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Original article

Design, synthesis and characterization of some bioactive conjugates of curcumin with glycine, glutamic acid, valine and demethylenated piperic acid and study of their antimicrobial and antiproliferative properties

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Abstract

The monoesters of curcumin, a symmetric diphenol with valine and glycine have been prepared by a novel solid phase synthesis and its diesters with valine, glutamic acid and demethylenated piperic acid have been prepared by solution phase method. The assessment of their antimicrobial and anticancer (antiproliferative) activities suggested that diesters of curcumin are relatively more active than curcumin itself due to their increased solubility, slow metabolism and better cellular uptake. Furthermore, significant observation was that monoesters of curcumin have even better antimicrobial activity than their corresponding diesters, emphasizing the role of free phenolic group. The conjugate of curcumin with demethylenated piperic acid in which methylenedioxy ring was open also shows enhanced activity than the corresponding piperic acid conjugate, emphasizing the role of free phenolics in the transport or in the binding processes.

Keywords: Curcumin; Piperic acid; Valine; Glutamic acid; Antimicrobial; Antiproliferative

1. Introduction

Curcumin [diferuloylmethane; 1,7-bis-(4-hydroxyl 3-methoxyphenyl)-1,6-heptadiene-3,5-dione; Scheme 1(I)] is the major pigmentary component of turmeric (*Curcuma longa*), a spice commonly used in India, better known as ''Indian solid gold'' due to its numerous therapeutic activities, its pharmacological safety and its colour. Curcumin occurs in turmeric along with its demethoxy and bis-demethoxy derivatives (curcuminoids). Curcumin has been shown to suppress carcinogenesis of the skin, liver, lung, colon, stomach and breast [1,2]. It has also been shown to inhibit the proliferation of a wide variety of tumor cells in culture and promote apoptosis through cleavage of BID (a Bcl-2 family member protein) [3], cytochrome *c* release, down regulation of bcl-2 and activation of caspases [3]. It has been shown to lower blood cholesterol, promote wound healing, prevent skin wrinkling, inhibit inflammation, suppress rheumatoid arthritis and inhibit human immunodeficiency virus replication [4,5]. Curcumin mediates wide variety of therapeutic effects through the regulation of the transcription factors, nuclear factor kappa B [6–9] and activator protein, suppression of $I_KB\alpha$ kinase and c-jun N-terminal kinase and inhibition of expression of cyclooxygenase (COX) 2 [10,11], Cyclin D1, adhesion molecules, matrix metalloproteases [12] inducible nitric oxide synthase, HER2, epithelial growth factor (EGF) receptor, bcl-2, bcl-xl, and tumor necrosis factor (TNF) [13]. Pharmacologically, curcumin is quite safe and doses as high as 8 g/day have been administered orally to humans with no side effects.

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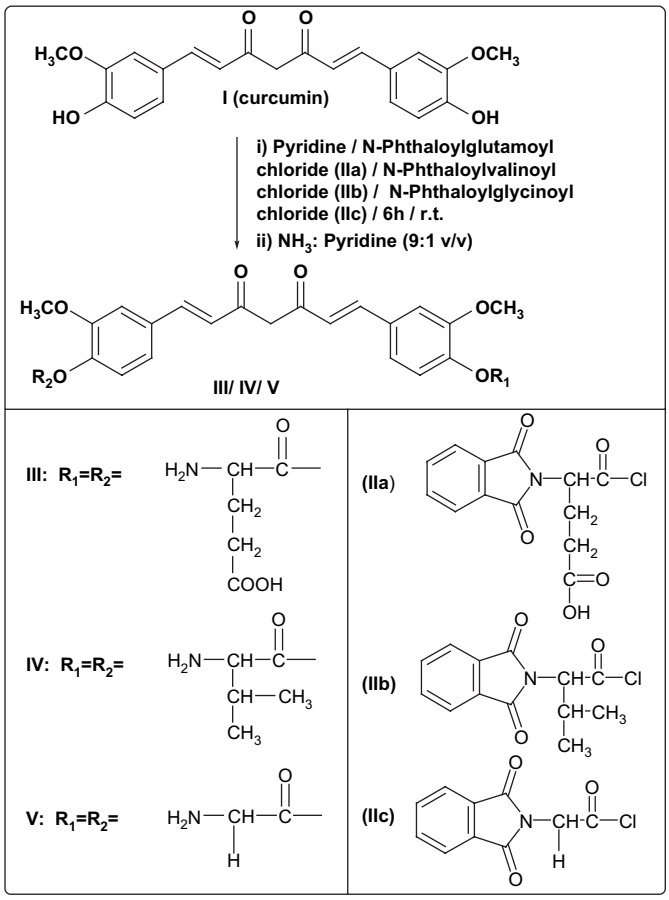


Scheme 1. Synthesis of 4,4′-(di-*O*-glutamoyl)-curcumin (**III**), 4,4′-(di-*O*-valinoyl) curcumin (**IV**) and 4,4′-(di-*O*-glycinoyl) curcumin (**V**).

Numerous studies suggest that Trikatu, an ayurvedic preparation containing black pepper (*Piper nigrum*), long pepper (*Piper longum*) and ginger (*Zingiber officinalis*), has a bioavailability-enhancing effect [22]. Since curcumin belongs to the same family as ginger, it has similar enhancing activity [23]. It has been shown that curcumin acts in both ways, i.e., as a antioxidant (antiproliferative) and as a good oxidant and causes apoptosis of a number of different cancerous cells in vitro [20,22,23]. We have earlier studied the piperic acid conjugate of curcumin [19,20]. However, we have now used demethylenated piperic acid for making a conjugate in order to assess the role of methylenedioxy group and whether free phenolics further enhance the activity by providing anchor with the active site of receptor.

The traditional knowledge that black pepper (piperine) when mixed with turmeric (curcumin) enhances the activity of latter several times led us to link the two covalently.

