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IN VITRO ANTIOXIDANT ACTIVITY, ANTIMICROBIAL AND PRELIMINARY CYTOTOXIC ACTIVITY OF *CYNOMETRA RAMIFLORA*- A MANGROVE PLANT

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ABSTRACT

A mangrove medicinal plant *Cynometra ramiflora* (Family: Leguminosae) was selected to investigate the bioactivities namely antioxidant, antimicrobial and preliminary cytotoxic activity using methanol and chloroform extracts of the leaves and stems, respectively. In 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 50% inhibitory concentration (IC_{50}) of the methanolic stem extract was found to be 31.62 µg.mL^{-1}. Reducing power of the same extract demonstrated consistent increase in a concentration-dependent manner and was comparable with quercetin while ferric reducing antioxidant power (FRAP) assay revealed potential total antioxidant capacity (84.0 mM Fe (II)/g of extract). In addition, the presence of total phenolics (96.2 mg GAE/g of extract), total flavonoids (166.4 mg QE/g of extract) and tannins content (80.4 mg GAE/g of extract) were determined in the methanolic stem extract. The chloroformic stem extract exhibited moderate antimicrobial activity against a number of bacterial strains while the MIC values of extracts were in the range from 62.5 to 500µg.mL^{-1}. The methanolic stem and leaf extracts demonstrated strong lethality in preliminary cytotoxicity assay using brine shrimp nauplii where the 50% lethal concentration (LC_{50}) values were 1.596 and 4.613 µg.mL^{-1} respectively. It can be therefore concluded that the methanolic extracts of *C. ramiflora* possess potential antioxidant, antimicrobial and strong preliminary cytotoxic activity and could be further exploited for prospective scientific exploration towards bioactive principles.

Keywords: *Cynometra ramiflora*, antioxidant, antimicrobial, cytotoxic

INTRODUCTION

Medicinal plants are the imperative sources of indigenous medical systems (**Ahmed *et al.*, 2009**) and they serve the primary health care needs of more than 80% of the people in the world (**Hassan *et al.*, 2009**). Plant derived natural products have been the single most productive source of leads for the discovery of novel drugs (**Cragg and Newman, 2013**). However, the global efforts remain still invigorated by the numerous attempts to analyze bioactivity and search for bioactive compounds from plant sources. In this context, mangrove ecosystem offers a large number of relatively untapped species in an exclusive climatic condition with unique metabolic profile.

Cynometra ramiflora Linn (Family: Leguminosae) is small to medium sized tree growing in the Sundarbans mangrove forest of Bangladesh. It is also distributed in the coastal mangrove system of India, Malaysia, Sri Lanka, China, Philippines, New Guinea and Australia (**Siraj *et al.*, 2013**). The plant is locally known as Shingra (**Hasan, 2000**) and also referred to as Balitbitan, Belangkan, Gal Mendora etc. (**Siraj *et al.*, 2013**). Historically, various plant parts of *C. ramiflora* such as leaves, roots and seeds are used in folk medicine to help cure a variety of diseases such as hypertension, diabetes, gout and hypercholesterolemia (**Muhtadi *et al.*, 2014**). Leaves are used as an anti-herpetic (any of several viral diseases) and roots are purgative (an agent for removing the bowels). Seed-derived oil is used to make lotion for skin diseases (**Siraj *et al.*, 2013**). Apart from the traditional use, *C. ramiflora* has been the subject to modern experimental endeavor during the last decade, particularly in bioassay-guided activity analysis. Different extracts of *C. ramiflora* have been reported to have antioxidant (**Bunyapraphatsara *et al.*, 2003; Muhtadi *et al.*, 2014**), antihyperglycemic (**Tiwari *et al.*, 2008**), cytotoxic (**Uddin *et al.*, 2011; Muhtadi *et al.*, 2014**), antibacterial and antinociceptive (**Siraj *et al.*, 2013**) activity. However, limited research have been done on chemical constituents and systematic bioactivity screening of *C. ramiflora*, only Muhtadi et al. **(2014)** reported that this plant possessed caffeic acid, apigenin and 3-(2,3,4-trihydroxyphenyl)-7-hydroxycoumarin.

The nature of the bioactivity analysis is permutative due to the uses of various plant parts and employing different solvent systems to extract the bioactive natural products. Therefore there remains the scope to analyze the bioactivity of *C. ramiflora*. Here we report the antioxidant, antimicrobial and preliminary cytotoxic activity of methanolic and choloroformic leaf and stem extract of *C. ramiflora*.

