April 30, 2018

PharmacologyOnLine

Archives • 2018 • vol.1 • 24-44

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FINGERPRINTING AND COMPARATIVE ANTIOXIDANT PROPERTIES OF ROOTBARK AND LEAF EXTRACTS OF *CALLIANDRA PORTORICENSIS*

Falode J A[1,2]*, Obafemi T O [3], Akinmoladun A C [2], Olaleye M T [2], Boligon A A[4], Athayde M L[4]

[1]Department of Biochemistry, Federal University, Oye-Ekiti, EkitiState, Nigeria.

[2]Department of Biochemistry, Federal University of Technology, Akure, Ondo State,Nigeria.

[3]Department of Biochemistry, AfeBabalola University, Ado-Ekiti, EkitiState, Nigeria.

[4]Phytochemical Research Laboratory, Department of Industrial Pharmacy, Federal University of Santa Maria, Build 26, Room 1115, Santa Maria, CEP 97105-900, Brazil.

john.falode@fuoye.edu.ng

Abstract

Calliandra portoricensis is widely used in South Eastern part of Nigeria for the treatment of prolong pregnancy and snakebite. In the present study, high-performance liquid chromatography with diode-array detection (HPLC-DAD) was used to quantify the flavonoids, phenolic acids and carotenoids present in the methanol leaf and root bark extracts of *C.portoricensis*. The phytochemical screening of the extracts from leaf and root bark of *C. portoricensis*for bioactive principles (secondary metabolites) was conducted. The preliminary phytochemical screening of *C. portoricensis*showed that saponins, flavonoids, alkaloids, terpenoids and cardiac glycosides were present in the extract from both the leaf and root back, while phlobatannins, steroids and anthraquinones were absent; but tannins are present only in the leaf extract but absent in the root bark extract. Both extracts exhibited remarkable contents of phenols and flavonoids, the root bark and leaf extract have 705.12 mg tannic acid equivalents (TAE) g^{-1} and 1448.73 mgTAE g^{-1}, respectively; the root bark and leaf extracts had a good total antioxidant capacity (TAC) of 251.153 mMascorbic acid equivalents (AAE) mg^{-1} and 274.652 mMAAE mg^{-1}, respectively. The leaf extract showed a good reductive potential than the root bark, while in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, the root bark extract showed better power in scavenging this radical. The abilities of the extracts to scavenge 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)(ABTS)radical, hydroxyl radical ($^{\cdot}OH$), chelate reactive metals and scavenge nitric oxide ($^{\cdot}NO$) radicals, the lipid peroxidation assay, anti-hemolytic, and anti-denaturation assays were assessed, and the leaf extract exhibited an upper hand in all these assays.

Keywords: *Calliandra portoricensis, extracts, antioxidant, anti-hemolytic, anti-denaturation, flavonoids.*

Introduction

Calliandraportoricensis(Jacq.)Benth.is a straggling perennial shrub and belongs to the family Mimosaeae[1]. The shrub or little tree is native to Central America, and most specifically to Mexico, Panama, and to the West Indies. It is used in Nigeria folklore medicine as a laxative/worm expeller [2] and an abortifacient in humans [3]. The plant has also been reported to have anticonvulsant [4, 5], antidiarrheal, antispasmodic, antipyretic, antirheumatic and analgesic[6] activities in humans. In addition, *C.portoricensis*has also been reported to exhibit anticholigenic, antacid, antiulcer, molluscidal and ovucidal activities in laboratory animals[6]. The plant extracts have been reported to have antimicrobacterial activities against the following organisms: *Escherichia coli, Staphylococcus aureus, Streptococcus faecium*and *Candida albicans*[5]

Traditional herbalists in south eastern Nigeria have found the leaves and roots of *Callianda protoricensis* (*eriagbor* in Igbo) very useful and effective in neutralizing viperian venom[7]. *C. protoricensis* has been found to possess strong antisickling properties as reported by Amujoyegbe *et al.,* [8]

Though the genus *Calliandra* consists of many species distributed worldwide, the only other species growing in Nigeria is *Calliandra haematocephala.* This has been extensively evaluated for its chemical constituents. Three acylatedquercetin rhamnosides were recently reported from the leaves and stem of *C. haematocephala* and their structures were established as quercitrin 2''-*O*-caffeate, quercitrin 3''-*O*-gallate and quercitrin 2'',3''-di-*O*-gallate[9].

Moreover, caffeic and betulinic acids were previously reported[10]. Compounds such as myricitrin, quercitrin, myricitrin 2''-*O*-gallate, quercitrin 2''-*O*-gallate, myricitrin 3''-*O*gallate and myricitrin 2'',3''-di-*O*-gallate, exhibited moderate to strong radical scavenging properties on lipid peroxidation, hydroxyl radical, superoxide anion generation and DPPH radical in comparison with that of quercetin as a positive control *in vitro*[9]. Despite these reported biological activities of *C. portoricensis,* no detailed investigation of the chemical constituents has been reported. The dried root of *C. portoriscensis* was pulverized and extracted with dichloromethane, the dichloromethane extract was fractionated by flash column chromatography from which fraction 1 eluted with n-hexane was obtained and subjected to gas-chromatography/mass-spectrometry (GC-MS) analysis. The results of the GC-MS analysis, phytochemical screening and preliminary antimicrobial activity of the hexane fraction of the root extract of *C. portoricensis* were reported.

Materials and methods

The plant material *C.portoricencis* (leaves and root bark), were obtained from a farm settlement in Esa Oke, Osun state, Nigeria. These were authenticated in the herbarium of Botany Department, University of Ibadan. They were washed thoroughly with distilled water and allowed to air dry. The air-dried leaves and root bark were pulverized and then subjected to solvent extraction.

Extraction

The ground root bark and leaves were put in a glass container and were extracted with 80% methanol. The extraction process was left for about 72 h and agitated about 24-hourly for the extraction of the bioactive components into the solvent and the solution was decanted.

Principle of extraction

The solution of extract and solvents were put into the rotary evaporator flask and the machine was set up, the temperature used was 37°C. When solution was heated to the required temperature, the solvents started to evaporate and were distilled with the use of a distiller attached to the evaporator, and the pure solvent was collected in the provided flask also in the rotary evaporator. The process continued until all the solvents were removed from the solution and only the pure bioactive components of the plants that are been sought are remaining. After the whole process, the extracts that were able to air dry were left in the open air in the laboratory and the others were freeze dried with a freeze drier until a concentrated extract was obtained. The extracts were then transferred into different bottles of known weights and kept in a refrigerator until further use.

Chemicals

Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, Tannic acid, Quercetin, Ascorbic acid,

Mannitol and Folin-Ciocalteau reagent were obtained from Sigma-Aldrich, USA. All other chemicals and reagents used were of analytical grade and obtained from standard suppliers.

