Original Research Article

Study of Collateral Sensitivity of Multidrug resistant Cell lines to Curcuminoids

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A B S T R A C T

Drug resistance is a major clinical problem in treating human cancers with conventional chemotherapeutic drugs. Many dietary phytochemicals have been found to be involved in anticancer activity for various types of cancer. Turmeric, Curcuma longa L. of zingiberaceae family is widely cultivated spice in India and other Asian countries. Turmeric is rich in curcuminoids like curcumin (C), Demethoxycurcumin (DMC), Bisdemethoxycurcumin (BDMC). Curcuminoids are recognized for their broad spectrum of biological activities. Each curcuminoids vary in chemical structures physicochemical characteristics as well as the functional properties. This study focused on extraction, identification and purification of curcuminoids by TLC and column chromatography. Each curcuminoids were characterized by UV spectroscopy, HPLC, GC-MS, FTIR, NMR spectroscopy. We studied curcuminoids were effectively cytotoxic to MDR cell lines such as KBChR-8-5 cell lines. Among three curcuminoids analysed BDMC were more potent cytotoxic than C, DMC on MDR cell lines. RR value of BDMC was >1 indicating BDMC kills KBChR8-5 cells more effectively than parental KB cell lines.

Introduction

Chemotherapy is the treatment of cancer with one or more cytotoxic drugs ("chemotherapeutic agents") as part of a standard regimen. Over time, cancer cells become more resistant to chemotherapeutic treatments. To overcome drug resistance most obvious response that widely employed is to use combination drug therapy. The general rationale for choosing drugs to combine is to use one drug which is active against the tumor when used individually and to combine another drug that have different mode and site of action to produce synergistic addictive effect of reversing drug resistance.
Phytochemical agents are plant-based chemicals, these include the plant derived toxins and their pigments, they act as mitotic inhibitors, interfere with cell cycle, they have different target site in cell, inhibits certain signalling pathways to terminate the cancer cell growth. (Wu, 2006). More than 10,000 phytochemicals have been described and among them flavanoids include about 6000 compounds. These phytochemicals play a major role in chemotherapy for cancer patients.

These phytochemicals have the advantage of being dietary compounds that are less toxic to animals, plentiful and inexpensive. (Govindarajan, 1980). Dietary phytochemicals have been found to be very promising in reversing the resistance to anticancer. They are present in the herbal constituents, fruits, berries and spices. Spices include mint, rosemary, garlic, piper nigrum, curcumin, ginseng, onion. (Bansal et al., 2009).

*Curcuma longa* L. (turmeric) is typical of the herbaceous plant with thick and fleshy rhizomes and leaves in sheaths that characterize the family Zingiberaceae (Govindarajan, 1980). Curcumin (C), main colouring substance in *Curcuma longa* L. and two related compounds, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), were altogether known as curcuminoids (Govindarajan, 1980). The chemical structures of three curcuminoids are shown in Figure 1. The total of curcuminoids which are about 4-6%, turmeric also contains 2-4% essential oil and 2-3% of fixed oil and various volatile oils, including turmerone, atlanstone, and zingiberone. Other constituents include sugars, proteins and resins. The value of the turmeric products is based on their curcuminoid content and estimated based on its absorbance at 420nm (Merina Benny Antony, 2003). Curcuminoids are polyphenols having a pronounced yellow color. They have poor solubility in water at acidic and physiological pH, curcuminoids are soluble in dimethyl sulfoxide (DMSO), acetone and ethanol. (Schiefier, 2002). The traditional uses of turmeric or natural curcuminoids in folk medicine are multiple, and some of these including antioxidant, anti-cancer, anti-inflammatory, anti-fungal, anti-parasitic, anti-venom, anti-mutagenic activity in vitro, (Peret-Almeida, 2005). Since the curcuminoid pigments vary in chemical structures, it is possible that the physico-chemical characteristics as well as the functional properties and biological role of curcuminoids would vary among them. As compounds DMC and BDMC are not commercially available, therefore it is important to obtain pure pigments and characterize them individually to study their biological properties.

