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Phytochemical, Metal Analysis, and *in-vitro* Antioxidant Activity of Ethanol Leaf Extract of *Alchornea cordifolia* (Schumach and Thonn) Mùll Arg (Euphorbiaceae)

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ABSTRACT

Although plants are notable reservoirs of pharmacologically and toxicologically bioactive phytoconstituents of medicinal importance, they also contain certain concentrations of some heavy metals that are reportedly of great health concern worldwide necessitating their continuous evaluations. The study aimed to perform the qualitative and quantitative phytochemical investigation of the ethanol leaf extract of *Alchornea cordifolia* Schumach and Thonn) Mùll Arg (Euphorbiaceae) *(A. cordifolia)* vis-à-vis its heavy metals content and *in-vitro* antioxidant effects using standard procedures in attempts to establish the plant's medicinal safety level. The phytochemical analysis revealed the presence of phenols, flavonoids, tannins, glycosides, cardiac glycosides, saponins, alkaloids, and carbohydrates of varying amounts and reference levels following thin-layer chromatography. Elemental analysis showed the presence of cobalt, iron, zinc, manganese, copper, sodium, calcium, and magnesium whose concentrations were all below the WHO/FAO recommended permissible limits except for cadmium and nickel, which was above their permissible limits even as the lead was below detectable level. The *in-vitro* antioxidant activities were concentration-dependent. The plant did not only contain many heavy metals below standard recommended permissible levels, it also demonstrated some antioxidant potentials to herald it relative safety. However, the presence of cadmium and nickel levels above the recommended permissible levels calls for its cautious usage while necessitating further investigations.

Keywords: *Alchornea cordifolia*, *In-vitro* antioxidant, Phytochemical, Heavy metals

Introduction

Medicinal plants and their products have remained alternative sources of remedy and healing among the large population of people worldwide. [1]According to the World Health Organization, [2] million of people rely directly or indirectly on medicinal plants as their major source of primary health care and remediation against different diseases, increasing demand, and interest. A report by Balunas and Kinghorn, [3] has shown that there is a growing effort by investigators to discover and develop drugs from medicinal plants with the adoption of a multifaceted approach, including botanical investigation, and phytochemistry, biological, and molecular techniques toward its effective utilization as alternative therapies. Most medicinal and therapeutic activities of plants might be due to their unique inherent phytochemical constituents, [4] of which are secondary metabolites derived from the primary metabolic processes of plants. Secondary metabolites are important in defending plants against pathogenic microorganisms and ensuring that the plants survive adverse environmental and climatic conditions. [5]

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Again, medicinal plants are the main sources of mineral elements required for most biochemical activities in human and animal bodies. [6] Among such elements are heavy metals often transferred to the man from the soil through consuming parts of plants and/or their products. [7] Essential or trace elements are very vital for most physiological activities, deficiencies of which often manifested in various health abnormalities. [8] The number of heavy metals absorbed from the soil most times depends on the geochemical characterization of the soil, the bioavailability of the element, and the ability of the plant to accumulate it. [7] Aside from soil, plants also acquire these elements from atmospheric dust, fertilizers, herbicides, and rainwater. [9] With increasing human activities that generate a large volume of both industrial and domestic wastes daily coupled with poor wastes disposal management and deteriorating climatic conditions, plants tend to accumulate more of these heavy metals that portend danger to both soil flora and fauna, including man who represents the final consumer at the top of the food chain. [10] Heavy metal toxicity represents one of the most environmentally induced health challenges and a major pollutant worldwide. [11] Given these challenges, World Health Organization recommended that medicinal plants intended for human and animal consumption should undergo evaluations for the presence of heavy metals, pesticides, and other contaminants. [12] Most heavy metals inflict damage to the body's systems through the induction of oxidative stress and reactive oxygen species, which often impair major physiological functions to induce most inflammatory, degenerative, cardiovascular, aging, and nervous diseases in man and animals. [13] Antioxidant substances, both natural and synthetic, help scavenge these free radicals and have proved to be useful in treating and managing oxidative stress-dependent diseases. [14] Some plants have phytoconstituents with antioxidant capabilities, which has positioned