Valine is an essential, naturally occurring non-polar, hydrophobic, aliphatic amino acid. During the period of valine deficiency, all of the other amino acids (and proteins) are less well absorbed by the GI tract but valine is

1. actively absorbed and used directly by muscle as an energy source;
2. not processed by the liver before entering the blood stream.

Glutamic acid enhances the flavor of foods and tobacco [24]. It is reported to be useful in muscular dystrophy [25] and accelerates wound healing and ulcer healing. Glutamine plays prominent role in circulating amino acid. It plays a unique role in the transfer of nitrogen from peripheral tissues to the liver.

Glycine is of major importance. Recently, a glycine gated chloride channel has been identified in neutrophils that can attenuate increase in intracellular calcium ions and diminish oxidant damage mediated by these white blood cells. Thus glycine may be a novel antioxidant.

The structure–activity relationship studies of curcumin molecule with respect to its multiple biological activities so far have indicated that the presence of two phenyl rings with a 7-C linker having β-diketo function (C═O groups as hydrogen acceptors and C-4 as hydrogen donor) is the basic necessity [26]. However, unsaturation in the linker (conformational flexibility) is important for its antitumor/anticancer activity but not for redox regulatory or apoptotic activities [27,28]. THC (tetrahydrocurcumin) has even better antioxidant property than curcumin. The curcumin conjugates reported with modification at active methylene group or even involving C═O did not show any enhanced activity. The only alternative was to use biodegradable (ester) linkage at phenolic hydroxyls.

In our earlier experiments we have observed that conjugates (phenolic esters) of curcumin with some amino acids show much better antibacterial, antifungal and antioxidant properties [14–21], which may be attributed to their better solubility, enhanced accumulation in the cells, resulting from better cellular uptake and decreased metabolic rate. Evidently, the carrier proteins for amino acids, the necessary building blocks of proteins, must be responsible for dragging the curcumin molecule along (drug smuggling). Since, for its physiological activity, specially antioxidant character, presence of free phenolics has been found to be essential, we designed the preparation of monoesters so that both advantages may be exploited. With this intention monoesters of curcumin with glycine and valine have been prepared and characterized.

A recent joint finding by US, Greek and Chinese workers [29] has indicated that dimethyl curcumin (DMC), i.e., with the two free phenolics methylated has better anticancer activity in vitro, i.e., in cultured human cancer cells. However, it may be that mechanism of antioxidation and apoptosis is entirely different. Regarding mechanism of apoptosis by curcumin it is known that it takes place through mitochondrial disintegration and release of cytochrome *c* through caspase activation. However, still more work is required before definite conclusion can be reached. Free radical formation in the case of DMC may be through another mechanism involving tertiary radicals. Keeping in mind the physiological activities of amino acids, glycine, glutamic acid and valine, the mono- and diester with both phenolics of curcumin has now been prepared, characterized and tested for antipathogenic activities. The diester with demethylenated piperic acid has been prepared to check the enhancement in bioavailability due to availability of two free phenolics. These ester linkages are biodegradable, therefore could get hydrolysed by esterase

enzymes releasing curcumin at target site and thus these conjugates may be acting as prodrugs.

2. Materials and methods

All solvents used were triply distilled and autoclaved prior to use. Curcumin, glycine, valine, glutamic acid and piperine were purchased from Merck-Schuchardt, Germany. The Muller–Hinton broth, agar and sterile discs were purchased from Hi Media laboratory Ltd., Mumbai, India.

Muller–Hinton broth and agar have been selected for testing aerobic and facultative anaerobic bacterial isolates; for fastidious organisms such as *Streptococci* and *Peptococci*, the agar was supplemented with 5% defibrinated blood. The microsusceptibility test was standardized at pH 7.4, agar–broth was incubated in an ambient air incubator at 37 °C.

The inoculum was prepared from broth culture that has been incubated for 4–6 h, when growth was considered in the logarithmic phase. Amoxyclav was taken as standard antibacterial. Fluconazole was taken as a standard antifungal drug. The microorganisms were obtained from a city clinic from the diagnosed patients and were subcultured on Sabouraud dextrose agar slant and incubated at 30 °C for 10–12 days. The sterile discs with 6 mm diameter were further sterilized and charged with compounds as per requirements. After drying the discs were stored at 4 °C.

2.1. Determination of zone of inhibition by Kirby–Bauer's method [30]

The antibacterial susceptibility test was done by determining the zone of inhibition by Kirby Bauer's method. The curcumin conjugates viz. **III–V**, **IX**, **X**, **XVII** and demethylenated piperic acid (**XV**) were dissolved in acetone to make a solution of 120 μmol/ml. From this stock solution serial dilutions have been done to 20, 10, 5, 2.5 and 1.25 μmol/ml in sterile test tubes. Sterilized filter discs were soaked with some solutions and subsequently dried to remove excess solvent. Different bacteria viz. *Enterobacter cloacae*, *Staphylococcus saprophyticus*, *Micrococci*, *Klebsiella aeruginosa* and *Escherichia coli* were selected and 1 ml of each bacterial broth culture was added in M–H plates and spreaded with the help of sterile spreader. Charged sterile disks were placed aseptically over the inoculated plates using the sterile forceps. All the procedures were done under laminar flow. Then the plates were incubated at 37 °C for 24 h in upright position. The zone of inhibition was measured by using the scale.

2.2. Determination of MIC by the microdilution broth susceptibility test

Different concentrations (20, 10, 5, 2.5 μmol/ml) of all the compounds were prepared in sterile dry test tubes to determine minimum inhibitory concentration (MIC). Nutrient broth was prepared using Muller–Hinton broth (M391) and 4.9 ml of it was taken in each test tube and was sterilized after plugging. After cooling 0.1 ml of each dilution was added to the test tube and the final volume was made up to 5.0 ml. The test tubes were shaken to uniformly mix the inoculums with the broth. The tubes were incubated at 37 °C for 18 h. Appearance of any turbidity shows that the compound is not able to inhibit the growth of bacteria, while no turbidity indicates the inhibition of microorganism by the sample.