Chemical, apparatus and general procedures for HPLC

All chemical were of analytical grade. Acetonitrile, formic acid, gallic acid, chlorogenic acid, ellagic acid, rosmarinic acid and caffeic acid purchased from Merck (Darmstadt, Germany). Quercetin, isoquercitrin, rutin, catechin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile (ACN) and ethyl acetate (EtAc) purchased from Merck (Darmstadt, Germany). Tocopherol, β-carotene and lycopene were acquired from Sigma Chemical Co. (St. Louis, MO, USA).High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 x 250 mm) packed with 5µm diameter particles. The mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20, 30, 50, 60, 70, 20 and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively (Boligon et al., 2012) with slight modifications. *C.portoricensis* (leaves and root) methanolicextractwas analyzed and dissolved in ethanol at a concentration of 20 mg/mL. The presence of ten antioxidants compounds was investigated, namely, gallic, chlorogenic, caffeic, ellagicandrosmarinic acid, catechin, quercetin, isoquercitrin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 ml/min, injection volume 50 µl and the wavelength were 254 nm for gallic acid, 280 nm for catechin, 327 nm for caffeic, ellagic, rosmarinic and chlorogenic acids, and 366 nm for quercetin, isoquercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for kaempferol, quercetin, isoquercitrin, catechin and rutin; and 0.030 – 0.250 mg/ml for gallic, caffeic, rosmarinic, ellagic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 400 nm). Calibration curve for gallic acid: Y = 13548x + 1259.7 (r = 0.9999); catechin: Y = 12730x + 1265.3 (r = 0.9997); rosmarinic acid: Y = 11947x + 1493.6 (r = 0.9995); caffeic acid: Y = 13248x + 1359.3 (r = 0.9990); chlorogenic acid: Y = 12661x + 1375.6 (r = 0.9995); ellagic acid: Y = 11985x + 1265.9 (r = 0.9998); rutin: Y = 12845 + 1365.7 (r = 0.9999); quercetin: Y = 13560x + 1192.6 (r = 0.9991), isoquercitrin: Y = 14273x + 1275.4 (r = 0.9998) and kaempferol: Y = 14253x + 1238.9 (r = 0.9997). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve [11, 43].

Quantification of carotenoids HPLC-DAD

Carotenoids analysis was carried at reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 150 mm) packed with 5 µm diameter particles. The mobile phase consisted of mixtures of ACN: H_2O (9:1, v/v) with 0.25% triethylamine (A) and EtAc with 0.25% triethylamine (B). The gradient started with 90% A at 0 min to 50% A at 10 min. The percentage of A decreased from 50% at 10 min to 10% A at 20 min. The flow-rate was 0.8 ml/min and the injection volume was 40µl. Signals were detected at 450 nm, following the method described by Janovik et al. (2012) with slight modifications. Solutions of standards references (tocopherol, β-carotene and lycopene) were prepared in HPLC mobile phase at a concentration range of 0.035 - 0.350 mg/ml. The samples were analyzed at a

concentration of 20 mg/mL, carotenoids were identified and quantified in the extracts by comparison of retention times and UV spectra with the standard solution. All chromatography operations were carried out at ambient temperature and in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10 r/S, respectively, where r is the standard deviation of the response and S is the slope of the calibration curve[10, 11].

Phytochemical screening

The extracts were screened for the presence of alkaloids, saponins, tannins, phlobatannins, anthraquinones, steroids, terpenoids, isoprenoids, flavonoids and cardiac glycosides [12, 13, 14, 15].

***In vitro* phytochemical and antioxidant test**

DPPH (1, 1, diphenyl 2-picryl hydrazyl) assay:

The antioxidant activity by DPPH assay was assessed using the stable free radical DPPH. To 1 ml of various concentrations of the extract was added 1 ml of DPPH 0.1mM in the test tube. Ascorbic acid was used as the standard for comparison. After incubation for 30 min in the dark at room temperature, absorbance was recorded at 517 nm. The percent DPPH radical scavenging was calculated with the equation:

DPPH radical scavenging (%) = [(control absorbance - sample absorbance)/control absorbance] × 100.

The experiment was carried out in triplicate [16].

ABTS radical cation decolorization assay:

ABTS also constitutes a relatively stable free radical, which decolorizes in its non-radical form. The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was determined according to the method of Re et al. (1999) [44]. In this method, an antioxidant was added to a pre-formed ABTS radical solution and after a fixed time period, the remaining $ABTS^{\bullet+}$ was quantified spectrophotometrically at 734 nm [17]. $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), and allowing the mixture tostand in the dark at room temperature for 6 h before use. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Prior to the assay, the solution was diluted in phosphate buffer (pH7.4) to give an absorbance at 734 nm of 0.700±0.02 in a 1 cm cuvette and equilibrated to 30°C, the temperature at which all the assays were performed. Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of resveratrol solutions in ethanol at different concentrations (10-30 µg/mL). The absorbance was recorded 30 min after mixing and the percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger. The extent of decolorization was calculated as percentage reduction of absorbance. For the preparation of a standard curve, different concentrations of $ABTS^{\bullet+}$ (0.033–0.33 mM) were used. $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r 2:0.9899):

Absorbance (λ_{734}) = 2:5905 × ½$ABTS^{\bullet+}$

The scavenging capability of test compounds was calculated using the following equation:

$ABTS^{\bullet+}$ Scavenging effect (%) = [1- (As = Ac)] × 100

where A_C is absorbance of a control lacking any radical scavenger and A_S is absorbance of the remaining $ABTS^{\bullet+}$ in the presence of a scavenger[17].

Nitric oxide radical scavenging:

The nitric oxide radical scavenging capacity of the fractions was measured by Griess reaction [18]. Various concentrations were prepared. Sodium nitroprusside (2.5 mL, 10 mM) in phosphate buffered saline (PBS) was added to 0.5 mL different concentrations of extracts. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent: (1% (w/v) sulfanilamide, 2% (v/v) $H_3PO_4$and 0.1% (w/v) naphthylethylenediamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of fractions. Sodium nitroprusside in PBS (2 mL) was used as control. The nitric oxide radicals scavenging activity of the

extractss and ascorbic acid was calculated according to the following equation:

Percentage of inhibition = [(Ao– A1) / Ao] x 100,

Where Ao is the absorbance of sodium nitroprusside in PBS (without extracts and ascorbic) and A1 is the absorbance in the presence of the fractions and ascorbic acid.

Reductive potential

Ferric cyanide (Fe^{3+}) reducing antioxidant power assay:

Reducing power of the extracts were measured by the direct reduction of $Fe^{3+}(CN-)6$ to $Fe^{2+}(CN-)6$ and was determined by absorbance measurement of the formation of the Perl's Prussian Blue complex following the addition of excess Fe^{3+} [42], as described previously [19, 20]. Different concentrations of extracts in 0.5mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%),the mixture was incubated at 50°C for 20 min. After 20 min incubation, the reaction mixture was acidified with 1.25 ml of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added to this solution and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates greater reduction capability [19, 20].

Hydroxyl radical scavenging activity assay:

The hydroxyl radical ($^{\cdot}OH$) scavenging activity was measured by the method of [21]. The hydroxyl radical was generated in a mixture of 1.0 mL of 0.75 mM 1,10-phenanthroline, 2.0 mL of 0.2 M sodium phosphate buffer (pH 7.4), 1.0 mL of 0.75 mM $FeSO_4$ and 1.0 mL of H_2O_2 (0.01%, v/v). After addition of 1.0 Ml sample solution, the mixture was incubated at 37°C for 30 min. Then, the absorbance of the mixture at 536 nm was measured. Deionized water and VC were used as the blank and positive control, respectively. The scavenging activity on $^{\cdot}OH$ was calculated by the following equation:

Scavenging activity (%) = (Abssample – Absblank)/(Abso – Absblank) x 100

where Abso is the absorbance of the deionized water instead of H_2O_2 and sample in the assay system.

