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Isolation, Purification and Identification of Curcuminoids from Turmeric (*Curcuma longa* L.) by Column Chromatography

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Abstract

Turmeric, *curcuma longa* L of Zingiberaceae family is a widely cultivated spice in India and other Asian countries. Turmeric is rich in curcuminoids, and recognized for their broad spectrum of biological activities, curcuminoids vary in chemical structures, physico-chemical characteristics as well as the functional properties. This study focused on screening of solvents for extraction of curcuminoids, isolation and purification of curcuminoids by column chromatography followed by purity analysis by HPLC. Different solvents were used for extraction, among them acetone showed maximum yield of each curcuminoids. Various solvent at different polarity were pre-tested in TLC for separation of curcuminoids, chloroform:methanol at 95:5 showed better resolution of R_f value at 0.75, 0.55, 0.27, as Curcumin(C), Demethoxycurcumin(DMC), Bisdemethoxycurcumin(BDMC) respectively. The acetone extract was subjected to silica gel column chromatography with chloroform: methanol at increasing polarity. Yield of each curcuminoid from column was determined and total curcuminoids of individual fractions of each curcuminoids were determined by UV spectrophotometry. Crystallization of each compound was done using chloroform: methanol (5:2) at 5°C. The isolated curcuminoids (C, DMC, and BDMC) showed single peaks at retention times of 10.81, 12.79, 13.03 min respectively on HPLC.

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Key Words: *Curcuma longa*, Curcumin(C), Demethoxycurcumin(DMC) and bisdemethoxycurcumin(BDMC), UV spectroscopy, HPLC

Introduction

Curcuma longa L. is typical of the herbaceous plant with thick and fleshy rhizomes and leaves in sheaths that characterize the family Zingiberaceae [1]. Turmeric is native to tropical South Asia and needs temperatures between 20° C and 30° C, and a considerable amount of annual rainfall to thrive [2]. Turmeric is commonly known for its medicinal values in the Indian traditional systems of medicine [3]. Curcumin (C), main coloring substance in *Curcuma longa* and two related compounds, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), are altogether known as curcuminoid [1]. The chemical structures of three curcuminoids are shown in Figure 1. The total of curcuminoids which is about 4-6%, turmeric also contains 2-4% essential oil and 2-3% of fixed oil and various volatile oils, including turmerone, atlantone, and zingiberone. Other constituents include sugars, proteins and resins. The value of the turmeric products is based on their curcuminoids content and estimated based on its absorbance at 420 nms [3].

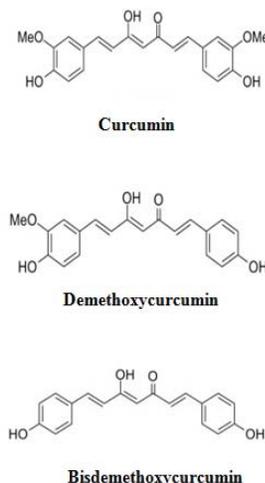


Figure 1

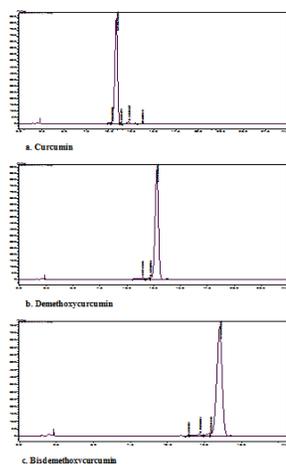


Figure 2: HPLC profile of purified curcuminoids.

A. Curcumin, B. Demethoxycurcumin, C. Bisdemethoxycurcumin shows single peak at the retention time of 11.2, 12.7, 13.4 min respectively.

