Biotransformation of curcumin for improved biological activity and antiproliferative activity on acute HT-29 human cell lines

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Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a natural hydrophobic polyphenol compound isolated from rhizomes of the herb Curcuma longa Linn. It has diverse pharmacological properties including antiinflammatory, antioxidant, antiproliferative and antiangiogenic activities. However, use of curcumin is limited due to its poor aqueous solubility resulting in reduced concentration of active molecule at target site and low systemic bioavailability. In the present study, conjugated glycosyl-curcumin was synthesized in good yield by biocatalytic method using β-glucosidase enzyme obtained from almonds and found to be enantioselective. The glucosilated curcumin product showed promising pharmacokinetic and biological (antioxidant, antimicrobial & antiproliferative) properties.

Keywords: Curcuma longa, phytochemicals, pharmacokinetic, rhizomes

Introduction

In the recent past, considerable public and scientific interest has been developed in the use of phytochemicals derived from dietary components to treat human diseases. A large number of natural products or the dietary components have been evaluated as potential chemo-preventive agents and also as the source of novel pharmacophores for wide variety of diseases. Polyphenols present in the medicinal plants are found to be the good source for potential pharmacological active molecules1-3. Curcumin, a major component in the rhizome of herb Curcuma longa Linn., is traditionally used as herbal medicine since centuries and has shown to have remarkable pharmacological activity against various malignant diseases, diabetes, allergies, arthritis, Alzheimer’s disease and other chronic diseases4-9. US Food and Drug Administration (FDA) considers curcumin as generally recognized as safe (GRAS) compound4. However, curcumin and other curcuminoids are poorly soluble in water, lowering their bioavailability, which limits their use in food and pharmaceutical agents8-9. There is an intense search to overcome the problems associated with hydrophobic nature of curcumin and other curcuminoids. Recent studies have shown that attempts are made to enhance in vitro and in vivo efficacies of curcumin through structural modifications of the molecule or through new formulations10-11. In the present study, we report synthesis of curcumin glycoside conjugate catalyzed by almond β-glucosidase and its enhanced biological activity. The synthesized curcumin-β-di-glucoside was used for in vitro studies and evaluated for its solubility (partition coefficient), followed by its antibacterial and antiproliferative activities.

Materials and Methods

Chemicals and Cultures

The Mueller Hinton broth and DMEM (Dulbecco’s Modified Eagles agar Medium) were purchased from Hi Media Laboratories Ltd., Mumbai, India. Curcumin was a gift from Asian Herbex Ltd., Hyderabad, India. Almond β-glucosidase (activity ≈2.18 units mg⁻¹) was purchased from Sigma-Aldrich (USA). The cancer cell lines A549 (lung cancer), MCF-7 (breast cancer) and HT-29 (colon cancer) used were obtained from National Center for Cell Science (NCCS), Pune, India. All common chemicals used in this study were of reputed make and obtained from the local dealers. The microorganisms and media components used in bioassays were procured from American Type Culture Collection Center (ATCC), VA 20108, USA and HiMedia Ltd., Mumbai, India.

Procedure for Glucosidation

Synthesis of the curcumin-bis-β-D-glucoside (curcumin-4’-O-D-glucopyranoside) was achieved by
modified method of Vijay Kumar et al. Curcumin (250 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) with constant stirring, to which a phase transfer catalyst (0.1 M of benzyl tri-ethyl ammonium chloride) dissolved in 10 mL of sodium acetate buffer (0.01 M, pH 7.5) was added. To the above reaction mixture, glucose (244.5 mg) and β-glucosidase (24.5 mg) were added and incubated in shaking incubator at 40±2°C, 150 rpm for 35 h. The product formation was intermittently monitored by TLC and HPLC. After completion, the reaction was quenched by keeping in a boiling water bath for 10 min. The product formed in the reaction mixture was extracted using ethyl acetate and concentrated by vacuum evaporation to get the crude product, which was further purified by column chromatography using silica gel (60-100 mesh size). Thus, the concentrated final product (25% yield) curcumin-bis-β-D-glucoside was tested by HPLC and the structure was confirmed by NMR, ESI-MS spectral data. 1H NMR (DMSO-d6), 3.1–3.4 (m, 8H), 3.6 (m, 3H), 3.77 (s, 6H, OCH3), 4.31 (s, 1H), 4.5 (t, 2H, J = 5.5Hz), 4.93 (d, 2H, J = 7 Hz), 4.98 (d, 2H, J = 5 Hz), 5.06 (s, 2H), 5.25 (d, 2H, J = 4 Hz), 6.04 (s, 1H), 6.81(d, 2H, J = 16 Hz), 7.05 (d, 2H, J = 8.5 Hz), 7.18 (d, 2H, J = 8 Hz), 7.32 (s, 2H), 7.52 (d, 2H, J = 16 Hz); ESI-MS, m/z 715 [M+Na]+.