2.3. In vitro antifungal test

For antifungal testing the compounds were prepared in acetone at initial concentration of 120 μmol/ml. *Aspergillus fumigatus* was obtained from patients. *Candida albicans* and *Candida parapsilosis* were isolated from the patients having chest infections on Sabouraud dextrose agar without antibiotics while *Penicillium notatum* was obtained from NCL (National Chemical Laboratory), Pune, India.

A standardized inoculum was prepared by counting microconidia; cultures were grown on Sabouraud dextrose agar for 48 h at 37 °C. Sterile saline solution (0–85%) was added to the slant and the culture was gently swabbed with cotton tipped applicator to dislodge conidia from the hyphal mat. The suspension was transferred to a sterile tube and volume was adjusted to 5 ml with sterile saline solution. Sabouraud dextrose agar was poured to depth of 5 mm in 90 mm Petri dishes and stored at 4 °C. The plates were dried, standardized suspension was poured and uniformly spread by means of swab sticks. The excess inoculum was decanted.

2.4. Cell culture

KB cells and HeLa cells, obtained from NCCS (National Centre for Cell sciences), Pune, India, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Israel) supplemented with 10% heat inactivated fetal calf serum (Biological Industries, Israel) and **IX** Penstrep antibiotic solution (Biological Industries, Israel) and incubated in fully humidified 5% CO_2 incubator at 37 °C.

2.5. MTT assay for cell proliferation

Both the types of cells were seeded at a density 5×10^3 per well in 96-well plates (BD Falcon). For measuring the effect of curcumin and its bioconjugates, the stock (10 mmol) was diluted in complete media to its final concentrations. After 16 h of seeding old medium is replaced with new curcumin and its conjugate added media. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich, USA) was added in each well making final concentration of **IX** from the stock of 5 mg/ml (10×) and incubated in 5% CO_2 incubator at 37 °C for further 4 h. After 4 h the medium was removed and crystals were dissolved in 200 μl DMSO (Sigma–Aldrich, USA). MTT assay was performed after 48 h of transfection, optical density was measured with ELISA plate reader (Biorad 840) at 570 nm. The comparative results are shown in Figs. 1 and 2.

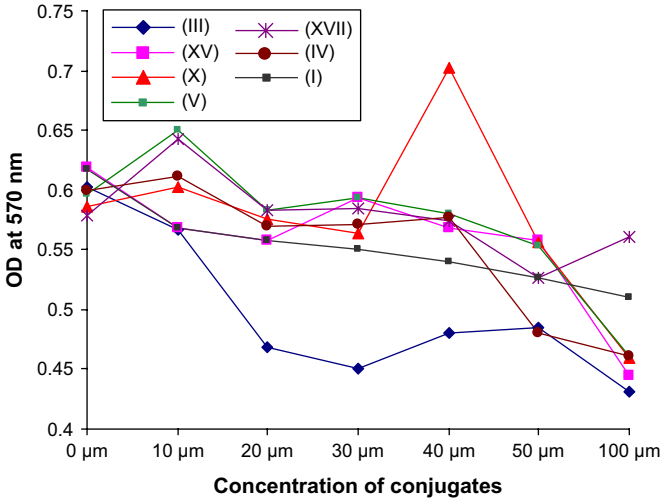


Fig. 1. Effect of curcumin bioconjugates on HeLa cancer cell lines (human origin).

2.6. 1,7-Bis-(4-O-glutamoyl-3-methoxy phenyl) 1,6-heptadiene-3,5-dione (*III*)

Curcumin (0.368 g, 1 mmol) was taken in dry pyridine and mixed with *N*-phthaloyl-glutamoyl chloride (0.55 g, 2 mmol) and stirred at r.t. for 8 h. After completion of the reaction as indicated by TLC, the reaction mixture was poured into crushed ice and repeatedly extracted with ethyl acetate. The organic layer was concentrated and treated with ammonia/pyridine (9:1 v/v) to remove phthaloyl group. The organic layer was concentrated and purified by silica gel column chromatography using DCM/methanol gradient. Yield: 40% (0.250 g); R_f: 0.9 (DCM/MeOH 9:1); UV λ_{max} (MeOH): 290 nm. Anal. Found: C, 59.39; H, 5.40; O, 30.67; N, 4.40. Calcd. for $C_{31}H_{34}O_{12}N_2$: C, 59.42; H, 5.47; O, 30.64; N, 4.47%. ^{1}H NMR ($CDCl_3$) δ ppm = 2.06–2.18 (m, 8H, 3′,4′-C of glutamate), 3.45 (m, 2H, CH–NH), 3.70 (s, 6H, $-OCH_3$), 4.46 (s, 2H, C_4–H), 4.09 (m, 2H, CH–NH_2), 6.53 (d, 2H, C_2–H and C_6–H), 6.83–7.11 (m, 6H, Ar–H), 7.54 (d, 2H, C_1–H and C_7–H), 11.2 (s, 2H, HO–C=O).

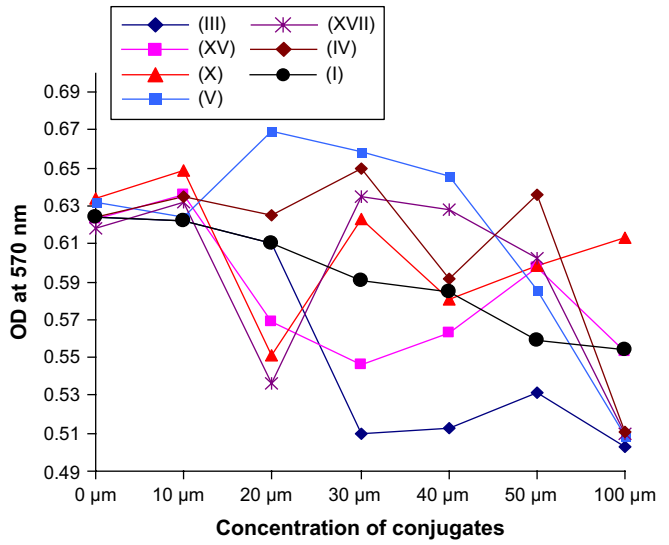


Fig. 2. Effect of curcumin bioconjugates on KB cancer cell lines (human origin).

