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In vitro evaluation of free radical scavenging activity of *Codariocalyx motorius* root extract

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ABSTRACT

Objective: To determine the phenolic content in *Codariocalyx motorius* root extract and to evaluate its antioxidant properties using various *in vitro* assay systems. **Methods:** The antioxidant activity was evaluated based on scavenging of 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radicals, superoxide anions, nitric oxide, hydrogen peroxide, peroxynitrite, reducing power and by inhibition of lipid peroxidation which was estimated in terms of thiobarbituric acid reactive substances. **Results:** The root extract of the *Codariocalyx motorius (C. motorius)* exhibited potent total antioxidant activity that increased with increasing amount of extract concentration, which was compared with standard drug such as quercetin, butylated hydroxytoluene, tocopherol at different concentrations. The different concentrations of the extracts showed inhibition on lipid peroxidation. In addition, the extracts had effective reducing power, free radical scavenging, super oxide anion scavenging, nitric oxide scavenging, lipid peroxidation, and total phenolic content depending on concentration. High correlation between total phenolic contents and scavenging potential of different reactive oxygen species (r^2=0.831-0.978) indicated the polyphenols as the main antioxidants. **Conclusions:** Codariocalyx motorius (*C. motorius*) root possess the highly active antioxidant substance which can be used for the treatment of oxidative stress-related diseases.

1. Introduction

Free radicals are produced either from by normal cellular processes or from external sources such as exposure to radiation, cigarette smoking, air pollutants and industrial chemicals. The most common reactive oxygen species (ROS) include: the superoxide anion ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). The oxidation induced by ROS may result in cell membrane disintegration, membrane protein damage and DNA mutations which play an important role in aging and can further initiate or propagate the development of many diseases, such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, coronary heart diseases and arthritis[1]. Under normal conditions, the antioxidant defence system within the body can easily scavenge free radicals that are produced. In human diseases an increased free radical generation occurs and resulted in lipid peroxidation of the membrane lipids, causing damage to phospholipids of cell membrane, lipoprotein by propagating a chain reaction cycle[2]. Therefore, antioxidants with free radical scavenging activities may have great significance in the protection and therapeutics of diseases involving free radicals[3,4].

Recently, there has been a considerable interest in finding natural antioxidants from plant materials, though they are accepted to be safe[5,6]. All higher plants have characteristic compounds, such as vitamins, carotenoids, and phenolics, have exhibited antioxidant property through scavenging of free radicals. Numerous studies have demonstrated that the consumption of plants and vegetables with high phenolic content can reduce the risk of cardio- and cerebrovascular diseases and cancer mortality[7,8]. Phytochemicals having antioxidant properties are associated with a lower risk of mortality from many of the diseases[9].

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Codariocalyx motorius (*C. motorius*) belongs to the Fabaceae family and is also known as Telegraph plant[10]. It is widely distributed throughout Bangladesh, Bhutan, Cambodia, China, India and Indonesia[11]. In Siddha medicine, it is used as antidote, cardiac-tonic and wound healing ointment[12]. It has also been used in curing snake bite poisons. In China, it has been widely used in Ayurvedic medicine as '*Codariocalyx motorius ohashi*' and the main constituent of herbal tea[13]. It also been reported to contain few alkaloids including hypaphorine, phenethylamines and 5-Methoxy-N,N-dimethyltryptamine[12]. To the best of our knowledge no other study on biological properties, including antioxidant properties was carried out so far on this plant. The main objectives of the present study were to determine the phenolic content and screening of free radical scavenging activities of *C. motorius* root extract.

2. Materials and methods

2.1. Plant material

Fresh root of *C. motorius* were collected in November' 2011 from the Kolli hills/ Kollimalai, Namakkal district of Tamil Nadu and authenticated in National institute of Herbal Science, Chennai, India (Accession No: PARC/2012/1085). The plant material was deposited at the Department of Biotechnology, SRM University.

2.2. Polyphenol extraction from roots

The collected roots were washed and dried in shade for a week and powdered. Every dried and powdered root sample (5 g) was extracted using soxhlet apparatus, at 30 ℃ for 6 h and acidified with 0.01 mol/L H_2SO_4 as previously described[14]. The aqueous solution obtained was extracted three times with butanol (200 mL), using a separatory funnel. The three alcoholic phases which contain all the polyphenol compounds were combined and the butanol extract was then brought to dryness using a rotating evaporator at 35 ℃. The dry matter obtained was dissolved with deionized water (100 mL) and extracted with ethyl acetate (3×10 mL). The organic layer containing the phenolics was finally dried.

