ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Evaluation of antimicrobial activity of curcuminoids isolated from turmeric

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Abstract

In a search for plant extracts with potent antimicrobial activity, turmeric (*Curcuma longa*) was extracted to get volatile oil and total curcuminoids .Further total curcuminoids portion was isolated by column chromatography to get curcumin , bisdemethoxycurcumin and demethoxycurcumin. The isolated curcuminoids were identified and characterized by melting point, thin layer chromatography, infra red spectroscopy, nuclear magnetic resonance and mass spectrometry. All the three isolated curcuminoids were evaluated for antibacterial activity by Agar diffusion method against medically important bacteria viz. *B. subtilis, K. pneumonia, E. coli, Enterobacter aerogenes, Pseudomonas aeruginosa, S. auresus* and *P. Mirabilis.* Two fungi were selected viz. *Aspergillus niger* and *Candida albicans* for antifungal potential of three isolated curcuminoids by Agar well diffusion method. Out of three curcuminoids, curcumin showed better antibacterial as well as antifungal activity as compare to bisdemethoxycurcumin and demethoxycurcumin. Kanamycin was used as standard drug for antibacterial activity and Fluconazole as standard for antifungal activity.

Key-Words: *Curcuma longa*, Total curcuminoids, Curcumin, Bisdemethoxycurcumin, Demethoxycurcumin, Antibacterial activity, Antifungal activity.

Introduction

Now a days multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases [1, 2]. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions [3].This situation forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance [4], there is a constant need for new and effective therapeutic agents [5]. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants [6, 7]. Several screening studies have been carried out in different parts of the world. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the worlds [8, 9].

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Curcuma longa L. (turmeric) is a medicinal plant that botanically is related to Zingiberaceae family[10]. Turmeric powder, derived from the rhizome of *Curcuma longa*, is commonly used as a spice, food preservative, and food-coloring agent [11-13]. It also has a long history of therapeutic uses. Turmeric extract is an oleoresin consisting of a volatile oil (light) fraction and a yellow–brown colour (heavy) fraction. It contains a number of curcuminoids, monoterpenoids and sesquiterpenoids. The compounds showing yellow colour are three curcuminoid compounds; curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcumin , a yellow bioactive pigment, is the major component of turmeric[13-15]. It has been shown that curcumin have a wide spectrum of biological activities such as antifungal[10], antidiabetic[11], antioxidant[13,14], antiinflammatory[16,17], anticancer[18], antiallergic[19], antiprotozoal[20] and antibacterial activities[10,12,21]. The volatile oil of *C. longa* reported for anti-inflammatory [22], antibacterial [23, 24] and antifungal [24] activities. The present study has been focused to isolate curcuminoids from turmeric (*C.longa*) for their antimicrobial properties.

Material and Methods

Plant Materials: The rhizome of *C.longa* L.(turmeric) were collected from Haridwar (Uttarakhand).The sample was identified by qualified Taxonomist. Fresh rhizomes were cleaned and cut into small pieces and air dried for 2 days. The dried sample was again dried in a hot air oven at 50°C for 24 hrs., then ground into powder and pass through a sieve with nominal mesh size of 2 mm. in diameter. First of all turmeric powder (10.00gm.) was put in a 500 ml. round bottom flask. The distilled water (100 ml.) was added. The mixture was distilled at a rate of 2-3 ml/min for 05 hours. The volatile oil portion was extracted out. After extraction of volatile oil by hydro-distillation, the marc was further macerated with 600 ml. of 95% ethanol on a shaker with 210 rpm at room temperature until the last extract was colorless .The ethanol extracts were combined and filtered. The filtrates were concentrated under reduced pressure at 50° C using a rotary vacuum evaporator. The extract was further evaporated on a boiling water bath until constant weight was obtained (Total curcuminoids portion) [25-28].

Isolation and identification of Curcuminoids [29-36]

The total curcuminoids portion extracted from *Curcuma longa* was further isolated by column chromatography and identified by TLC techniques.

Column chromatography

The curcumenoids extracted from *Curcuma longa* were subjected to Column chromatography using different solvent systems. The fractions collected were further chromatographed using column of silica gel G. The general techniques applied in column chromatography includes-

1. Pre-column preparation.
2. Elution.
3. Identification of fractions.

Pre-column preparation

The pre column preparation includes adsorption of selected extract / fraction, charging and saturation of column.

