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In Vitro Antioxidant Activity and Total Polyphenolic Content of *Cyperus rotundus* Rhizomes

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Abstract: In this study, Antioxidant activity of *Cyperus rotundus* rhizomes extract (CRRE) was evaluveted in a series of *in vitro* assay involving free radicals and reactive oxygen species and IC_{50} values were determined. CRRE exhibited its scavenging effect in concentration dependent manner on superoxide anion radicals, hydroxyl radicals, nitric oxide radical, hydrogen peroxide, and property of metal chelating and reducing power. The extract was also studied for lipid peroxidation assay by thiobarbituric acid–reactive substances (TBARS) using young and aged rat brain mitochondria. The extract was also effective in preventing mitochondrial lipid peroxidation induced by $FeSO_4$/ ascorbate in concentration dependent manner. The results obtained in the present study indicate that *C. rotundus* rhizomes extract can be a potential source of natural antioxidant.

Keywords: Antioxidant, *Cyperus rotundus* rhizomes, Iron chelation, Lipid peroxidation, Polyphenols, Radical scavenging.

Introduction

Cyperus rotundus, vernacularly called "Nagarmotha" is a medicinal plant belonging to the family of the *Cyperaceae* and appearing among Indian, Chinese, Japanese natural drugs used as home remedy against spasms, stomach disorders and irritation of bowel[1]. In Indian system of medicine, the rhizome of the plant has been recommended for use in several clinical conditions like fever and arthritis.[2] The rhizomes are cooling, intellect promoting, nervine tonic, diuretic, antiperiodic, and used to treat diarrhoea, dysentery, leprosy,

bronchitis, amenorrhea and blood disorders.[3] The rhizome is reported to possess analgesic, anti-inflammatory, antipyretic activity[4]. They form an ingredient of poly herbal formulation Abana and health food Amrita Bindu, useful for prevention of nitrosamine induced depletion of antioxidant defense.[5] The phytochemical investigations of *C. rotundus* rhizomes have revealed the presence of polyphenol[6,] flavonol glycoside[7], saponin[8] , vitamin – C[9], sesquiterpenoids[10] , and essential oil[11].

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-) and hydroxyl ($^{\cdot}OH$) radical and hydrogen peroxide (H_2O_2) are often generated as by products of biological reactions or from exogenous factors[12].These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells [13] including DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease, and Parkinsons disease[14, 15, 16]. Recent investigations have shown that the antioxidants with free-radical scavenging properties of plant origins could have great importance as therapeutic agents in aging process and free radical mediated diseases including neuro degeneration[17,18]. Plant extracts[19] and plant products such as flavonoids and other polyphenolic constituents have been reported to be effective radical scavengers and inhibitors of lipid peroxidation[20, 21]. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant. In view of its wide use and its chemical composition, the ethanolic extract of *C. rotundus* rhizomes was determined for its *in vitro* anti oxidative activities.

Experimental

Materials and methods

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. Catechin was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade available commercially.

Plant collection

The rhizomes of the *Cyperus rotundus* were collected from Thiruvaiyaru, Thanjavur District, Tamil Nadu, India. The collected rhizome was identified and authenticated by botanist Dr. M. Jegadeesan, Associate Professor, Department of Environmental and Herbal Sciences, Tamil University, Thanjavur, Tamilnadu. A Voucher specimen (R.N.264) was deposited in Herbarium of our department. The rhizome was shade dried at room temperature for 15 days and make in to coarse powder.

Preparation of rhizomes extract

The coarse powder (75 g) was extracted exhaustively in a Soxhlet apparatus with mixture of ethanol: water (7:3 ratio) for 72 h. The extract was then concentrated *in vacuo* until the solvent was completely removed. The yield of ethanol extract was found to be 12.32 g.

Phytochemical analysis

Estimation of total polyphenol content

The total polyphenol content (g/100 g extract) present in the CRRE was analyzed using the Folin - Ciocalteu reagent method[22] by Singleton *et al.* Extract solution (0.1 mL

containing 1000 µg) was transferred to a 100 mL Erlenmeyer flask, and then the final volume was adjusted to 46 mL by the addition of distilled water. Afterward, 1 mL of Folin-Ciocalteu Reagent (FCR) was added into this mixture, and after 3 min, 3 mL of Na_2CO_3 (2%) was added. Subsequently, the mixture was shaken on a shaker for 2 h at room temperature, and then absorbance was measured at 760 nm. Catechin was used as the standard for calculation.