Alcohol and acetone are good extractants of curcuminoids and the yields can also be expected to be high because of extraction of non-flavor components. Acetone as solvent was slightly superior to alcohol and ethylene dichloride, the curcuminoids content also is on the high side, suggesting selective extraction. (Govindarajan, 1980). Soxhlet extraction of turmeric powder with acetone gave a yield of about 5.0% containing 43% curcuminoids in 4 to 6 hours. (Revathy et al., 2011). 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 for chromatographic separations (Gupta et al., 1999). HPLC method was sensitive,
precise, and accurate for detection and quantification of curcuminoids in the extract of rhizome *Curcuma longa* L. (Sompol *et al.*, 2009). Characterization of compounds by GC-MS, FTIR, NMR confirms the identity of curcuminoids.

To investigate the basis of drug resistance in vitro, drug resistance cell lines have been isolated by exposing various cancer cells to increasing amounts of chemotherapeutic agents such as adriamycin, daunomycin, colchicine, doxorubicin, actinomycin-D, taxol, vinca alkaloids etc. (Gottesman and Ling, 2006). The experiments were carried out on colchicine selective cell line (KBChR8-5) as resistant cell line in comparison with KB cell line (HeLa derivative) as parental cell line.

A phenomenon of drug-resistant cells is that the development of resistance to one or multiple drugs. When drug-resistant cell lines is resistant to one agent but show greater sensitivity to an alternate agent than seen in the original parental cell line phenomenon called collateral sensitivity. Collateral sensitivity is abbreviated as (RR) for resistance ratio. Drug resistance phenotypes are usually assessed in terms of relative resistance (RR) values for a panel of cytotoxic anti-tumor drugs (RR patterns).

In the present study we have demonstrated extraction, identification, purification and characterization of curcuminoids from turmeric rhizome. Further we have examined the cytotoxic properties of individual curcuminoids on drug resistance cell line and its relative resistance for each compound. The mechanism of curcuminoids and its role in collateral sensitivity is yet to be studied in future.

**Materials and Methods**

*Curcuma longa* L. (Turmeric) rhizome were collected from five different places in India – Nizam, Rajapuri, Assam - Lakhadong variety, Erode and Allepey.

**Sample Preparation**

Fresh rhizomes were cleaned by washing 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 into small pieces, powdered by electronic mill.

**Extraction of curcuminoids**

20g of turmeric powder was taken into a thimble and placed in a soxhlet apparatus and extracted using acetone as solvent 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. Extracts were analysed for curcuminoids by HPLC analysis.

**Separation of curcuminoids by TLC using different solvent system**

Acetone extract of turmeric rhizome from Assam variety 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**

Acetone extract of Assam variety was subjected to column chromatography in silica gel (60-120 mesh) glass column. About 5gm of crude Curcuminoids were mixed with 8gm of silica gel and loaded on to the column of 46x2 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 fractions with similar 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 spectrophotometer at 420 nm.

**Purification of each curcuminoids**

The individual Curcuminoids collected from the column chromatography was dissolved in methanol and heated. After complete dissolution chloroform was added 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.

**Characterization of Curcuminoids**

**Estimation of Curcuminoids:**

by HPLC analysis

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. 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 wavelength of 420nm. (Cooper et al., 1994).

**Gas chromatography - Mass Spectrometer (GC-MS)**

Molecular weight of compounds was analysed by GC-MS using JOEL GCMATE II GC –Mass spectrometer. Each curcuminoids were dissolved in methanol, the solution was then injected with the help of a microsyringe into injection port of GC system coupled with mass spectrometer. Perfluorokerosene (PFK) is used as reference sample (Biemann, 1962).

**Fourier Transform - Infra Red Spectroscopy (FTIR)**

Curcuminoids samples are dispersed in 100mg KBr and pressed to form a pellet and analysed on Perkin Elmer Spectrum One FT-IR instrument. (Colthup, 1990).

**Nuclear Magnetic Resonance Spectroscopy (NMR)**

Spectra of $^{13}$C and $^1$H were determined in DMSO-d$_6$, operating at 200 and 50 MHz.
respectively, using JEOL GSX-400 NMR spectrometer. Tetramethyl silane (TMS) was used as internal standard. (Slichter, 1990).