MATERIALS AND METHODS

Drugs and chemicals

The solvents and chemicals used in the present investigation were of analytical grade from Merck (Darmstadt, Germany) unless otherwise stated. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), and Folin-Ciocalteu's reagent were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

Collection of plant material

Fully grown *C. ramiflora* was collected from Dhangmaree, Chadpai Range of the Sundarbans East Division situated at Bagerhat district, Bangladesh on 16th December, 2011 and collected plant samples were sent to Bangladesh National Herbarium, Dhaka, Bangladesh for taxonomical identification.

Preparation and extraction of plant material

C. ramiflora leaf and stem were separated from each other and then cleaned by gentle washing with distilled water followed by air drying for several weeks. The dried material was ground into coarse powder with a motorized plant grinder

(Capacitor Start motor, Wuhu Motor Factory, China). The powder was kept in a dry, cool and dark place in a suitable airtight container until analysis commenced. About 120 gm of powdered leaf and 160 gm of powdered stem was soaked into 440 mL and 500 mL petroleum ether respectively, in a clean, flat-bottomed glass container for a period of 5 days with occasional stirring and shaking. It was then filtered and after this first filtration, the remaining residues (approx. 115 gm powdered leaf and 157 gm powdered stem) were soaked into 400 mL and 470 mL chloroform respectively, kept for a period of 6 days and then filtered; then final remaining residues (113 gm of powdered leaf and 155 gm of powdered stem) were soaked into 390 mL and 450 mL methanol respectively, kept for a period of 6 days with occasional stirring and shaking and then filtered. Coarse plant material was separated from the mixture by pouring through a clean cloth filter. These extracts were passed through filter paper, and the filtrates were evaporated, yielding the chloroformic and methanolic extracts, respectively. The extraction process yielded six extracts among which chloroform and methanol extracts of leaf and stem were used in this study.

Determination of DPPH free radical scavenging activity

In determining DPPH free radical scavenging activity, different concentrations of the extracts were prepared in the range from 1.57 to 400 $\mu g.mL^{-1}$ and then 2 mL of 0.004% DPPH solution was added in each test tube. The test tubes were allowed to stand in the dark for 30 min to complete the reaction and then absorbance was recorded at 517 nm (**Gupta *et al.*, 2003**). The decrease in absorbance with respect to the blank was also measured. Control was prepared in the same way as the sample except addition of sample or standard. Percent scavenging activity was calculated using the formula: scavenging activity = $(A_0-A_1)/A_0 \times 100\%$, where A_0 is the absorbance of control, and A_1 is the absorbance of sample or standard. The experiment was carried out in triplicate.

Determination of reducing power

The reducing power of the extract was evaluated according to the method of **Oyaizu** et al (1986). According to this method different concentrations of extracts (25-400 $\mu g.mL^{-1}$) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Blank was prepared same as sample without addition of extract or standard. Quercetin was used as standard. Reducing power of the extract was compared with standard quercetin by drawing curve plotting absorbance against concentration.

Determination of total antioxidant activity

Total antioxidant activity in the form of the FRAP assay was carried out according to the method of **Benzie and Strain** (1996). The stock solutions included 300 mM acetate buffer (3.1 gm $C_2H_3NaO_2$ hydrate and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM $FeCl_3$ hydrate solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution and 2.5 mL $FeCl_3$ hydrate solution and then warmed at 37°C before using. Extracts of 0.2 mL were allowed to react with 3 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve. All determinations were performed in triplicate.

Determination of total phenolic, flavonoid and tannin content

Total phenolic content of the extracts was determined by using Folin-Ciocalteau assay (**Peteros and Mylene, 2010**) where extract or standard solution (25 to 250 $\mu g.mL^{-1}$) of 1 mL was added to distilled water (9 mL), and then 1 mL of FC reagent (10 times diluted with distilled water). After 5 minutes; 10 mL 7% Na_2CO_3 was added to the mixture, kept for 30 minutes and then the absorbance was measured at 750 nm using UV spectrophotometer. The percentage of total phenolics was calculated from the calibration curve of gallic acid plotted by using the similar procedure as the extracts and expressed as mg gallic acid equivalent (GAE)/g dried plant material.

Total flavonoid content of the extracts was determined by using an aluminium chloride colorometric assay (**Peteros and Mylene, 2010**) where extract or standard solution (25 to 400 $\mu g.mL^{-1}$) of 1 mL was added to distilled water (5 mL); 0.3 mL 5% $NaNO_2$ then added to the mixture followed by addition of 0.6 mL 10% $AlCl_3$ and 2 mL 1M NaOH after 5 min. Then absorbance was measured at 510 nm; percentage of total flavonoids was calculated from the calibration curve of quercetin plotted by using the similar procedure as the extracts and expressed as mg quercetin equivalent (QE)/g dried plant material.