Metal (Fe^{2+}) chelating ability assay:

The *in vitro* Fe^{2+} chelating ability of plant extract was assayed according to the method of [22], with slight modification [23]. Briefly, 900 µl of aqueous $FeSO_4$ (500 µM) and 150µl of mesocarp extract were incubated for 5min at room temperature. Seventy eight microlitres (78 µl) of 1,10-phenanthroline (0.25% w/v, aqueous) was added.The absorbance of the orange colour solution was read at 510nm with a spectrophotometer. The principle of the assay is based on distruption of o-phenanthroline - $Fe2^{+}$complex in the presence of chelating agent. The *in vitro* of Fe^{2+} chelating ability of the sample was calculated by using the following formula:

Chelating ability(%) = (Acontrol - A sample)/Acontrol x 100

Where Acontrol = The absorbance of the control (reaction mixture in the absence of sample) ($FeSO_4$ alone); Asample = The absorbance of the reaction mixture(sample,$FeSO_4$ and 1,10-phenanthroline).

***In vitro* inhibition of lipid peroxidation assay:**

In vitro inhibition of lipid peroxidation was estimated according to the method of Ruberto and Baratta [24]. In this assay, egg yolk homogenate served as lipid rich medium and $FeSO_4$ acted as initiator of lipid peroxidation. Briefly, 50µl of plant extract was mixed with 0.25 ml 10% egg yolk. This was followed by the addition of 10 µl $FeSo_4$ (0.07M aqueous).The mixture was incubated at room temperature for 30min.This was followed by the addition of 0.75 ml of glacial acetic acid(5%v/v aqueous) and 0.75ml of thiobarbituric acid 0.8% in sodium dodecyl sulphate (SDS) 1.1%.The mixture was incubated in a boiling water bath (90°C) for 60 min, cooled and centrifuged at 3000rpm.One milliliter(1ml) of the pink colour supernatant was read at 532 nm on a spectrophotometer. Ascorbic acid was used as reference standard was treated the same way as the extract.

In vitro inhibition of lipid peroxidation = (Acontol – Asample)/Acontrol x 100

Determination of total phenolic content:

The total phenolic content of the extract was determined according to the method described [25]. In this assay, the phenolic group present in plant extract interacts with Folin-Ciocalteau in alkaline medium using Na_2CO_3 solution giving a blue colour, which has maximum absorption at 685nm and correlates with total phenolic content. 0.1ml of aqueous

extract of the plant of different concentrations (2 - 10%) was rapidly mixed with 0.1 ml of FolinCiocalteu reagent followed by the addition of 0.3ml sodium carbonate (15%,w/v) solution. The mixture was incubated in the dark for 30min.The absorbance of the blue colour was read at 760 nm after 30 min on a spectrophotometer. The total phenolic content was extrapolated from a standard curve using tannic acid (graded doses, 50 – 250 µg/ml) as a standard phenol.

Determination of total flavonoids:

TFC of the leaf extract was determined using the method described by Kumaran and Karunakaran[26], with slight modification. Briefly, 0.5 ml of extract solution (1 mg/ml) andstandard (quercetin) at different concentrations were taken in test tubes. 3.0 ml of methanol followed by 0.1 ml of 10% aluminum chloride solution was added into the test tubes. Two hundred millilitresof 1M potassium acetate solution was added to the mixtures in the test tubes. Furthermore, each reaction test tube was then immediately diluted with 2.8 ml of distilled water and mixed to incubate for 30 min at room temperature to complete the reaction. The absorbance of pink colored solution was noted at 415 nm using a spectrophotometer against blank methanol. TFC of the extract was expressed as quercetin equivalents (QE) after calculation using the following equation:

$C=(c \cdot V)/m$,

where, C =total flavonoid contents, mg/g plant extract in QE, c = concentration of quercetin obtained from calibration curve (mg/ml), V =the volume of the sample solution (ml), m= weight of the sample (g). All tests were conducted in triplicate.

Total antioxidant activity

The total antioxidant capacity of the extracts/fractions was determined with phosphomolybdenum using ascorbic acid as the standard. The assay was based on the reduction of Mo (vi) to Mo (v) by the extracts/fractions and the subsequent formation of a green phosphomolybdate (v) complex at acidic pH.0.1ml of the extract/fraction (100 µg/ml) solution was combined with 3ml of reagent (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90min. After the samples had cooled to room temperature, absorbance of the aqueous solution of each was read at 695nm against blank in a spectrophotometer. The blank solution contained 3ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions as the rest of the sample. The antioxidant capacity was expressed as the equivalent of ascorbic acid[27].

Anti-hemolytic assay:

Anti-hemolytic activity was assessed by following the spectrophotometric method [28, 29]. From a normal healthy individual, 5 ml of blood was taken and centrifuged at 1500 rpm for 3 min. Pellet of blood was washed three times in sterile phosphate buffer saline solution (pH 7.2). Obtained RBC pellets were washed twice and then diluted to 20% cell suspension with PBS.

Test

500 µl of RBC + 500 µl of extract (different concentrations) + 250 µl of $HgCl_2$ (5 µM) were incubated at 37°C for 3h in a water bath. Thereafter, 2 ml of PBS was added and centrifuge at 2000 g for 10 min. Absorbance of the supernatant was read at 540 nm. For the control, extract was replaced with PBS. Tannic acid was used as standard.

$$\text{Calculation} = \frac{A_{Control} - A_{Test}}{A_{Control}}$$

Anti-denaturation activity:

The method of Williams et al. (2008) [30], was employed for the anti-denaturation assay. A solution of 0.2%w/v of BSA was prepared in Tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. Different concentrations of various fractions of the extract were prepared. 50 µl of these different concentrations was transferred to Eppendorf tubes using 1ml micro pipette. 5ml of 0.2% W/V BSA was added to all the above Eppendorf tubes. The standard consisted 10µg/ml of diclofenac sodium in methanol with 5ml 0.2% W/A BSA solution, quercetin, tannicacid. The test tubes were heated at 72°C for 5 min and then cooled for 10 min. The absorbance of these solutions was determined by using UV/Vis Double beam spectrophotometer (Elico SL -196) at a wave length of 660 nm. The percentage inhibition of

precipitation (denaturation of the protein) was determined on a percentage basis relative to the control using the following formula:

$$\text{Inhibition of denaturation (\%)} = \frac{(\text{Abs of control} - \text{Abs of extract})}{\text{Abs of control}} \times 100$$

Results

HPLC analysis

HPLC fingerprinting of *C.portoricensis* (leaves and root) methanolic extract revealed the presence of the gallic acid (t_R = 10.15 min; peak 1), catechin (t_R = 15.09 min; peak 2); chlorogenic acid (t_R = 21.56 min; peak 3), caffeic acid (t_R = 25.73 min; peak 4), ellagic acid (t_R = 29.88 min; peak 5), rosmarinic acid (t_R = 35.12 min; peak 6), rutin (t_R = 39.73 min; peak 7), isoquercitrin (t_R = 44.91 min; peak 8), quercetin (t_R = 51.26 min; peak 9) and kaempferol (t_R = 57.04 min; peak 10) (Figure 1andTable1). The HPLC analysis revealed that flavonoids (quercetin, isoquercitrin, rutin and kaempferol), tannins (catechin) and phenolics acids (gallic, chlorogenic, ellagic, rosmarinic and caffeic acids) arepresentintheextractofC.*portoricensis*.

The phytochemical screening revealed the presence of alkaloids, saponins, tannins, flavonoids and glycosides with steroidal rings (Table 2).