Cell culture

The KB and KB-ChR-8-5 human cervical carcinoma cell lines were purchased from NCCS Pune, Maharashtra, India. Both the cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 2 mM L-glutamine, 10% fetal calf serum (FCS), 1.5 g/L sodium bicarbonate, 1 mM non-essential amino acid and 1.0 mM sodium pyruvate. Only the KBChR8-5 cell line was routinely maintained in 10 ng/ml colchicines and subsequently grown in colchicine-free medium for one week prior to drug treatment. The cell lines were maintained in a humidified incubator with an atmosphere of 5% air and 5% CO₂ at 37°C.

MTT assay procedure

The MTT [3-(4,5-dimethyl-2-thiazoly)-2, 5-diphenyl-2H-tetrazolium bromide] assay was used to determine drug sensitivity. (James et al., 1987). KB, KBChR8-5 cells in the exponential growth phase were harvested by trypsinization and seeded into 96-well plate at a concentration of 3500 cells per well. The cells were treated in triplicate with gradient concentration of curcuminoids and doxorubicin (reference drug) ranging from 0.01 µg, 0.1 µg, 1.0, 10 and 100 µg and incubated at 37°C for 24 hours. The IC₅₀ value defined as the drug concentration required to reduce cell survival to 50% as determined by the relative absorbance of MTT. IC₅₀ value was calculated by (mean absorbance in test wells) / (mean absorbance in control wells) x 100.

Collateral sensitivity

Collateral sensitivity is assessed invitro by determining the cytotoxicity (IC₅₀) of a compound against a parental line relative to its MDR subline. It was calculated as the ratio of compounds IC₅₀ for parental cell divided by its IC₅₀ for MDR cells.

Results and Discussion

The percentage of composition of Curcuminoids in 5 different turmeric samples collected were determined by HPLC is summarized in table1. The identity of each peak was confirmed by determination of retention times by spiking with standards. Assam variety showed optimum amount of individual Curcuminoids.

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.

Column chromatography

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 fractions collected show the percentage of total Curcuminoids present in the fraction.
Table 1 Evaluation of Curcuminoids of *Curcuma longa* collected from different places in India by HPLC

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample Collected Place</th>
<th>Curcumin [C] %</th>
<th>Demethoxycurcumin [DMC] %</th>
<th>Bisdemethoxycurcumin [BDMC] %</th>
<th>Total % of Curcuminoids*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nizam</td>
<td>23.6</td>
<td>8.4</td>
<td>4.8</td>
<td>37.0</td>
</tr>
<tr>
<td>2.</td>
<td>Rajapuri</td>
<td>26.4</td>
<td>8.7</td>
<td>4.4</td>
<td>39.6</td>
</tr>
<tr>
<td>3.</td>
<td>Assam</td>
<td>23.9</td>
<td>10</td>
<td>6.1</td>
<td>40.1</td>
</tr>
<tr>
<td>4.</td>
<td>Erode</td>
<td>13.7</td>
<td>6.2</td>
<td>7.0</td>
<td>26.9</td>
</tr>
<tr>
<td>5.</td>
<td>Allepey</td>
<td>14.8</td>
<td>6.8</td>
<td>6.3</td>
<td>27.9</td>
</tr>
</tbody>
</table>

*The total extract of the turmeric samples were determined as described in the text, all extracts were done using acetone as solvent. The percentage composition of each curcuminoid was estimated and the results are the average of three experiment.

Table 2 Separation of Curcuminoids by TLC using different solvent system

<table>
<thead>
<tr>
<th>TLC mobile Phase</th>
<th>Ratio</th>
<th>Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Benzene:ethylacetate</td>
<td>18 : 2</td>
<td>0.79</td>
</tr>
<tr>
<td>Dichloromethane:methanol</td>
<td>19 : 1</td>
<td>0.8</td>
</tr>
<tr>
<td>Chloroform:methanol</td>
<td>19 : 1</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Each plate was developed to a height of about 8cm.
C = curcumin, DMC = demethoxycurcumin, BDMC = bisdemethoxycurcumin.

