate cytotoxicity (using brine shrimps lethality test) and antioxidant and free radical scavenging activity of ethylacetate extract of *Calliandra portoricensis* root bark.

MATERIALS AND METHODS:

Chemical: Thiobarbituric acid, sodium nitroprusside, sodium dodecyl sulphate and sulphanilic acid were purchased from Sigma Co. (St. Louis, USA), 2-deoxy-D-ribose, L-ascorbic acid, ammonium molybdate, trichloroacetic acid, 95% methanol and n-hexane from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals and reagents used were of analytical grade.

Plant collection and extraction: About 500g root of *C. portoricensis* was collected and authenticated from the medicinal plant garden of the Botany Department University of Ibadan, Nigeria. The root was freshly harvested, washed and the peeled barks were air-dried and pulverized using a hammer mill (Trapp TRF 80, Trapp Metallurgical, Brazil), and thereafter powdered at room temperature. The powdered samples (500g each) were suspended and extracted in 2.5L of methanol (w/v) and kept at 25^{o}C for 3 days. The extracts were filtered through Advantech -4B filter paper (Tokyo Roshi Kaisha Ltd., Japan).

The extraction of the residue was repeated twice under the same conditions. The methanol extract was first dried using a vacuum rotary evaporator (N-1000; EYLA, Tokyo, Japan) in a water bath at 40°C.

The crude methanol extract *Calliandra portoricensis* root bark was extracted sequentially using n-hexane, chloroform, ethylacetate and methanol as solvents. The ethylacetate extract obtained from the above method was used for further analysis.

In vitro Antioxidant Activity:

1. **Determination of Total Antioxidant Capacity:** The antioxidant activity of the ethylacetate extracts of *Calliandra portoricensis* root bark was evaluated by the phosphor- molybdenum method according to the procedure of Prieto *et al.*, [20]. 0.3 ml (the conc. of the solution is 5 µM/ml) of extract was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95^{o}C for 90 min and cooled to room temperature. Finally, absorbance was measured at 695 nm using a spectrophotometer against blank. The total antioxidant capacity was expressed as the number of equivalents of ascorbic acid.

2. **Nitric oxide (NO) Radical Scavenging Assay:** Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction [21, 22]. The reaction mixture (3 ml) containing sodium nitroppruside (10 mM) in phosphate buffer saline (PBS) and the extract from (100 - 400) µg/ml was incubated at 25°C for 150 mins.

   After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test preparations. Ascorbic acid, was used as a positive control.

3. **Hydroxyl Radical Scavenging Assay:** The assay was performed as described by Halliwell *et al.*[23] with some modifications. All solutions were prepared fresh essentially 1.0 ml of the reaction mixture contained 28 mM 2-deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 500 µl of various concentrations of methanol extract of *calliandra portoricensis* root bark (100-400 µg/ml), 200µM Fecl3 and 1.04 mM EDTA (1:1 v/v), 1.0 mM H_2O_2 and 1.0 mM ascorbic acid. After incubation period of 1 h at 37^{o}C, 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) were added and incubated at 100^{o}C for 20 min. After cooling, Absorbance was measured at 532nm against sample blank and ascorbic acid was used as a positive control.

4. **Estimation of Lipid Peroxidation:** A modified thiobarbituric acid reactive species (TBARS) assay[24] was used to measure the lipid peroxide formed using liver mitochondria as lipid rich media[25]. The liver mitochondria which was obtained by method of Schneider (1984) in combination with that of Johnson and Lardy [26].

5. **Brine Shrimp Lethality Bioassay:** The toxic potentiality of the ethylacetate extract of *Calliandra portoricensis* root bark was evaluated using Brine Shrimp lethality bioassay method [27] where 3 graded doses (viz, 10, 100, and 1000ppm) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine solution with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment. DMSO was used as solvent and also as a negative control. The median lethal concentration LC_{50} of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. And LC_{50} values at 95% confidence interval for statistically significant comparisons of potencies less than 100 ppm or between 0-100ppm was considered as potent [28].

Statistical analysis: Experimental results are expressed as Mean ± SD of three parallel measurements (n=3). Statistical evaluation was done by using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The statistical significance was at a $p<0.05$.