2.7. 1,7-Bis-(4-O-valinoyl-3-methoxy phenyl) 1,6-heptadiene-3,5-dione (*IV*)

Curcumin (0.368 g, 1 mmol) was taken in dry pyridine and mixed with *N*-phthaloyl-valinoyl chloride (0.53 g, 2 mmol) and stirred at r.t. for 8 h. After completion of the reaction as indicated by TLC, the reaction mixture was poured into crushed ice and repeatedly extracted with ethyl acetate. The organic layer was concentrated and treated with ammonia/pyridine (9:1 v/v) to remove phthaloyl group. The organic layer was concentrated and purified by silica gel column chromatography using DCM/methanol gradient. Yield: 45% (0.254 g); R_f: 0.9 (DCM/MeOH 9:1); UV λ_{max} (MeOH): 295 nm. Anal. Found: C, 65.74; H, 6.71; O, 22.60; N, 4.91. Calcd. for $C_{31}H_{38}O_8N_2$: C, 65.71; H, 6.76; O, 22.59; N, 4.94%. ^{1}H NMR ($CDCl_3$) δ ppm = 1.03 (d, 12H, CH–$(CH_3)_2$), 2.65 (m, 2H, 3′C of valine), 3.70 (s, 6H, $-OCH_3$), 4.09 (s, 2H, C_4–H), 4.19 (m, 2H, CH–NH_2), 6.53 (d, 2H, C_2–H and C_6–H), 6.82–7.13 (m, 6H, Ar–H), 7.56 (d, 2H, C_1–H and C_7–H).

2.8. 1,7-Bis-(4-O-glycinoyl-3-methoxy phenyl) 1,6-heptadiene-3,5-dione (*V*)

It was prepared as reported in Ref. [15].

2.9. Demethylenated piperic acid from piperic acid (*XV*)

Piperic acid (0.654 g, 3 mmol) was taken in DCM. PCl_5 (0.762 g, 3.5 mmol) dissolved in DCM was added slowly. The reaction mixture was refluxed for 2 h to give geminal dichloride. DCM was evaporated and the residue was taken in water and again refluxed for 2 h to give catechol. The solution was cooled and the solid precipitate was collected which was again washed with cold water and crystallized from ethanol. Yield: 80% (0.494 g); R_f: 0.46 (DCM/MeOH 9.5:0.5); m.p.: 148 °C; UV λ_{max} (MeOH): 340 and 250 nm. Anal. Found: C, 64.00; H, 4.72; O, 31.10. Calcd. for $C_{11}H_{10}O_4$: C, 64.07; H, 4.75; O, 31.06. ^{1}H NMR ($CDCl_3$) δ ppm = 5.03 (s, 2H, aromatic C–OH), 5.64 (d, 1H, –CH=CH–C=O), 6.38 (d, 1H, –CH=C), 6.58–7.78 (m, 4H, olefinic and aromatic), 6.73 (d, 1H, Ar–H), 7.25–7.51 (d, 1H, CH=CH=C=O), 11.2 (s, 1H, HO–C=O).

2.10. Demethylenated piperoyl chloride (*XVI*)

To demethylenated piperic acid (0.824 g, 4 mmol) was added redistilled thionyl chloride slowly over a period of 45 min while heating and shaking in a water bath. The reaction mixture was further refluxed for 30 min and thionyl chloride was removed at reduced pressure. Yield: 60% (0.537 g).

2.11. 1,7-Bis-[4-O-demethylenated piperoyl-3-methoxyphenyl]-1,6 heptadiene-3,5-dione (**XVII**)

Curcumin (0.736 g, 2 mmol) was taken in dry pyridine and mixed with demethylenated piperoyl chloride (1.008 g, 4.5 mmol) and stirred at r.t. for 6 h. After the completion of reaction as indicated by TLC, the reaction mixture was poured onto crushed ice and repeatedly extracted with EtOAc. The organic layer was concentrated and purified on a silica gel column using a DCM/methanol gradient. Yield: 45% (0.667 g); R_f: 0.74 (DCM/MeOH 9.5:0.5); UV λ_{max} (MeOH): 395 and 295 nm. Anal. Found: C, 69.34; H, 4.83; O, 25.80. Calcd for $C_{43}H_{36}O_{12}$: C, 69.29; H, 4.87; O, 25.78. ^{1}H NMR ($CDCl_3$) δ ppm = 3.65 (s, 6H, $-OCH_3$), 4.15 (s, 2H, C_4-H), 4.65 (d, 2H, $O{=}C-CH_2$), 5.03 (s, 4H, aromatic C–OH), 5.65 (d, 2H, $-CH{=}CH-C{=}O$), 6.38 (d, 2H, $-CH{=}C$), 6.72–7.80 (m, 8H, olefinic and aromatic), 6.90 (d, 1H, Ar–H), 7.18–7.48 (d, 2H, $CH{=}CH{=}C{=}O$), 7.48 (d, 2H, C_1-H and C_7-H).

2.12. p-Nitrophenyl ester of chloroacetic acid

To chloroacetic acid (0.190 g, 2 mmol) dissolved in dry dioxane (8 ml), *p*-nitrophenol (0.280 g, 2 mmol) was added dropwise. The reaction mixture was made basic by the addition of 0.5 ml pyridine and 0.5 ml TEA (triethylamine). After stirring for 10 min dicyclohexyl carbodiimide (DCC) (1.03 g, 5 mmol) was added. The reaction mixture was stirred for 2 h under nitrogen atmosphere (*caution: protection from moisture*) and monitored on TLC. The completion of reaction was assessed by total consumption of starting reagent. $R_f = 0.88$ (DCM/MeOH 9.5:0.5).