Table 3 Silica Gel Column chromatography elution profile

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

Each fraction contains 40 mL.
C = curcumin, DMC = demethoxycurcumin, BDMC = bisdemethoxycurcumin.
Elution by chloroform and chloroform:methanol 98:2.
In our study percentage of total curcuminoids present in fractions were collected at 84%, 86%, 80.6% of C, DMC, and BDMC respectively.

**Characterization of curcuminoids**

**High - Performance Liquid Chromatography (HPLC)**

The purity profile of isolated individual curcuminoids were analysed by HPLC. C, DMC, BDMC showed single peaks at retention times of 10.81, 12.79 and 13.03 min respectively. The identity of each peak was confirmed by determination of retention times and by spiking with standards. Purity profile for each curcuminoids were calculated as 99%, 98%, 95% purity for C, DMC, BDMC respectively.

**GC-MS**

The full scan mass spectra of each curcuminoids isolated were detected shows molecular weight of each individual compounds of C, DMC, BDMC was found to be 368.31, 337.77, and 307.95 respectively.

**FT-IR analysis**

The presence of absorption bands of specific stretching regions obtained at their respective frequency confirms the structure of compounds. IR absorption bands (cm\(^{-1}\)) For C showed 3413.76 (O-H str); 1629.14 (conjugated C=C str); 1585.60 (α,β-unsaturated C=O str); 1455.64 (aromatic ring str); 1288.65, 1028.68 (C=O-CH\(_3\) str); 1140.52 (C-OH str).

For DMC showed 3326.98 (O-H str); 1627.30 (conjugated C=C str); 1575.24 (α,β-unsaturated C=O str); 1437.33 (aromatic ring str); 1263.07, 1025.21 (=C-O-CH\(_3\) str); 1134.67 (C-OH str).

For BDMC showed 3230.06 (O-H str); 1629.40 (conjugated C=C str); 1575.64 (α,β-unsaturated C=O str); 1508.90 (aromatic ring str); 1165.85 (C-OH str).

**NMR analysis**

\(^{13}\)C NMR chemical shift (ppm): for curcumin 183.6, 149.8, 148.4, 141.1, 126.8, 123.5, 121.5, 116.1, 111.8, 101.2, 56.1. DMC- 183.7, 183.5, 160.27, 149.82, 148.47, 141.16, 140.83, 130.78, 126.82, 126.29, 123.61, 121.27, 116.39, 116.18, 111.77, 101.40, 56.1. BDMC - 183.66, 160.26, 140.82, 130.78, 126.30, 121.25, 116.38 and 101.41.

The presence of signal at 56.1 in C and DMC denotes the presence of methoxyl group while there is no methoxyl group present in BDMC.

\(^{1}\)H NMR chemical shift (ppm): for curcumin 9.65, 7.57, 7.32, 7.16, 6.84, 6.77, 6.06, 3.84. DMC- 10.03, 9.68, 7.57, 7.55, 7.33, 7.16, 7.14, 6.84, 6.78, 6.74, 6.04, 3.84. BDMC- 10.04, 7.57, 7.53, 6.83, 6.71, 6.04. Signal corresponding to the methoxyl group at 3.84 is found in C and DMC and absent in BDMC.

**MTT assay**

The dose response curve and the effect of three curcuminoids and doxorubicin on KB, KBChR8-5 cell lines were determined by cytotoxicity assay. Dose response cytotoxicity profiles for three curcuminoids (C, DMC, BDMC) and doxorubicin were established for KB, KBChR8-5 cell lines were shown in figure 2 A, B. The percent of viable cells was calculated to determine the IC\(_{50}\).
**Table 4** Cytotoxicity of three curcuminoids and reference drug (doxorubicin) in KB, KBChR8-5 and HeLa cell lines

<table>
<thead>
<tr>
<th>Drugs</th>
<th>KB (mean µM)</th>
<th>KBChR8-5 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>18.3</td>
<td>19.2</td>
</tr>
<tr>
<td>DMC</td>
<td>22.8</td>
<td>26.7</td>
</tr>
<tr>
<td>BDMC</td>
<td>7.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.11</td>
<td>0.09</td>
</tr>
</tbody>
</table>

C = Curcumin, DMC = Demethoxycurcumin, BDMC = Bisdemethoxycurcumin.