2.3. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

To determine the DPPH scavenging activity of the extract, 3 mL of 60 μM DPPH in ethanol was added to different concentration of CMRt (1-1 000 μg/mL) and then incubated at room temperature (dark condition) for 15 min. Absorbance was read at 517 nm using a Genesys-5-spectrophotometer (Spectronic Instruments, Rochester, NY). butylated hydroxytolune (BHT) was used as a standard. The percentage of DPPH radical scavenging activity was calculated by comparing the absorbance values of control (not treated with the extract). All determinations were performed six times.

2.4. ABTS cation radical scavenging activity

The total antioxidant activity of *Codarioalyx motorius* root extract (CMRt) was measured by the ABTS radical cation-decolorization assay[15]. ABTS was prepared by reacting 5 mL of 7 mM ABTS and 80 μL of 140 mM potassium persulphate and the mixture was allowed to stand in the dark at room temperature for 12-16 h before use. ABTS (1 mL) was added to glass test tubes containing 50 μL of CMRt (1-1 000 μg/mL) and mixed by vortex mixer for 30s. Absorbance was measured at 734 nm after 2 min. BHT was used as a standard. The percentage of radical scavenging activity was calculated by comparing the absorbance values of control (not treated with the extract). All determinations were performed six times.

2.5. Scavenging of superoxide radical

The scavenging activity towards the superoxide radical was measured in terms of inhibition of generation of $O_2^{\bullet -}$. The assay was performed using alkaline DMSO method[16]. Potassium superoxide and DMSO were allowed to stand in contact for 24 h and the solution was filtered immediately before use. Filtrate (200 μL) was added to 2.8 mL of an aqueous solution containing NBT (500 μM), EDTA (10 μM) and potassium phosphate buffer (10 mM). Test compounds (1 mL) at various concentrations (1-1 000 μg/mL) were added and the absorbance was recorded at 560 nm against a control in which pure DMSO was added instead of alkaline DMSO. The results were compared with the reference antioxidant quercetin.

2.6. Nitric oxide radical scavenging assay

The nitric oxide generation capacity was quantified by the Griess Illosvoy reaction using sodium nitroprusside (SNP)[17]. The reaction mixture contained 10 mM SNP, phosphate buffer (pH 7.4) and different concentration of the extract (1-1 000 μg/mL) and incubated for 2 hours at room temp. Then 1 mL of 0.33% sulfanilamide (in 20% glacial acetic acid) was added and allowed to stand for 5 min. Then 1 mL of 0.1% napthylethylenediamine dihydrochloride was added and the mixture was further incubated for 30 min. Quercetin was used as the reference compound. The color developed was measured spectrophotometrically at 540 nm against a blank sample.

2.7. Peroxynitrite scavenging assay

An Evans Blue bleaching assay was used to measure peroxynitrite scavenging activity[18]. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, different concentration of plant extract (1-1 000 μg/mL) and 1 mM peroxynitrite in a final volume of 1 mL. After incubation for 30 min, the absorbance was measured at 611 nm. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. The

effect was compared with the standard antioxidant α-tocopherol.

2.8. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity was measured using FOX reagent as previously described[19]. An aliquot of 50 mM H_2O_2 incubated with various concentrations (1-1 000 μg/mL) of the extract for 30 min at room temperature. Then 10 μL of methanol and 0.9 mL of FOX reagent was added (9 volumes of 4.4 mM BHT in methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was incubated at room temperature for 30 min. The absorbance was measured at 560 nm. The effect of scavenging was compared with the standard antioxidant α-tocopherol.

2.9. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging capacity was quantified by Fenton reaction[20]. The reaction mixture contains 2.8 mM of 2-deoxy-2-ribose in phosphate buffer (pH 7.4), 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2, 100 μM ascorbic acid and different concentrations of the extract (1-1 000 μg/mL) or reference compound was incubated for 1 h at 37 ℃. Then 0.5 mL of the reaction mixture was added to 1 mL 2.5% TCA, then 1 mL 1% TBA was added and the mixture was incubated at 90 ℃ for 15 min then the absorbance was measured at 532 nm. Percentage inhibition was calculated by comparing the test and blank solutions. The effect of scavenging was compared with the standard antioxidant α-tocopherol.

