ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

EVALUATION OF PHYTOCHEMICAL, ANTIBACTERIAL, ANTIOXIDANT, AND CYTOTOXIC PROPERTIES OF *COCCINIA CORDIFOLIA* LEAVES

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Manuscript Info

Manuscript History:

Received: 14 June 2015
Final Accepted: 19 July 2015
Published Online: August 2015

Key words:

Coccinia cordifolia, Antibacterial activity, Minerals, Antioxidant, Cytotoxicity

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Abstract

There are numerous medicinal plants that have the potential to treat many diseases, one of them is *Coccinia cordifolia* (Cc) popularly known as Telakucha in Bangladesh. Biochemical screening revealed the presence of carbohydrates, proteins, lipids, vitamins, minerals, phenol and flavonoid in moderate concentration. The antibacterial activity of the methanol and ethanol extracts of Cc were evaluated against Gram positive bacteria (Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Staphylococcus pyogenes), Gram-negative bacteria (Escherichia coli, Agrobacterium species, Shigella dysenteriae, Shigella sonnei, Salmonella typhi) using disc diffusion assay. Antioxidant potential of the ethanol extract of the plant leaves was evaluated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, reducing power and total antioxidant capacity. The extract showed significant activity in all antioxidant assays compared to the reference antioxidant ascorbic acid in a dose dependent manner. Cytotoxicity was studied by brine shrimp lethality bioassay. Water extract showed less antibacterial activity as compared to other extractants like ethanol and methanol. Methanol extract showed highest antibacterial activity compared to the ethanol extract. The DPPH free radical scavenging effect of the ethanol extract was compared with standard antioxidant ascorbic acid. IC_{50} values were found 3000 µg/ml for the extract and 98±8.9 µg/ml for ascorbic acid. Total antioxidant activity was also found to increase in a dose dependent manner and also showed strong reducing power. Methanol extract showed more toxic effect than the ethanol extract. These results suggest that *C. cordifolia* may act as a chemopreventative agent, providing antibacterial, antioxidant properties and offering effective protection from free radicals.

INTRODUCTION

Plants, the most wonderful gift from nature have been used as an origin of drugs. Various types of drugs are obtained from them. These types of plants are known as medicinal plants (Yadav et al, 2010). *Coccinia cordifolia* (Synonym: *Coccinia indica*, *Coccinia grandis*, Family: Cucurbitaceae) commonly known as "ivy gourd" is available

in wild form and is native of Asia and Central Africa, distributed in Australia, China, India and Bangladesh. Every part of this plant is valuable in medicine and various preparations have been mentioned in indigenous system of medicine for various skin diseases, bronchial catarrh, bronchitis and Unani systems of medicine for ringworm, psoriasis, small pox, scabies (Perry, 1980) and other itchy skin eruptions and ulcers (Behl et al, 1993). In recent years, there has been a growing interest in trace element concentrations in the environment and they are considered a factor indispensable for its proper functioning. These elements are contained in enzymes and activate them, thereby in an essential way influencing the biochemical process in cells (Lozak et al, 2002). The antimicrobial compounds found in plants are of interest because antibiotic resistance is becoming a worldwide public health concern especially in terms of food-borne illness and nosocomial infections (Anderson et al., 2001, Hsueh et al., 2005, Lin et al., 2005, Mora et al., 2005, Navon et al., 2005, Vattem et al., 2004). Chatterjee (2008) reported that the leaf of *C. cordifolia* contains potential natural antioxidants. Antimicrobial activities of *C. grandis* leaf and fruit extracts against several bacterial and fungal strains have also been reported (Dewanjee et al., 2007, Farrukh et al., 2008). An antioxidant may be defined as any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate. A free radical is defined as any atom or molecule possessing unpaired electrons. There is an increased evidence for the participation of free radicals in the etiology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, aging; etc (Deshpande et al., 2001). There is a limited research has been done to evaluate the potentiality of *C. cordifolia* plant. The objective of the present study was to investigate the antibacterial, antioxidant, and cytotoxic properties of the different fractions of *Coccinia cordifolia.* Therefore, systematic research with medicinal plants may open the door of many therapeutic choices.

MATERIALS AND METHODS

Plant Material

The leaves of *C. cordifolia* were collected in December, 2014 from Rajshahi city in Bangladesh and authenticated by Botany Department, Rajshahi University. The leaves were initially dried under shade and grinded.

