International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 916-924
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Phytochemical Quantification and Antioxidant Potential of *Curcuma karnatakensis* [White turmeric] – An endemic taxon

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Received: 8 Oct 2018 / Accepted: 10 Nov 2018 / Published online: 1 Jan 2019
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Abstract
Plants are the rich source of various phytochemicals. Therapeutic values of medicinal plants such as anti-cancerous, antioxidant, antimutagenic and anti-inflammatory are mainly due to the presence of various phytochemicals. Alkaloids, phenols and terpenoids are among the important phytochemicals synthesized by plants whose therapeutic activities are well established. The genus *Curcuma* is known for its medicinal value since ancient time. The present work is an attempt to analysis the phytochemicals present in rhizomatous rootstock of *Curcuma karnatakensis*, which is an endemic taxon of the Western Ghats. Rootstocks of *C. karnatakensis* were collected from two localities having different geographical features. The extraction is carried out by following infusion and centrifugation. Among the various solvents used for the extraction, methanol (90%) was found to be best for the extraction. Standard preliminary tests were conducted to detect various phytochemicals in the extracts. Further, quantification of selected phytochemicals in the samples was carried out following standard methods using UV-Vis spectrophotometer. Among the phytochemicals analyzed in both the samples, phenols were found to be more in quantity than others were. Antioxidant potential of the extracts was also determined by using DPPH, ABTS and NO scavenging assays. The present study is a step forward to identify the medicinal importance of the taxon by analyzing various phytochemicals and antioxidant potential.

Keywords
Curcuma karnatakensis, Endemic, White turmeric, Phytochemicals, Antioxidant.

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1. INTRODUCTION
Plants are the rich source of phytomedicines obtained in the form of secondary metabolites. Various phytochemicals synthesized through secondary metabolism by plants are used in drug development and treating several ailments including cancer, cardiovascular diseases and even HIV [1]. The source of these bioactive compounds includes all the

parts of the plants body which are more ecofriendly in nature and much safer. Alkaloids, phenols, flavonoids, tannins, lignins and terpenoids are among the important phytochemicals synthesized by plants whose curative properties are well established [1, 2, 3].

Apart from their therapeutic values, some of these compounds are known to scavenge free radicals that are generated because of various biochemical reactions in living tissues. Accumulation of free radicals results in oxidative stress, which can damage every component of the cell including proteins, enzymes and even DNA leading to cell death. In this context, antioxidants are the potential compounds that help neutralizing the harmful free radicals in living systems. A balance between the free radicals and antioxidants is essential for proper physiological function. An imbalance if created that can be rectified by application of external source of antioxidants, which can assist in counteracting the effects of oxidative stress.

The genus *Curcuma* is well known for its medicinal properties since ages including its antioxidant potential [4]. Various pharmocological activities attributed to *Curcuma* species are due to the presence of curcuminoids-phenolic compounds [5]. Antioxidant efficacy of curcuminoids of various species of *Curcuma* is well established [5, 6].

Curcuma karnatakensis Amalraj, Velayudhan and Muralidharan, an endemic to Karnataka is less known medicinally and an under exploited taxon. Amalraj *et al*., reported it for the first time in 1991[7] from Hirehalli Village, Karnataka which comes under Western Ghats range. Later Kotresha *et al.,* in 2008[8] have collected the same species from Dharwad, Karnataka – a plain region with different climatic conditions. Unlike the other species of *Curcuma*, the dried rhizomatous rootstock yield white powder as *C.zedoaria* which is commonly known as white turmeric. Pharmacological and ethno medicinal properties of *C.zedoaria* are well established [9]. Tejavathi *et al.,* [10] have reported varied contents of curcuminoids in the above two samples of *C. karnatakensis* and attributed this variation to their different habitats in which they are growing. Hence, the aim of the present study is to quantity other phytochemicals and to analyze the antioxidant potential of these two samples.

2.MATERIALS AND METHODS

Plant source and Sample Preparation

Sample A form Dharwad and B from Hirehalli were collected from their natural habitats and maintained in the department garden. The authenticated herbarium specimens of the two samples are deposited in the Department of Botany, Bangalore University, Bangalore 560056, India (Voucher no's: 202 and 203).