Total tannin content in plant extract was determined by using Folin-Denis method as described by Polshettiwar et al. 2007 (**Polshettiwar and Ganjiwale, 2007**). Extract solution (1 ml of 100 $\mu g.ml^{-1}$) was mixed with 7.5 mL distilled water and 0.5 mL FC reagent. After 5 min, 1 ml of 35% sodium carbonate was added and the final volume was adjusted to 10 mL with distilled water. The mixture was allowed at room temperature for 30 minutes and absorbance was measured at 725 nm using spectrophotometer. Gallic acid was used to prepare the standard curve.

Screening for antimicrobial activity

Ten strains of microorganisms were tested in this study. Five Gram-positive bacteria include *Bacillus cereus* (ATCC 14579), *Bacillus megaterium* (ATCC 14581), *B. subtilis* (ATCC 6059), *Micrococcus luteus* (ATCC 4698), *Staphylococcus aereus* (ATCC 25923) and five Gram-negative bacteria including *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27833), *Shigella dysenteriae* (ATCC 26131) and *Salmonella typhi* (ATCC 13311). These strains were collected from the Microbiology Laboratory, Khulna University, Bangladesh, as pure cultures were used. The bacterial isolates were cultivated in nutrient broth at 37 °C for 24 hours.

Antimicrobial activity of *C. ramiflora* extracts was tested by disc diffusion method (**Bauer *et al.*, 1966**). Bacterial strains were maintained on the nutrient agar medium. The sterile filter paper discs were prepared by adding desired concentration (250 and 500 µg/disc) of extracts on the disc with the help of a micropipette. Standard tetracycline disc (30 µg/disc), discs containing extracts and control discs were then impregnated, incubated overnight at 37˚C, checked for the zone of inhibitions and then diameters of inhibition zone were measured in millimeters(mm). Each measurement was carried out in triplicate.

Determination of MIC values

The extracts that showed antimicrobial activity in disc diffusion were later tested to determine the MIC value for each bacterial sample by using broth macrodilution method (**Nascimento *et al.*, 2000**) according to the Clinical and Laboratory Standards Institute (CLSI) protocol (**CLSI, 2010**). Briefly, Bacterial samples were grown in nutrient broth for 6 hours. Approximately 100 µL of these cultures containing 10^6cells/ml was inoculated in separate tubes with nutrient broth supplemented with different concentration of the extracts ranging from 7.8 to 500 $\mu g.mL^{-1}$. Afterwards 24 hours incubation at 37°C, the MIC of each sample was determined by measuring the optical density in the spectrophotometer (620 nm), comparing the sample readout with the non inoculated nutrient broth. Tetracycline was used as standard (0.05-2 µg/ml).

Brine shrimp lethality bioassay for preliminary cytotoxic activity

The eggs of the brine shrimp, *Artemia salina*, and sea water were collected from BRAC prawn hatchery, Sreeghat, Bagerhat, Bangladesh. Followed by 24h hatching, eggs matured and these were then called nauplii. *C. ramiflora* extracts were dissolved in DMSO and was added in test tubes in such a way that each tube contained 4 mL of sea water with different concentrations of extracts ranging from 5 to 320 $\mu g.mL^{-1}$. The final volume for each test tube was adjusted to 10 mL with artificial sea water and 10 living nauplii were introduced into each tube. After observing test tubes in the subsequent 24 hours, the number of survived nauplii was recorded (**Apu *et al.*, 2010**). The percentage of dead nauplii in the test and standard group was established by linear correlation when logarithm concentration versus percentage of mortality was plotted and LC_{50} value was calculated using Graphpad Prism Version 6.01 (GraphPad Software, Inc., USA).

RESULTS AND DISCUSSION

DPPH-scavenging assay

A number of methods are available for the determination of free radical scavenging activity but the DPPH assay employing the stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience (**Concepcion *et al.*, 1998**). The method is based on the reduction of 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound) in the presence of antioxidant substances having hydrogen donating groups (RH) such as phenolics, flavonoids compounds due to the formation of non radical DPPH-H form (**Paixao *et al.*, 2007**). Figure 1(A) and (B) shows the dose-dependent curve of DPPH radical scavenging activity of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* respectively. The leaf extracts of chloroformic and methanolic solvent system exhibited 50% inhibition (IC_{50}) at a concentration of 537.03 and 97.72 $\mu g.mL^{-1}$ while the values for stem extracts in the same solvent systems were found to be 606.7 and 31.62 $\mu g.mL^{-1}$ respectively and standard quercetin showed 8.95 $\mu g.mL^{-1}$ (Table 1). This experiment showed that the stem methanolic extract showed the higher antioxidant activity than the other extract, which could be due

to their presence of phenolic components of sample and the leaf extract of same solvent system showed moderate activity. It has been reported that free radical scavenging activity is greatly influenced by the phenolic components of samples (**Cheung *et al.*, 2003**). Chloroform extracts of both leaf and stem exhibited very poor scavenging activity that could be attributed due to absence of active antioxidant compounds (**Almey *et al.*, 2010**) and influence of the polarity of extracting solvent system (**Moure *et al.*, 2001**).