Preparation of Extract

The shade-dried leaves were coarsely powdered and extracted with 95% methanol and ethanol by a Soxhlet apparatus at 45^{o}C. The solvents were completely removed by rotary evaporator and obtained greenish gummy exudates. These crude extracts were used for further investigation.

Biochemical analysis

The pH of plant extract was determined by the conventional procedure using a pH meter. Moisture content was determined by the conventional procedure ICOMR (AOAC, 1980). Ash content was determined following the method of AOAC (Ranganna, 1986). The total protein and water-soluble protein were determined by themicro-Kjeldahl method and spectrophotometrically Lowry (Lowry, 1951), respectively. Total phenol and lipid content were determined colorimetrically, (Bray and Thorpe, 1954; Bligh and Dyer, 1959) respectively. Total flavanoid content was determined by aluminum chloride colorimetric assay (Atanassova, 2011). Soluble carbohydrate was determined with the Anthronemethod (Sadasivam and Manickam, 1992). Vitamin C was determined by the method as described by (Bessey and king, 1993). Vitamin A was determined by the method as describe by Jayaraman (Jayaraman, 1981). Minerals were determined using an atomic absorption spectrophotometer (Analyst 200, Perkin Elmer, Waltham, MA, USA) was used to analyze the calcium content of the samples, the molybdate method (Onwuka, 2005) was used to analyze Manganese, Iron , Zinc, while the potassium content of the samples were determined using a flame photometer.

Phytochemical screening of methanol extract

To identify the phytochemicals of methanol extract, chemical tests were carried out by standard methods of analysis (Evans and Trease., 1989, Gibbs, 1974).

Test microorganisms

Nine cultures of bacteria were used in this study, among these were four Gram-positive (Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Staphylococcus pyogenes) and five Gram-negative (Escherichia coli, Agrobacterium species, Shigella dysenteriae, Shigella sonnei, Salmonella typhi) bacteria.

Antibacterial assay

The disc diffusion method (Bauer et al., 1966) was used to test antibacterial activity against nine bacteria. Solutions of known concentration (mg/ ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with

known amounts of the test substances using micropipette. Discs containing the test materials were placed on nutrient agar medium uniformly seeded with the pathogenic test microorganisms. Standard antibiotic discs (Azithromycin 15µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then kept at low temperature (4^{o}C) for 24 hrs to allow maximum diffusion. The plates were then incubated at 37^{o}C for 24 hrs to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out three times and the mean of the reading is required (Bauer et al., 1966). The antibacterial activity of ethanol and methanol extract of leaf was determined at a concentration of 200µg/ disc.

Antioxidant activity test

DPPH free radical scavenging activity

The free radical scavenging capacity of the extracts was determined using DPPH (Braca et al., 2001). DPPH solution (0.004% w/v) was prepared in 95% methanol. Ethanol extract of *C. cordifolia* was mixed with 95 % methanol to prepare the stock solution (5 mg/mL). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and *C. cordifolia* leaves extract was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (UV – visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95% methanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation:

$$\% \text{ scavenging Activity} = \frac{\text{Absorbanc of the control - Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis (Viturro et al., 1999).

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto et al (Prieto et al., 1999). The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^{0}C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (UV – visible spectrophotometer) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Reducing power

The reducing power of *C. cordifolia* was determined according to the method described by Oyaizu (Oyaizu, 1986). Different concentrations of *C. cordifolia* extract (10– 250 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(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. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean ± standard deviation.

Toxicity testing against brine shrimp

Hatching shrimp: Brine shrimp eggs, *Artemia salina* leach were hatched in artificial seawater prepared by dissolving 38g of sea salt in 1L of distilled water. The pH of the solution was adjusted to 8.5. After 48h incubation at room temperature (26-30^{o}C) under constant aeration, the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with a pipette. Nauplii were separated from eggs by aliquoting them three times in small beakers containing seawater.