Rhizomatous root stocks of both the samples were excised from the healthy plants and washed thoroughly under running tap water for 60min and are air-dried for 8 days (Fig.1A-1C). The air-dried samples were cut into small pieces and weighed separately. Extraction was performed by grinding one gram of the sample with 10ml of 90% methanol for a required period to be homogenized in a pestle and mortar. The extract was taken in 15ml plastic centrifugation tube and centrifuged for 20min at 6000 rpm. Supernatant is collected and used for the analysis.

Phytochemical Screening

Preliminary screening for the presence and absence of various phytochemicals were conducted by following the standard methods [11, 12].

Estimation of Alkaloids

Alkaloids in the extracts were estimated by following Shamsa *et al.,* [13] method with slight modification. One ml of the extract was taken in a separating funnel and washed with 10ml of chloroform for three times by adjusting the p^H to 6.4 with 0.1N NaOH. Then 7.5 ml of BCG solution and 5ml of phosphate buffer were added to this solution. The mixture was shaken and extracted with chloroform by vigorous shaking. The extract was then collected in a 10ml volumetric flask and diluted to various concentrations with chloroform. The absorbance was measured at 470nm in Uv-Vis spectrophotometer (Elico) against the blank. Atropine sulphate was used as standard. The data were expressed as mg/Atropine sulphate equivalent (AsE) per gram of extract.

Estimation of Total Phenols

The total phenolic contents were estimated as per Folin-ciocalteau method with slight modification [14]. One ml of different concentration of extracts ranging from 25 to 100 µg/ml was mixed with a 5ml of Folin-ciocalteau reagent (diluted 1:10 v/v). 1 ml of sodium carbonate (20%) was added to this mixture after 5 min. The mixture was allowed to stand in dark for 30min at room temperature. Absorbance was measured against the blank at 765nm with the help of Uv-Vis spectrophotometer (Elico). Quantification was done on the basis of the standard curve plotted for various concentrations of gallic acid. Total phenolic content was expressed as mg/gallic acid equivalent (GAE) per gram of extract.

Estimation of Tannins

Amount of tannins in the extracts was determined by following Bray and Thorpe method [15] with slight modification. The extracts of various concentrations of 1 ml mixed with 3 ml of 4% vanillin in methanol and added 1.5ml of con. Hcl. After allowing the mixture for 15 min to react, absorbance of samples was measured against the blank at 700nm. Tannic acid was used as standard. The results were expressed as mg/tannic acid equivalent (TAE) per gram of extract.

Estimation of Terpenoids

Total Terpenoids in the extracts of two samples was determined by Ghorai *et.al.,* [16] method with slight modification. 1 ml of extracts of various concentrations was mixed with 3 ml of chloroform. Sample mixture was thoroughly Vortexed and left for 3 min. Absorbance of the samples was measured against the blank at 470nm. Linalool was used as standard. The results were expressed as mg/Linalool equivalent (LE) per gram of extract.

Antioxidant assay

DPPH assay

The DPPH radical scavenging activity of extracts of two samples was determined by using the method of Braca *et.al.*[17] with slight modification. The extracts were dissolved in an appropriate volume of 90% methanol to get the concentrations ranging from 25 to 100 µg/ml. 3ml of reaction mixture consisting of 1ml of methanolic plant extracts at different concentrations and 2 ml of 0.002% of methanolic solution of DPPH was kept in dark for 30 min. Absorbance at 517nm was determined using Uv-Vis spectrophotometer. Ascorbic acid was used as standard. The percent inhibition was calculated by using the following formula

$$\% \text{ Scavenging activity} = \frac{[(Ac-As)]}{Ac} x100$$

Where, Ac is Absorbance of the control, As is Absorbance of the samples

ABTS assay

Free radical scavenging activity of the samples was determined by ABTS radical's cation decolorization assay with slight modification of Katalinic *et.al.,* [18] method. ABTS radical cations ($ABTS^+$) were produced by the reaction of ABTS solution (2mM) with 17mM potassium per sulphate. The mixture was allowed to stand in dark at room temperature for 12-16 h before use. The radicals were stable in this form for more than 2 days when stored in dark. The $ABTS^+$ solution was diluted with methanol (90%) to get an absorbance of 0.70±0.02 at 734 nm. 2ml of diluted $ABTS^+$ solution was added to different concentrations of extracts ranging from 25 to 100µg/ml. Ascorbic acid was used as standard and absorbance was recorded at 734nm. The scavenging ability of the sample was calculated according to the following equation