Free radical scavenging assays

Superoxide anion scavenging activity assay

The scavenging activity of the CRRE towards superoxide anion radicals was measured by the method of Liu *et al* [23] (1997). Superoxide anions were generated in a non-enzymatidc phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 mL of NBT (300 µM) solution, 0.75 mL of NADH (936 µM) solution and 0.3 mL of different concentrations of the extract. The reaction was initiated by adding 0.75 mL of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100],$$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction. This method was recommended by Yu *et al* [24] (2004). Reaction mixture contained 60 µL of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 µL of 0.17 M H_2O_2, and 1.5 mL of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat[25] (1964). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the extract was estimated by replacement titration[26]. Aliquot of 1.0 mL of 0.1 mM H_2O_2 and 1.0 mL of various concentrations of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 mL of 2 M H_2SO_4 and 7.0 mL of 1.8 M KI. The mixed solution was titrated with 5.09 mM NaS_2O_3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as

$$\% \text{ Inhibition} = (V_0 - V_1) / V_0 \times 100$$

where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract), V_1 was the volume of NaS_2O_3 solution used in the presence of the extracts.

Metal chelating activity assay

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of Dinis *et al* [27] (1994). To 0.5 mL of extract, 1.6 mL of deionized water and 0.05 mL of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 mL ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

$$\text{Chelating rate } (\%) = (A_0 - A_1) / A_0 \times 100$$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu [28](1986). The extract (0.75 mL) at various concentrations was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Lipid peroxidation inhibition assay

Young (3-4 months, 120-150 g) and aged (22-24 months, 380-410 g) Wistar albino rats were anaesthetized with Thiopentone sodium (50 mg / kg); brain was excised and washed with 0.95 NaCl solution. Tissue homogenates were prepared in ice-cold 3 mM Tris buffer containing 250 mM sucrose and 0.1 mM EDTA (pH 7.4). Differential ultra centrifugation and their protein content characterized the mitochondrial fraction. Based on the Liu and Ng method[29] (2000), the inhibition of lipid peroxidation assay was determined. 0.5 mL mitochondrial fraction, 0.1 mL of 10 mM $FeSO_4$, and 0.1 mL of 0.1mM ascorbic acid were taken to incubate with 0.3 mL of the extract (10 to 1000 µg) at 37°C for 1 h. The reaction was then stopped by addition of 0.75 mL of 28% (w/v) trichloroacetic acid (TCA) and 0.5 mL of 1% (w/v) thiobarbituric acid (TBA), successively. The mixture was then heated at 100°C for 45 min. After centrifugation, all precipitated proteins were removed and the color of the malondialdehyde (MDA)-TBA complex in the supernatant was detected at 532 nm. The values of MDA were expressed as nmol/mg of protein.

Statistical analysis

Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a linear regression algorithm.

Results and Discussion

Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with aging.

Amount of total phenolic compounds

Phenolics are the most wide spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[30]. Therefore, in the present study, total phenolic content present in extract was estimated using modified Folin- ciocalteau method. Values are expressed as catechin equivalents 73.27 ± 4.26 g of dried rhizomes extract.

Superoxide anion scavenging activity

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, $^{\cdot}OH$, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation[31].The superoxide anion radical scavenging activity of *C. rotundus* rhizome extract assayed by the PMS-NADH system was shown in Table 1. The superoxide scavenging activity of CRRE was increased markedly with the increase in concentrations. Thus, higher inhibitory effects of the rhizomes extracts on superoxide anion formation noted herein possibly renders them as a promising antioxidants. The half inhibition concentration (IC_{50}) of CRRE was 0.031 mg mL^{-1}. These results suggested that CRRE has a potent superoxide radical scavenging effects.

Table 1. Radical scavenging activity of ethanol extract of *Cyperus rotundus* rhizomes at different concentrations.

Concentration μg mL^{-1}	Superoxide radical scavenging, %	Hydroxyl radical scavenging, %	Nitric oxide radical scavenging, %
10	31.25 ± 2.18	36.27 ± 2.22	12.53 ± 1.59
20	43.18 ± 2.58	48.68 ± 2.38	22.18 ± 1.14
50	72.46 ± 3.01	81.24 ± 1.98	31.52 ± 2.16
100	89.63 ± 2.27	97.06 ± 3.21	38.27 ± 2.27
250	-	-	52.41 ± 2.11
500	-	-	59.62 ± 3.75
750	-	-	67.28 ± 2.69
1000	-	-	75.34 ± 2.55
IC_{50} (mg mL^{-1})	0.031	0.021	0.428

Data are presented as the mean ± SD of each triplicate test.