2.10. Determination of reducing power

To determine the reducing power of the extracts[21], various concentrations of the extract (1-1 000 μg/mL) was added with 1% potassium ferricyanide and incubated for 30 min at 50 ℃. At the end of the incubation period, 10% TCA was added to arrest the reaction and then centrifuged for 10 min at 4 700 × *g*. The upper layer of the centrifuged solution was mixed with 0.1% $FeCl_3$ and the absorbance was read at 700 nm. The percentage reducing power was calculated by comparing the absorbance values of control (not treated with the extract). All determinations were performed six times. The results were compared with the reference compound BHT.

2.11. Lipid peroxidation

2.11.1 Preparation of rat brain homogenate

Adult male Wistar rats of approximately weighing about 200-250 g were used. The animals were fed ad libitum with normal laboratory pellet diet (Hindustan Lever Ltd., India) and water. Animals were maintained under a constant 12 h light and dark cycle and at an environmental temperature of 21-23 ℃. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The rats were deprived of food 12 h prior to sacrifice. The animals were euthanized and sacrificed by cervical decapitation, dissected and the whole brain except cerebellum was removed quickly. It was further processed to get 10% homogenate in 0.15 M KCl using Teflon homogenizer. The homogenate was filtered to get the clear solution and used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation.

2.11.2 In vitro lipid peroxidation determination

The extent of lipid peroxidation in rat homogenate was measured in vitro in terms of formation of thiobarbituric acid reactive substances (TBARS). Different concentrations of the extract were made up with ethanol and expressed in terms of dry weight (μg/mL). The samples were individually added to the brain homogenate (0.5 mL). This mixture was incubated with 0.15 M KCl (100 μL). Lipid peroxidation was initiated by adding 100 μL of 15 mM $FeSO_4$ solution. The reaction mixture was incubated at 37 ℃ for 30 min. An equal volume of TBA: TCA (1:1, 1 mL) was added to the above solution followed by the addition of 1 mL BHT. This final mixture was heated on a water bath for 20 min at 80 ℃ and cooled, centrifuged and absorbance was read at 523 nm using a spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by comparing the absorbance of the test with those of controls not treated with the extract as per the formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.12. Total phenolic assay

The amount of total phenolics was measured using the Folin-Ciocalteu reagent method[22]. One mL of CMRt was taken into test tubes and mixed with 1 mL of 95% ethanol, 5 mL water and then 0.5 mL of 1N Folin-Ciocalteu reagent was added. After 5 min, 1 mL of 5% Na_2CO_3 was added and the reaction mixture was allowed to stand for 60 min before the absorbance at 725 nm was measured. A standard curve was established for each assay using 50 to 500 μg of gallic acid in 95% ethanol and expressed as gallic acid equivalent (GAE) (mg of gallic acid equivalent/gm of leaf extract).

2.13. Correlation analysis

The total phenolic content as gallic acid equivalents of the polyphenolic extract was correlated with the antioxidant capacity as determined by teducing power, inhibition of lipid peroxidation, $ABTS^+$ assay and DPPH scavenging assays and evaluated by linear regression analysis.

3. Results

The root extract of the plant *C. motorius* was used to assess the free radical scavenging activity.

3.1. DPPH scavenging assay

The antioxidant activity of polyphenol extract prepared from CMRt was evaluated using an alcoholic solution of the stable free radical DPPH. The free radical scavenging activity of CMRt was found to be dose-dependent, and results were compared with BHT. The percentage of DPPH scavenging capacity of CMRt, indicated the sensitivity of the assay towards water-soluble antioxidants. The IC_{50} value of CMRt and BHT for DPPH scavenging activity was found as 78 and 192 μg/mL, respectively (Figure 1).