Elution

The charged column was then eluted with different mobile phase with gradual increase in polarity / non polarity. The fractions were collected on the basis of color and were dried in rotatory evaporator (Buchi make). The dried fractions were weighed and recovered using methanol / acetone. All the fractions were subjected to TLC for the identification of desired spots.

Identification by TLC

Identification of the extracts and collected fractions was done by using suitable TLC plates viz. pre coated normal / reverse phase TLC plates, solvent system, and detecting reagents.

Procedure:

The dried fractions were dissolved in acetone / methanol / chloroform in 5 mg/ml concentration and applied on pre coated TLC plate using capillary and developed in a TLC chamber using suitable mobile phase. The developed TLC plate was removed and dried in hot air oven for few seconds and sprayed with anisaldehyde. The sprayed plate was placed in hot air oven for 1 minute for the development of color in separate spots. The curcuminoids extracted from *Curcuma longa* was subjected to fractionation using different columns (COLUMN -1 to COLUMN -4) to isolate the chemical constituents.

Isolation / fractionation using Column -1

Requirements

Stationary phase: - Silica gel G (60-120 mesh)

Mobile phase: - Petroleum ether, chloroform, methanol

Charged matter:- Curcuminoids extracted from *Curcuma longa* (150 gm.)

Solvent system:- Chloroform: Ethanol: Glacial Acetic Acid (95:5:1)

Volume of each fraction:- 500-1000 ml

Procedure: The column was first eluted with 100% petroleum ether. The polarity was gradually increased with chloroform and methanol. The fractions were collected on the basis of color, dried in rotary evaporator and weighed. Fraction CC-1/Fr-3 contains curcumin which was dissolved in 12 litres of methanol and was refluxed for half an hour. The methanolic extract was concentrated on water bath to half of the original volume and was kept overnight for crystallization of pure curcumin. The curcumin crystals obtained was then filtered under reduced pressure. The curcumin obtained by this was 98.5% pure which was advent through melting point and HPLC. While fractions CC-1/Fr-8 and CC-1/Fr-9 contains both Bisdemethoxycurcumin and Demethoxycurcumin which was further subjected to column chromatography.

Isolation / fractionation using Column-2

Requirements

Stationary phase: - Silica gel G (230-400 mesh)

Mobile phase: - Petroleum ether, chloroform, methanol

Charged matter: - Combined fraction i.e. CC-1/Fr-8 and CC-1/Fr-9 (30.2 gm.)

TLC solvent system: - Chloroform: Ethanol: Glacial Acetic Acid (95:5:1)

Volume of each fraction: - 250-500 ml

Procedure: On the basis of TLC, the fractions CC-1/Fr-8 and CC-1/Fr -9 were combined (30.2 gm.) and were subjected to column chromatography for further purification. The fraction was dissolved in methanol and adsorbed on silica gel and charged in MPLC. The column was first eluted with 100% petroleum ether. The polarity was gradually increased with chloroform and methanol. The column was eluted in the same way as described earlier. The fractions CC-2/Fr-5, CC-2/Fr-6 and CC-2/Fr-7 contained Demethoxycurcumin whereas fractions CC-2/Fr-8 and CC-2/Fr-9 contained Bisdemethoxycurcumin which were further purified.

Isolation / fractionation using Column -3

Requirements

Stationary phase: - Silica gel G (60-120 mesh)

Mobile phase: - Petroleum ether, chloroform, methanol

Charged matter: - Combined fraction i.e CC-2/Fr-8 and CC-2/Fr-9 (9.00 gm.)

TLC solvent system: - Chloroform: Ethanol: Glacial Acetic Acid (95:5:1)

Volume of each fraction: - 250-500 ml

Procedure: On the basis of TLC, the fraction CC-2/Fr-8 and CC-2/Fr-9 were combined (9.00 g) and was subjected to column chromatography for further purification. The fraction was dissolved in methanol and adsorbed on silica gel and charged in MPLC. The column was first eluted with 100% petroleum ether. The polarity was gradually increased with chloroform and methanol. The column was eluted in the same way as described earlier. Fraction CC-3/Fr-5 contains Bisdemethoxycurcumin with 98.99% purity was advent by HPLC.

Isolation / fractionation using Column-4

Requirements

Stationary phase: - Silica gel G (60-120 mesh)

Mobile phase: - Petroleum ether, chloroform, methanol

Charged matter: - Combined fraction i.e. CC-2/Fr-5, CC-2/Fr-6 and CC-2/Fr-7 (12.00 gm.)

