Phytopharmacology

Inforesights Publishing

Antioxidant and anti-inflammatory activities of proteins isolated from eight *Curcuma* species

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Received: 5 August 2012, **Revised**: 21 September 2012, **Accepted**: 24 September 2012

Abstract

Curcuma species are used in traditional Indian medicine for their antimicrobial, anticancer, anti-inflammatory properties. The present study evaluated the bioactivity of proteins in aqueous extracts of rhizomes of eight *Curcuma* species (*C. aeruginosa, C.amada, C. aromatica, C. brog, C. caesia, C. malabarica, C. rakthakanta* and *C. sylvatica*), in comparison with *C.zedoaria.* The purified native proteins had a molecular weight of 66 kDa, which was resolved by SDS-PAGE as 12 and 14 kDa proteins. Proteins isolated from all species showed significant antioxidant activity which was found to be heat stable. *C. brog*, *C.amada* and *C.caesia* proteins showed highest antioxidant potential. The proteins also exhibited high anti-inflammatory activity at a dose level of 100mg/kg bw, when assayed by the carrageenan rat paw model system. The study indicated that these activities could contribute significantly to the pharmacological properties of these species.

Keywords: *Curcuma* species; rhizomes: soluble proteins; antioxidant activity; anti-inflammatory activity

Introduction

Curcuma species belongs to the family Zingiberaceae comprising of about 80 rhizomatous species, which occur widespread throughout the tropics of Asia, Africa and Australia. Many species of the genus are used in traditional systems of Indian and Chinese medicine particularly as an anti-inflammatory agent and in the treatment of flatulence, jaundice, and gastric ailments (Purseglove et al., 1981). *Curcuma longa*, the most commonly utilized species, as well as *C. zedoaria*, and *C. xanthorrhiza* possesses a wide range of medicinal properties (Srimal., 1997; Luthra et al., 2001; Wilson et al., 2005; Panda., 2010). Most of the studies on the pharmacological constituents in these species have focussed on organic compounds such as curcumin, phenolics, terpenoids, flavonoids which are present in the rhizomes, while very few investigations have been carried out on the aqueous principles. Biologically active proteins have been reported from Zingiberaceae plants. Proteins having significant antioxidant activity were found to be present in aqueous extracts of *C. longa* rhiz-

omes. A water soluble peptide, turmerin, and a Turmeric Antioxidant Protein (TAP) have been isolated from *Curcuma longa.* These proteins were powerful antioxidants which could inhibit lipid peroxidation and scavenge free radicals (Srinivas et al., 1992; Cohly et al., 2003; Selvam et al., 1995). Others include an antifungal protein from *C. longa* (Wang and Ng., 2005; Petnual et al., 2010), haemaglutinating proteins (lectins) from *C. amarissima* (Kheeree et al, 2010) and *C. zedoaria* (Tipthara et al, 2007).

There are several other *Curcuma* species which are less known viz. (*C.aeruginosa, C.amada, C.aromatica, C.brog, C.caesia, C.malabarica, C.rakthakanta* and *C.sylvatica*) but are also pharmacologically active and are traditionally used as remedies for infections, inflammations and gastric conditions. Their medicinal properties have been correlated with the presence of phytochemicals such as phenolics, essential oils, flavonoids etc in the rhizomes. The presence of bioactive proteins has not been reported in these species. In the present study, proteins were isolated from aqueous extracts of rhizomes of eight lesser known starchy *Curcuma* species and evaluated for antioxidant and anti-inflammatory activity. Proteins were isolated from *C.zedoaria* using the same procedure and the activity was compared.

Materials and Methods

Plant Material

The species (*C. aeruginosa, C. amada, C. aromatica, C. brog, C. caesia, C. malabarica, C.rakthakanta, C. sylvatica* and *C. zedoaria*) were collected from the National Bureau of Plant Genetic Resources (Regional Station) Trichur, Kerala. The crop was raised in the farm of Central Tuber Crops Research Institute, Trivandrum. The rhizomes were harvested after 8 months, cut into small pieces and shade dried for 48 h. The dried samples were ground to a fine powder and used for extraction of protein.