$$\% \text{ Scavenging activity} = \frac{[(Ac-As)]}{Ac} x100$$

Where, Ac_{is} Absorbance of the control, As is Absorbance of the samples

Nitric Oxide scavenging activity

The ability of extracts to scavenge nitric oxide radicals was determined by following Sreejayan and Rao's method [19] with the slight modification. Sodium nitroprusside (10mm) in phosphate buffer saline was mixed with different concentrations of methanolic extracts ranging from 25 to 100µg/ml and incubated at 25°c for 30 min. 1 ml of Griess reagent (1% sulfanilamide, 2% H_3Po_4 and 0.1% N- (1-naphthyl) ethylenediamine dihydrochloride) was added to the mixtures. The absorbance of the chromophore thus formed was measured at 546nm. Ascorbic acid was used as standard. The NO scavenging activity was expressed as the inhibition percentage and calculated as per the following formula

$$\% \text{ Inhibition} = \frac{[(Ac-As)]}{Ac} x100$$

Where, Ac_{is} Absorbance of the control, As is Absorbance of the samples.

Statistical Analysis

All estimations and assays were carried out in triplicate. Data were expressed as means ± standard deviation and subjected to one-way analysis of variance test (ANOVA). Significant F ratios between the group means were analyzed by Duncan's multiple range at $p \leq 0.05$.

3. RESULTS AND DISCUSSIONS

Phytochemicals synthesized by plants play an important role in growth and reproduction in addition to providing protection against pathogens and predators [20]. Phytochemicals apart from their beneficiary effects to plants, they form an important source for various pharmacological effects on human beings. Preliminary screening of the methanol extracts of two samples have shown the presence of various phytochemicals (Table1). Alkaloids and polyphenols have a wide range of pharmacological activities among the various phytochemicals synthesized by plants and used in both traditional and modern medicines. In the present study, quantification of alkaloids, phenols, tannins and terpenoids were conducted following standard methods in the methanol extracts of rootstocks of two samples of *C. karnatakensis* (Table 2).

Selection of a solvent and the method of extraction play a crucial role in the estimation of biologically active compounds since they are available in small quantity. Solvents with a wide range of polarity were used commonly for the extraction. Recovery of polyphenols from the plant extracts is influenced by their solubility in the solvents. Furthermore, solvent polarity will play a key role in increasing phenolic solubility. Methanol, ethanol, acetone, chloroform, hexane and ethyl acetate are among the important solvents commonly used for extraction of bioactive compounds from biological materials. However, methanol in water at different concentrations has been considered as a better solvent for more consistent extraction of active compounds since, several active components are readily soluble in methanol [21-23]. Addition of water to methanol has enhanced the extraction efficiency significantly from the tubers of *Kirkia wilmsii* and other selected medicinal plants since water tends to increase the polarity [24, 25]. Methanol (90%) was found to be better solvent for the extraction of phytochemicals in the present study. Ethanol and water mixtures are also considered to be best for extraction of wide range of phenols because of their acceptability for human consumption [26]. Alkaloids were found to be 0.144±0.008 mg/AsE/g in sample A and 0.247±0.0014 mg/AsE/g in sample B. *Curcuma angustifolia* was reported to have highest alkaloid content of 162.9±0.6mg/g in the methanol extract of rhizomes than *Curcuma longa* and *Curcuma caesia* [27]. However, presence of new Quinoline alkaloid was reported in the rhizome extracts of *Curcuma longa* by Wang *et.al.,* [28]. Alkaloids constitute one of the important therapeutic agents and are used to treat various diseases. Further, alkaloid derivatives of plant origin are widely used as antimicrobial agents by inhibiting DNA topoisomerase [29]. Polyphenols have gained a lot of interest in the recent years because of their antioxidant potential. In addition to this, they also play an important role as antinutrients, due to their ability to reduce digestibility of proteins, either by direct precipitation or by inhabitation of enzyme activity [30]. Sample A has 3.5±0.003 mg/GAE/g of total phenols; whereas sample B contains 3.53±0.001 mg/GAE/g. *Curcuma longa* was reported to have more content of phenols than *Curcuma angustifolia* and *Curcuma caesia* [27]. Curcuminoids, a group of phenolic compounds, present in the members of *Curcuma* reported to have wide range of medicinal uses. Sarangthem and Haokip [27] and Datta [31] have found high content of curcuminoids in *Curcuma longa* among the other species of *Curcuma* studied. Extensive phytochemical and pharmacological studies were conducted in *Curcuma longa* to estimate the curcuminoids and to study their biological activities [32,33]. However lowest content of curcuminoids among the *Curcuma* species is reported in *C. karnatakensis* [10].