Steps involved in solid phase synthesis of monoesters of curcumin

1. suspended dried long chain alkyl amine on CPG resin (LCAA–CPG) (200 mg, loading 60 μmol/100 mg, 120 μmol of NH_2 gp, pore size 500 Å) in dry dimethylformamide (DMF) (2 ml) and loaded on a column.
2. Dissolved chloroacetic acid (0.190 g, 2 mmol) in dry dioxane (8 ml) containing dry pyridine (0.5 ml) and *p*-nitrophenol (0.280 g, 2 mmol) and stirred for 20 min in a round bottom flask.
3. To it was added dicyclohexyl carbodiimide (DCC) (1.030 g, 5 mmol). After few minutes dicyclohexylurea began to precipitate. The reaction mixture was stirred for 2.5 h under nitrogen atmosphere (*caution: protection from moisture*) and monitored on TLC. The completion of reaction was assessed by total consumption of starting reagent. $R_f = 0.88$ (DCM/MeOH 9.5:0.5).
4. Removed dicyclohexylurea by filtration and added the supernatant to LCAA–CPG (200 mg) suspended in DMF on column.
5. To it was added 0.5 ml of triethylamine (TEA); a bright yellow colour due to the release of *p*-nitrophenol developed immediately. The reaction mixture was kept for 8 h.
6. The supernatant was removed and chloroacetic acid derivatised LCAA–CPG was washed with dry DMF, methanol followed by ether (2 × 3 ml). The derivatisation of chloroacetic acid as linker to LCAA–CPG was confirmed by ninhydrin test of the solid support which was negative indicating no free amino group and was ready for further synthesis.
7. Sodium salt of curcumin (2 mmol) dissolved in dry pyridine (10 ml) was passed through column repeatedly and was kept overnight.
8. The column was washed with pyridine, methanol followed by water (2 × 5 ml). It was again washed with methanol and ether (2 × 2 ml).
9. Phthaloyl glycinoyl chloride (2 mmol) dissolved in pyridine (5 ml) was passed through column with little addition (5 mg) of DMAP as catalyst and was kept overnight.
10. Column was washed with water (2 × 2 ml) followed by methanol (2 × 2 ml).
11. To the column was added 5 ml solution of ammonia and pyridine (ammonia/pyridine 9:1) and was kept for 5–10 min.
12. Column was again washed with water (2 × 2 ml) followed by methanol (2 × 2 ml).
13. HI (2 ml) was added to the column and kept for 12 h.
14. The monoester was eluted from the solid support with methanol along with HI, and methanol was distilled off.
15. The remaining DCM solution was added and it was washed with aqueous KI (10%) and DCM repeatedly until all the colours were removed from DCM layer to remove any I_2 and compound was extracted with DCM.
16. The DCM solution was washed with cold 5% aq. sodium bicarbonate (10 ml) solution followed by water (10 ml) to remove any HI.
17. It crystallized from DCM.

2.13. Monoglycinoyl curcumin (**IX**)

Yield: 50% (0.025 g); UV λ_{max} (MeOH): 360 and 295 nm; R_f: 0.6 (DCM/methanol 9.5:0.5). The pure product was characterized by Anal. Found: C, 64.94; H, 5.41; O, 26.35; N, 3.30. Calcd. for $C_{23}H_{23}O_7N$: C, 64.93; H, 5.45; O, 26.33, N, 3.29. ^{1}H NMR ($CDCl_3$) δ ppm = 3.70 (s, 6H, $-OCH_3$), 4.13 (s, 2H, C_4-H), 4.49–4.61 (m, 4H, CH_2-NH_2), 5.03 (s, 1H, –OH), 6.53 (d, 2H, C_2-H and C_6-H), 6.85–7.08 (m, 6H, Ar–H), 7.53 (d, 2H, C_1-H and C_7-H).

2.14. Monovalinoyl curcumin (**X**)

Monovalinoyl curcumin was also prepared by protocol as stated above. During the synthesis the amino function of valine was protected with phthalic anhydride as *N*-phthaloyl glycine and the carboxy function was activated with thionyl chloride to get the corresponding acid chlorides. This *N*-protected and carboxy activated amino acid was reacted with sodium salt of curcumin on solid support to give the ester of valine with curcumin. The phthaloyl group of protected amino acid

was removed using ammonia. HI was used to deblock the monoester from the solid support by cleaving the ethereal bond and thus selective esterification of curcumin was accomplished. UV λ_{max} (MeOH): 370 and 295 nm; R_f: 0.68 (DCM/methanol 9.5:0.5). The pure product was characterized by Anal. Found: C, 66.81; H, 6.20; O, 23.99; N, 2.99. Calcd. for $C_{26}H_{29}O_7N$: C, 66.80; H, 6.25; O, 23.96; N, 3.0. ^1H NMR ($CDCl_3$) δ ppm = 1.03 (d, 6H, CH–$(CH_3)_2$), 2.65 (m, H, 3 °C of valine), 3.70 (s, 6H, –OCH_3), 4.09 (s, 2H, C_4–H), 4.19 (m, H, CH–NH_2), 5.03 (s, 1H, –OH), 6.53 (d, 2H, C_2–H and C_6–H), 6.82–7.13 (m, 6H, Ar–H), 7.56 (d, 2H, C_1–H and C_7–H).