Curcuminoids are polyphenols having a pronounced yellow color. They have poor solubility in water at acidic and physiological pH, and also hydrolyze rapidly in alkaline solutions. Curcuminoids are soluble in dimethyl sulfoxide (DMSO), acetone and ethanol. They are readily decomposed when exposed to bright light, high temperature or oxidative conditions [4]. The traditional uses of turmeric or natural curcuminoids in folk medicine are multiple, and some of these including antioxidant, anti-inflammatory properties, anti-carcinogenic effects and hypoglycemic effects in humans [2]. Although the chemical structure of curcumin was determined in the 19th century, the immense value of this molecule is being realized now with several extensive studies on its pharmaceutical and nutraceutical potential [5]. The content of total curcuminoids in turmeric powder plays an important role in its antioxidant activity and effectiveness of the product [4]. The content of curcuminoids may vary in turmeric rhizome grown in different agro-climatic zones [7]. Curcuminoids are recognized for their broad spectrum of biological activities, the potential use of curcumin in the prevention of cancer is the subject of intensive laboratory and clinical research [8]. Recently it was reported that the effect of curcuminoids was examined on the proliferation of MCF-7 human breast tumour cells that demethoxycurcumin was the best inhibition of MCF-7 cells followed by curcumin and bisdemethoxycurcumin [9]. Many of these properties could be enhanced through improving bioavailability of curcuminoids by different approaches and subject of intensive laboratory and clinical research [10]. Eventhough, curcumin has more pharmacological properties, the amount of total curcuminoids absorbed by the animal systems far less. Mixing of curcumin with standardized essential oil of turmeric enhances the absorption and bioavailability in animal system [11].

The choice of solvents for extraction is restricted to the few solvents of defined purity allowed by national and international food laws in the processing of food materials. From the processing angle, choice is dictated by the efficiency of extraction of characteristics components of the individual

spices, optimization in respect of yield of oleoresin with desirable handling properties, and finally the ease and economy of desolventizing to the permitted residual solvent level in the final product, and recovery of solvent. Hexane, acetone, alcohol, and ethylene dichloride have generally been used in the extraction of oleoresins of spices. From consideration of solubility of active constituents, the curcuminoids are poorly soluble in the hydrocarbon solvents. Alcohol and acetone are good extractants and the yields can also be expected to be high because of extraction of non-flavor components. Soxhlet extraction of turmeric powder with acetone gave a yield of about 5.0% containing 42% curcuminoids in 4 to 5 hours. Acetone as solvent was slightly superior to alcohol and ethylene dichloride, the curcuminoids content also is on the high side, suggesting selective extraction. The results of extraction with acetone have, however, been reported to give high yields of curcuminoids than alcoholic extraction [1].

A number of studies are undertaken to separate curcuminoid pigments by thin layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), and column chromatography (CC). The stationary phase most commonly used is silica gel with different solvent systems including benzene, ethyl acetate, ethanol, chloroform, acetic acid, hexane, and methanol for chromatographic separations [6, 12]. HPLC method was sensitive, precise, and accurate for detection and quantification of curcuminoids in the extract of rhizome *Curcuma longa* [13]. Separation by high-performance liquid chromatography (HPLC) was done mostly on reverse phase employing mixtures of water, acetonitrile, ethanol, and methanol [8]. Since the curcuminoid pigments vary in chemical structures, it is possible that the physico-chemical characteristics as well as the functional properties would vary among them. As compounds DMC and BDMC are not commercially available, it could be important to obtain these pigments in high purity for detailed studies on their chemical and physiological attributes [14]. Therefore it is important to obtain pure pigments and characterize them individually to study their biological properties [15].

The present study describes screening of solvent system for extraction of curcuminoids from turmeric rhizome using non-polar to polar solvent for complete extraction, and isolation, identification and purification of curcuminoids by column chromatography followed by purity analysis by HPLC.

Materials and Methods

Curcuma longa (Turmeric) rhizome were collected from Assam - Lakhadong variety. All solvents / Chemicals used were of AR / HPLC grade and obtained from E-Merck. The reference standard of Curcumin was purchased from Sigma Chemicals Co. U.S.A.

Methods

Extraction of curcuminoids

Fresh rhizomes were cleaned washed with deionised water, sliced and dried in the sun for one week and again dried at 50°C in a hot air oven for 6 hours. Dried rhizomes were cut in small pieces, powdered by electronic mill. Approximately 20gm of sample were taken into a thimble and placed in a soxhlet apparatus, were set up with various solvent from non-polar to polar. 150ml of solvent was added and extracted

according to their boiling point for 6 hours. The solvents used were Hexane(B.P=69° C), Chloroform (B.P = 61°C), Ethyl acetate (B.P=77°C), Methanol (B.P=65°C), and Acetone (B.P=56.53°C). And one sample was extracted with hexane for 2 hours and hexane extract was discarded and the powder was re-extracted with methanol for 6 hours. After completion of extraction the dark brown extract was then cooled, filtered, concentrated using rotary evaporator, and finally by vacuum suction to get a crude dried extract which was black orange in color. Each raw sample of turmeric was extracted by the same method and yield was calculated.