Brine shrimp assay: The bioactivity of the extracts was monitored by the brine shrimp lethality test (Meyer et al., 1982). Samples were dissolved in dimethylsulfoxide (DMSO) and diluted with artificial sea salt water so that final

DMSO concentration did not exceed 0.05%. 50 ml of 2000 mg of the plant extract was placed in one sample tube and a two-fold dilution carried out down the column of sample tubes. The last sample tube was left with sea salt water and DMSO only to serve as the drug-free control. The total volume was adjusted to 5 ml with sea salt water. 100 ml of suspension of nauplii containing 10 larvae was added into each tube and incubated for 24h. The tubes were then examined under a magnifying glass and the number of dead nauplii in each tube counted. Experiments were conducted with control (vehicle treated), and different concentrations of the test substances in a set of three tubes per dose. Vincristine sulfate was used as a positive control in all experiments.

RESULTS

Biochemical analysis

Chemical compositions such as pH, moisture, ash, total sugar, total protein, water soluble protein, lipid, total phenol, total flavonoid vitamin C, and vitamin A of *C. cordifolia* plant is shown in table 1. The minerals content were determined in three stages of leaves like immature mature and ripen. The processed samples demonstrated significant amount of minerals present in leaves of the *C. cordifolia* is shown in Table 2.

Phytochemicals screening

The crude methanolic extract of the *C. cordifolia* plant leaves revealed the presence of phytochemicals such as flavonoids, saponins, tannins, terpenoids and Glycosides (Table 3). The presence of these active ingredients of this plant was somewhat similar to the herbal tea containing, Ficus deltoidea, *Orthosiphon stamineus* and *Stevia rebaudian*. This shows that the *C. cordifolia* plant can be also used to produce herbal tea.

Antibacterial activity of *C. cordifolia* leaves extracts

The in vitro antibacterial activity of *C. cordifolia* plant leaves extracts is shown in Table 4. The plant leaves extract showed high activity against Shigella dysenteriae, Agrobacterium species, Staphylococcus aureus, Escherichia coli, and Staphylococcus pyogenes (13.0, 11.8, 12, 10.2 and 9.2mm, respectively) from its methanol extract. These bacteria are food borne pathogens and therefore, methanolic extract of the plant can be useful for food preservation. Methanol extract of the plant showed moderate activity against Bacillus cereus, Shigella sonnei and Salmonella typhi, Bacillus subtilis (4.0, 5.2, 7.6 and 7.8mm, respectively) whereas ethanol extract was moderately active against Gram positive and Gram-negative bacteria, which were used in this study. Water extract showed moderate activity against Shigella dysenteriae, Shigella sonnei and Salmonella typhi (8.8, 7.6and7.8mm, respectively) and no activity against E. coli, B. cerus, B. subtilis, S. pyogenes, S. aureus and Agrobacterium sp.

Antioxidants Screening

The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of ethanol extract of the plant is given in Fig. 1. This activity was increased by increasing the concentration of the sample extract. DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The IC_{50} value of the extract was 3000 µg/ml, as opposed to that of ascorbic acid (IC_{50} 98 µg/ml), which is a well-known antioxidant.

Total antioxidant capacity of the *C. cordifolia* extract, expressed as the number of gram equivalents of ascorbic acid, is shown in Table 5. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm.

Fig.2. shows the reductive capability of the plant leaves extract compared to ascorbic acid. The reducing power of extract of *C. cordifolia* was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased.

Cytotoxicity Test

The crude methanol and ethanol extracts of *Coccinia cordifolia* leaves showed better cytotoxic activity with LC_{50}values of 103.25, 157.46 µg/ml in comparison with vincristine sulphate as standard whose LC_{50}value is 7.88 µg/ml (Table 6 and 7). In addition the methanol extract is more toxic than the ethanol extract.

Table 1: Chemical compositions of *Coccinia cordifolia* plant leaves

Chemical compositions	Amount
pH	5.68±0.12
Moisture (gm %)	57.42±4.0
Ash (gm %)	1.22±0.30
Total sugar content (gm %)	1.50±0.22
Total Protein (%)	3.5±0.25
Water soluble Protein (gm %)	2.35±0.14
Lipid (gm %)	2.00±0.11
Total phenol (mg/100 gm)	28.97±2.04
Total flavonoid (mg/100 gm)	83.91±6.26
Vitamin C (mg %)	2.20±0.12
Vitamin A (IU)	180.00±6.0

Values are mean ± S.D. of triplicate analyses.