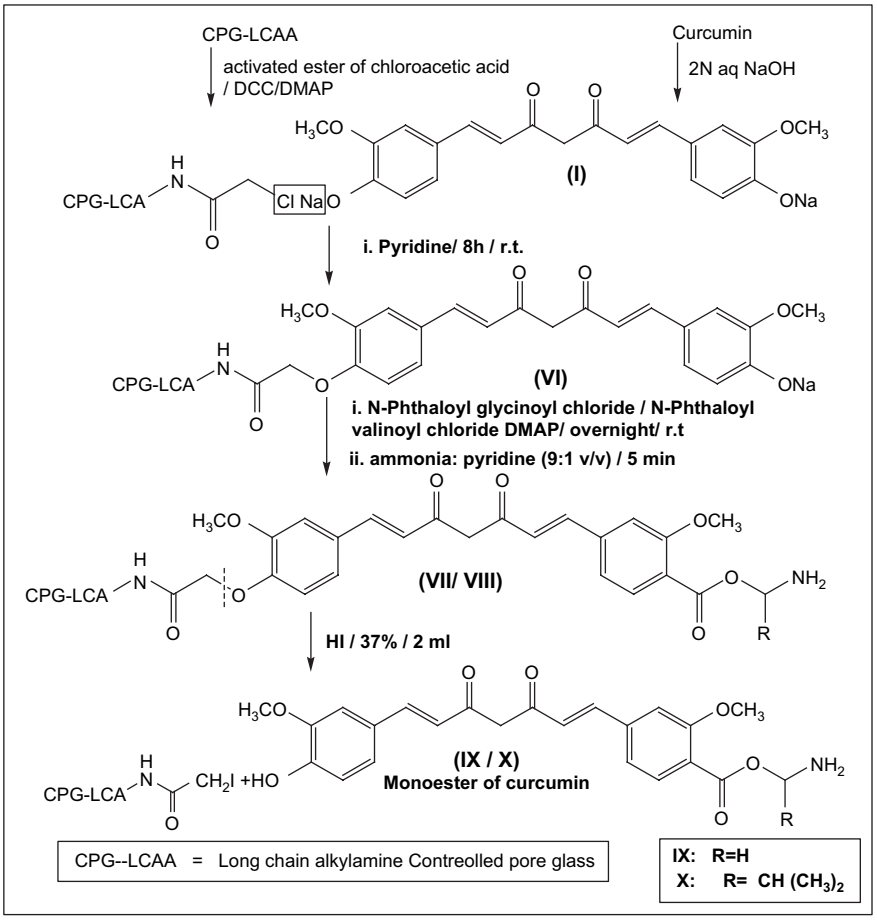
3. Results and discussion

Different curcumin conjugates viz. 4,4′-(di-*O*-glutamoyl)-curcumin (**III**), 4,4′-(di-*O*-valinoyl) curcumin (**IV**), 4,4′-(di-*O*-glycinoyl) curcumin (**V**), monoglycinoyl curcumin (**IX**), monovalinoyl curcumin (**X**), 4,4′-(di-*O*-demethylenated piperoyl) curcumin (**XVII**) and demethylenated piperic acid (**XV**) were prepared and these were tested for their antimicrobial activities vis-a-vis curcumin and piperic acid. The monoesters of curcumin have been prepared for the first time which have one phenolic free while the other was attached with suitable ligand.

During the synthesis of diesters, the –NH_2 group of amino acids (glycine, valine and glutamic acid) was protected as respective *N*-phthaloyl derivatives. The carboxyl group was activated by treating with thionyl chloride to get the corresponding acid chlorides. Curcumin was taken in dry pyridine and treated with *N*-phthaloyl chlorides of respective amino acids in 1:2.5 molar proportion and the mixture was stirred for 6 h. Phthaloyl group was removed using ammonia and the product was purified using column chromatography (Scheme 1).

Synthesis of monoesters of curcumin, a symmetrical diphenolic compound with glycine/valine has been carried out by anchoring one of its free phenolic group to an insoluble polymeric solid support resin (CPG–LCAA) via a 2-carbon linker by solid phase. However, the protocol of reactions used was the same as that used in the case of diesters, but due to changed kinetics of solid phase reactions, the reaction conditions vary (Scheme 2).

One of the phenolic groups of curcumin was attached to LCAA–CPG (long chain alkyl amine controlled pore glass) via chloroacetic acid as a linker; the carboxy function of chloroacetic acid was esterified (activated) by reaction with *p*-nitrophenol by the addition of dicyclohexyl carbodiimide (DCC) in the presence of pyridine and triethylamine to make the medium basic to get the corresponding activated ester.



Scheme 2. Synthesis of monoglycinoyl curcumin (**IX**)/monovalinoyl curcumin (**X**) on CPG–LCAA.

The activated ester of chloroacetic acid was reacted with amino function of LCAA–CPG using DCC/DMAP to get the amide bond, a well-established strategy in peptide synthesis. To this was added sodium salt of curcumin, a symmetrical diphenolic compound, which reacts with chloroacetic acid derivatised LCAA–CPG, such that one phenolic forms the bond while the other remains free for further synthesis.

The *N*-phthaloyl chlorides of glycine and valine were reacted with sodium salt of curcumin on solid support to give the corresponding monoesters of curcumin. The phthaloyl group was removed using ammonia. HI was used to deblock the monoester from the solid support by cleaving the ethereal bond and thus selective esterification of curcumin with glycine and valine was accomplished (Scheme 2).

In summary we have developed a novel strategy for the selective esterification of symmetrical diphenolic compound like curcumin using solid phase synthesis. This strategy can be used further for selective synthesis of monoesters of any symmetrical diphenolic compound. Another advantage of the procedure is that after deblocking the resin is left activated, i.e., with a $-CO-CH_2I$ group attached to its terminal NH_2 group and can be used as such for another cycle.

Piperic acid was obtained by alkali hydrolysis of piperine and selective cleavage of methylenedioxy group was accomplished by traditional method of halogenation followed by hydrolysis [31,32]. Halogenation was done with phosphorus pentachloride to give geminal dichloride and the subsequent hydrolysis to catechol (Scheme 3).