RESULTS:

Total Antioxidant Activity/Capacity: The antioxidant activity of ethylacetate extract of *Calliandra portoricensis* root bark at 100, 200, 300 and 400µg/ml concentrations was measured by the phospho-molybdenum method. Total antioxidant capacity of the ethylacetate extracts of *Calliandra portoricensis* was calculated using the standard curve of ascorbic acid ($y = 1.413x - 1.1606$; $R^2 = 0.992$) and is expressed as number of equivalent of ascorbic acid per gram of plant extract (AAE). The total antioxidant capacity of ethylacetate extract of *Calliandra portoricensis* was found to be 2.362 µg/mg of plant extract (expressed as ascorbic acid equivalents) which was comparable to that of ascorbic acid(1 µg/mg) as shown in **table 1**.

TABLE 1: TOTAL ANTIOXIDANT CAPACITY OF THE ETHYLACETATE EXTRACT OF *CALLIANDRA PORTORICENSIS* ROOT BARK THE EXTRACT IS SIGNIFICANTLY(*P<0.05) HIGHER THAN STANDARD (VITAMIN C)

Samples	EC_{50} Effective concentration	Equivalent to ascorbic acid (µg/mg plant material)
Ethylacetate extract	336.61	2.362
Vitamin C (ascorbic acid)	795.2	1

Nitric Oxide Scavenging Activity of the Ethylacetate Extract of *Callaiandria portoricensis* Root Bark: The effect of the ethylacetate extract of *C. portoricensis* on the inhibition of nitric oxide production was assessed and measured by Griess reaction [21, 22]. **Table 2** shows results of nitric oxide scavenging activity of the methanol extract of *Calliandra portoricensis* root bark and vitamin C as standard at varying concentrations of 100, 200, 300, and 400 µg/ml. Sodium nitroprusside in aqueous solution at physiological pH generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitric oxide. The ethylacetate extract of *Calliandra portoricensis* root

bark in this study showed significant inhibition of NO with IC_{50} value of 364.175µg/ml compared to vitamin C with IC_{50} value of 231.31 µg/ml as standard. This indicates that the ethylacetate extract of *Calliandra portoricensis* root bark is a good scavenger of nitric oxide.

TABLE 2: NITRIC OXIDE SCAVENGING ACTIVITY OF ETHYLACETATE EXTRACT OF *CALLIANDRA PORTORICENSIS* ROOT BARK

Concentration of sample (µg/ml)	% scavenging activity (µg/ml) EECPRB	IC_{50}(µg/ml)	% scavenging activity (µg/ml) Vitamin C	IC_{50}(µg/ml)
100	28.254±0.242*	364.175	20.433±0.191*	231.31
200	51.114±0.153*		61.264±0.134*	
300	71.522±0.202*		76.558±0.399*	
400	93.611±0.192*		98.235±0.49*	

Values are means±SD values differ significantly at *p<0.05 n=5

Hydroxyl Radical Scavenging Activity: The effect of methanol extract of *C. portoricensis* root bark on the inhibition of hydroxyl radical production was assessed by the iron (II)-dependent deoxyribose damage assay. **Table 3** presents the results of the effects of examined methanol extract as well as known antioxidant (vitamin C) on $OH^{\cdot}$ radical production at varying concentrations of 100, 200, 300, and 400µg/ml. The ethylacetate extract of *Calliandra portoricensis* root bark in this study significantly inhibited HO^{-} Radical formation thus preventing the degradation of 2-deoxy-2-1 ribose with IC_{50} value of 313.52µg/ml compared with vitamin C IC_{50} of 228.78µg/ml as standard. The percentage of hydroxyl radical scavenging activity increased with the increasing concentration of extract and known antioxidants. At a concentration of 400 *µ*g/mL, the extract shows the maximum inhibitory effect of about 93.05% which was comparable to that of L-ascorbic acid (82.2%). Thus, the ethylacetate extract of *Calliandra portoricensis* could scavenge hydroxyl radicals effectively.