Chemicals

Bovine Serum Albumin (BSA), Egg Albumin, trypsinogen, α lactalbumin, trypsin, transferrin, glucose, 2, 2, Diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6 Tripyridyl -s-triazine (TPTZ) was purchased from Sigma Chemical Co. (St Louis MO) chemicals. All other chem.-icals used were of analytical grade.

Extraction of soluble protein from rhizomes

Dry rhizome powder (25 g) was extracted twice with distilled water for 2h using a rotary shaker. The solution was centrifuged at 10,000xg for 30min and the clear supernatant was precipitated with 3 volumes of acetone. The precipitate was air dried and then extracted with cold 10 % TCA and centrifuged at 10000xg for 15min. The supernatant containing polysaccharides was decanted and the protein residue was collected, washed with acetone until acid free and then air dried. Total protein was estimated by the method of Lowry et al and total carbohydrate, by the phenol-sulphuric method of Dubois (Lowry et al., 1951; Dubois et al., 1956).

Gel filtration and Electrophoresis

Gel filtration

Sepharose CL 6B pre equilibrated with appropriate buffer (phosphate buffer 0.05 M containing 0.1 M NaCl) was used for gel filtration. A mixture of dextran blue and potassium dichromate were used for the determination of void volume and total volume. The column was calibrated using transferrin, bovine serum albumin, egg albumin and trypsin. After washing the column with buffer, 200 µl of protein was loaded on to the column and 2 ml fractions were collected with a flow rate of 1ml/min using the elution buffer (0.05 M phosphate buffer containing 0.3 M NaCl, pH 6.8). Protein in the fractions was estimated by measuring the absorbance at 280nm. Molecular weight was calculated from the standard graph plotted with log molecular weight of marker proteins on X axis and Kav on Y axis. Kav is the fraction of the stationary gel volume which is available for diffusion of a given solute species.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Samples for analysis were dissolved in sample buffer, and boiled for 3min, and centrifuged. The supernatant was loaded on to the gel. Electrophoresis was performed in a Genei gel electrophoresis unit, with 12% gels, at a current of 20mA. Molecular weight markers used were α lactalbumin (14.2 kDa), trypsinogen (24 kDa), egg-albumin (45 kDa) and Bovine Serum Albumin (66 kDa). The gel was stained overnight with Coomassie blue, destained and photographed. The molecular weight of the separated proteins was determined using Alpha Innotech gel documentation system (California, USA).

Antioxidant activity

Antioxidant activity was determined by measuring DPPH free radical scavenging activity and ferric reducing power.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Chung et al (2002) with some modifications. Aliquots of diluted samples was mixed with 100 µl of Tris-HCl buffer (1 M, pH 7.9) and 1200 µl of DPPH solution (80 µm in methanol). The reaction system was incubated in the dark for 20 min and decrease in absorbance at 517 nm was measured. Controls were run with DPPH and without addition of test sample. The half-inhibition concentration values (IC_{50}), the volume of test protein at which the inhibition of DPPH radical is 50%, were calculated.

Reducing power

The ferric reducing power of the protein was determined by the method of Benzie and Strain (1999). Absorbance of three ml of FRAP (Ferric Reducing Antioxidant Power) reagent

(prepared fresh by mixing 10 mM TPTZ (2,4,6 Tripyridyl -s-triazine) in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer, pH 3.6 in 1:1:10) was measured at 593 nm against distilled water as blank. Test solution containing 1 mg protein was added to the FRAP reagent and increase in absorbance at 1, 2, 3 and 4 min was measured. Resultant absorbance of the test protein at specific time was calculated by reducing the absorbance value of FRAP reagent at time zero. Absorbance at min 4 was considered for the calculation of concentration of ferrous ion released by the test protein. Increase in absorbance is an indication of conversion of Fe^{3+} of the reagent to Fe^{2+} and iron reducing power of the test compound. Increasing concentration of ammonium ferrous sulphate was used as the source of ferrous ion (50 µM- 1000 µM Fe^{2+}). A standard graph was prepared by plotting absorbance of standard solution at 4 min against concentration of ferrous ion in µM. Concentration of Fe^{2+} released by the test protein was calculated from the graph. The EC_{50} values (the effective concentration at which the A700 of the Prussian blue complex is 0.5) of the sample were determined.