them as potential sources of antioxidants that could effectively scavenge free radicals from the body and ameliorate pathological damages caused by them. [15] The growing interest in plants as potential antioxidants might be due to the increased toxicity associated with the use of synthetic antioxidants. [16] *Alchornea cordifolia (A. cordifolia)*, which is an evergreen shrub and herb belonging to the family *Euhorbiaceace* inhabiting tropical African countries, is a highly valued medicinal plant for the treatment of many disease conditions like diarrhoea, skin diseases, anemia, infectious and inflammatory conditions. [17,18] Although there are reports of the pharmacological activity of *A. cordifolia* such as its hepatoprotective, antioxidant, and, spasmolytic activities, [19, 20, 21] there is a need for more investigations of the plants, especially concerning its safety levels in the face of its widespread traditional medicinal use and heavy metal content. Therefore, this study aimed to investigate the phytochemical and heavy metals contents vis-à-vis the *in-vitro* antioxidant activity of ethanol leaf extract of *A. cordifolia* in attempts to determine its safety level as a medicinal plant.

Materials and Methods

Plant Preparation and Extraction

Mr Patrick Ekwuno of the Department of Botany, College of Biological Science, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria, authenticated the leaves of *A. cordifolia* collected from Aikplia village in Ugbokolo, Okpokwu Local Government Area, Benue State from January to December 2020 with a deposited voucher number (401). The leaves were air-dried within the laboratory following proper washing under a running tap to remove sand and other solid contaminants before pulverizing into a powder ready for use.

Extraction of the powdered leaves was via the use of Soxhlet apparatus involving 500 g of the powder and 2.5 L of absolute ethanol with continuous refluxing at 60^{o}C, for 16 hours to obtain a filtrate consequently concentrated to dryness at reduced pressure by a rotary evaporator.

Qualitative and Quantitative phytochemical screening

Qualitative phytochemical analysis to determine the presence of bioactive compounds was according to Harborne [22] and Trease and Evans. [23]

Total flavonoid content Determination

The total flavonoid content of ethanol leaf extract of *A. cordifolia* was determined by the Aluminium chloride colorimetric method of Zhishen *et al.* [24] with the addition of 0.5 ml aliquot of the extract, standard solution of quercetin, 2 ml of distilled water, and subsequently, 0.15 ml of sodium nitrite (5% w/v). After 6 minutes, 0.15 ml 10% $AlCl_3$ solution was added and allowed to stand for another 6 minutes, thereafter, 2 ml 4% NaOH was then added to the mixture and the volume was made up to 5 ml with distilled water. This was thoroughly mixed and allowed to stand for 15 minutes for absorbance reading at 510 nm. Total flavonoid content = Milligram of quercetin equivalent per gram of the sample measured in triplicates calculated from the calibration curve using the equation.

Total Phenol content Determination

The total phenolic content of ethanol leaf extract of *A. cordifolia* was via the modified Spectrophotometric method of Wolfe *et al.* [25] involving mixing the extract with 2.5 ml 10% Folin Ciocalteu reagent and 2 ml of Na_2CO_3. The resulting mixture was vortex for 15 seconds and incubated at 40^{o}C for 30 minutes for color development before absorbance reading at 765 nm with UV-spectrophotometer. Total phenolic content was expressed as mg/g of gallic acid equivalent.

Total Tannin content determination

The total tannin content determination followed the method described by Pearson [26] with the addition of 2.5 ml of the extract into a 50 ml volumetric flask as well as the same volume of tannic acid into another 50 ml flask. Both flasks then had 1.0 ml of Folin-Denis reagent and 2.5 ml of saturated Na_2CO_3 solution followed by dilution to 50 ml mark and incubated at room temperature for 90 minutes. The absorbance reading was at 725 nm. Total tannin content was expressed as mg/g of Gallic acid equivalent.