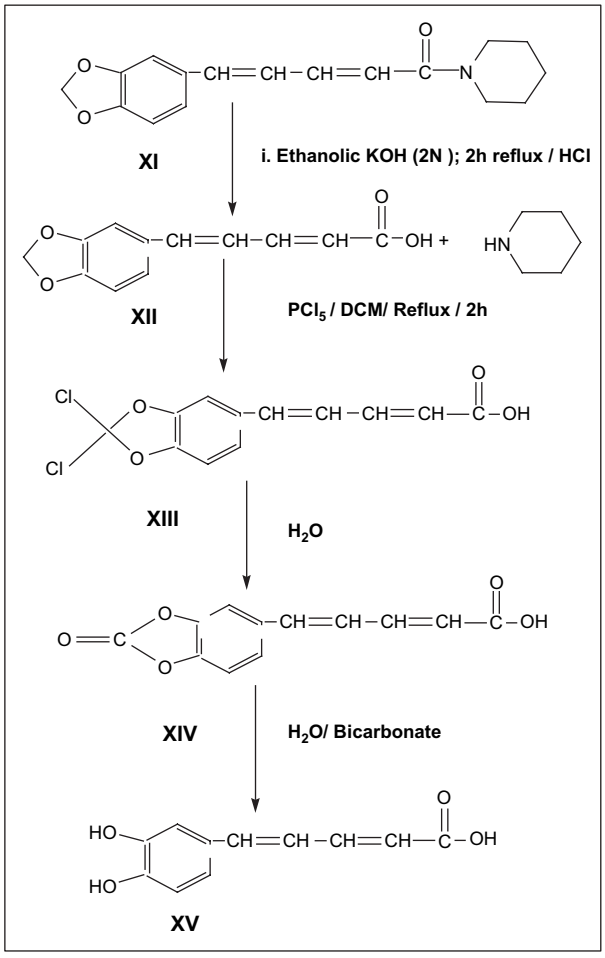
Curcumin was directly attached to demethylenated piperic acid [19,20] (Scheme 4). Different conjugates of curcumin viz. 4,4′-(di-*O*-valinoyl) curcumin, 4,4′-(di-*O*-glutamoyl) curcumin, 4,4′-(di-*O*-demethylenated piperoyl) curcumin and monoesters of curcumin with glycine and valine were prepared and characterized.

3.1. Antibacterial activity

Susceptibility test in vitro was performed on multiresistant bacteria specially causing secondary infection in human beings, e.g. *E. cloacae*, *S. saprophyticus*, *Micrococci*, *K. aeruginosa* and *E. coli*. The results are tabulated in Tables 1 and 2.

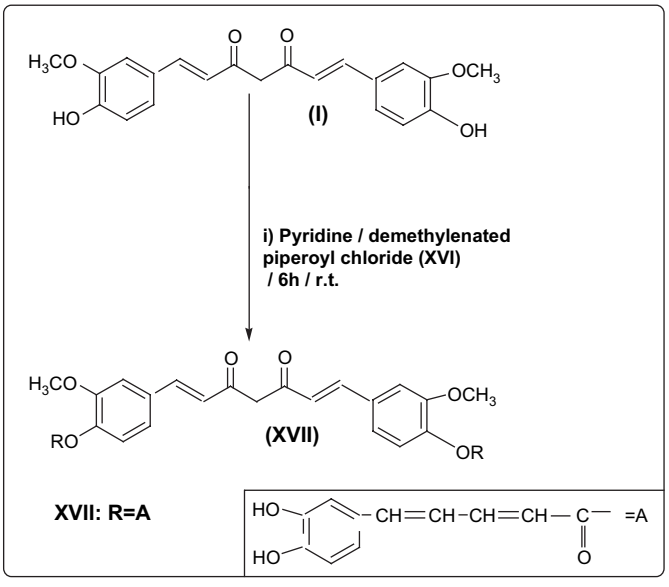
The antibacterial activity of curcumin bioconjugates including diesters as well as monoesters was compared with curcumin itself along with demethylenated piperic acid and its conjugate with curcumin by microdilution broth susceptibility test method. The lowest concentration of curcumin bioconjugates in μmol/ml that prevented in vitro growth of microorganism has been represented as correlated with zone of inhibition Table 1 and MIC (minimum inhibitory concentration) shown in Table 2.

Each test was performed in triplicate and the MICs reported represent the result of at least two repetitions. Six conjugates (**III**–**V**, **IX**, **X**, and **XVII**) show good positive result on multiresistant organisms while demethylenated piperic acid (**XV**) with methylenedioxy ring open shows better results than piperic acid (**XI**). The most encouraging results were obtained in the case of monoesters (**X**) having MIC of 2.5 μmol/ml against



Scheme 3. Synthesis of demethylenated piperic acid.

Micrococcus and *E. cloacae* while Amoxyclav, the best marketed antibiotic, shows MIC of 10 μmol/ml showing that monovalinoyl curcumin (**X**) is four times more effective than Amoxyclav, a marketed drug at the similar concentrations.



Scheme 4. Synthesis of curcumin demethylenated piperoyl conjugate (**XVII**).

Table 1
Antibacterial activity of curcumin bioconjugates (numerals show zone of inhibition in mm) for concentrations (20, 10, 5, 2.5 μmol/ml) of each bioconjugate against bacterial strains

Name of bacteria	I	III	IV	V	IX	X	XI	XV	XVII
Micrococci	–	18,16	16,13	15,13,10	–	**26**,**20**,**19**	–	15	16,12
Klebsiella aeruginosa	–	9	–	–	**18**	18	8	–	11
Staphylococcus saprophyticus	–	–	14,12	14,12,10	–	–	–	10	10
Enterobacter cloacae	–	–	**20**	12	–	**25**	–	18	18,14,12
Escherichia coli	10	–	**20**	–	12	13,11	–	–	16

(–) Resistant; curcumin (**I**); 4,4′-(di-*O*-glutamoyl)-curcumin (**III**); 4,4′-(di-*O*-valinoyl) curcumin (**IV**); 4,4′-(di-*O*-glycinoyl) curcumin (**V**); monoglycinoyl curcumin (**IX**); monovalinoyl curcumin (**X**); piperic acid (**XI**); demethylenated piperic acid (**XV**); 4,4′-(di-*O*-demethylenated piperoyl) curcumin (**XVII**). Values given in bold indicate significant inhibition obtained.