The other two phytochemicals quantified in the present study are tannins and terpenoids. Sample A has shown 0.234±0.009 mg/TAE/g of tannins and 0.489±0.0189 mg/LE/g of terpenoids. Whereas, sample B contains 0.234±0.023 mg/TAE/g of tannins and 0.508±0.008 mg/LE/g of terpenoids. Tannins and terpenoids are indicated in various Physiological and pharmacological activities such as NO inhibitory effects, antitumour activity, anti-inflammatory and anticoagulutive effects [31,34,35]. Besides, tannins have also been shown to form complexes with dietary proteins and carbohydrates as well as with enzymes [36,37]. Mukherjee *et.al.,* [38] have opined that phenolics and terpenoids are the promising anthelmintics as they boost the immunity against the invading parasites by virtue of their binding capacity with proteins [38]. Quantification of biologically active compounds in the methanolic extracts of two samples of *C. karnatakensis* paves the way for further pharmacological studies in the taxon that is otherwise underexploited in its medicinal value.

Since both the samples have shown to contain significant quantities of phenols, naturally occurring antioxidants, antioxidant potential of the methanolic extracts were analyzed by DPPH, ABTS and NO radical scavenging assays since any single method cannot fully evaluate the antioxidant capacity of a particular compound. Tabart *et.al.,* [39] have confirmed that different methods can give widely divergent results because of different principles involved in each method. DPPH is a stable free radical widely used for

determination of antioxidant capacity of plant extracts. It is simple, sensitive and reproducible procedure [40]. The reaction is mainly dose dependent. DPPH scavenging activity has proportionately increased with the increase in the concentration of the extracts. The extracts were able to reduce radical DPPH to the yellow colored diphenyl picrylhydrazine. The IC_{50} value of DPPH scavenging by sample A and B are shown in the Table 3. The results have indicated that sample B has shown highest scavenging activity at the concentration of 25µg/ml with IC_{50} value of 160.52±0.64µg/mL as against the IC_{50} value of 207.70±0.49µg/mL for sample A. However, the antioxidant capacity of sample A was found to be better with IC_{50} value of 211.32±0.01µg/ml as compared to sample B with the IC_{50} value of 489.90±0.1µg/ml in ABTS assay. Lack of correlation between antioxidant activities determined in the same sample with different assays was also reported by Juntachote and Berghofer, Yang *et.al.*, Mata *et.al.,* and Shalaby andShanab in various plants [41-44]. Kopjar *et.al.,* [45] have suggested that the different results thus obtained for various methods may be because of the differences in the activity of different phenolic compounds with the reagents applied for their antioxidant determination. Structure of the phenolic compounds present in the extracts is a key determinant of their radical scavenging activity [45]. The other reason for various results may be because of rapid reaction of phenols with ABTS, but their actions with DPPH radical differ from compound to compound [18, 45]. However, the results obtained for nitric oxide scavenging activity correlates with the ABTS scavenging activity of the samples in the present study. Extract of Sample A with IC_{50} value of 120.00±0.081µg/ml has better antioxidant potential than sample B with IC_{50} value of 126.50±0.081µg/ml as per NO scavenging activity in the present study. Studies on NO scavenging activity of curcuminoids by Sreejayan and Rao [19] have shown that curcumin is a potent scavenger of NO. In all the three assays conducted in the present study, ascorbic acid was used as standard and scavenging of the radicals is concentration dependent. Various results obtained for the two samples of the same taxon may be due to their different habitats, sample A growing in plains, whereas sample B growing in Western Ghats region. Antioxidant potential of *Curcuma longa* was attributed to geographical and climatic conditions by Arya *et.al.,* [46]. Akbar *et.al.,* [47] have found variations in the curcumin content in 131 genotypes collected from different agro climatic zones of India. It is well established that polyphenols are greatly responsible for the antioxidant potential of the plant extracts [48]. Hence, the correlation between the phenolic contents and the antioxidant potential of the samples was studied. Present study is in accordance with the total phenol content and the antioxidant activity of the extracts [49-51]. However, Yu *et.al.,* [52] have found no correlation between the content of their main antioxidant compounds and the radical scavenging activity. Linear correlation between the phenolic contents and the antioxidants activity of the two samples was observed in all the three assays conducted in the present study with the correlation coefficients are above $R^2 = 0.9$[53].