Determination of total Saponins Content

The total saponin contents of *A. cordifolia* answered the method of Makkar *et al.* [27] with the dissolution of 0.1 g of the extract in 50 % methanol with the addition of 0.25 ml of 8% Vanillin reagent followed by 2.5 ml of 72% (v/v) of sulphuric acid, and incubated at 60^{o}C in a water bath for 10 minutes, before cooling with ice to take the absorbance reading at 544 nm against a blank that does not contain the extract. Total saponins concentration = Milligram diosgenin equivalents per g weight from a standard calibration curve obtained from 0.5 mg/ml of diosgenin in 50% aqueous methanol.

Total Alkaloid Determination

The method of Harborne [22] used in the determination of the total alkaloid content of *A. cordifolia* involved the addition of 5 g of the sample to 80 ml of 10% acetic acid in ethanol in a 250 ml beaker that was covered and allowed to stand for 48 hours; and filtered and concentrated at 60^{o}C on a water bath to one-quarter of the original volume before adding concentrated ammonium hydroxide drop by drop to the mixture until precipitation was formed. The alkaloid was dried and weighed after allowing it to settle and precipitate with the filtration and washing with dilute ammonium hydroxide thereafter.

Thin Layer Chromatographic Phytochemical Constituent Determination

Following the determination of the various phytochemical constituents using the chemical method, we further isolate specific bioactive components through the thin layer chromatographic technique. Briefly, the method involved the dissolution of the extract in methanol and subjected to thin-layer chromatography on silica gel "G" (Merck, India) coated (0.5 mm thickness) plates using hexane: ethyl acetate (8:2 v/v) as a mobile phase. Specific sprays were used to determine the various components of flavonoids (Aluminium spray), an alkaloid (Dragendorff Spray), Steroids, Triterpenes (Lieberman Burchard Spray), and phenol (Ferric Chloride Spray) in the extract with the viewing of the spot through the ultraviolet lamp. The equation for the Reference Value (RF) was:

$$RF = \frac{\text{Distance traveled by the Spot from the base}}{\text{Distance traveled by solvent front from the base}}$$

Metals Determination Using Atomic Absorption Spectrophotometer

The digestion process involved the use of Nitric - hydrochloric acid (3:1) with the addition of 0.5 g of the extract, and 7.5 ml of 65% HNO_3 to 2.5 ml of 30% HCl acid before heating for 4 hours at 120^{o}C in a Microwave Plasma- Atomic Emission Spectroscopy (MP-AES) 42000 Agilent Technology), and later allowed to cool before adding 20 ml of deionized water for filtration with Whatman 1 filter paper to make it up to 50 ml with deionized water in 50 ml volumetric flask ready for individual heavy metals analyses with Atomic Absorption Spectrophotometer.

In vitro Antioxidant Analysis

Ferric Ion Reducing Antioxidant Power Assay

The modified method of Oyaizu[28] used to determine the reducing power of ethanol leaf extract of *A. cordifolia* involved the use of different concentrations of the extract ranging from 100 – 500 µl in mixtures with 2.5 ml of 20 mM phosphate buffer and 2.5 ml 1 % of potassium ferrocyanide (w/v), which was incubated at 50^{o}C for 30 minutes followed by 2.5 ml of 10% trichloroacetic acid and 0.5 ml of 0.1% ferric chloride addition. The absorbance was measured at 700 nm 10 minutes thereafter with ascorbic acid as the standard in triplicates.