HPLC analysis for Carotenoids

Calibration curve:

Tocopherol was: Y = 34721x + 1046.9 (r = 0.9999); t_R 13.81 min. LOD = 0.023 µg/mL and LOQ = 0.075 µg/mL.

β-carotene: Y = 27732x + 1156.8 (r = 0.9988); t_R 23.59 min. LOD = 0.031 µg/mL and LOQ = 0.099 µg/mL.

Lycopene: Y = 31058x + 1527.1 (r = 0.9999); t_R 29.14 min. LOD = 0.028 µg/mL and LOQ = 0.091 µg/mL. (Figure 2, table 2).

Discussion

Phytochemical screening of *C. portoricensis* showed that saponins, flavonoids, alkaloids, terpenoids and cardiac glycosides were present in the extract from both the leaf and root back, while phlobatannins, steroids and anthraquinones were absent; but tannins was present only in the leaf extract but absent in the root bark extract, this result corroborates the findings by Onyeama *et al.,* (2012)[7]. The functions of alkaloids in plants are mostly unknown and their importance in plants metabolism is debatable. Most alkaloids are very toxic and, therefore, have the potential to function in chemical defense cache of plants against attack by herbivores and micro-organisms[31] and consequently, because of its very toxic nature, may be harmful to man and animals. Anti-protozoan, anti-cancer, anti-inflamatory and analgesic properties of alkaloids have been documented by [32]. There have been reports of antibacterial, cholesterol lowering activity, immune-stimulant and anti-oxidant activity of saponins[33]. Flavonoids and polyphenolic compounds were present in both leaves and roots of *C. portoricensis*. Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to reactive oxygen species (ROS), allergens, virus and carcinogens.

HPLC is a chromatographic technique which is used to separate, identify, quantify and purify individual components of a mixture. HPLC fingerprinting has become an important quality control tool for herbal samples. It provides quantitative and qualitative information to researchers as well as enables the screening of samples for the presence of new compounds[34]. The HPLC analysis as displayed in Tables 1&2 and Figures 1&2 correspondingly revealed that flavonoids (quercetin, isoquercitrin, rutin and kaempferol), tannins (catechin) and phenolics acids (gallic, chlorogenic, ellagic, rosmarinic and caffeic acids) arepresentin theextractofC. *portoricensis*. Also, some carotenoids like α-Tocopherol, β-Carotene and Lycopene were found through the HPLC fingerprinting. Both the leaves and root bark extracts showed a higher concentration of β-Carotene when compared with others.

Tannins are generally known to be useful in the treatment of inflamed or ulcerated tissues and have remarkable activity in cancer prevention[35]. Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity [36, 37, 38]. Flavonoids also lower the risk of heart diseases. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. Cardiac steroids are widely used in the treatment of congestive heart failure. They help in increasing the force

of contraction of the heart (positive ionotropic activity) in heart failure patients.

Phytochemical screening of the plant leaf extract revealed the presence of flavonoids and polyphenols, and more specifically 2-Hydroxy-4-methoxy benzoic acid, a secondary metabolite which probably carries out nucleophilic attack on some of the functional groups of the numerous hydrolytic enzymes and non-enzymatic polypeptides involved in snake venom toxicity, thereby neutralizing the venom[7].

Both extracts as shown in Table 4 exhibited remarkable contents of phenols and flavonoids, the root bark and leaf extract have 705.12and 1448.73mg/gTAE, respectively; the root bark and leaf extracts had a good total antioxidant capacity (TAC) of 251.153 and 274.652 mMAAE/mg respectively, which showed commendable antioxidant activities.

The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. The reductive potential of both extracts are shown in Figure 3, and this portrays the leaf extract as the best in this regard, but still low when compared with the quercetin standard, while Figure 4 shows the DPPH radical scavenging activity, and the root bark extract shows a better power in scavenging this radical, but not as high as the tannic acid standard. The abilities of the extracts to scavenge ABTS radical was shown in Figure 5, the leaf extract demonstrated a better ability to scavenge this radical than the root bark extract. The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage in adjacent biomolecules[39]. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins, their capacities to forage hydroxyl radical (OH) was illustrated in Figure 6, where the leaf extract had a very good potential to hunt down this radical even comparable to the standard mannitol.

Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms, since it reduces the concentration of the catalyzing transition metal in LPO[40]. The abilities to chelate reactive metals and scavenge nitric oxide (NO) radicals are demonstrated in Figures 7 and 8. The leaf extract performs better in these activities than the root bark extract, although in iron chelation activity, at high concentration, there is almost no significant difference in the activities of the extract and the standard, and also in the NO scavenging assay, the extracts at all concentrations showed comparable activities but different from the standard.

Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver[41]. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. The lipid peroxidation assay was depicted by Figure 9, and it showed the leaf extract having more power to reduce lipid peroxidation than the root bark extract.

The assays to confirm that the extracts possess anti-inflammatory properties were also done and reported in Figures 10 and 11. These are the antihemolytic and antidenaturation assays. Quite a number of extracts have been demonstrated to protect and stabilize red blood cells that were exposed to a combined hypotonic and hemolytic induced stress.

The degree of inhibition of BSA denaturation increased with the decrease in the concentration of both the extracts as stated by[30] that the anti-denaturation of the drug will be more at lower concentration. Both extracts showed a concentration dependent increase in both antihemolytic and antidenaturation activities, but the leaves extract demonstrated high potentials than the root bark extract.

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Table 1. Composition of *C.portoricensis*methanolic extract.

Compounds	leaves mg/g	root mg/g	LOD µg/mL	LOQ µg/Ml
Gallic acid	0.72 ± 0.03 a	0.79 ± 0.01 a	0.024	0.079
Catechin	0.69 ± 0.01 a	1.81 ± 0.02 b	0.019	0.063
Chlorogenic acid	2.85 ± 0.01 b	1.78 ± 0.01 b	0.007	0.023
Caffeic acid	0.98 ± 0.02 c	4.15 ± 0.03 c	0.035	0.115
Ellagic acid	2.41 ± 0.01 d	2.26 ± 0.01 d	0.042	0.138
Rosmarinic acid	3.06 ± 0.01 e	0.83 ± 0.01 a	0.021	0.070
Rutin	0.75 ± 0.02 a	2.19 ± 0.03 d	0.028	0.091
Isoquercitrin	1.83 ± 0.01f	0.87 ± 0.02 a	0.010	0.032
Quercetin	2.32 ± 0.01 d	3.05 ± 0.01 e	0.015	0.049
Kaempferol	1.17 ± 0.03 c	1.74 ± 0.01 b	0.013	0.042

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.01$.

Table 2 – Carotenoids compositions

Carotenoids	*C. portoricensis* (leaves) mg/g	*C portoriensis* (root) mg/g
Tocophero	0.71 ± 0.01 a	0.95 ± 0.02 a
β-Carotene	1.98 ± 0.03 b	1.84 ± 0.01 b
Lycopene	2.26 ± 0.03 c	1.02 ± 0.03 a

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$

Table 3. Phytochemical constituents of *C.portoricensis* leaf and root bark extracts.

Constituents	Result in leaf	Result in root bark
Saponin	+	+
Tannins	+	-
Flavonoids	+	+
Alkaloids	+	+
Phlobatannins	-	-
Steroids	-	-
Isoprenoids/Terpenoids	+	+
Anthraquinones	-	-
Cardiac glycosides	+	+

Present = +, Absent = -.

Table 4. Total phenols, flavonoid contents and total antioxidant capacity of *Calliandra portoricensis*[$].