Effect of temperature on antioxidant activity

The protein solutions were heated in a water bath at temperatures ranging from 60 – 100^{o} C for 10 min, cooled to room temperature and assayed for antioxidant activity by DPPH free radical scavenging assay and ferric reducing power.

Anti inflammatory activity

The anti inflammatory activity was measured by the carrageenan induced rat paw oedema model system. Male Wistar rats (150-170 g) were used for the assay. The animals were maintained in the animal home in the Department of Biochemistry, University of Kerala, Trivandrum and fed with standard Hind Lever diet and water *ad libitum*. Groups of six animals were used in each test group.

Inflammation of the hind paw was induced by injection of 0.1 ml of a 1% w/v solution of carrageenan into the subplantar surface of the right hind paw of the rat (Winter et al., 1962; Cashin et al., 1977). The oedema (inflammation) was assessed as the difference between zero time linear circumference of the injected paw and its circumference at 3 h after administration of carrageenan (Akah and Nwambie., 1999). The proteins were freshly dissolved in dimethyl sulfoxide and the volume made up with sterile water and administered i.p. 1 h before inducing inflammation. The paw volume was measured initially and at 3hr, 5 hr and 24 hr. Control rats received an equivalent amount of vehicle. The average oedema (paw circumference) and percentage inhibition of oedema were calculated (Oriowao., 1982). The anti-inflammatory effect of the extract was evaluated as the degree of oedema inhibition.

Statistical analysis

The data was expressed as the mean ± standard deviation (SD) of triplicates and then analysed using SPSS.17 (SPSS Inc. Chicago, Illinois, USA). One- way analysis of variance (ANOVA) and Duncans multiple range test ($p<0.05$) were used to determine the significance of the difference between means.

Results

Yield of soluble protein in Curcuma rhizomes

Soluble proteins were isolated from dry rhizomes by aqueous extraction, followed by acetone precipitation. The precipitate was extracted with TCA in order to remove polysaccharides, and the residue was collected by centrifugation. The yield of the crude protein, so obtained, varied from 1.3 to 5.3 % between species (Table 1). The highest yield was obtained from *C. aromatica*, followed by *C. sylvatica* and *C. brog*.

The protein preparations contained approximately 1.0–4.4 % carbohydrate, indicating that they were glycoproteins. Higher carbohydrate content was present in *C.aeruginosa* (4.4%) and *C. malabarica* (2.9%) while *C. zedoaria* contained 2.2 %. The results are presented in Table 1.

Gel filtration and SDS PAGE

The protein samples were subjected to gel filtration on Sepharose CL 6B. The major fraction eluted as a single peak (Fig 1) and constituted >90% of the total proteins present in

Table 1. Yield and composition of protein isolated from *Curcuma* rhizomes.

Species	Yield (%)	Protein (%)	Carbohydrate (%)
C. aeruginosa	1.3	75 ± 1.5 [c]	4.4 ± 0.15 g
C. amada	2.7	72 ± 0.6 [a, b]	0.88 ± 0.01 [a]
C. aromatica	5.3	72 ± 1.0 [a, b]	1.3 ± 0.05 [c]
C. brog	3.5	72 ± 1.5 [a, b]	1.5 ± 0.01 [d]
C. caesia	2.9	78 ± 1.5 [d]	1.1 ± 0.05 [b]
C. malabarica	2.7	73 ± 0.6 [a, b]	2.9 ± 0.05 [f]
C. rakthakanta	2.2	71 ± 0.6 [a]	1.1 ± 0.06 [b]
C. sylvatica	3.7	72 ± 1.5 [a, b]	1.5 ± 0.03 [d]
C. zedoaria	4.3	74 ± 1.5 [b, c]	2.2 ± 0.06 [e]

[*] Values are the mean of triplicate analysis ± standard deviation. Mean values followed by different letters in a column are significantly different ($p \leq 0.05$).