Determination of Total Antioxidant Capacity (TAC)

The measurement of the total antioxidant capacity of the extract was via the phosphomolybdenum method, according to Prieto *et al.* [29]. The molybdate reagent was prepared by measuring 1 ml each of 0.6 M

Sulphuric acid, 28 Mm sodium phosphate, and 4 Mm Ammonium molybdate in 20 ml of distilled water and made up to 50 ml with water. Thereafter, different concentrations of the extract (100 – 500 µl) were measured into each test tube containing 3 ml of distilled water and 1 ml of the molybdate reagent solution and incubated at 95^{o}C for 90 minutes before normalization to room temperature for 20-30 minutes before reading the absorbance at 695 nm. The sample was prepared in triplicate and the average was recorded with ascorbic acid as the reference standard.

Determination of Hydrogen Peroxide Scavenging Activity

The method of Ruch *et al.* [30] adopted in the evaluation of the scavenging ability of hydrogen peroxide by the extract involved the dissolution of various concentrations of the extract (0.0312 – 0.5 ng) in 1 ml distilled water and mixed with 0.6 ml of 4 Mm hydrogen peroxide (H_2O_2) solution in 0.1 M phosphate buffer (PH 7.4) before incubation for 10 min before reading the absorbance at 230 nm against a blank solution containing the extract without H_2O_2. The amount of hydrogen peroxide radical inhibited by the extract was:

$$H_2O_2 \quad RSA = \text{Absorbance of Control} - \text{Absorbance of}\frac{\text{Extract}}{\text{Absorbance}} \text{ control x } 100$$

Determination of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The method of Liyana-Pathirana and Shahidi [31] was adopted in determining the scavenging activity of the extract in DPPH free radicals with the prepared 0.135 Mm of DPPH in methanol mixed with 1 ml of various concentrations of the extract from 0.025 - 0.5 in methanol, properly mixed and left in the dark for 30 minutes. The ascorbic acid solution was prepared separately as the reference standard. The measurement of the absorbance of both the test extract sample and ascorbic acid was at 517 nm. The ability of the extract to scavenge DPPH radicals was:

$$\text{DPPH} \quad \text{RSA} = \text{Absorbance of Control} - \text{Absorbance of}\frac{\text{Extract}}{\text{Absorbance}} \; control \; X100$$

Data analysis

Where applicable, descriptive statistics using the GraphPad program (GraphPad Prism, Version 7.04, www.graphpad.com) were used for the analysis of the data obtained from this study. Values are presented as percentages and mean with the standard error of the mean at $p<0.05$ level of statistical significance.

Results and Discussion

Table 1 showed the results of the qualitative phytochemicals constituents of the ethanol leaf extract of *A. cordifolia*, which revealed the presence of flavonoid, phenol, tannin, alkaloid, saponins, glycosides, steroids, triterpenes, and carbohydrates with no anthraquinone. Quantitative total flavonoid, phenols, tannin, saponins, and alkaloid of ethanol leaf extract of *A. cordifolia* were as in Table 2 showing varying amounts of total phenols (110.75 mg/g gallic equivalent to 11.1%), total flavonoid (102.77 mg/g quercetin equivalent to 10.3%), total tannins (72.33 mg/g gallic equivalent to 7.2%, total alkaloids (46.29 mg/g equivalent to 4.6%), and total saponins (30.08 mg/g diosgenin equivalent to 3.0%), respectively.

The presence of flavonoids, tannins, phenols, alkaloids, steroids and triterpenes, glycosides, cardiac glycosides, saponins, and carbohydrates in agreement with an earlier report [35, 36] except for the recorded steroids and saponins in the present study. The difference in extraction solvent, seasons of the part of the plant harvest, stage of development, and the geochemical constituents of the soil could be responsible for these results. [37]