HPLC
The concentrations of curcumin and curcumin-bis-β-D-glucoside were analyzed by HPLC (Gilson LC system), using analytical C18 column 3.9 mm x 150 mm, 5 μm particle size. The system was run isocratically using mobile phase acetonitrile:water (80:20) (pH adjusted to 3.5 with acetic acid) at a flow rate of 1 mL min⁻¹ and the sample detection was done using UV/VIS-155 detector at 254 nm. The mobile phase was filtered through 0.5 μm nylon membrane filter and the solvent was degassed ultrasonically before use.

Determination of Partition Coefficient
The partition coefficients of curcumin and curcumin-bis-β-D-glucoside in the n-octanol/water system were studied by the shake-flask method and the concentration of the compounds in two phases was determined by HPLC. The log P value was calculated as follows:

\[
\log P = \frac{A_o - A_i}{A_i} \times \frac{V_o}{V_w}
\]

Where A_o and A_i represent the area percentage of the molecule in the aqueous phase before and after partitioning, and V_o and V_w are the volumes of water and octanol, respectively. Log P values were calculated as an average of three independent measurements.

Antioxidant activity
The hydrogen donating or free radical-scavenging activity of the curcumin and curcumin-bis-β-D-glucoside was evaluated by the reduction of 1,1-diphenyl-1-picrylhydrazyl (DPPH) as described by Wayne et al. To the solution of DPPH (0.6 mM), specific dilutions of each test samples (both curcumin and curcumin-bis-β-D-glucoside) were added and reaction mixture was incubated in dark place for 30 min at room temperature. The decrease in absorbance was measured at 520 nm against their respective blanks in UV-spectrophotometer (Perkin Elmer). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (0.1 mg mL⁻¹), an antioxidant agent, was used as positive control. The radical scavenging activity of the test samples at different concentrations were evaluated by comparing with positive control. The experiments were performed in triplicates for each concentration and IC₅₀ values were determined by the interpolation of the dose-response curves (concentration of the extract to scavenge 50% of the DPPH radicals).

Tyrosinase Enzyme Inhibition Assay
The tyrosinase inhibition assay was performed using L-3,4-dihydroxyphenylalanine (L-DOPA) (0.5 mM) as substrate. To the different concentrations of the curcumin and curcumin-bis-β-D-glucoside in DMSO, 2 mL of 0.1 M sodium phosphate buffer (pH-6.0) containing mushroom tyrosinase enzyme (25 KU) was added and the reaction mixture was incubated for 5 min at room temperature. The reaction was initiated by adding L-DOPA (0.5 mM) and the reaction was monitored by measuring the change in absorbance at 490 nm for 1 min (formation of L-DOPA chrome). Kojic acid was used as the reference inhibitor compound to study tyrosinase activity. All the analyses were performed in triplicate for each concentration and IC₅₀ values of tyrosinase inhibition activity were determined.

Antimicrobial Assay
The antimicrobial activity of curcumin and curcumin-bis-β-D-glucoside was tested against following microbial strains: Staphylococcus aureus...
(ATCC 29737), Bacillus cereus (ATCC 14603), Escherichia coli (ATCC 10536), Klebsiella pneumoniae (ATCC 13883) and Pseudomonas aeruginosa (ATCC 25619), and pathogenic fungi like Candida albicans (ATCC 53324), and Aspergillus fumigatus (ATCC 204305). Mueller Hinton agar and potato dextrose agar (PDA) were used to culture the selected bacteria and fungi, respectively. Each culture was transferred from slants stored at 4°C to 10 mL of Mueller Hinton broth (bacteria) and potato dextrose broth (fungi) tubes and incubated overnight at 37±2°C for bacteria and two days at 30±2°C for fungi. After incubation both bacterial and fungal inoculums were used for determining minimum inhibitory concentration (MIC).