Reducing power assay

Reducing capacity is associated with antioxidant activity (**Meir *et al.*, 1995**). The reducing power of the extracts was determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)_6]^{3-}$ to ferro cyanide $[Fe(CN)_6]^{4-}$. The reducing power of the extracts increases with the increase in amount of sample. Figure 2(A) and (B) shows the reductive capabilities of the plant extract compared to standard quercetin. The data represented here (Table 1) showed that the methanolic extracts have strong reducing power than the chloroformic extract. These could be attributed due to the influence of solvent system (**Moure *et al.*, 2001**) and presence of active reductones (**Duh, 1998**). It has been published that the phenolic components in plants may act in a similar fashion as reductones and terminating free radical chain reaction (**Liu and Yao, 2007**). Lu and Foo, (2001) reported that a direct correlation exists between polar polyphenols and the reducing power observed in phosphomolybdenum method (**Lu and Foo, 2001**).

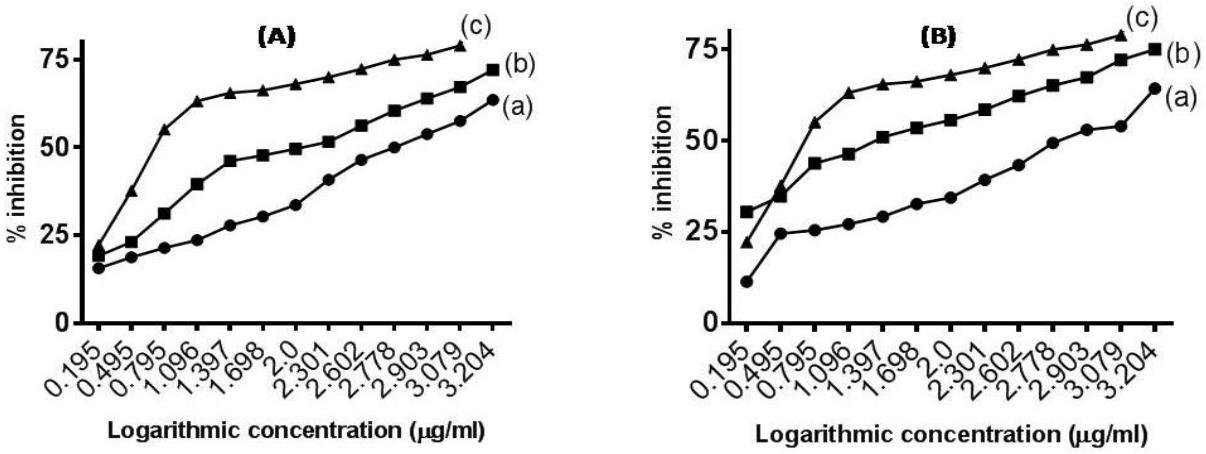

Figure 1 DPPH free radical scavenging activity of chloroform (a) and methanolic (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard quercetin (c).

Results of total antioxidant capacity, total phenol, flavonoid and tannin content assay

In this study, the total antioxidant potential of sample was determined using FRAP assay. In this assay, the antioxidant efficiency of the leaf extracts under investigation was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. A linear calibration curve of Quercetin, in the range of 6.25-100 µg.mL^{-1} with a coefficient of determination (R^2) value of 0.991 was obtained [Figure 3 (A)]. The results were expressed in µM Fe (II)/g of sample (Table 1). The results were expressed in µM Fe (II)/g of sample (Table 1). Chloroformic leaf and stem extracts of *C. ramiflora* showed the antioxidant capacity of 22.2 and 50 µM Fe (II)/g sample while leaf methanolic extract showed the moderate antioxidant capacity of 57.8 µM Fe (II)/g and stem methanolic extract showed the potential antioxidant capacity of 84.0 µM Fe (II)/g.