The thin layer chromatograms of the ethanol leaf extract of *A. cordifolia* showed four components of flavonoids (0.13, 0.35, 0.53, and 0.67), three components of phenols (0.35, 0.60, and 0.83), Steroids, and Triterpenes (0.76, 0.88, and 0.93), and two components of alkaloids (0.48 and 067), respectively, as in Table 3. The results also validated the obtained qualitative and quantitative analysis in corroboration of the finding of [38] who reported the presence of different components on a chromatogram that included alkaloid, ellagic acids, diisopentenyl guanidine, triisopentenyl guanidine, β sitosterol, daucosterol, and 2-ethylhexyl phthalate. These constituents reportedly possess anti-inflammatory and immunomodulated activity. [39, 40]

The concentrations of each heavy metal element found in the extract are as in Table 4 with high concentrations of cadmium (2.03 ± 0.54), nickel (2.16 ± 0.8), and calcium (9000 ± 0.00) above the WHO/FAO permissible limit of 0.3 mg/kg, 1.5-1.63 mg/kg, and 614 mg/kg, respectively, even as lead (Pb) concentration was below the detection limit.

Apart from bioactive compounds conferring most of the pharmacological and therapeutic activities on plants, elemental compositions of the plants also play significant roles in modulating some of these activities exhibited by plants. [6] Essential elements like Fe, Zn, Cu, and Mn, which are often involved in many physiologic and metabolic processes of the body, could interact with phytoconstituents such as flavonoids and polyphenols to alter their biological activities. [41] Therefore, plants containing some of these useful elements with therapeutic potential and relevance could sometimes pose a risk to human and animal health when present at high dangerous concentrations. [42] Our results revealed the presence of iron (Fe), manganese (Mn), zinc (Zn), magnesium (Mg), copper (Cu), and sodium (Na^+) at concentrations below permissible levels while the concentrations of cadmium (Cd), calcium (Ca), and nickel (Ni) were above the permissible level according to WHO/FAO, [43] respectively. Cadmium, a non-essential metal, has no known useful physiological activity in the body [44] but poses a serious toxicological effect on human and animal health who may consume Cd-laden plants as recorded in this study, with toxic effects on the kidney, liver, vascular, and immune system. [45]

Table 1: The qualitative phytochemical constituents of ethanol leaf extract of *Alchornea cordifoli*

Constituents	Inference
Carbohydrates	+
Anthraquinone	-
Glycosides	+
Cardiac glycosides	+
Saponins	+
Steroids and Triterpenes	+
Tannins	+
Flavonoid	+
Alkaloids	+
Phenol	+

Table 2: The quantitative analysis of ethanol leaf extract of *Alchornea cordifolia*

Constituents	Concentrations in µg /ml	% Contents
Saponins	30.08	3.0
Alkaloids	46.29	4.6
Tannins	72.33	7.2
Flavonoids	102.77	10.3
Phenol	110.75	11.1

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Table 3: The Thin-layer chromatographic profiles of ethanol leave extract of *Alchornea cordifolia*

Constituents	Specific sprays	Reference (Rf) Value
Flavonoids	Aluminium chloride	0.13, 0.35, 0.53, 0.67
Alkaloids	Dragendorff	0.48, 0.67
Phenol	Ferric chloride	0.35, 0.60, 0.83
Steroids and Triterpenes	Lieberman-Burchard	0.76, 0.88, 0.93

Table 4: Heavy metals elemental analysis of the ethanol leaf extract of *Alchornea cordifolia*

Metals/Trace Element	Measured Values (mg/kg)	Reference Values (mg/kg)
Cobalt (Co)	2.57 ± 1.06	3.50
Iron (Fe)	28.50 ± 2.27	48.0
Copper (Cu)	5.97 ± 0.08	20-150
Manganese (Mn)	213.3 ± 6.63	2000.0
Zinc (Zn)	16.19 ± 0.27	50.0
Nickel (Ni)	2.17 ± 1.44*	1.5-1.63
Cadmium (Cd)	2.23 ± 1.3*	0.3
Sodium (Na)	10000 ± 0.00	51340
Magnesium (Mg)	1875 ± 0.01	2000
Calcium (Ca)	9000 ± 0.00*	614
Lead (Pb)	ND	10.00

Data with asterisks were higher than WHO/FAO recommended values (WHO/FAO, 2009).
ND – Not detected

Similarly, nickel is required by the body in very small quantities for insulin production but at high concentrations, it affects the peripheral tissues and reproductive system [44] as well as the cardiovascular system, [46] with most of its toxicity attributed to its interference in zinc and calcium metabolism. [47] Our finding, therefore, showed the need for a detailed toxicological evaluation of this plant to ascertain its safety.