**Determination of MIC**

The agar well diffusion method was used to assay the curcumin and curcumin-bis-β-D-glucoside for antimicrobial activity18-19. Different bacterial and fungal (cell/spore suspension) cultures as prepared above were spread on Mueller Hinton agar and PDA medium, respectively. Six wells (7 mm each) were bored in each plate using an aseptic well borer. The test compounds (curcumin and curcumin-bis-β-D-glucoside) and two positive controls (gentamicin against bacteria and fluconazole against fungi) were dissolved in 90% DMSO and serially diluted to get concentrations of 2-1000 µg mL⁻¹. An equivalent amount of DMSO (0.1 mL) was included in each plate as negative control. Each experiment was performed in duplicate and repeated thrice. The MIC was reported as the lowest concentration of test samples capable of inhibiting the growth of each bacteria and fungi (to give inhibition zone) used in the study.

**Cytotoxicity Evaluation**

Toxicity of curcumin and glucosylated curcumin against cancer cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, based on mitochondrial reduction of yellow coloured MTT tetrazolium dye to a highly purple coloured product formation. 1x10⁴ Cells (counted by Trypan blue exclusion dye method) in 96-well plates were incubated with curcuminoids with a series of concentrations (10, 20, 40, 60 µM mL⁻¹) that were tested for 48 h at 37±2°C; the cells were cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640), Minimum essential medium (MEM) and Dulbecco’s modified eagle medium (DMEM), with 10% Fetal bovine serum (FBS) medium. The above media were replaced with 90 µL of fresh serum free medium and 10 µL of MTT reagent (5 mg mL⁻¹) and plates were incubated at 37°C for 4 h. Thereafter the above medium was replaced with 200 µL of DMSO and incubated at 37°C for 10 min. The absorbance at 570 nm was measured on a spectrophotometer (Spectra Max Molecular devices) and IC₅₀ values were determined from plot between % inhibition (from control) versus concentration²⁰-²².

**Statistical Analysis**

All experiments were conducted in triplicates and the results were presented as the mean of three independent experiments ±standard error.

**Results and Discussions**

**Glucosidation of Curcumin**

In the present study, curcumin-bis-β-D-glucoside was synthesized and characterized by spectral analysis ¹H NMR and ESI-MS. Thus from their coupling constant, it was confirmed that the configurations of the anomeric carbons were defined as β for all the glucose molecules. Therefore the structure of the glucosylated product was established as curcumin-bis-β-D-glucoside. The curcumin bioc conjugate synthesized can act as a potent prodrug as the glucosyl bond formed gets hydrolyzed at the target sites to liberate pharmacological active molecule curcumin²⁵. When compared to earlier reports on direct glucosidation of curcumin, the present methodology developed involves a one-step glucosyl conjugation to curcumin by β-glucosidase enzyme, which showed good yield (25%) and enantioselectivity²⁶-²⁸. Despite curcumin demonstrated therapeutic efficacy and safety, the poor bioavailability of it in systemic circulation continues to be highlighted as a major concern in wider pharmacological therapeutic applications²³. From the literature, it is well known that the glycosylation of the hydrophobic compounds allows the conversion of water-insoluble compounds into corresponding water-soluble derivatives, which could probably improve the bioavailability and pharmacological properties of the compound²⁹.

**Antioxidant/Radical Scavenging and Tyrosinase Inhibition Activity**

The compounds curcumin and curcumin-bis-β-D-glucoside showed potent antioxidant activity by reduction in absorbance (A₅₇₅ nm) of the added
DPPH at 25°C, which was also indicated by IC$_{50}$ values as 32.86 µM and 22.25 µM, respectively (Table 1). In the DPPH test, the antioxidant is able to donate an H atom to reduce radical DPPH into non-radical biphenyl picryl hydrazine. The lower IC$_{50}$ value of curcumin-bis-β-D-glucoside, therefore, suggests a more promising hydrogen donating ability by conjugating curcumin with glucose molecule. The free radical scavenging activity of curcuminoids studied may be attributed mainly due to the compound’s pharmacophore, as all these compounds have phenolic hydroxyl group and β-diketone structure. The results reveal that the curcumin-bis-β-D-glucoside show a promising antioxidant activity when compared to curcumin and was equivalent to trolox (a water-soluble derivative of vitamin E) (Table 1).