TLC solvent system: - Chloroform: Ethanol: Glacial Acetic Acid (95:5:1)

Volume of each fraction: - 250-500 ml

Procedure: On the basis of TLC, the fraction CC-2/Fr-5, CC-2/Fr-6 and CC-2/Fr-7 were combined (12.00 g) and was subjected to column chromatography for further purification. The fraction was dissolved in methanol and adsorbed on silica gel and charged in MPLC. The column was first eluted with 100% petroleum ether. The polarity was gradually increased with chloroform and methanol. The column was eluted in the same way as described earlier. Fraction CC-4/Fr-5 contains Demethoxycurcumin with 97.32% purity was advent by HPLC.

Identification and characterization

The isolated compounds were identified and characterized by:

1. Melting point
2. Thin layer chromatography
3. Infrared spectroscopy
4. Nuclear magnetic resonance spectroscopy
5. Mass Spectrometry

Qualitative Anti-bacterial Studies [37-39]

Method followed: Agar Diffusion Method

Working procedure

Preparation of test and standard solutions:-

The test solutions of the curcuminoids were prepared in distilled DMSO at a concentration of 1, 5 and 20 mg / ml. Kanamycin was used as standard and was dissolved in distilled DMSO to get a final concentration of 30 µg / ml. DMSO (0.1 ml) was used as solvent control.

Microorganisms used

The *Bacillus subtilis* (NCIM 2439), *Klebseilla pneumonia* (NCIM 2065), *Escherichia coli* (NCIM 2345), *Enterobacter aerogenes* (NCIM 2340), *Pseudomonas aeruginosa* (NCIM 2200), *Staphylococcus auresus (*NCIM 2079) and *Proteus mirabilis* (NCIM 2241) strains were employed for the present study. The microorganisms were maintained by sub-culturing and used at regular intervals in nutrient agar medium.

Preparation of Inoculums

The suspensions of all the organisms were prepared as per Mac-Farland Nephelometer Standard. A 24 hrs old culture was used for the preparation of bacterial suspension. Suspensions of organisms were made in sterile isotonic solution of sodium chloride (0.9% w/v) and the turbidity was adjusted.

Culture medium

The following media were used for the antimicrobial studies:

Nutrient broth: 37 gm of readymade powder was dissolved in 1 ltr of distilled water; pH was adjusted to 7.8 and sterilized by autoclaving at 15 lbs for 15 min.

Nutrient Agar: The sterilized medium was cooled to 40º C and poured into petridishes to obtain 4-6 mm thickness. The media was allowed to solidify at R.T (room temperature).

Procedure

A sterile borer was used to prepare cups of 10 mm diameter in the agar media spread with the microorganisms. 0.1 ml of inoculums (of 10^4 to 10^6 CFU / ml population prepared from standardized culture, adjusted with peptone water) was spread on the

agar plate by spread plate technique. Accurately measured (0.1 ml) solution of each sample and standard samples were added to the cups with a micropipette. All the plates were kept in a refrigerator at 2 to 8 ºC for a period of two hours for effective diffusion of test compounds and standards. Later, they were incubated at 37 ºC for 24 hrs. The presence of definite zones of inhibition around the cup indicated antibacterial activity. The solvent control was run simultaneously to assess the activity of DMSO, which was used as a solvent for extracts. The diameter of the zone of inhibition was measured and recorded.

Qualitative Anti-Fungal Studies [40-46]

The *in-vitro* antifungal activity by agar well diffusion method was standardized using fluconazole. This method is based on diffusion of antifungal component from reservoir hole to the surrounding inoculated Sabouraud dextrose agar medium, so that the growth of fungus is inhibited as zone around the hole. Two fungi were selected viz. *Aspergillus niger* and *Candida albicans.*

Fungi used

Standard cultures of *Candida albicans* (NCIM 3471) and *Aspergillus niger* (NCIM 545) were employed for the present study. The fungi were maintained by sub culturing and used at regular intervals.

Sample preparation

Samples were dissolved in DMSO to get final concentrations of 5mg/ml, 10mg/ml & 15mg/ml.

Culture medium

Sabouraud dextrose agar medium (Hi Media) was used for preliminary antifungal activity. The medium was prepared by dissolving in water and autoclaving at 121^0 C for 15 minutes.

Standard Preparation

Fluconazole standard was prepared at a final concentration of 10µg/ml in sterile distilled water.