The results of total penolics, flavonoids and tannins content are presented in Table 1. In this study, total phenolics, flavonoids and tannins content were determined using calibration curves of standard [Figure 3(B), (C) and (D)]. The values of phenolics were found to be 6.3 and 84.8 mg in the leaf and 17.2 and 96.2 mg of GAE/g of dried plant material in the stem of chloroform and methanolic solvent system respectively (Table 1). The amount of total flavonoids content in leaf was found to be 68.4 and 86.2 mg and in stem 84.4 and 166.4 mg QE/g of dried plant material in chloroform and methanol respectively (Table 1). Total tannins content in leaf was 12.2 and 65 mg and in stem 23.2 and 80.4 mg GAE/g of dried plant material in chloroform and methanol respectively (Table 1). Analyzing the results, it has shown that the leaf and stem methanolic extracts have greater content of these compounds than the choloroformic extract. Polyphenolic compounds, like flavonoids, tannins and phenolic acids are commonly found in plants have been reported to have biological effects, including antioxidant activity (**Brown and Rice-Evan, 1998**) due to the presence of number of hydroxyl groups and their attachment with the aromatic ring, particularly in ortho-para position of phenolic structure, enhancing antioxidative capability of plant extract (**Sroka, 2005**). Phenolic compounds have the ability to donate electron that results the conversion of highly reactive free radicals to nonreactive stable molecules. Phenolic compounds not only neutralize lipid free radicals but also prevent the decomposition of highly reactive species (**Javanmardi *et al.*, 2003; Li *et al.*, 2009**). Flavonoids have a significant role in scavenging different reactive oxygen species like hydrogen peroxide, hydroxyl, peroxyl, superoxide anion etc. Tannin possesses strong antiradical and antioxidative properties especially due to large number of hydroxyl groups connected to the aromatic ring (**Sroka, 2005**). Tannins have also been reported to possess anticarcinogenic and antimutagenic potentials as well as antimicrobial properties (**Amarowicz 2007, Szollosi and Varga, 2002**). Considerable amount of total phenolic, flavonoid and tannin content was found in the methanolic extracts which are positively related with DPPH method, phosphomolybdenum method and FRAP assay, whereas chloroformic extract exhibited relatively lesser content and showed lower antioxidant activity.

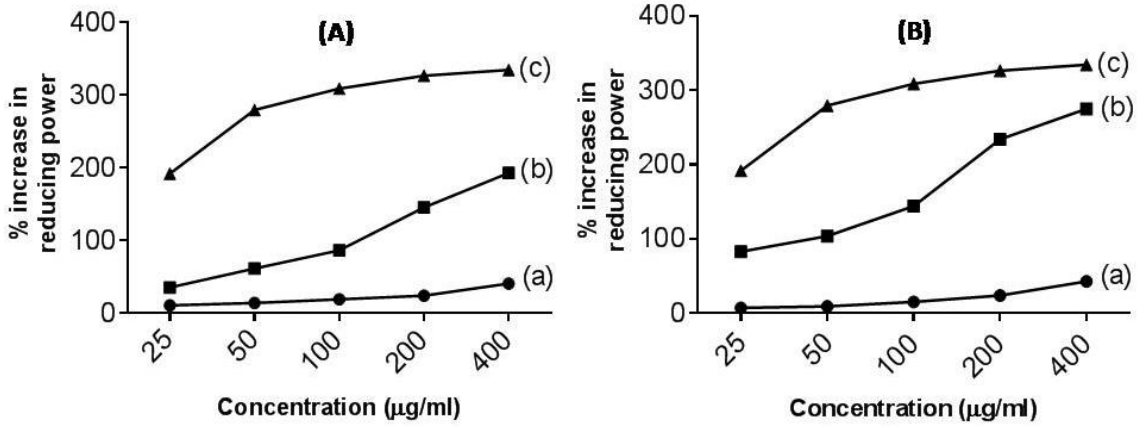

Figure 2 Reducing power of chloroform (a) and methanol (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard Quercetin (c).