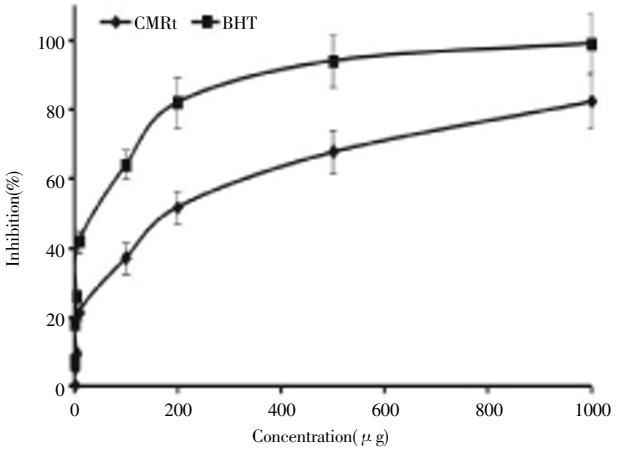

Figure 1. DPPH radical scavenging activity of CMRt at different concentrations. BHT was used as positive control.

3.2. ABTS scavenging assay

In the ABTS scavenging assay, the CMRt showed a dose-dependent effect and the results were compared with known antioxidant, BHT. The IC_{50} value of CMRt and BHT was found as 9 and 10 μg/mL, respectively (Figure 2). This result may suggest the high level of phenolic antioxidants in the extract.

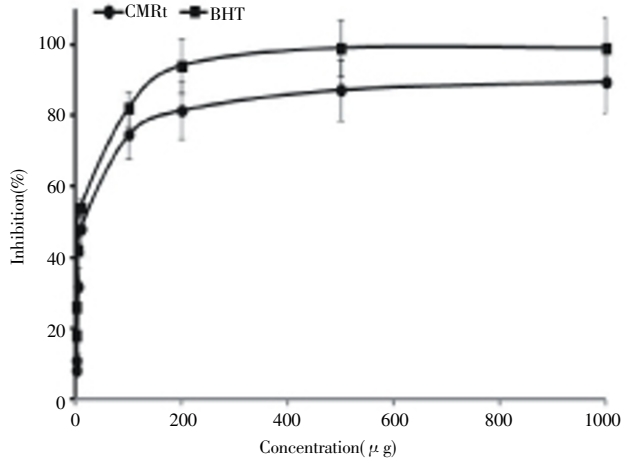

Figure 2. ABTS scavenging activity of different concentration of CMRt. BHT was used as positive control.

3.3. Superoxide scavenging activity

C. motorius root extract displayed a dose-dependent effect in scavenging superoxide radicals and the results were compared with known antioxidant quercetin. The IC_{50} value for superoxide scavenging activity was found as 195 μg/mL (Figure 3). At 500 μg/mL, the percentage of inhibition was found as 65%.

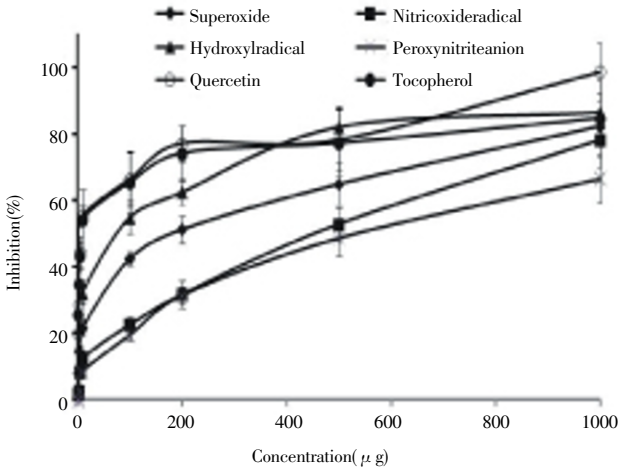

Figure 3. Superoxide, nitric oxide, hydroxyl radical, peroxynitrite anion scavenging activity of different concentrations of CMRt. Quercetin and tocopherol was used as positive control.

3.4. Nitric oxide radical scavenging activity

The CMRt showed a dose-dependent inhibition of nitric oxide with an IC_{50} of 473 μg/mL (Figure 3). Quercetin was used as a reference compound and 9 μg/mL quercetin was needed for 50% inhibition. At 500 μg/mL, the percentage of inhibition was found as 53%.

3.5. Peroxynitrite anion scavenging

Figure 3 shows that the peroxynitrite scavenging activity of the CMRt is in concentration-dependent manner. At 500 μg/mL, the scavenging activity of tocopherol was about 77%. The IC_{50} of extract was found to be 515 μg/mL and that of the reference compound α-tocopherol was 9.2 μg/mL.