Hydroxyl radical scavenging activity

Activity of the rhizomes extract on hydroxyl radical has been shown in Table 1. Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule it contacts[32] and is known to be capable of abstracting hydrogen atoms from membrane lipids[31]and brings about peroxidic reaction of lipids. CRRE exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC_{50} value was found to be 0.021 mg mL^{-1}.

Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleiotropic mediator of physiological process such as smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities[33]. Although nitric oxide and superoxide radicals are involved in host defense, over production of these two radicals contributes to the pathogenesis of some inflammatory diseases[34]. Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules, peroxynitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases. CRRE significantly inhibited nitric oxide in a dose dependent manner (Table 1) with the IC 50 being 0.428 mg mL^{-1}. The result indicated that the extract might contain compounds able to inhibit nitric oxide and offers scientific evidence for the use of the rhizomes in the indigenous system in inflammatory condition.

Hydrogen peroxide scavenging activity assay

As shown in Figure 1, CRRE also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 1.642 mg mL^{-1}. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects[35]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by CRRE may at least partly result from its antioxidant and free radical scavenging activity.

Metal chelating activity

Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals[36, 37]. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the CRRE, indicating its chelating activity with an IC_{50} of 0.192 mg mL^{-1} (Figure 2). Chelating agents that forms bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion[38.] This study shows that CRRE has a marked capacity for iron binding, suggesting the presence of polyphenols that has potent iron chelating capacity.

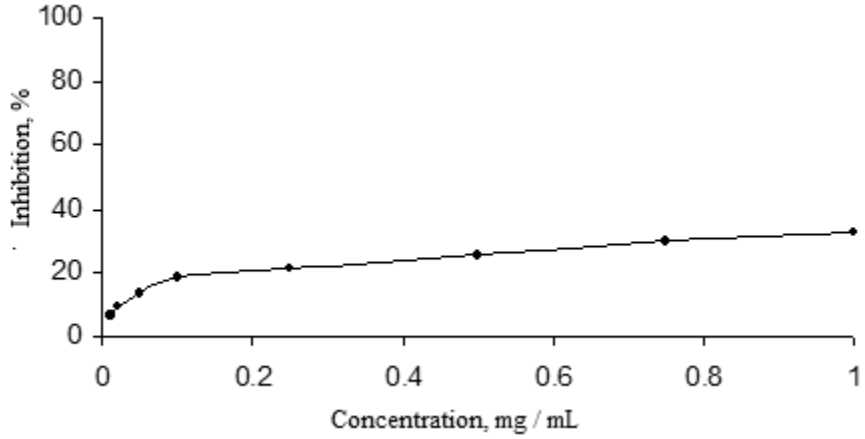


Figure 1. H_2O_2 scavenging activity of *C. rotundus* rhizomes ethanol extract at different concentrations. Each value represents means ± SD (n=3).

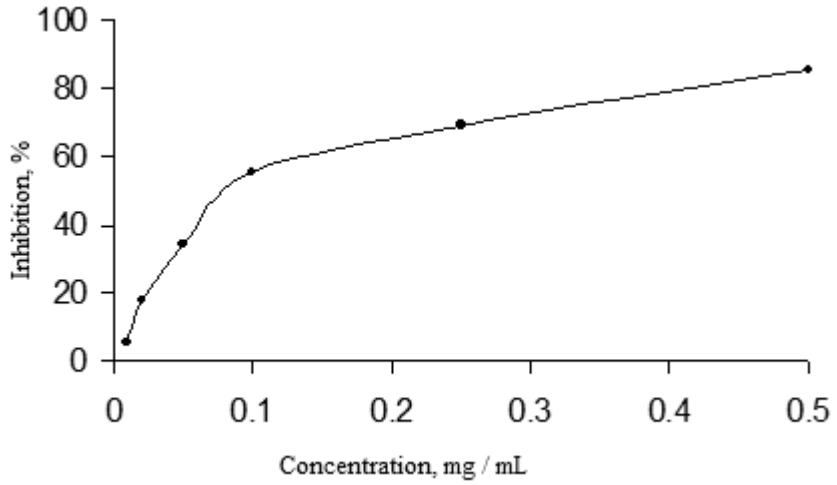


Figure 2. Ferrous ion chelating activity of *C. rotundus* rhizomes ethanol extract at different concentrations. Each value represents means ± SD (n=3).