Calliandra portoricensis	Total phenols (mg TAE g^{-1})[A]	Total flavonoids (µg QE mg^{-1})[B]	Total antioxidant capacity (mM AAE mg^{-1})[C]
Root Bark	705.115	11555.7	251.153
Leaves	1448.73	17555.7	274.652

[$]Values are expressed as mean ± SD (n =3); [A]TAE-tannic acid equivalents;[B]QE-quercetin equivalents; [C]AAE-ascorbic acid equivalents.

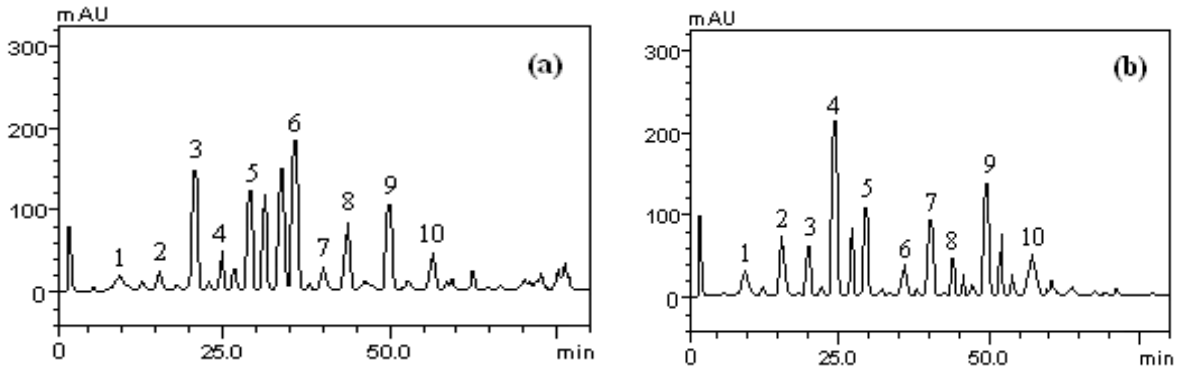


Figure 1. Representative high performance liquid chromatography profile of *Caliandra portoricensis* leaves (a) and root (b),detection UV was at 327nm.Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), rosmarinic acid (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercetin (peak 9) and kaempferol (peak 10).

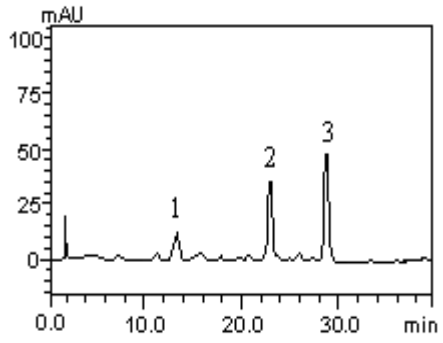
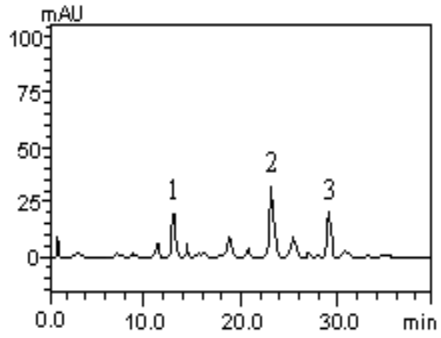
***Caliandra portoriensis* (leaves)**	***Caliandra portoriensis* (root)**
	

Figure 2 - High performance liquid chromatography plants carotenoids profile. Tocopherol (peak 1), β-carotene (peak 2) and lycopene (peak 3), detection UV was at 450 nm.