Table 2: Minerals content of three different stages of *Coccinia cordifolia* leaves

Parameters	**Stages of Maturation**		
	Immature	**Mature**	**ripen**
Potassium (ppm)	5.0211	4.7118	5.2068
Calcium (ppm)	5.0292	8.9244	8.3069
Manganese (ppm)	0.2466	0.2660	0.2785
Iron (ppm)	0.2953	0.3374	0.3769
Zinc (ppm)	0.0220	0.0138	0.0184
Heavy metal contents			
Arsenic(ppb)	2.8112	3.3224	3.4653
Lead (ppm)	0.1084	0.1987	0.1535

Table 3: Qualitative analysis of *Coccinia cordifolia* plant leaves extract

Phytochemicals	Methanolic extract
Phenols	+
Flavonoids	+
Saponins	+
Tannins	+
Lignin	-
Terpenoids	+
Glycosides	+

+ indicates presence; - indicates absence

Table 4: Antibacterial activity of *Coccinia cordifolia* leaves extracts

Bacterial Cultures	Water extract(200 µg/ml)	Ethanol extract(200 µg/ml)	Methanol extract(200 µg/ml)	Azithromycin (15 µg/ml)
	Zone of inhibition			
	Gram-positive bacteria			
Staphylococcus aureus	-	7.2±0.01	12.±0.1	28±0.3
Bacillus cereus	-	5.6±0.02	4±0.1	27±0.2
Bacillus subtilis	-	5.2±0.2	7.8±0.01	28±0.4
Staphylococcus pyogenes	-	5.4±0.02	9.2±0.1	26±0.3
	Gram-negative bacteria			
Escherichia coli	-	6.0±0.1	10.2±0.2	28±0.1
Agrobacterium species	-	7.2±0.2	11.8±0.2	29.6±0.2
Shigella dysenteriae	8.8±0.2	6.2±0.2	13±0.1	29±0.4
Shigella sonnei	7.6±0.1	9.6.0±0.2	5.2±0.1	28.2±0.1
Salmonella typhi	7.8±0.2	6.6±0.01	7.6±0.2	26±0.5

'-' indicates no measurable zone. Values are mean ± SD of 3 replicates

Table 5: Total antioxidant capacity of the ethanol extract of *Coccinia Cordifolia* plant leaves

Material	Concentration (µg/mL)	Equivalent to ascorbic acid
Ethanol extract of *C. cordifolia*	30	1.26±0.15
	50	2.34±0.20
	75	3.43±0.32
	100	3.91±0.34
	125	3.93±0.38
	150	3.96±0.35

Values are expressed as the mean ± SD of three independent observations.

Table 6: Concentration dependent cytotoxic potential of crude methanol extract of *Coccinia cordifolia* against Brine shrimps nauplii

Treatment	Conc. (µg/ml)	Total No's of shrimps tested	Total No's of shrimps killed	% mortality	LC_{50} (µg/ml)
Methanol extract of *C. cordifolia*	20	10	01±0.5	10	103.25
	40	10	02±0.57	20	
	80	10	03±0.0	30	
	100	10	06±1.0	60	
	160	10	08±0.5	80	
	200	10	100±0.0	100	

Conc. Standard drug; vincristine sulphate whose LC_{50} value is 7.88; Values are expressed as the mean ± SD of three independent observations.

Table 7: Concentration dependent cytotoxic potential of crude ethanol extract of *Coccinia cordifolia* against Brine shrimps nauplii

Treatment	Conc. (µg/ml)	Total No's of shrimps tested	Total No's of shrimps tested	% mortality	LC_{50} (µg/ml)
Ethanol extract of *C. cordifolia*	20	10	01±0.57	10	157.46
	40	10	02±0.5	20	
	80	10	02±0.5	20	
	100	10	04±0.57	40	
	160	10	06±1.0	60	
	200	10	100±0.0	100	

Conc: Concentration. Values are expressed as the mean ± SD of three independent observations. Standard drug; vincristine sulphate whose LC_{50} value is 7.88.