**Figure 1** Chemical Structure of three Curcuminoids

![Chemical Structure of three Curcuminoids](image)
**Figure 2** Cytotoxic activity of Curcuminoids and doxorubicin on KB and KBChR8-5 cell lines

**Figure 2a** Percentage growth against KB cells

![KB cell line graph](image)

**Figure 2b** Percentage growth against KBChR8-5 cells

![KB-CHR-8-5 graph](image)

Doxo – Doxorubicin.
The IC\textsubscript{50} value of C, DMC, BDMC and Doxorubicin in KB, KBChR8-5 cell lines is described in table 4.

**Collateral sensitivity of Curcuminoids**

Relative resistance (RR) value has been calculated by dose response curve of that drug on MDR subline compared to parental cells. The RR value of three curcuminoids and doxorubicin on KB, KBChR8-5 cell lines were calculated shows 0.953, 0.853, 1.5, 1.2 for C, DMC, BDMC and doxorubicin respectively.

Cancer is a major worldwide public health problem. Resistance of cancer cells to multiple chemotherapeutic drugs (a mechanism termed MDR) is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure. Recently it was reported that the effect of curcuminoids was examined on the proliferation of MCF-7 human breast tumor cells that demethoxycurcumin was the best inhibition of MCF-7 cells followed by curcumin and bisdemethoxycurcumin (Simon, 1998). Bisdemethoxycurcumin (BDMC) is active for modulation of MDR-1 gene expression (Anuchapreeda, 2004).

Among five turmeric varieties analyzed Assam variety showed optimum amount of total extracts. Curcumin (C) was found to be the major compound in the extracts of all varieties followed by demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC). Assam variety could be the best turmeric variety for further isolation and separation of curcuminoids.

The Rf value of curcuminoids shown in TLC 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.

In column chromatography the separation is achieved by elution with chloroform and methanol with increasing polarity. The UV spectroscopy analysis of fraction shows the total curcuminoids present as 84%, 86%, 80.6% of C, DMC, and BDMC respectively. Further purification results in curcumin as bright yellow needle shaped crystals, DMC as light yellow crystals, BDMC as reddish orange color crystals.

The structure and purity of isolated curcuminoids were determined by GC-MS, FTIR, and NMR. The purity of curcuminoids were analysed by HPLC from the percentage calculation using peak area and retention time, it was found to be > 95% purity for each compound.

MTT assay was used to determine the relative cytotoxicity of curcuminoids in KB and KBChR8-5 cell lines. Each curcuminoids have different IC\textsubscript{50} values in the range of 5-25\textmu M. The IC\textsubscript{50} value of drugs on MDR subline were nearly equivalent to that of parental cell line. There was a decrease in the viability of individual cell line with increasing concentration of curcuminoids treated.

Collateral sensitivity is assessed *in-vitro* by determining the cytotoxicity (IC\textsubscript{50}) of a compound against a parental line relative to its MDR subline. It is calculated as the ratio of compounds IC\textsubscript{50} for parental cell divided by its IC\textsubscript{50} for MDR cells. A RR value >1 indicates that the compound kills MDR cells more effectively than parental cells. (Hall *et al.*, 2009). The RR value for C and DMC was <1 and BDMC was 1.5.
which indicates BDMC kills KBChR8-5 cells more effectively than parental KB cells. MDR cell population is collaterally sensitive to BDMC. RR value for C and DMC were <1 indicating that cells are resistance to that drug relative to parental cell lines. Relative drug resistance of KBChR8-5 cells for curcuminoids was calculated by ratio of IC$_{50}$ for MDR cells divided by IC$_{50}$ for parental cell. The relative drug resistance was 1.04 and 1.17 times more resistant to C and DMC respectively than parental cell line. Therefore BDMC can act as collateral sensitive drug for drug resistant cell lines.

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