TABLE 3: HYDROXYL RADICAL SCAVENGING ACTIVITY OF ETHYLACETATE EXTRACT OF *CALLIANDRA PORTORICENSIS* ROOT BARK

Concentration of sample (µg/ml)	% scavenging activity (µg/ml) EECPRB	IC_{50}(µg/ml)	% scavenging activity (µg/ml) Vitamin C	IC_{50}(µg/ml)
100	80.88±0.156*	313.52	34.4±0.160*	228.78
200	88.83±0.215*		45.94±0.188*	
300	90.06±0.191*		78±0.162*	
400	93.05±0.134*		82.2±0.061*	

Values are means±SD values differ significantly at *p<0.05 n=5

Effect of Extract on Lipid Peroxidation (LPO): The *in vitro* lipid peroxidation effect of methanol extract of *Calliandra portoricensis* root bark on the mitochondria was assayed. Membrane lipids undergo rapid non enzymatic peroxidation when incubated in the presence of ferrous sulphate with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen with TBA absorbing at 532 nm (Kosugi *et al*., 1987). The extract was observed to exhibit strong lipid peroxidation inhibition with percentage inhibition of (37.600, 41.267, 58.761, and 70.049%) at concentrations of 100, 200, 300, and 400 µg/ml respectively (**Table 4**). The ethylacetate extract conferred 50% protection at the concentration of 51.92µg/ml on lipid peroxidation induced by $FeSO_4$ in liver mitochondria.

TABLE 4: THE EFFECT OF ETHYLACETATE EXTRACT OF *C. PORTORICENSIS* ROOT BARK ON LIPID PEROXIDATION VALUES DIFFERS SIGNIFICANTLY AT P<0.05, n=5

Sample	Concentration (µg/ml)	% inhibition Mean ±SD	IC_{50}(µg/ml)
Extract	100	37.600±0.116*	51.92
	200	41.267±0.127*	
	300	58.761±0.102*	
	400	70.049±0.092*	

Brine shrimp lethality: The ethylacetate extract of *Calliandra portoricensis* root bark were found to be potent against brine shrimps with LC_{50} value of 0%. The brine shrimps lethality was found to be concentration dependent.

TABLE 5: EFFECTS OF ETHYLACETATE EXTRACT OF *CALLIANDRA PORTORICENSIS* ROOT BARK ON BRINE SHRIMPS

Dose level ppm	Initial Nauphili	Number Survive after 24hrs	Number Died after 24hrs	Average Number died after 24hrs	% mortality
1000	30	0	30	30	100
100	30	0	30	30	100
10	30	1	29	29	96.67
Control	30	21	9	9	30

DISCUSSION: The relation between diseases and free radicals has been proved by many studies. UV light, radiation, smoking, alcohol consumption, stress and high cholesterol consumption can increase the process of cell oxidation [29]. This study aimed to establish a platform for *in vitro* evaluation of antioxidant capacity of ethylacetate extract of *Calliandra portoricensis* root bark.

In the present study, the total antioxidant capacity of the ethylacetate extract of *Calliandra portoricensis* root bark was 2.362 expressed as antioxidant ascorbic acid equivalent (AAE) compared to 1 for ascorbic acid (standard) (Table 1). This shows that the ethylacetate extract has more antioxidant properties than ascorbic acid due to the presence of flavonoids, polyphenols etc. of *Calliandra portoricensis* root bark [30]. The active components of the ethylacetate extract of *Calliandra potoricensis* root bark could be isolated and purified, for drug developments and/or as food supplement to boost intrinsic biological antioxidant capacity thereby equipping biological systems' with avalanche of antioxidant that can mitigate/reduce the menace free radicals.

The nitric oxide (NO) scavenging ability of the ethylacetate extract of *Calliandra portoricensis* root bark using sodium nitroprusside as a source of nitric oxide was also investigated in the present study. Nitric oxide (NO) is an essential molecule required for several physiological processes like neural signal transmission, immune response, vasodilation and control of blood pressure [31]. However, high concentration of NO may result in several pathological conditions including cancer [32]. Sodium nitroprusside in aqueous solution at physiological pH generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitric oxide.

The ethyl acetate extract of *Calliandra portoricensis* root bark in this study showed significant inhibition of NO with IC_{50} value of 364.175µg/ml compared with 231.31µg/ml for ascorbic acid as standard (Table 2). The activity exhibited by the extract is significantly higher than those previously reported for some extracts [33-35]. The result obtained, indicates that the ethylacetate extract of *C. portoricensis* is a good scavenger of nitric oxide and is complementary to its total antioxidant capacity.

Hydroxyl radical is the most reactive among reactive oxygen species (ROS); it has the shortest half life compared with others and is considered to be responsible for much of the biological damage in free radical pathology [36]. The radical has the capacity to cause strand breakage in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity [37]. The ability of the extract to 50% inhibition of hydroxyl radical was evaluated using the deoxyribose in determining the rate constant of hydroxyl radical reactions [38].