3.6. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the CMRt correlated well with increasing concentration. The effect was compared with the standard antioxidant α-tocopherol and the IC_{50} values of the extract and α-tocopherol were 91 and 9.2 μg/mL, respectively (Figure 3). However as anticipated, the activity of α-tocopherol was relatively more pronounced than the extract.

3.7. Hydrogen peroxide scavenging activity

This assay shows the abilities of the extract to inhibit hydroxyl radical-mediated deoxyribose degradation in an Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The root extract strongly scavenged hydrogen peroxide with the IC_{50} being 210.7 μg/mL, which has been greater than that of

mannitol (400 μg/mL) (Figure 4).

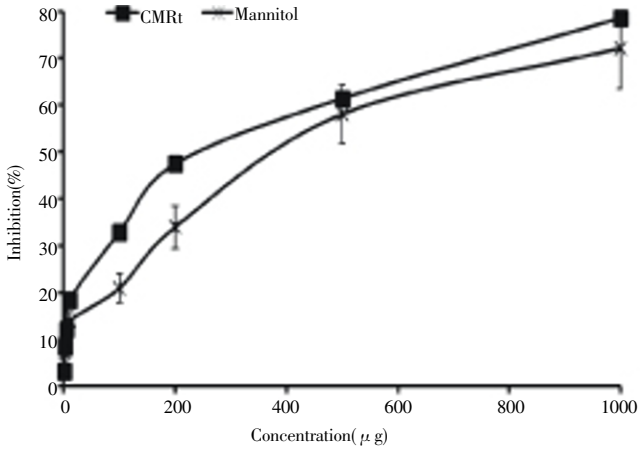

Figure 4. Hydrogen peroxide scavenging assay of different concentrations of CMRt. Mannitol was used as positive control.

3.8. Determination of reducing power

The effect of CMRt on reducing power was studied according to the reaction of Fe^{3+} to Fe^{2+}. The results were compared with the reference compound BHT (Figure 5). The IC_{50} was found as 77 μg/mL, which was higher than that of the reference compound BHT (IC_{50} = 9 μg/mL). The results indicate that maximum reducing power (92%) was observed at 500 μg/mL of CMRt.

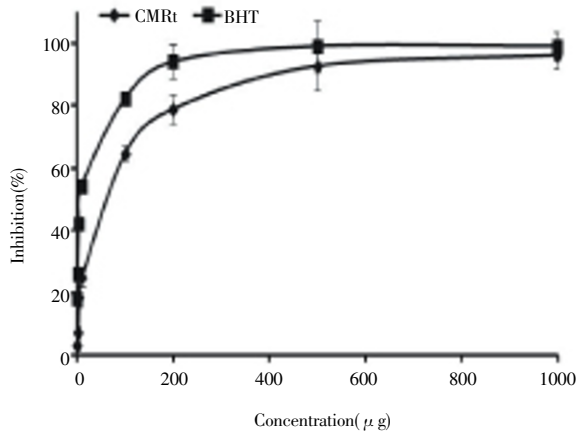

Figure 5. reducing power of different concentration of CMRt which was estimated by potassium ferricyanide method. BHT was used as positive control.

3.9. Inhibition of lipid peroxidation

The lipid peroxidation inhibition of CMRt was shown in Figure 6. The extract significantly reduced the accumulation of lipid peroxides in a dose-dependent manner and IC_{50} was found to be 169.6 μg/mL. At 500 μg/mL, the percentage inhibition was found to be 76%.

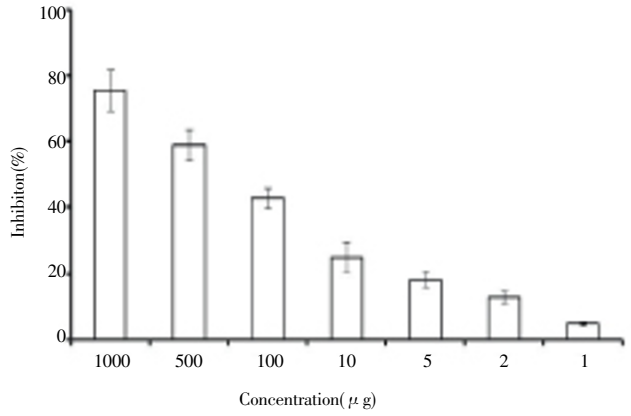

Figure 6. Lipid peroxidation inhibition by CMRt.
The malondialdehyde (MDA) was quantitatively estimated by thiobarbituaric acid method.