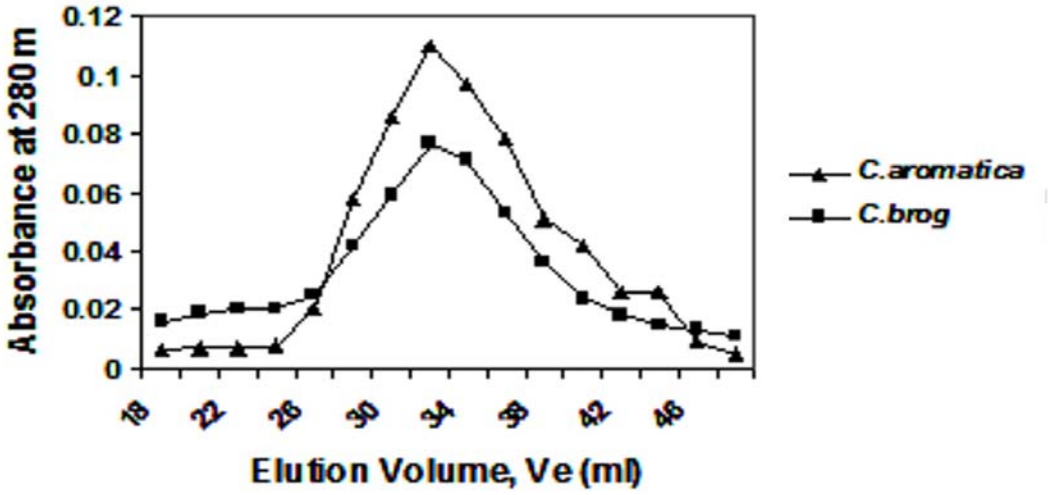

Figure 1. Elution profile of rhizome proteins in *Curcuma* (a) *C.aromatica* (b) *C. brog* subjected to gel filtration chromatography on sepharose CL 6B.

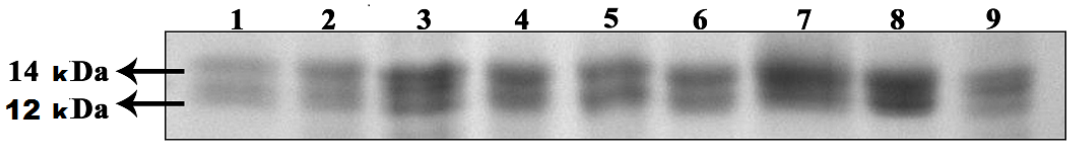

Figure 2. SDS Polyacrylamide Gel Electrophoresis of *Curcuma* rhizome proteins. Lanes 1 to 9: 1- *C. aeruginosa*, 2- *C. amada*, 3- *C. aromatica*, 4- *C. brog*, 5- *C. caesia*, 6- *C. malabarica*, 7- *C. rakthakanta* , 8- *C. sylvatica*, 9- *C. zedoaria.*

the extracts. Molecular weight corresponded to 66 kDa .The proteins in this fraction were analysed using SDS PAGE. Two major proteins of molecular weight 12 kDa and 14 kDa were seen in SDS-PAGE (Fig 2). These proteins were found to be present in all these *Curcuma* species.

Antioxidant activity

The antioxidant activities of the purified proteins from the different species were determined by measuring the DPPH free radical scavenging activity and ferric reducing power. The results are given in Fig 3. The DPPH scavenging activity (expressed as IC_{50} values) of the proteins ranged from 0.70 to 1.8 mg. Protein extracts from *C.brog, C.amada*, and *C. caesia* had low IC_{50} values of 0.70, 0.73, 0.80, showing high DPPH scavenging activity which were comparable with that of *C. zedoaria* (IC_{50} 0.84). Proteins from these four species also possessed higher iron reducing power. EC_{50} (FRAP) values ranged from 1.4 to 3.8, compared to 1.0 mg in *C. zedoaria*. The ferric reducing power was found to increase linearly with time (data not shown).