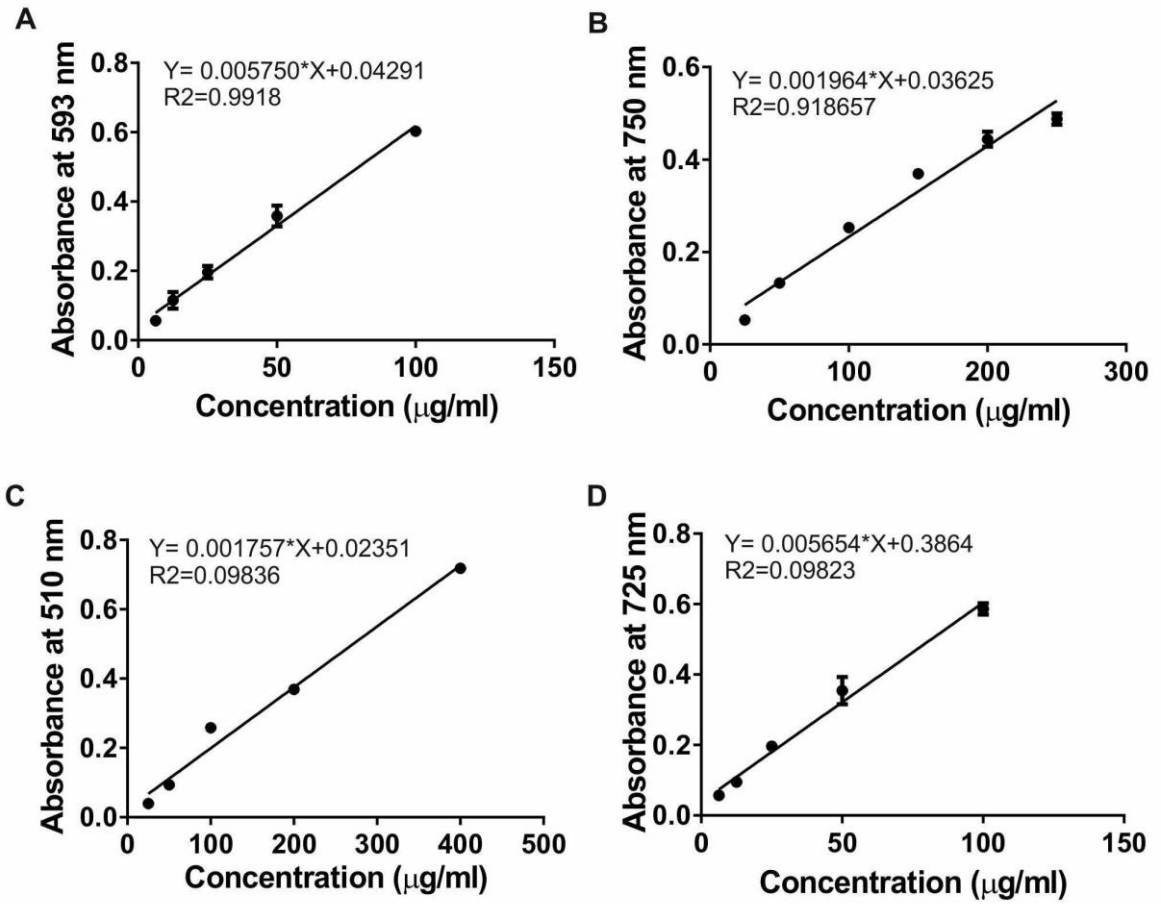

Figure 3 Standard calibration curve of, (A) Quercetin for determining total antioxidant power; (B) Gallic acid to estimate total phenolic content; (C) Quercetin to measure total flavonoid content and (D) Gallic acid to determine total tannin content of *C. ramiflora* leaf and stem, respectively.

Antimicrobial Screening

In this study, we have tested choloformic and methanolic leaf and stem extracts (250 and 500 µg/disc) of *C. ramiflora* for their antimicrobial activity against ATCC strains of some Gram-positive and Gram-negative bacteria. Standard antibiotic discs of Tetracycline (30 µg/disc) were used for comparison purpose. Among these extracts stem chloroformic extract showed better activity than the other extracts; however the leaf chloroformic extract failed to demonstrate any zone of inhibition against all the tested bacterial strains. Table 2 showed that the stem methanolic extract of *C. ramiflora* (250 µg/disc and 500 µg/disc) showed highest antimicrobial activity against *S. aereus* (17 mm and 18 mm) and *P. aeruginosa* (16 mm and 18 mm) respectively. Zone of inhibition of stem chloroformic extract of other strains includes, *B. cereus* (10 mm and 13 mm), *B. megaterium* (14 mm and 15 mm), *B. subtilis* (15 mm and 17 mm), *Micrococcus* (15 mm and 17 mm), *E. coli* (14 mm and 16 mm) and *S. dysenteriae* (9 mm and 15 mm) respectively. Stem methanolic extract of *C. ramiflora* showed moderate activity against seven bacterial strains and the zone of inhibition ranged between 6 to 12 mm and 7 to 14 mm, at the doses of 250 and 500 µg/disc, respectively (Table 3). Leaf methanolic extract showed the lowest antimicrobial activity against tested bacterial strains. Literature suggested that, the size of inhibitory zones <8 mm were considered as not active against microorganisms (**Bhalodia and Shukla, 2011**).

The MIC method was applied on extracts which showed antimicrobial activity in the disk diffusion method. The MIC values ($\mu g.mL^{-1}$) of the extracts against the experimental organisms obtained are represented in Table 2 and 3. The results revealed variability in the inhibitory concentrations of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* against tested bacteria. The results revealed variability in the inhibitory concentrations of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* against tested bacteria. The stem chloroformic extract showed activities in the range of MIC value 15 to 250 $\mu g.mL^{-1}$ (Table 2). The lowest MIC value was found to be that of stem chloroformic extract 15 $\mu g.mL^{-1}$ and 16 $\mu g.mL^{-1}$ against *S. aereus* ATCC 25923 and *P. aeruginosa* ATCC 27833. The stem methanolic extract showed activities in the range of MIC value 63.1 to 482 $\mu g.mL^{-1}$ (Table 2) and leaf methanolic extract showed 133 to 500 $\mu g.mL^{-1}$ (Table 3).