Table 1: Preliminary phytochemical analysis in the extracts of rhizomatous rootstocks of two samples of *C.karnatakensis*

Sl.No	Chemical Constituents	Tests	Solvents				
			Hexane	Ethyl acetate	Methanol	Pet. ether	Water
1	Alkaloids	Mayer's test	+	+	+	+	+
		Wagner's test	+	+	+	+	+
		Dragendorff's test	+	+	+	+	+
2	Phenols	Ferric chloride test	-	-	+	-	+
		Zinc-hydrochlooride reduction test	-	-	+	-	-
3	Favonoids	Alkaline reagent test	+	+	+	+	-
		Ferric chloride test	-	-	+	-	+
4	Terpenoids	Salkowski's test		+	+		
5	Tannins	Lead acetate test Ferric chloride test	+	+	+	+	+

Sl.No	Chemical Constituents	Tests	Solvents				
			Hexane	Ethyl acetate	Methanol	Pet. ether	Water
			+	+	+	+	+
6	Carbohydrates	Molisch's test	+	+	+	+	+
		Fehling's test	+	+	+	+	+
7	Proteins and Amino acids	Millon's test	+	+	+	+	+
		Ninhydrin's test	+	+	+	+	+
8	Saponins	Foam test	-	-	-	-	-
9	Phlobotannins	HCL test	-	-	+	-	-
10	Glycosides	Borntrager's test	-	-	-	-	+

Table 2: Quantification of phytochemicals in rhizomatous rootstocks of *C.karnatakensis.*

Phytochemical	Sample A	Sample B
Phenols	3.5±0.003 (mg/GAE/g) ^{b}	3.53±0.001 (mg/GAE/g)^{a}
Alkaloids	0.144±0.008 (mg/AS/g) ^{b}	0.247±0.0014 (mg/AS/g)^{a}
Tannins	0.234±0.009(mg/TAE/g)^{b}	0.234±0.023(mg/TAE/g)^{a}
Terpenoids	0.489±0.0189 (mg/LE/g)^{b}	0.508±0.008 (mg/LE/g)^{a}

Data represents the mean ± standard deviation (n=3), Mean± SE followed by different letters within the columns are significantly different (P≤0.05) using Duncan's multiple range test.

Table 3: Antioxidant potential of *C.karnatakensis*

Assay	Sample A(µg/mL)	Sample B(µg/mL)
DPPH	207.70 ± 0.49	160.52 ± 0.64
ABTS	211.32 ± 0.01	489.90 ±0.1
NO	120.00 ± 0.081	126.50 ±0.081

Data represents the mean ± standard error (n=3)

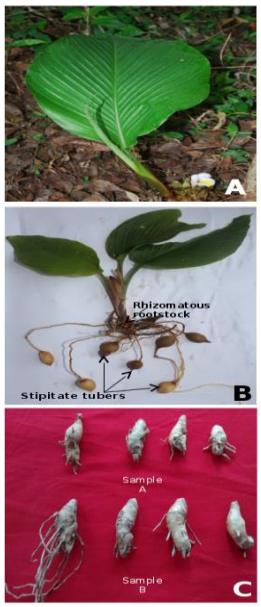

Figure 1: *Curcuma karnatakensis*
A-Plant in natural habitat with inflorescence
B-Plant with rhizomatous rootstock and stipitate tubers
C-Rhizomatous rootstocks of sample A and sample B

4. CONCLUSION

Estimation of phytochemicals and analysis of antioxidant potential of the rhizomatous rootstocks of two samples of *Curcuma karnatakensis* is the first attempt to provide a basis for further pharmacological studies in the taxon. From the previously mentioned data, both the samples contain significant amount of phytochemicals whose curative properties are well established. Furthermore, both the samples have exhibited significant antioxidant potential as confirmed by various assays. Though synthetic antioxidants comprising curcuminoids and chalcone are developed, natural antioxidants are always score better when it comes to human consumption [5].

5. ACKNOWLEDGEMENT

Authors are thankful to UGC, New Delhi for granting BSR Faculty fellowship to one of the author (DHT). The present work was carried out under this scheme.

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