The ethyl acetate extract of *Calliandra portoricensis* root bark significantly prevented the degradation of 2-deoxy-2- ribose with IC_{50} of 313.52µg/ml compared with 228.78µg/ml for L-ascorbic acid as standard (Table 3). The percentage of hydroxyl radical scavenging activity increased with the increasing concentration of extract and known antioxidants (Table 3). This is similar to the NO scavenging activity of *Cyperus rotundus* and water extract of *Propolis* [39, 40]. Therefore, ethylacetate extract of *Calliandra portoricensis* has the potential to scavenge hydroxyl radicals at the stage of initiation and protect the biomembrane from H^+ abstraction that might lead to lipid peroxidation.

Lipid peroxidation is used as an indicator of oxidative stress in cells and tissues which underlies many pathological processes that lead to cancer, atherosclerosis, liver injury, aging, inflammation, neurodegenerative disease, and other diseases [41, 1].

Lipid peroxidation initiators are reactive oxygen species (ROS) such as hydroxyl (OH•) and peroxyl radicals (ROO•) and the superoxide anion radicals (O2•-), which are formed by exogenous chemicals factors and endogenous metabolic processes in the human body [42, 43]. Mitochondria are the most important intracellular source of ROS. Scavengers of active oxygen radicals might be beneficial for the prevention or cure of such diseases.

In the present study, the *in vitro* inhibition of lipid peroxidation effect of ethylacetate extract of *Calliandra portoricensis* root bark on the mitochondria was assayed by measurement of Malonialdehyde (MDA) which is a stable end product of free radical induced lipid peroxidation was used as a surrogate marker for oxidative damage to tissues (liver mitochondrial membrane). The extract was observed to inhibit lipid peroxidation in a concentration dependent mode with IC_{50} of 51.92µg/ml (Table 4). This is in agreement with the free radical scavenging assays which shows significant inhibition of free radicals (nitric oxide, hydroxyl radicals) and the total antioxidant capacity of the ethylacetate extract of *Calliandra portoricensis* root bark in the present study.

Brine shrimp lethality assay (BSLA) is a general bioassay, used routinely to assess toxicity of plant extracts which is indicative of cytotoxicity, antitumor, antibacterial activities, pesticidal effects and various pharmacologic actions [44,27]. The findings of this research have shown that the ethylacetate extracts of *Calliandria portoricensis* is toxic to brine shrimps on exposure for 24 hours in a dose dependent manner in which the tested animals (brine shrimps) were killed.

The LC_{50} obtained for the ethylacetate extract was 0.00% which falls within the lethality range (0-100) of biological compounds which is considered very toxic. The observed brine shrimp lethality activity of the extract may due to the presence of saponins, alkaloids, tannins, flavonoids and cardiac glycosides. This conforms to the work of Sedmak[45] and Chou *et al* [46] who conducted a brine shrimp lethality assay with the extract of *M. aeruginosa* isolated from the Solvene pond in Central Europe. And more recently the work of Siemuri *et al*.,[30] in which the aqueous and methanol extracts of *Calliandra portoricensis* root bark used in the brine shrimp lethality assay killed test animals (brine shrimp) at various doses of the extract. Moreover, the significant lethality of the ethylacetate extracts (LC_{50} values less than 100 ppm or µg/ml) to brine shrimp is indicative of the presence of potent toxic compounds of pharmacological importance which warrants further investigation. BSLA results may be used to guide the researchers on which crude plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds.

CONCLUSION: The results of the present study indicate that the ethylacetate extract of *Calliandra pororicensis* root bark exhibit interesting antioxidant properties via various *in vitro* model and also show moderate toxicity. This could be harnessed in drug development and/or food supplements or neutraceuticals that can be used in the management and treatment of various ailments. However, it is recommended that the doses carefully and clinically chosen. These results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity. It is therefore worthwhile that further studies be conducted to isolate and purify the active principle involved in the antioxidant activities of *Calliandra portoricensis* extract as well as to elucidate the mechanisms action of the active compounds.

Competing Interests: The authors declare that they have no competing interests and that the authors of this manuscript have no financial or personal relationship with any organization which could influence the work.

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