In this study, Chloroformic stem extract exhibited significant antimicrobial activity against all Gram-positive bacterial strains and three Gram-negative bacterial strains. Particularly, *S. aureus* was found to be more sensitive than the other bacterial strains which may be due to its cell wall structure and outer membrane (**Zaika, 1988**). Our results suggest that Gram-positive bacteria are generally more sensitive to the plant extracts and this was consistent with other previous studies (**Ceylan and Fung, 2004**).

Table 1 Summary of results related to DPPH-free radical scavenging power assay, reducing power, total antioxidant capacity, total phenolic, flavonoid and tannin content of chloroformic and methanolic leaf and stem extracts of *C. ramiflora*.

Name of the tests	Plant parts	Solvent systems	Values in respective units	Regression equation	R^2value
DPPH (IC_{50} value in $\mu g.mL^{-1}$)	leaf	Ch	537.03	y = 15.18x + 8.546	0.956
		Mt	103.0	y = 15.46x + 18.89	0.935
	stem	Ch	606.7	y = 13.88x + 11.37	0.925
		Mt	32.65	y = 12.69x + 30.78	0.959
	Standard (Quercetin)				
Total antioxidant capacity (µM Fe (II)/g)	leaf	Ch	22.2 ± 0.013	Quercetin calibration curve : y = 0.005x + 0.042	0.991
		Mt	57.8 ± 0.015		
	stem	Ch	50 ± 0.007		
		Mt	84 ± 0.009		
Total phenolic content (mg GAE/g of dry plant material)	leaf	Ch	6.3 ± 0.002	Gallic acid calibration curve : y = 0.002x + 0.036	0.968
		Mt	84.8 ± 0.023		
	stem	Ch	17.2 ± 0.019		
		Mt	96.2 ± 0.01		
Total flavonoid content (mg QE/g of dry plant material)	leaf	Ch	68.4 ± 0.016	Quercetin calibration curve : y = 0.001x + 0.023	0.984
		Mt	86.2 ± 0.028		
	stem	Ch	85.4 ± 0.026		
		Mt	166.4 ± 0.019		
Total tannin content (mg GAE/g of dry plant material)	leaf	Ch	12.2 ± 0.01	Gallic acid calibration curve : 0.005x + 0.037	0.988
		Mt	65 ± 0.011		
	stem	Ch	23.2 ± 0.009		
		Mt	80.4 ± 0.009		
Reducing Power (highest value at maximum Conc.)	leaf	Ch	0.592 ± 0.006	Standard (Quercetin) : 1.673 ± 0.012	
		Mt	1.238 ± 0.089		
	stem	Ch	0.685 ± 0.005		
		Mt	1.454 ± 0.386		

Legend: Ch – Chloroform, Mt– Methanol

Brine Shrimp Lethality Bioassay

In brine shrimp lethality bioassay, LC_{50} values of chloroformic and methanolic leaf extracts of *C. ramiflora* was found to be 8.273 and 4.613 $\mu g.mL^{-1}$ respectively and stem extract at the same solvent system was found to be 5.297 and 1.596 $\mu g.mL^{-1}$, compared to positive control vincristine sulphate (VS) with a LC_{50} value of 0.128 $\mu g.mL^{-1}$ (Table 3). Percent mortality of methanolic stem extract of *C. ramiflora* was proximal to the standard indicating the strong cytotoxic activity of this extract (**Peteros and Mylene, 2010**). The crude extracts resulting in LC_{50} values less than 250 $\mu g.mL^{-1}$ are considered significantly active (**Kabir *et al.*, 2012**).