Preparation of inoculum

The suspension of fungus was prepared as per Mac-Farland Nephlometer Standard. A 24 hour old culture was used for the preparation of fungus suspension. A suspension of fungus was made in a sterile isotonic solution of sodium chloride and the turbidity was adjusted such that it contained approximately 1.5 X 10^6 cells / ml. It was obtained by adjusting the optical density (650 nm) equal to 1.175 % barium chloride in 100 ml of 1% sulphuric acid.

Working procedure

An inoculum was prepared by suspending a single isolated colony in about 5ml of 0.9 %w/v of Normal saline. This is mixed slowly to achieve a smooth suspension. Later one drop of Tween-20 was added for filamentous fungi and the mould was broken by shaking. A sterile cotton swab was moisten in the inoculum suspension and excess of was removed by rolling the cotton swab on the inside of the tube, above fluid level 30 ml of sterile hot sabouraud agar medium was poured in each plate and allowed to harden on a level surface. A glass spreader was moistened in the adjusted inoculums suspension and surfaces of sabouraud dextrose agar plates were streaked in 4 different directions (at 90 º angels), so as to cover the entire surfaces. Using flamed sterile borer the medium was bored and the prepared test samples of three concentrations were taken and 0.1 ml each test sample was added in each bore. This procedure was carried out for the both fungi viz. *Candida albicans* and *A. niger*. The surface of Sabouraud agar plate was dried out 35 ^{0}C. Later bores were made using sterile cork borer. The above operation was carried out in aseptic condition and 0.1 ml test solution was added to the respective bore and 0.1 ml Fluconazole was taken as standard reference. A control having only DMSO was maintained in each plate. The plates were incubated at 35 ^{0}C for 48 hr. Later the values of zones of inhibition were recorded.

All the samples showing maximum zone of inhibition with concentration 15 mg/ml.

Results and Discussion

The isolated compounds were identified and characterized by:

IR spectra

IR spectra of isolated compounds showed characteristic bands- Curcumin, bisdemethoxycurcumin and demethoxycurcumin showed characteristic carbonyl peak in the range of 1600-1709 cm^{-1}.

Curcumin, bisdemethoxycurcumin and demethoxycurcumin showed the characteristic hydroxyl peak in the range of 3400-3520 cm^{-1}.

Mass spectra

Mass spectra of isolated compounds showed characteristic molecular ion peaks and the fragmented peaks- Observance of molecular ion peak of curcumin at m/e=368, base peak at m/e=177. Observance of molecular ion peak of bisdemethoxycurcumin at m/e=308, base peak at m/e=147. Observance of molecular ion peak of demethoxycurcumin at m/e=338, base peak at m/e=147.

Nuclear magnetic resonance spectroscopy

^{1}H NMR spectra:-

^{1}H NMR spectra of isolated compounds showed characteristic peaks:

Appearance of methoxy protons at δ 3.95 and 3.91 indicates the isolation of curcumin and demethoxycurcumin.

Absence of methoxy protons at δ 3.91-3.95 indicates the isolation of bisdemethoxycurcumin.

^{13}C NMR spectra

^{13}C NMR studies provided further confirmation of the compounds isolated.

Curcumin exhibited very good activity against *B. subtilis*, *E. coli*, *S. auresus* and *P. mirabilis*, whereas it showed moderate activity against *K. pneumonia*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*.

Bisdemethoxycurcumin exhibited good activity against *B. subtilis* and *S. auresus* whereas it showed moderate activity against *E. coli*, *P. mirabilis*, *K. pneumonia*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*.

Demethoxycurcumin exhibited some degree of activity against *B. subtilis*, *S. auresus*, *E. coli*, *P. mirabilis*, *K. pneumonia* and *Enterobacter aerogenes*. It was inactive against *Pseudomonas aeruginosa*.

Curcumin exhibited very good activity against both fungi, viz., *A.niger* and *C.albican*.

Bisdemethoxycurcumin exhibited moderate activity against *A.niger* and *C.albican*.

Demethoxycurcumin exhibited some degree of activity againgt *C.albican* and no activity against *A. niger*.