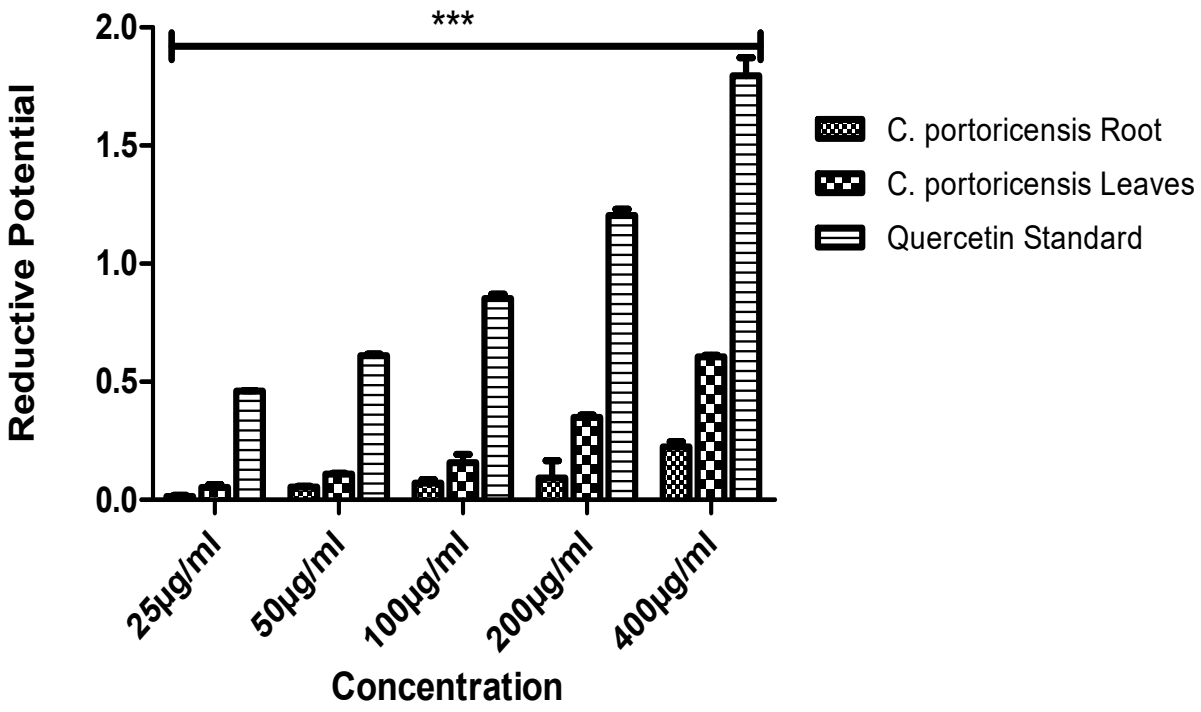


Figure 3. Reducing power of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

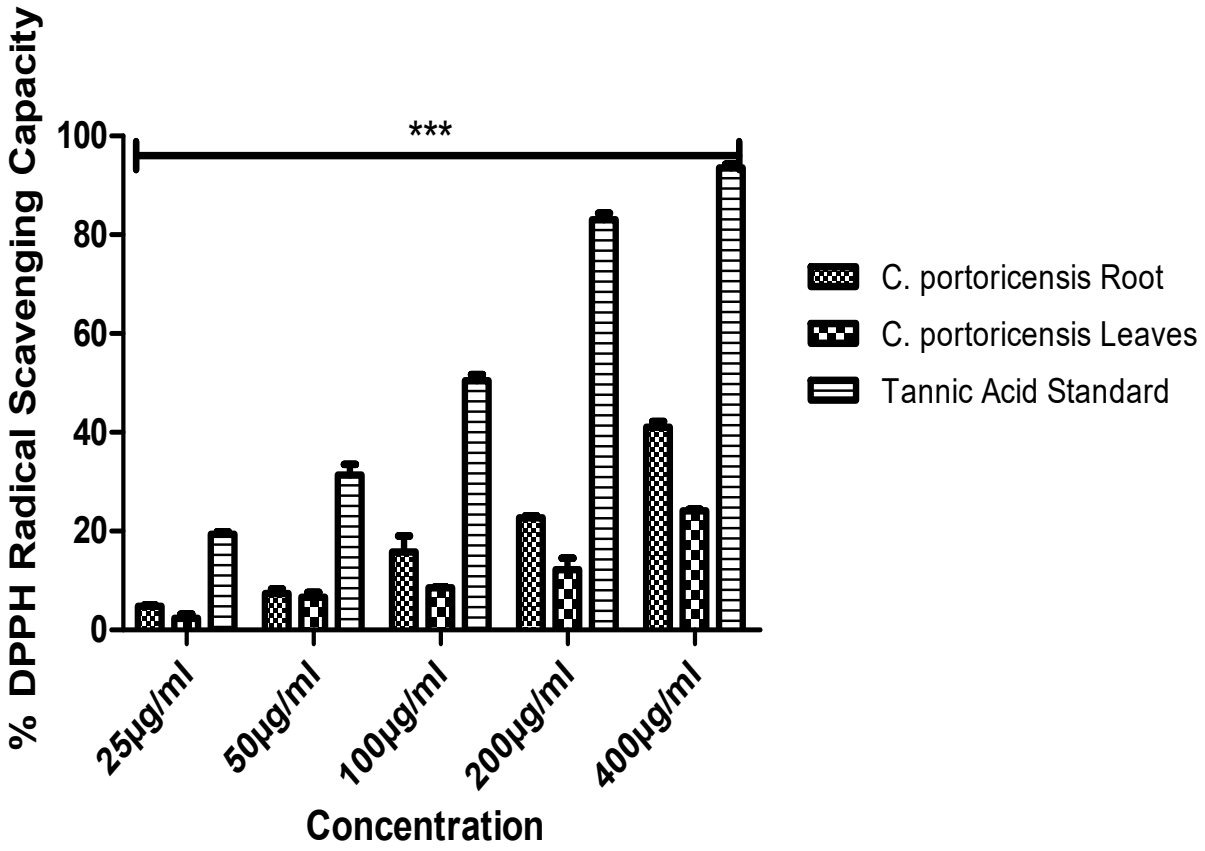


Figure 4. DPPH radical scavenging activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

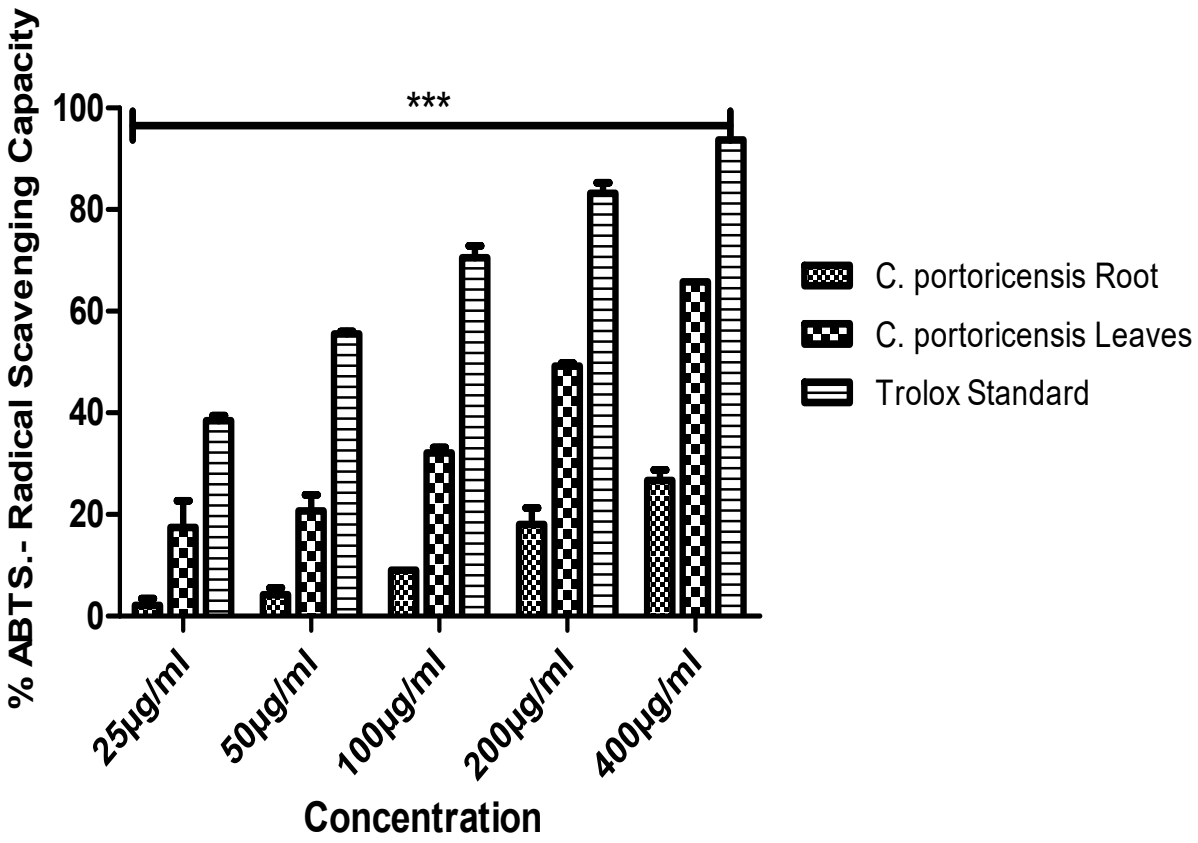


Figure 5: ABTS radical scavenging activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