Effect of temperature on antioxidant activity

The stability of the proteins to heat was studied by incubating the protein solutions at different temperatures (60, 70, 80, 90 and 100^{o} C) for 10 min and determining the antioxidant

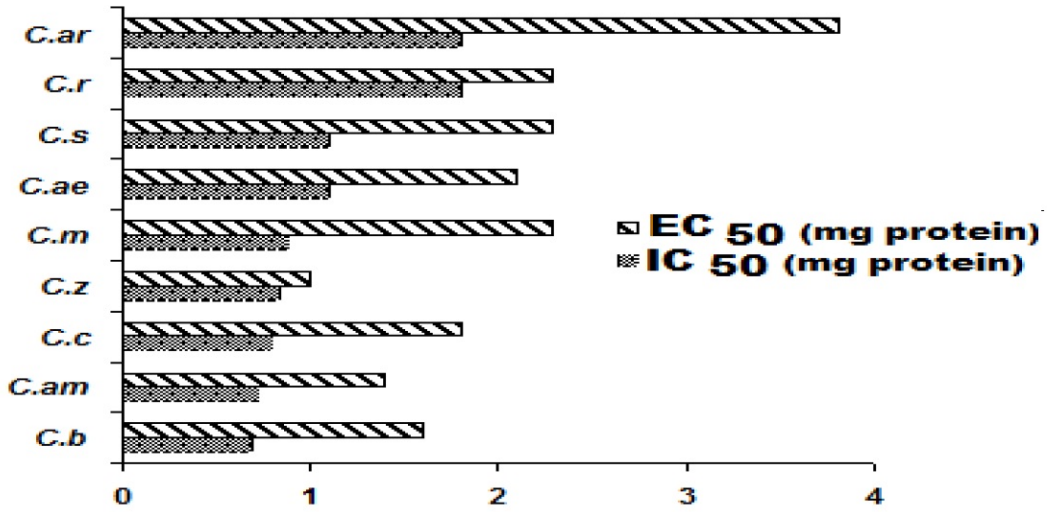

Figure 3. Antioxidant activity of protein in rhizomes of *Curcuma* species (Species on y axis represents; C.b - *C.brog*, C.am- *C.amada*, C.c -*C.Caesia*, C.z - *C. zedoaria*, C.m- *C.malabarica*, C.ae-*C.aeruginosa*, C.s - *C.sylvatica*, C.r- *C.rakthakanta* and C.ar- *C.aromatica*).

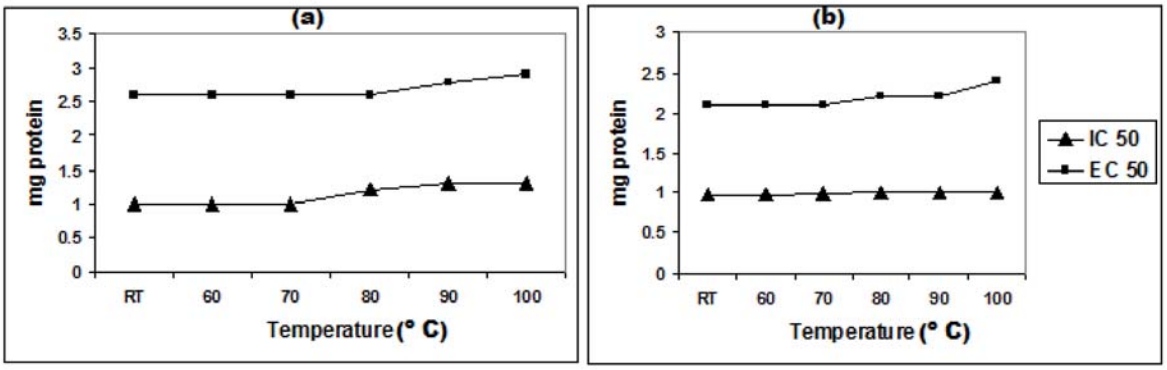

Figure 4. Heat stability of antioxidant activity of rhizome protein (a) *C.caesia* (b) *C.brog* at different temperatures.

Table 2. Anti inflammatory activity of *Curcuma* proteins.