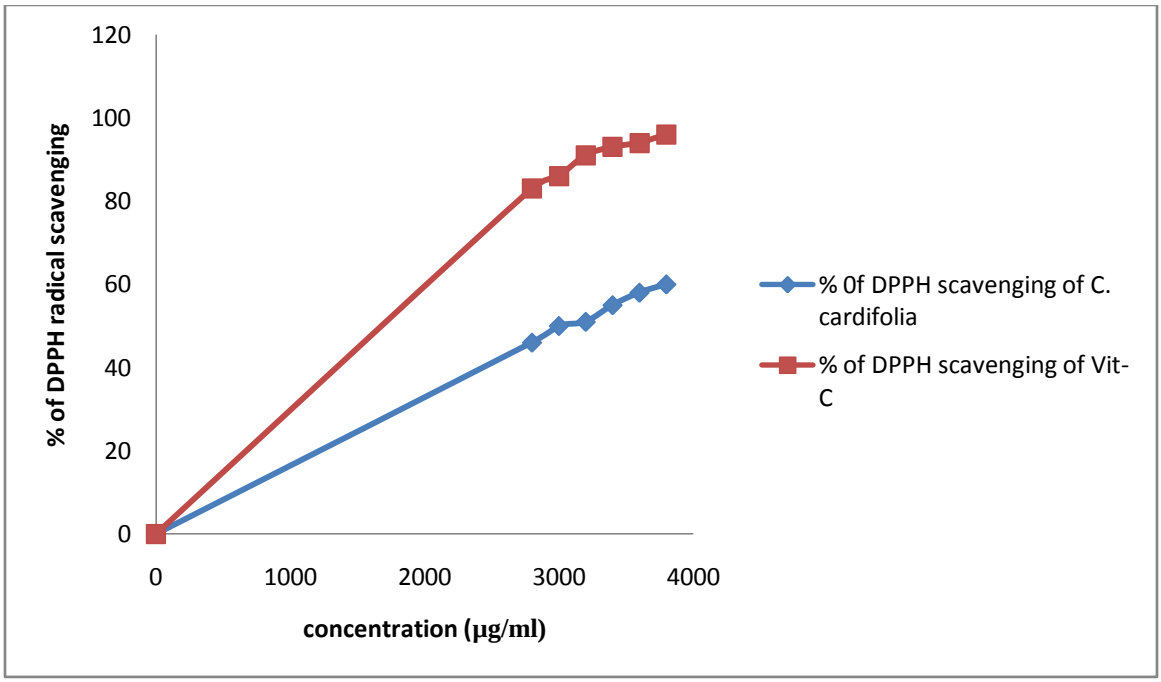

Fig 1: DPPH radical scavenging activity of the crude ethanol extract of *C. cordifolia* plant leaves. Values are the average of duplicate experiments and represented as mean± SD