3.10. Relationship between antioxidant activity and total phenolic content

A highly significant positive correlation was observed between the phenolic content of CMRt and the antioxidant activity (Figure 7 A-D). Values of the correlation coefficient (r^2) were 0.927, 0.978, 0.831 and 0.978 for reducing power, lipid peroxidation, ABTS and DPPH respectively. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the CMRt.

4. Discussion

It is thought that many stresses inherent in the modern life style may cause an increased incidence of diseases such as cancer, diabetes, heart diseases and hypertension. The rising incidence of such diseases is alarming and becoming a serious public health problem. Many synthetic drugs confer protection against oxidative damage but they have adverse side effects. An alternative solution to the problems was to consume natural antioxidants from food supplements and traditional medicines. Recently, many natural antioxidants have been isolated from different medicinal plants[23,24].

Plant substances continue to serve as viable source of drugs for the world population and several plant-based drugs are in extensive clinical use[25]. For the past few decades, number of plants and plant products has been traditionally used for the treatment of various diseases due to their free radical scavenging and antioxidant properties[26]. Plants contain antioxidant principles that can explain and justify their use in traditional medicine in the past as well as in the present. For the preparation of the herbal mixture of Indian traditional medicine, the antioxidant activity arising from their content of plants due to their active compounds is essential.

In the present study, we have analysed the antioxidant properties of an unexplored Indian medicinal plant, *C. motorius* by using different *in vitro* model systems[27]. The polyphenolic compound was extracted from the dried roots of the plant and compared with the standard antioxidants. This present study shows that significance of the plant in