Species	Dose (mg/kg body weight)	Difference in paw circumference (cm)	Percentage inhibition of oedema
Control	Vehicle	4.77± 0.25	--
C. aeruginosa	100	4.77 0.25	73
C. amada	100	1.29 0.02 [c]	75
C. aromatica	100	1.20 0.02 [b]	72
C. brog	100	1.35 0.01 [d]	75
C. caesia	100	1.20 0.02 [b]	75
C. malabarica	100	1.21 0.01 [b]	75
C. rakthakanta	100	1.20 0.01 [b]	67
C. sylvatica	100	1.58 0.02 [f]	72
C. zedoaria	100	1.35 0.01 [d]	77
Voveran	20	1.46 0.02 [e]	69

activity. The results showed that both DPPH free radical scavenging activity as well as reducing power was not affected even at high temperatures, indicating that the antioxidant activity was heat stable (Fig 4).

Anti-inflammatory activity

The anti-inflammatory activity of the proteins isolated from different species is given in Table 2. Injection of carrageenan in the rat paw, induced oedema which was found to be maximum after 3h. Proteins from all species produced significant anti-inflammatory activity at a dose of 100mg/kg bw (percentage of inhibition ranged from 67-75 % at 5h, which was almost comparable to that of *C. zedoaria* (77%). The anti-inflammatory activity was less than that of the standard drug Voveran which produced 69 % inhibition at a dose of 20mg / kg bw. The oedema suppressing effect was significant, $p<0.05$.

Good correlation was seen among different bioactivities viz, DPPH scavenging activity >< ferric reducing power ($R^2 = 0.580$), DPPH scavenging activity >< anti-inflammatory activity ($R^2 = 0.668$), ferric reducing power >< anti-inflammatory activity ($R^2 = 0.307$).

Discussion

Studies on the pharmacologically active compounds in *Curcuma* species have mainly focussed on organic constituents and very few studies have been carried out on the aqueous components except for reports on antioxidant proteins and polysaccharides in aqueous extracts of rhizomes in a few species, particularly *C. longa*. The bioactive proteins include an antifungal protein from *C. longa* (Wang and Ng., 2005; Petnual et al., 2010), haemagglutinating proteins (lectins) from *C. amarissima* (Kheeree et al., 2010), *C. aromatica* and *C. zedoaria* (Tipthara et al., 2007) and antioxidant enzymes from *C. longa* and *C. Comosa* (Ramadas and Srinivas., 2011; Boonmee et al., 2011). The mannose binding lectin exhibiting haemmaglutinating activity isolated from rhizomes of *C. zedoaria* was found to correspond to a molecular mass of 13 kDa (Tipthara et al., 2007). Lectins isolated from rhizomes of *C. longa*, corresponded to a molecular weight of 17 kDa exhibited antifungal, antibacterial and α-glucosidase inhibitory activity (Petnual et al., 2010). A water soluble peptide in *C. longa* which was present in a concentration of 0.1% dry weight, showed good antioxidant activity, inhibited α-amylase, was heat stable and insensitive to heat, trypsin and pepsin (Srinivas et al., 1992). Another 14kDa protein turmerin purified from *C. longa* showed potent antioxidant activity against venom phospholipase A2 induced free radical formation. β-turmerin, a 34 kDa protein also isolated from *C. longa* was a glycoprotein with strong antioxidant activity (Smitha et al., 2009).

The present studies showed that soluble proteins were present in rhizomes of all the eight *Curcuma* species studied, with yields ranging from 1.3 – 5.3 %. The purified native proteins had a molecular weight of 66 kDa, which was resolved by SDS-PAGE as 12 and 14 kDa proteins, and showed high degree of similarity between the species. These proteins showed significant antioxidant activity, (measured in terms of DPPH free radical scavenging activity and ferric reducing power) which was stable to heat. Among the species studied, the highest antioxidant potential was observed for *C. brog*, *C. amada, C. caesia* which was comparable to *C. zedoaria.* The proteins also exhibited high anti-inflammatory activity. These low molecular weight proteins resemble the antioxidant proteins present in rhizomes of *C. longa*, and could be exploited for their pharmacological activity. The positive effects of proteins from *C. longa* are reported to be comparable to that of curcumin. The solubility and heat stability of the proteins present in aqueous extracts of these *Curcuma* rhizomes could further enhance their pharmacological action in comparison with other bioactive phytochemicals such as phenolics, essential oils and flavonoids present in these species.

Acknowledgements

The authors are grateful to Kerala State Council for Science Technology and Environment for financial support and to the Director CTCRI for providing facilities.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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