The results of the ferric reducing power revealed low absorbance of the extract at 5 mg/ml (0.738 nm) than the standard ascorbic acid (1.673) (Figure 1). A concentration-dependent increase in percentage inhibitions occurred in the standard than in the extract following hydrogen peroxide scavenging activity of ethanol leaf extract of *A. cordifolia* (Figure 2). Similarly, a concentration-dependent increased total antioxidant capacity (TAC) occurred more in the ethanol leaf extract of *A. cordifolia* compared to the ascorbic acid as in Figure 3. The DPPH scavenging activity of the extract was dose-dependent, with a percentage inhibition of 68.98% at 0.5 mg/ml compared with 98.35% of the ascorbic acid used as standard (Figure 4).

Despite the limited toxicological investigation of *A. cordifolia*, there is information on its therapeutic and pharmacological potentials, including its antioxidant capacity and activity. [38, 36] Our results showed a dose-dependent increase in the reducing power of the extract, although the reducing power of the extract was far below the standard, indicative of the antioxidant property of the extract in agreement with the reports of Effo *et al.* [21].

The concentration-dependent increase in the total antioxidant capacity of the extract was an indication of the antioxidant capacity and potential of the plant. The hydrogen peroxide scavenging activity potential of the extract also revealed a dose-dependent increase in percentage inhibition of the peroxide in corroboration of the work of Osei *et al.*, [48] who reported a concentration-dependent increase in total antioxidant capacity and hydrogen peroxide inhibition assay of various solvent leaf extract of *A. cordifolia*. Both findings further support the antioxidant potential of *A. cordifolia* and its probable use as an antioxidant agent in the treatment and management of oxidative stress-induced disorders. The obtained percentage of DPPH scavenging activity of the extract further supported the antioxidant property of this plant as reported by other investigators. [36, 47, 21] However, the percentage inhibition of DPPH free radicals of ethyl acetate and acetone fraction of *A. cordifolia* reported by [36] was higher than the results obtained in this study. This discrepancy could be associated with differences in solvent polarity used for extraction and the geochemical soil characteristic, including the interaction of heavy metals [7, 21] reported similar antioxidant activity of aqueous extract of *A. cordifolia* leaves.

Therefore, the current antioxidant activity recorded in this study may be attributed to the presence of polyphenols and flavonoid content in the extract and agreed with earlier reports. [38, 48] On the other hand, the decline in antioxidants activities seen in this study concerning other reported investigations might be due to possible interference of the evaluated heavy metals reported in the present investigation, particularly, cadmium and nickel, including differences in the geochemical content of soil and the climatic condition of the study location. However, the antioxidant activity of the plant might be via the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and the enhancement of total antioxidant capacity. Although the plant demonstrated an *in-vitro* antioxidant, the levels were below those earlier reported by other investigators probably due to the recorded high cadmium and nickel contents, which calls for caution in the use of the plant for the treatment of diseases.