Estimation of curcuminoids: by HPLC analysis

Procedure

i) Preparation of Sample: Weighed accurately 25mg sample and dissolved in 25ml acetone. From this pipetted out 1ml and diluted to 5 ml with acetone. Filtered through 0.2µm membrane filter before injection.

ii) Chromatographic conditions

Samples were analysed by HPLC in a Shimadzer LC 20A0 liquid chromatograph system with SPD-M20AuV detector in isocratic mode. 20µl of sample was injected and the elution was carried out with gradient solvent systems with a flow rate of 1.0ml/min at ambient temperature. Column used was C18 (250X4.6mm), mobile phase 40% THF and 60% water containing 1% citric acid, pH adjusted to 3.0 using concentrated potassium hydroxide solution and measured in wave length 420nm.

Separation of curcuminoids by TLC using different solvent system:

Acetone extract were tested in TLC for presence of three curcuminoids. The TLC pre-coated silica gel (Merk-60 F254, 0.25mm thick) plate were developed using a Camag twin-trough glass tank which was pre-saturated with the mobile phase for 1 hour and each plate was developed to a height of about 10cm. The composition of mobile phase was optimized by using different mobile solvents of varying polarity. After development plates were removed and dried and spots were visualized in UV light.

Column chromatography:

Sample preparation

100 gm of fine powdered rhizome were subjected to soxhlet extraction and solvent used were acetone for 6 hours. The extract was filtered and concentrated in rotary evaporator, yielded olerosin was precipitated with petroleum ether and vacuum dried, this crude curcuminoid mixture contained curcumin, demethoxycurcumin, bisdemethoxycurcumin.

Silica gel column chromatography

Acetone extract was subjected to column chromatography in silica gel (60-120 mesh) glass column. About 5 gm of crude Curcuminoids were mixed with 8 gm of silica gel and loaded on

to the column of 46×2 cm and eluted with chloroform followed by chloroform:methanol with increasing polarity. All the collected fractions were subjected to TLC silica gel 60 F254 plate using chloroform:methanol (95:5) as the developing solvent system and detected as yellow spots. And similar fractions with the Rf values were pooled and the organic solvent was removed by rotary evaporator. The total curcuminoid content of each curcuminoid collected were analysed by UV spectrophotometry at 420 nm.

Purification of each curcuminoids:

The individual Curcuminoids collected from the column chromatography was dissolved in methanol and heated. After complete dissolution added chloroform to get the ratio methanol : chloroform 5:2 and kept at 5°C for overnight. The crystals obtained were separated by filtration. The crystals were precipitated with petroleum ether. The purity of Individual crystals were analysed in HPLC.

Results and Discussion

Curcuminoids have immense biological properties in which curcumin (C) is reported for so many medicinal properties. Recently the analogs of curcumin were reported for biological activities. Demethoxycurcumin (DMC) was the best inhibition of MCF-7 cells [9]. Bisdemethoxycurcumin (BDMC) is active for modulation of MDR-1 gene expression [17]. Compounds II and III are not commercially available. Therefore to study biological properties of individual curcuminoids we need isolate compounds at high purity. In our present study acetone was the suitable solvent for extraction of curcuminoids. The HPLC profile of extracted crude curcuminoids showed curcumin (C), and its analogues DMC and BDMC to be present in 22.8%, 14.2%, 6.5% respectively spiking with standards.