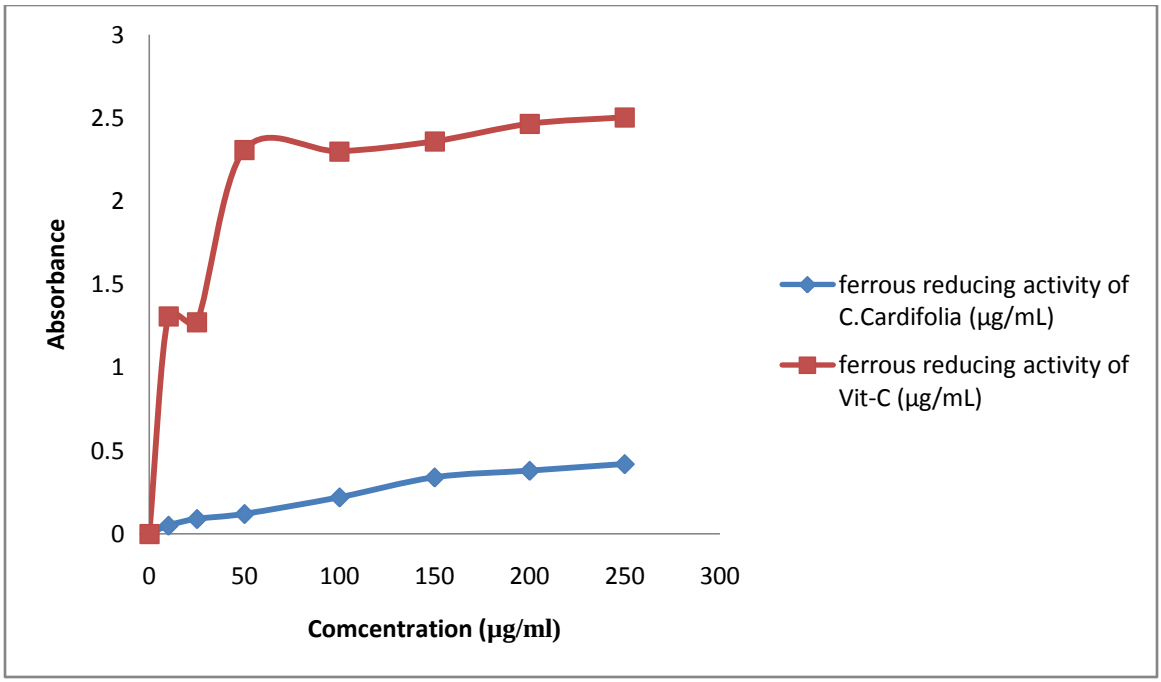

Fig. 2: Reducing powers of the crude ethanol extract of *C. cordifolia* plant leaves. Values are the average of duplicate experiments and represented as mean± SD.

Discussion

In traditional societies, nutrition and health care are strongly interconnected and many plants have been consumed both as food and for medicinal purposes. *Coccinia cordifolia* plant leaves contain phenolics, flavonoids, Vitamin C, Vitamin A, and the qualitative experiment of methanol extract showed the presence of saponins, flavonoids, terpenoids, tannins and glycosides. These compounds have been reported to possess antibacterial activity (Aziz *et al.*, 1998, El Gammal and Mansour, 1986, Cowan, 1999). Minerals demonstrated significant antibacterial effect (Lynda and Shelley, 2011) against both Gram positive and Gram negative bacteria is an indication of broad spectrum of activity and thus can be used to source of antibiotic substances for drug development that can be used in the control of these bacterial infections.

Phenolic compounds exert their free radical scavenging ability facilitated by their hydroxyl groups and the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity (Yi et al., 2007). Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases (Bravo, 1998). Antioxidant fights with free radicals and protect us from various disease. They exert their actions by either scavenging the reactive oxygen species or protecting the antioxidant defense mechanism. DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts (Nanjo et al., 1996). DPPH is a stable, nitrogen-centered free radical, which produces violet color in ethanol solution. It was reduced to a diphenylpicryl hydrazine, with the adding of the fractions in a concentration-dependent manner. The lessening in the number of DPPH molecules can be associated with the number of available hydroxyl groups. Ethanol extract showed significantly higher inhibition percentage (stronger hydrogen – donating ability).

The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes (Shahidi*et al.*, 1992). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). For the measurements of the reductive ability, it has been found that the Fe3+–Fe2+ transformation occurred in the presence of extract samples, which was postulated previously by Oyaizu (1986).

Earlier authors (Tanaka *et al.*, 1988) have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh *et al.*, 1999), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Figure 2 shows the reductive capabilities of the plant extract compared to ascorbic acid. The reducing

power of extract of *C. cordifolia* was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased.
The LC_{50}values obtained from brine shrimp lethality bioassay were 103.25, 157.46 µg/ml for methanol and ethanol extracts of *C. cordifolia*, respectively. Compared to positive control (vincristine sulphate, LC_{50} 7.88 µg/ml), all the fractions tested showed good cytotoxicity. The cytotoxic activity exhibited by the solvent fractions was promising and this clearly indicates the presence of potent bioactive compounds (Meyer et al., 1982). The brine shrimp cytotoxic assay revealed that the plant extract possesses strong toxicological effect and further investigation may leads to the isolation of active principle responsible for cytotoxic activity.

Conclusion

Based on the results obtained, it may be concluded that *C. cordifolia* plant leaves extracts demonstrated significant inhibition effects against gram-positive and gram-negative bacteria. The extract also reveals that all the fractions of the hydroethanolic extract of *C. cordifolia* showed strong antioxidant activity, reducing power ability and free radical scavenging activity due to the presence of phenolic, flavonoid compounds, and other phytochemicals. Crude methanol and ethanol extracts revealed potent cytotoxic action compared with vincristine sulphate. Therefore, it could be used as a source of antioxidant as well as chemotherapeutic agent. This is an ongoing study and further work is being carried to investigate its biological activities.

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