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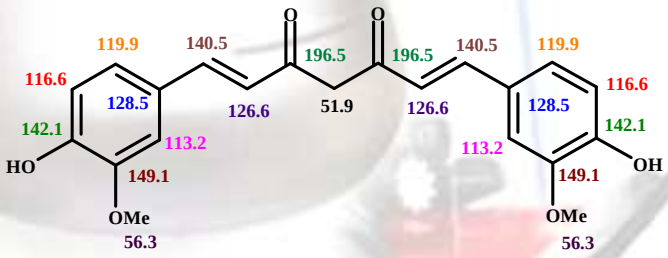
Table 1: IR spectral data of the isolated compounds

Compounds isolated	ν_{max} (cm^{-1})
Curcumin	3509 (-OH str),1627 (C=O str), 1602 (C=C str)
Bisdemethoxycurcumin	3518 (-OH str),1627 (C=O str), 1600 (C=C str)
Demethoxycurcumin	3500 (-OH w),1625 (C=O str), 1601 (C=C str)

Table 2: MASS spectral data of the isolated compounds

Compounds isolated	MASS Data
Curcumin	Molecular ion peak at m/e= 368, Base peak at m/e = 177 , Other peaks at m/e= 350, m/e=335, m/e= 272, m/e=159, m/e=117
Bisdemethoxycurcumin	Molecular ion peak at m/e= 308, Base peak at m/e = 147, Other peaks at m/e= 290, m/e=238, m/e= 160, m/e=120, m/e=65
Demethoxycurcumin	Molecular ion peak at m/e= 338, Base peak at m/e = 147, Other peaks at m/e= 256, m/e=191, m/e= 161, m/e=131, m/e=91

Table 3 : On the basis of ^{13}C-NMR spectral data of the isolated compounds

Compounds isolated	^{13}C- NMR
Curcumin	

ISSN: 0976-7126

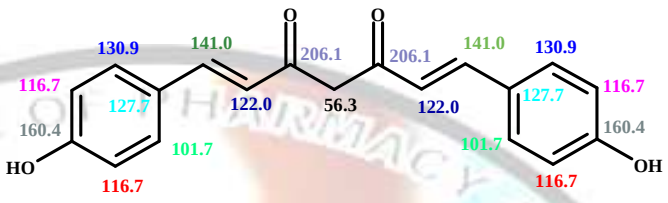
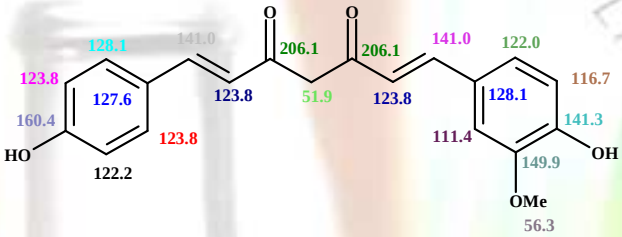
Bisdemethoxycurcumin	
Demethoxycurcumin	

Table 4: ^{1}H-NMR spectral data of the isolated compounds

Compounds isolated	^{1}H- NMR
Curcumin	($CDCl_3$) δ : 7.26-6.70 {m, Ar-H, (6H)}, 5.85 {s, (OH) 1H}, 3.95 {s, (2 X OCH_3) 6H}
Bisdemethoxycurcumin	($CDCl_3$) δ : 7.46-6.90{m,(Ar-H) 6H}, 5.95 {s, (OH) 1H}
Demethoxycurcumin	($CDCl_3$) δ : 7.26-6.70 {m, (Ar-H) 6H}, 5.95 {s, (OH) 1H}, 3.91{s, (OCH_3) 3H}

Table 5: Melting point AND R_f values of the isolated compounds

Isolated Compounds	Melting-Point (°C)	Rf Value
Curcumin	180	0.69
Bisdemethoxycurcumin	220	0.39
Demethoxycurcumin	160	0.51

ISSN: 0976-7126

Table 6: Antibacterial activity of the isolated compounds (Zone of inhibition in mm.)*

Isolated portion used	*B.subtilis*	*K.pneumonia*	*E.coli*	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*	*S.auresus*	*P.mirabilis*
Curcumin	20	15	20	13	14	19	17
Bisdemethoxy-curcumin	18	12	14	10	11	16	12
Demethoxy-curcumin	12	10	08	08	-----	14	06
Kanamycin	23	21	22	22	20	24	21
Control (DMSO)	-----	-----	-----	-----	-----	-----	-----

---- = No Activity, * Average of three readings

Table 7: Antifungal activity of the isolated compounds (Zone of inhibition in mm.)*

Extracted portion used	*A. niger*	*C. albican*
Curcumin	18	19
Bisdemethoxycurcumin	10	11
Demethoxycurcumin	-----	08
Fluconazole	23	22
Control (DMSO)	-----	-----

---- = No Activity, * Average of three readings