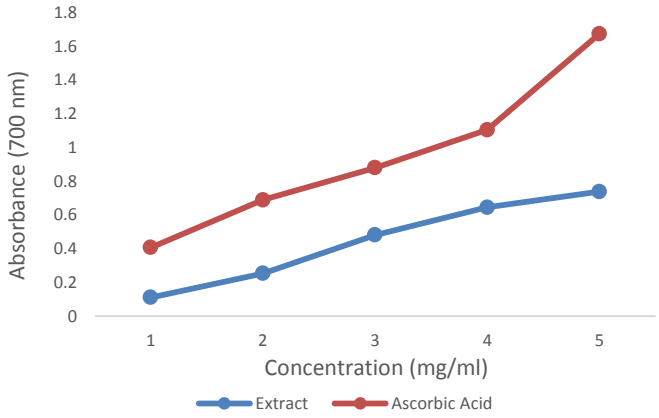

Figure 1: Ferric reducing power of the ethanol leaf extract of *Alchornea cordifolia*

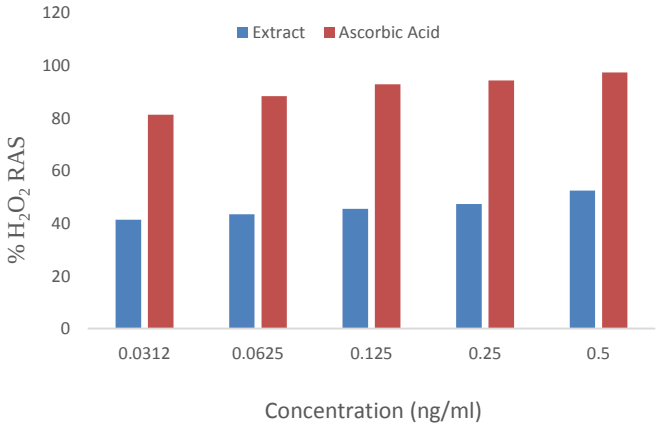

Figure 2: Hydrogen peroxide scavenging activity of ethanol leaf extract of *Alchornea cordifolia*

Conclusion

The study has therefore demonstrated the presence of important phytochemical compounds with antioxidants activity that could be helpful in the management of oxidative stress induced disorder. However, there should be caution in the use of this plant for therapeutics in view of the high concentration of cadmium and nickel recorded in this study**.**

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

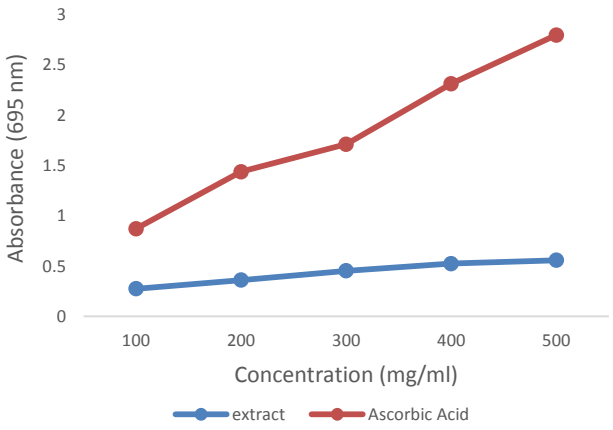

Figure 3: Total antioxidant capacity (TAC) of the ethanol leaf extract of *Alchornea cordifolia*

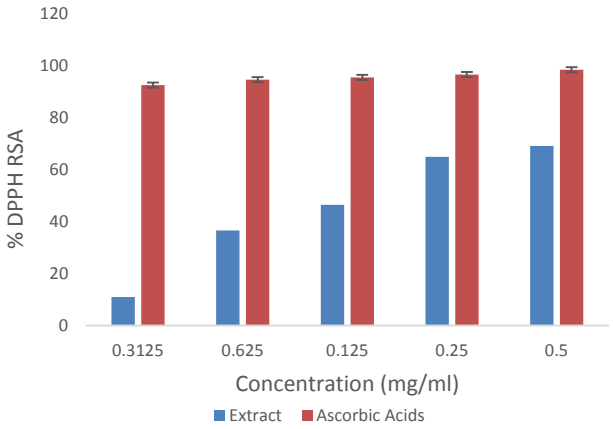

Figure 4: The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of ethanol leaf extract of *Alchornea cordifolia*

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