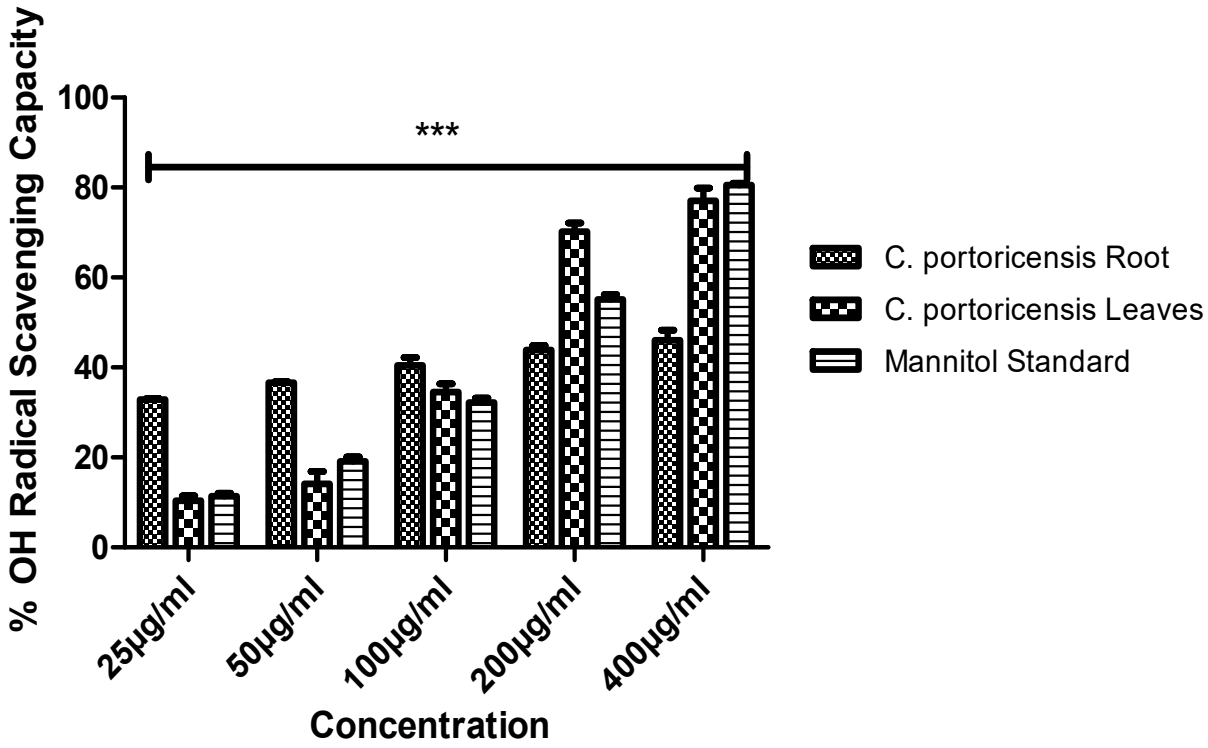


Figure 6: Hydroxyl radical scavenging activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

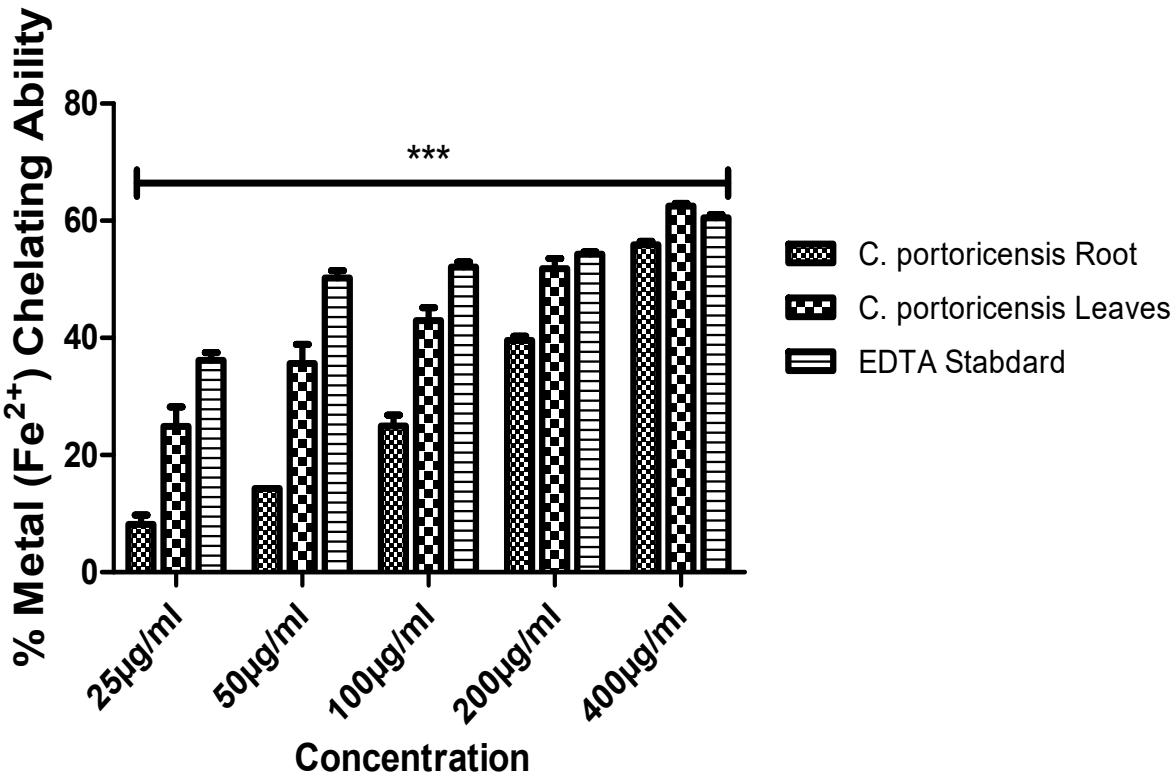


Figure 7. Metal (Fe^{2+}) Chelation activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

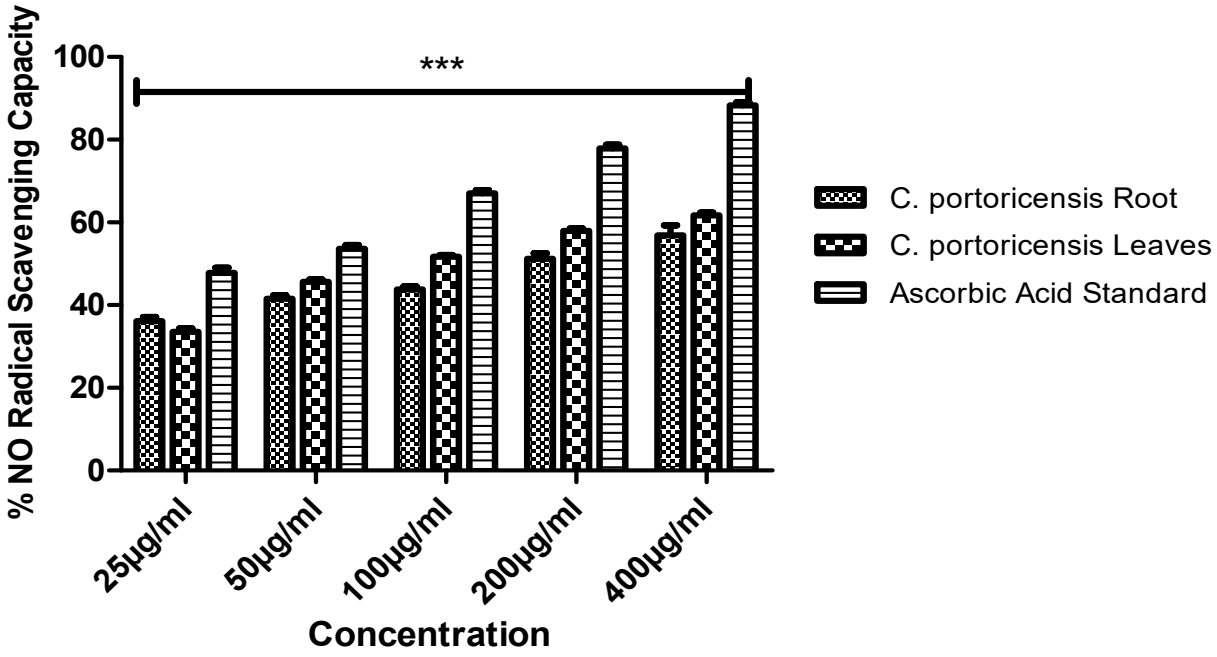


Figure 8. Nitric oxide radical scavenging activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