Reducing power activity

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of CRRE. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[39,40]. Figure 3 depicts the reductive effect CRRE. Similar to the antioxidant activity, the reducing power of CRRE increased with increasing dosage. The result shows that CRRE consist of hydrophilic poly phenolic compounds that cause the greater reducing power.

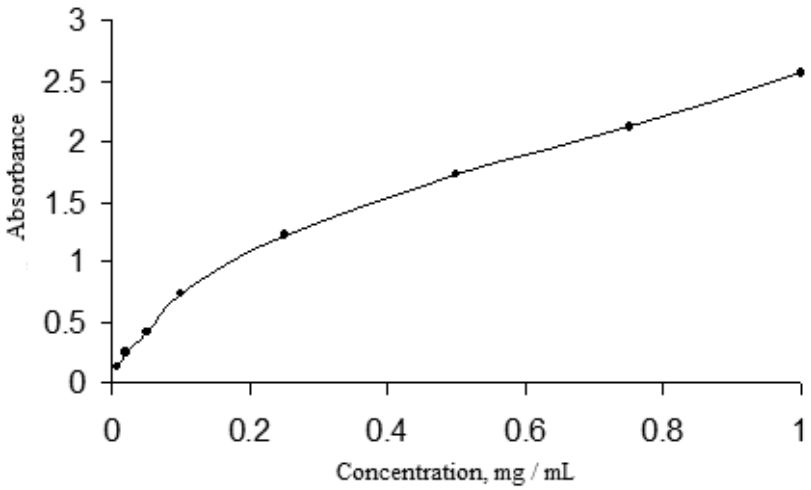


Figure 3. Reducing power of *C. rotundus* rhizomes ethanol extract at different concentrations. Each value represents means ± SD (n=3). High absorbance at 700 nm indicates high reducing power.

Lipid peroxidation inhibition activity

In addition to free radical scavenging activity, the rhizome extract was evaluated for its ability to protect biomembrane from oxidative damage. Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through $OH^{\cdot}$ radical by Fenton's reaction. CRRE inhibited $FeSO_4$/ascorbate induced lipid peroxidation in young and aged rat brain mitochondria in a dose dependent manner as given in Table 2. The inhibition could be caused by the absence of ferryl-perferryl complex or by scavenging the $^{\cdot}OH$ radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. The mitochondria of aged rats showed an elevated level of MDA content as compared to young rats. This result indicated that aging is associated with increased production of free radicals by mitochondria[41]. Addition of 0.01-1.0 mg mL^{-1} of CRRE to rat brain mitochondria significantly reduced MDA formation in a dose dependent manner. The result shows that CRRE has the capacity to prevent oxidative deterioration of mitochondrial membrane lipids. The beneficial effect of CRRE on lipid peroxidation is attributed to its phenolic content.

Table 2. Activity of ethanol extract of *Cyperus rotundus* rhizomes at different concentrations on ferrous sulphate induced lipid peroxidation in young and aged rat brain mitochondria.

Concentration	MDA (nmol mg $protein^{-1}$)	
µg mL^{-1}	Young	Aged
10	3.37 ± 0.22	7.75 ± 0.21
20	3.04 ± 0.25	7.25 ± 0.27
50	2.73 ± 0.31	6.81 ± 0.23
100	2.58 ± 0.15	6.62 ± 0.24
250	2.44 ± 0.18	5.94 ± 0.32
500	2.30 ± 0.23	5.51 ± 0.30
750	2.24 ± 0.38	5.15 ± 0.26
1000	2.19 ± 0.19	4.83 ± 0.22

Data are presented as the mean ± SD of each triplicate test.

Conclusions

The results obtained in the present study indicate that *C. rotundus* rhizomes extract exhibits free radical scavenging, reducing power and metal chelating activity. The overall antioxidant activity of CRRE might be attributed to its polyphenolic content and other phytochemicals constituents. The findings of the present study suggest that *C. rotundus* rhizomes could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases.

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