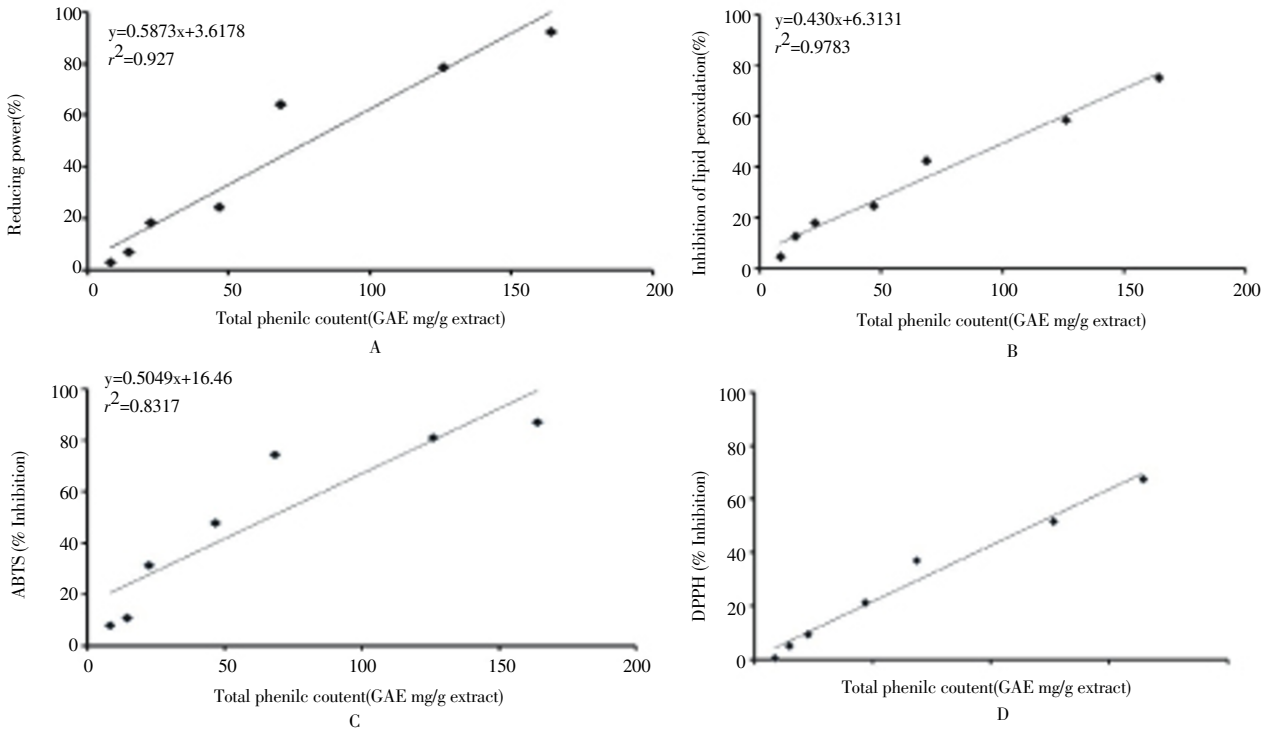

Figure 7. Correlation between total phenolic content as gallic acid equivalents (GAE mg/g extract) of CMRt and their antioxidant capacity as determined by reducing power (A), inhibition of lipid peroxidation (B), $ABTS^+$ assay (C), and DPPH scavenging assay (D).

medicinal use for its antioxidant properties.

The antioxidant activity reflected by the DPPH radical scavenging assay was clearly observed in CMRt in dose-dependent manner with BHT standard. Earlier reports suggested that the physico-chemical nature of the individual phenolics in the extract may be more important in contributing to the antioxidant activity[28]. Thereby, the percentage inhibition of ABTS free radical assay indicated the presence of water-soluble phenolic antioxidants in the extract.

The reducing capability of the CMRt was compared with the standard BHT. With the measurements of the reductive ability, we investigated the Fe^{3+}, Fe^{2+} transformation in the presence of CMRt. The reducing capacity of a compound also serves as a significant indicator of its potential antioxidant capacity[29]. The presence of reductants (antioxidants) in CMRt causes the reduction of Fe^{3+} (ferric cyanide complex) to Fe^{2+} (ferrous form). In this study, the reducing power of CMRt increased with increasing concentration.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage[30]. The conjugated dienes MDA-like products) generated during lipid peroxidation through a set of chemical reaction has been inhibited by the addition of CMRt extract to the reaction mixture. CMRt has been found to remove the hydroxyl radicals from the sugar thereby preventing the reaction. The MDA was quantitatively estimated by thiobarbituaric acid method which resulted in profound lipid peroxidation inhibition by CMRt.

Superoxide anions were generated *in vitro* enzymatically by hypoxanthine/xanthine oxidase system that reduces NBT and forms a blue coloured chromophore, formazone that can be measured at 560 nm. Upon addition of CMRt, there was a dose-dependent decrease in absorbance due to the antioxidants present in the extract thus indicating the consumption of superoxide anion in the reaction mixture. Percentage inhibition thus indicated the extent to which the CMRt was capable of inhibiting NBT reduction by the superoxide anion radical and thus its superoxide scavenging activity.

Nitric oxide was generated from sodium nitroprusside which in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Our results evidenced the inhibitory effect of CMRt on nitric oxide radical generated from sodium nitroprusside at physiological pH, which is indicative of its promising scavenging property.

Peroxynitrite ($ONOO^-$) is relatively stable compared to other free radicals but once protonated it forms the highly reactive peroxynitrous acid (ONOOH)[31]. Peroxynitrite bleaches Evans Blue by means of oxidization. According to the present results, CMRt inhibited Evans Blue bleaching by scavenging peroxynitrite and its activity was found to be greater than that of the reference gallic acid.

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups[32]. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects. From the results, it appeared that the H_2O_2 scavenging activity of the plant extract is negligible compared to that of the standard sodium pyruvate. Hence, metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation .

Phenolic constituents are very important components in plants because of their scavenging ability due to their hydroxyl groups. Numerous studies have been demonstrated

the biological activities of phenolic compounds, which are potential antioxidants and free scavengers[32,33]. It has also been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reagent. In this investigation, *C. motorius* root extract contained 241 GAE. These phenolic compounds may be the main direct contributor to the antioxidative action. It has been reported that upto 1 g of polyphenolic compounds (from a diet rich in fruits and vegetables) ingested daily has inhibitory effects on mutagenesis and carcinogenesis in humans[34].

Based on the results of the present study, it can be concluded that the root extract of *C. motorius* can be an effective and safer antioxidant source. With these antioxidant properties, the plant can be effectively used in the development of new pharmaceutical medicine for oxidative stress mediated problems. Further studies are required to throw light on the biological activity of the *C. motorius* and its bioactive compounds against various diseases.

Conflict of interest statements

We declare that we have no conflict of interest.

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