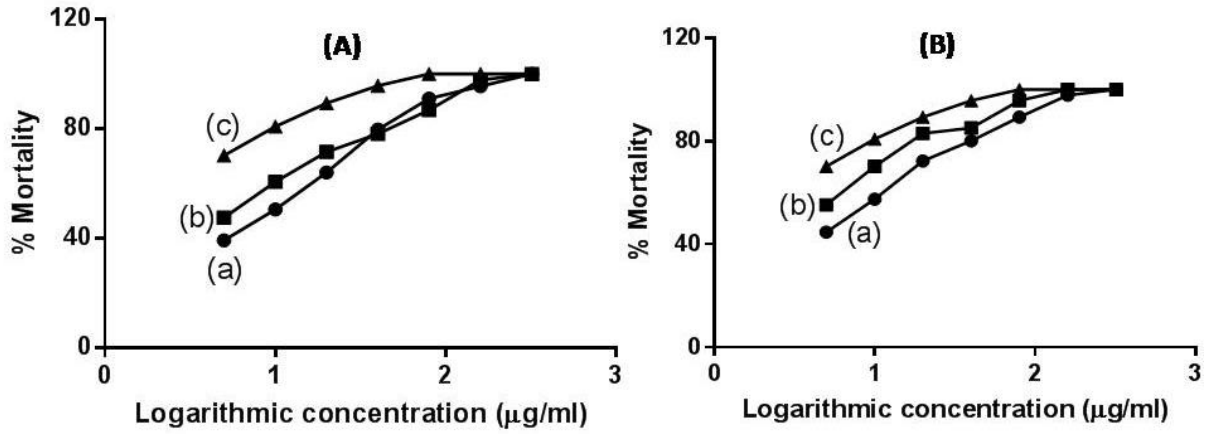

Figure 4 Brine shrimp lethality bioassay of chloroform (a) and methanolic (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard vincristine sulfate (c).

Table 2 Results of disc diffusion assay of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* and MIC values of the extracts against tested bacterial strains.

Tested Organisms	*C. ramiflora* leaves Mt-OH extract			*C. ramiflora* stem $CHCl_3$ extract			*C. ramiflora* stem Mt-OH extract			Positive control Tetracycline
	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)
	250 (µg/disk)	500 (µg/disk)		250 (µg/disk)	500 (µg/disk)		250 (µg/disk)	500 (µg/disk)		30 (µg/disk)
Gram-positive bacteria										
B. cereus (ATCC 14579)	7±1	9±1	411.6	10±2.6	13±1	150	8±1.7	11±1.8	482	32
B. megaterium (ATCC 18)	8±1	9.5±2.1	350	14±1	15±1	119.7	9±1.3	10±1	251	35
B. suntilis (ATCC 6059)	6±0.5	8±1	500	15±1.7	17±0.4	27	6±0	7±0	400	30
Micrococcus	6±0	8±1.7	415	15±1	17±0	101.1	6±0	8±0	462	34
S. aereus (ATCC 25923)	9±0	12±1.1	133	17±1	18±0	15	10±1	14±0	63.1	29
Gram-negative bacteria										
E. coli (ATCC 8739)	8±1.2	9±0	369	14±1.8	16±0	82.2	12±1.7	14±0	82	28
Klebsiella(ATCC 700603)	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27833)	7.5±1	11±0	321.6	16±1	18±0	16±0	10±1	13±0	70	32
S. dysenteriae (ATCC 26131)	-	-	-	9±1	15±0	250	-	-	-	25
S. typhi (ATCC 13311)	-	-	-	-	-	-	-	-	-	-

The brine shrimp lethality bioassay is normally conducted to draw inferences on the safety of the plant extracts and to further depict trends of their biological activities and considered as a useful tool for the preliminary assessment of toxicity (**Solis *et al.*, 1993**). The LC_{50} values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts. In this study, percent mortality of stem methanolic extract of *C. ramiflora* is nearer to the standard, indicating the extract could be a potential source of cytotoxic and/or pharmacologically active agent. This is in also agreement with the previous studies. Methanolic extracts of *Cynometra ramiflora had* selective cytotoxicity against three human cancer-cell lines (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) (**Uddin *et al.*, 2011**) and leaf ethanolic extract was most active against WiDR cell lines (**Muhtadi *et al.*, 2014**).

Table 3 Result of brine shrimp lethality bioassay of chloroformic and methanolic leaf and stem extracts of *C. ramiflora.*

Plant parts	Solvent system	LC_{50} value ($\mu g.mL^{-1}$)	Regression equation	R^2
leaf	**Ch**	**8.279**	**y = 35.50x + 17.40**	**0.963**
	Mt	**4.613**	**y = 29.32x + 30.51**	**0.979**
stem	**Ch**	**5.297**	**y = 31.26x + 27.34**	**0.963**
	Mt	**1.596**	**y = 24.45x + 45.03**	**0.979**
Vincristine sulfate (standard)	----------	**0.128**	**y = 64.64 + 16.39x**	**0.821**

CONCLUSION

Findings from the present study indicate potential antioxidant and strong cytotoxic activity of *C. ramiflora* stem. Confirmation of the bioactivity, particularly cytotoxic activity, in vitro and in vivo set up is the immediate step ahead. The presence of antioxidant and cytotoxic activity thus implicates *C. ramiflora* for potential neutral and pharmaceutical applications, respectively. Thus, bioactivity guided appropriate separation scheme might be employed for the identification of bioactive compound(s) in pure form.

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