Naturally occurring biomolecules are known to inhibit L-DOPA oxidation catalyzed by tyrosinase enzyme. This property of tyrosinase enzyme inhibition is of particular interest both in skin medications and cosmetics (hyper pigmentation). Lee et al. recently reported that some curcumin analogues exhibited inhibitory activity against tyrosinase. These reports attracted our interest to further study the inhibitory effect of curcumin-bis-β-D-glucoside on tyrosinase. In this study, curcumin-bis-β-D-glucoside showed an enhanced result with the IC$_{50}$, 52.12 µM for tyrosinase inhibition activity compared to that of natural polyphenolic compound curcumin (IC$_{50}$, 64.46 µM), whereas the reference compound kojic acid had IC$_{50}$, 29.15 µM (Table 1).

**Antimicrobial Activity**
Curcumin, the main yellow bioactive component of turmeric powder, has been shown to have several biologically active properties, such as, antioxidant, anticancer, antiviral, antimutagenic, and antibacterial activity. In vitro antimicrobial activity of curcumin and curcumin-bis-β-D-glucoside was compared with standard antibacterial and antifungal drugs gentamycin sulphate and fluconazole, respectively. Both the molecule curcumin and curcumin-bis-β-D-glucoside showed antibacterial activities with MIC ranging from 74.3-125.75 µg/mL and 21.7-25.25 µg/mL, respectively against the different bacterial strains tested. While both were active against all the tested bacterial strains, curcumin-bis-β-D-glucoside had shown the best results, having lower MIC values as compared to curcumin (Fig. 1). Therefore, the improved antibacterial effect of curcumin-bis-β-D-glucoside may be because of its increased solubility, enhanced cellular uptake, reduced metabolism and better binding to cell components.

**Cytotoxicity**
It has been reported that curcumin possesses a wide spectrum of antitumor properties due to the important role of β-diketones in the curcumin. However the molecular mechanism of the compound is still unclear. Previous studies by Liu et al. have shown that curcumin analogues acted as antiproliferative agent and were also responsible for ER (endoplasmic reticulum) stress-mediated apoptosis in lung cancer cells. We evaluated the potential anticancer activity of curcumin and curcumin-bis-β-D-glucoside in selected cancer cell lines (HT-29 colon cancer, A549 lung cancer & MCF-7 breast cancer) by MTT assay. The results show that both curcumin and curcumin-bis-β-D-glucoside induced cytotoxicity to colon cancer cells, while glucosidation of curcumin enhanced its inhibition activity to 2-fold as compared to the free curcumin (Fig. 2). Though the curcumin-bis-β-D-

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**Table 1—IC$_{50}$ values of curcuminoids on DPPH radical scavenging and tyrosinase inhibition activity**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$(µM)*</th>
<th>DPPH</th>
<th>Tyrosinase</th>
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<tbody>
<tr>
<td>Curcumin</td>
<td>32.86±4.80**</td>
<td>64.46±3.44*</td>
<td></td>
</tr>
<tr>
<td>Curcumin-bis-β-D-glucoside</td>
<td>20.25±3.80***</td>
<td>52.12±6.52**</td>
<td></td>
</tr>
<tr>
<td>Trolox</td>
<td>20.75±2.10**</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Kojic acid</td>
<td>NA</td>
<td>29.15±4.63*</td>
<td></td>
</tr>
</tbody>
</table>

IC$_{50}$ values were obtained from a minimum of 3 separate experiments performed in triplicates. A lower IC$_{50}$ indicates greater antioxidant activity. Statistical significant values: ***p<0.05, **p<0.01 and *p<0.1
The IC_{50} value of curcumin was 67 µM mL^{-1} and curcumin-bis-β-D-glucoside was 32.13 µM mL^{-1} against colon cancer cells. It was observed that glucosidation of curcumin predominantly improved the cancer cell killing activity specifically against colon cancer cell lines (HT-29), compared to the other two cancer cell lines (A549 lung cancer & MCF-7 breast cancer) tested (Fig. 2).

Conclusion

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was improved for its solubility and bioavailability by glycosylation to curcumin-bis-β-D-glucoside by a selective enzymatic route using β-glucosidase with good yields and enantioselectivity (β-form) in a simple and eco-friendly reaction conditions. Increased solubility of curcumin-bis-β-D-glucoside resulted in its synergistic effect of enhanced antioxidant, tyrosinase inhibition, antimicrobial activities and higher cytotoxicity on colon cancer cell lines, which may improve the medical value of curcumin. Thus, the curcumin-bis-β-D-glucoside gives a hope for a future potential drug molecule in treatment of various skin diseases and in cosmetic applications.

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References