Screening of solvent for extraction

Different solvents with varying polarity were used for extraction of curcuminoids from turmeric rhizome. Various solvents used were Hexane, Chloroform, Ethyl acetate, Methanol, Acetone. After concentrating each extract total yield were determined and percentage composition of individual curcuminoids present in the extract were analysed by HPLC shown in table1, the identity of each peak were confirmed by determination of retention times and by spiking with standards. Curcumin was found to be the major compound in all of the tested extracts followed by demethoxycurcumin and bisdemethoxycurcumin. In our present study acetone extract resulted with optimum amount of individual curcuminoids than in all other extract. The percentage of C, DMC, BDMC in acetone extract were 22.8%, 14.2%, 6.5%. Hence acetone extract can be good source for the isolation of individual curcuminoids.

Table 1. Screening of different solvents with varying polarity for extraction of Curcuminoids

Solvent	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin	Total	Total extract gm
Acetone	22.8%	14.2%	6.5%	43.5%	3.49
Chloroform	19.7%	12.15%	5.05%	36.9%	3.09
Hexane	6.5%	1.03%	0.04%	7.57%	0.90
Methanol	15.68%	9.90%	4.73%	30.3%	4.31
Ethylacetate	18.76%	11.6%	5.2%	35.5%	3.20
Hex/MeOH	18.1%	11.2%	6.1%	35.4%	3.62

The total extract of the turmeric samples were determined as described in the text. The percentage composition of each curcuminoid was estimated by HPLC and the results are the average of three experiments.

Table 2: Separation of curcuminoids by TLC using different solvent system

TLC mobile Phase	Ratio	Rf values		
		C	DMC	BDMC
Benzene:ethylacetate	18 : 2	0.79	0.69	0.61
Dichloromethane:methanol	19 : 1	0.8	0.7	0.6
Chloroform:methanol	19 : 1	0.75	0.55	0.27

Each plate was developed to a height of about 8cm.

C = curcumin, DMC = demethoxycurcumin, BDMC = bisdemethoxycurcumin.

Table 3: silica gel column chromatography elution profile.

Fractions numbers	Total volume collected (mL)	Curcuminoids present	Weight of extract (mg)	Percentage of total curcuminoid by UV spectroscopy
1 to 31	240	C	906.4	84%
32 to 40	360	C+DMC	173.5	22%
41 to 67	1080	DMC	597.5	86%
68 to 75	320	DMC+BDMC	192.7	46.6%
76 to 95	800	BDMC	390.5	80.61%

Each fraction contains 40 mL.

C = curcumin, DMC = demethoxycurcumin, BDMC = bisdemethoxycurcumin.

Elution by chloroform and chloroform:methanol 98:2.

Separation of curcuminoids by TLC using different solvent system.

Different compositions of mobile phase were tested in TLC for the separation of individual curcuminoids and its Rf values were determined shown in table 2. The desired resolution of separation was achieved using chloroform:methanol 95:5 as the mobile phase. The Rf value of curcuminoids were 0.75, 0.55, and 0.27, for C, DMC, BDMC respectively. Better resolution of Rf value showed that chloroform and methanol can be suitable solvent for the separation of compounds in column chromatography.

Column chromatography

In the present study acetone extract was precipitated with petroleum ether and yielded crude curcuminoids were subjected to column chromatography the elution was done using chloroform followed by chloroform: methanol with increasing polarity and the fractions obtained were tested with TLC. Fractions showed same pattern in TLC were pooled and concentrated. The composition of the fractions collected during column chromatographic separation of crude curcuminoids and the concentrated fractions were tested for determination of total curcuminoids by UV spectroscopy is shown in Table 3.

The UV spectroscopy analysis of fraction collected shows the percentage of total curcuminoids present in the fraction. In our study percentage of total curcuminoids present in fractions collected 84%, 86%, 80.6% of C, DMC, and BDMC respectively. Therefore further purification was done by repeated crystallization with chloroform and methanol. Finally

precipitated with petroleum ether. The purity profile of isolated individual curcuminoids were analysed by HPLC were shown in figure2, C, DMC, BDMC showed single peaks at retention times of 10.81, 12.79, 13.03 min respectively. The identity of each peak was confirmed by determination of retention times and by spiking with standards. Curcumin formed as bright yellow needle shaped crystals, Demethoxycurcumin as light yellow crystals, Bisdemethoxycurcumin as reddish orange colour crystals. These purified compounds were further studied for biological activities and pharmaceutical properties.

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