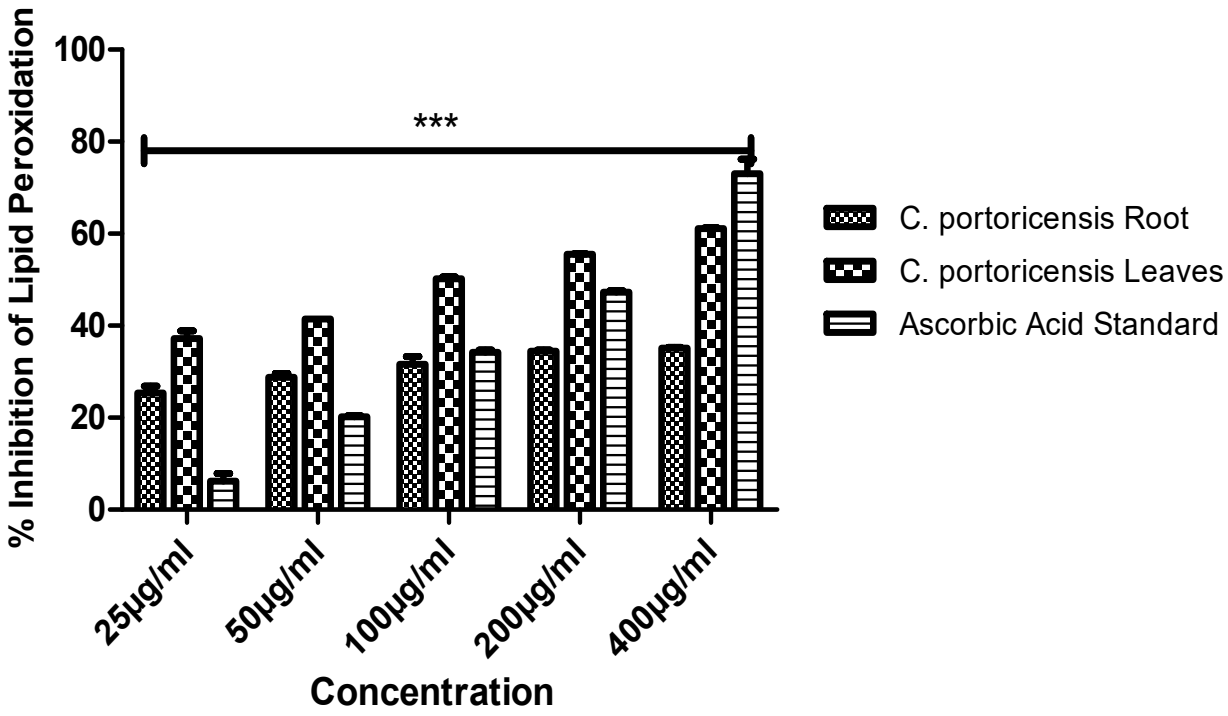


Figure 9: Inhibition of Lipid Peroxidation activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

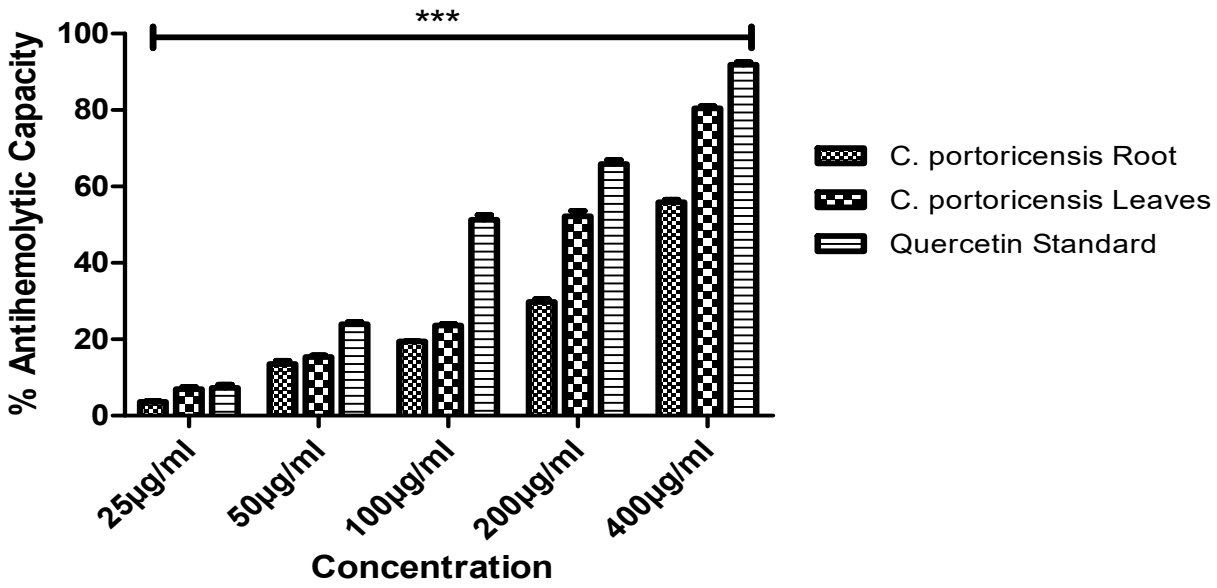


Figure 10: Antihemolytic activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.

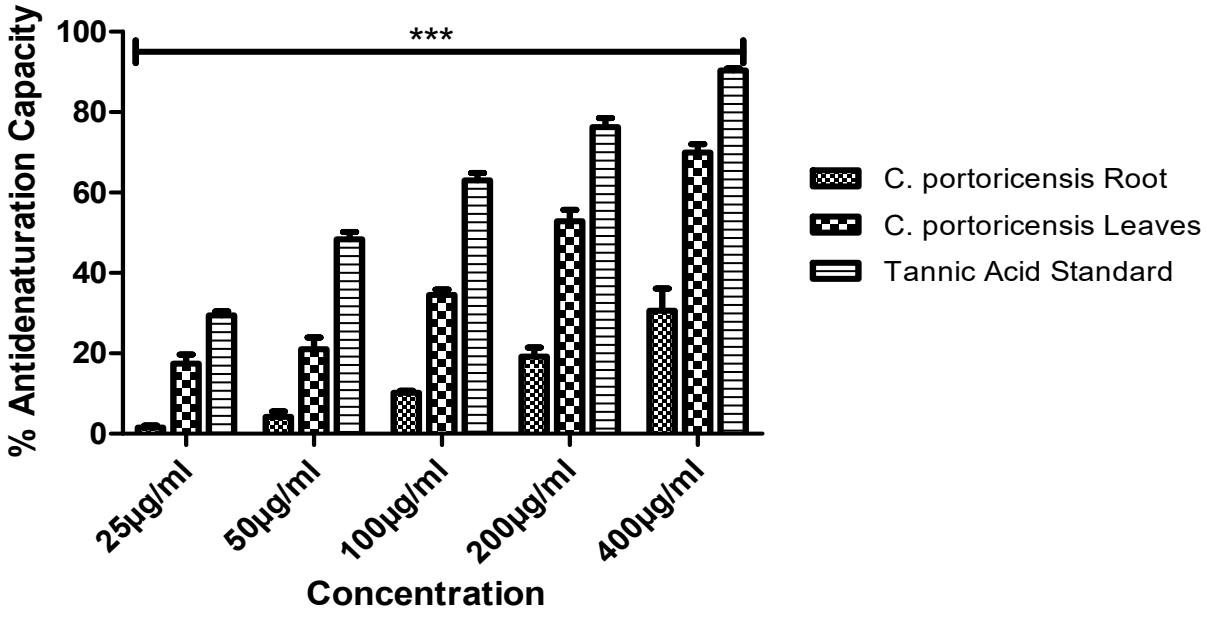


Figure 11: Antidenaturation activity of *Calliandra portoricensis* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations*.