The result of zone of inhibition are also encouraging. The disc containing 20 μmol of Amoxyclav was purchased and equal amount of conjugates were loaded on separate discs. The zone of inhibition of Amoxyclav was 20 mm while Monovalinoyl curcumin (**X**) had zone of 26 mm. The conjugates **III**, **IV**, **IX** and **XVII** showing MIC of 5 μmol/ml were also comparable to Amoxyclav which shows MIC of 10 μmol/ml.

These results suggest that diesters of curcumin have more antibacterial activity than curcumin itself which may be due to their increased solubility, better cellular uptake (bioavailability) and slow down of metabolic process due to masking of free phenolics. However, monoesters have even better activity than their corresponding diesters since monoesters have both the advantages, i.e., as a ligand helping in cellular uptake and a free phenolic for binding at active site. The role of free phenolics in binding and transport is further clear from the enhanced activity of the conjugate having demethylenated piperic acid in which methylenedioxy ring was open as compared to that of the corresponding piperic acid.

All the covalent bonds synthesized are biodegradable, i.e., hydrolysable with common enzymes present in living systems. This was checked in vitro by treating curcumin–glycine conjugate with chymotrypsin when complete hydrolysis was observed. This makes these derivatives as potent prodrugs which can get hydrolysed at the target sites. The amino acids are known to be transported through carrier proteins associated with cellular uptake and were linked with curcumin in order to facilitate the cellular uptake through receptor mediated endocytosis.

The enhancement in antimicrobial activity of these curcumin bioconjugates may be due to

Table 2
MIC (minimum inhibitory concentration) correlation diagram (in μmol/ml) of bioconjugates against bacterial strains

Name of bacteria	I	III	IV	V	IX	X	XI	XV	XVII	Amoxyclav
Micrococci	–	5	10	10	R	**2.5**	–	10	10	10
Klebsiella aeruginosa	R	20	R	R	5	5	20	R	20	12
Staphylococcus saprophyticus	–	R	10	R	R	R	R	20	20	10
Enterobacter cloacae	–	R	5	R	R	**2.5**	–	10	5	10
Escherichia coli	20	R	5	20	20	10	R	R	10	10

(R) Resistant; curcumin (**I**); 4,4′-(di-*O*-glutamoyl)-curcumin (**III**); 4,4′-(di-*O*-valinoyl) curcumin (**IV**); 4,4′-(di-*O*-glycinoyl) curcumin (**V**); monoglycinoyl curcumin (**IX**); monovalinoyl curcumin (**X**); piperic acid (**XI**); demethylenated piperic acid (**XV**); 4,4′-(di-*O*-demethylenated piperoyl) curcumin (**XVII**). Values given in bold indicate significant inhibition obtained.

(1) enhanced metabolic stability due to masking of phenolic hydroxyl groups and delay in their glucuronide formation during metabolism,
(2) better cellular uptake due to the transportation of the conjugate via the amino acid carrier protein, i.e., drug smuggling, and
(3) more solubility of the conjugates because of enhancement in polar character (hydrophilicity).

Since, the transporter proteins for many amino acids as well as peptides are known, the monoesters of curcumin with these amino acids or peptides can act as substrates for the carrier proteins. ABTO+ is one such example which is a known substrate for monovalinoyl curcumin and can be used for characterizing the ester by transporter expression system. However, these transport proteins for amino acids and peptides are known to be very promiscuous and can transport amino acid esters of many other compounds also.

3.2. Antifungal activity of curcumin bioconjugates

We have also evaluated the antifungal activity of curcumin and its bioconjugates against *A. fumigatus*, *C. albicans* (yeast), *C. parapsilosis* and *P. notatum*. The antifungal activity of curcumin bioconjugates was compared with the standard drug fluconazole where it was found comparable to *C. albicans* (yeast form). From the results shown in Table 3 we can infer that monoglycinoyl curcumin (**IX**) shows better activity against *A. fumigatus*, *C. albicans* (yeast), *C. parasilosis* and *P. notatum*, than its corresponding diester 4,4′-(di-*O*-glycinoyl) curcumin (**V**) vis-a-vis curcumin (**I**). Similarly demethylenated piperic acid (**XV**) shows better results than piperic acid (**XI**) in the case of *C. albicans* emphasizing the role of free phenolic groups as we have seen in antibacterial activity. The results are significant as fungal diseases are stubborn to most of the antifungal drugs.

3.3. Anticancer activity of curcumin bioconjugates on human cancer cell lines, HeLa (cervical cancer) and KB (oral cancer)

Antiproliferative property of curcumin is very well known. In traditional medicine also turmeric is not given to pregnant woman, as it may have detrimental effect on growth of fetus. Recent evidences suggest that the antiproliferative property is

Table 3
Antifungal activity of curcumin bioconjugates (numerals show zone of inhibition in mm) in concentrations (20, 10, 5, 2.5 μmol/ml) of each bioconjugate against fungal strains (fluconazole concentration was 20 μmol/ml)

Name of fungi	I	III	IV	V	IX	X	XI	XV	XVII	Fluconazole
Aspergillus fumigatus	–	–	–	–	**25**, **20**	–	–	–	–	–
Candida albicans	15	–	–	–	**20**, **18**	–	–	16	16	29
Candida parapsilosis	–	–	–	–	**14**	–	–	–	–	20
Penicillium notatum	–	–	–	–	**20**, **16**	12	–	–	–	–

(–) Resistant; curcumin (**I**); 4,4′-(di-*O*-glutamoyl)-curcumin (**III**); 4,4′-(di-*O*-valinoyl) curcumin (**IV**); 4,4′-(di-*O*-glycinoyl) curcumin (**V**); monoglycinoyl curcumin (**IX**); monovalinoyl curcumin (**X**); piperic acid (**XI**); demethylenated piperic acid (**XV**); 4,4′-(di-*O*-demethylenated piperoyl) curcumin (**XVII**). Values given in bold indicate significant inhibition obtained.

due to its ability to induce apoptosis. However, the molecular mechanism through which curcumin induces apoptosis is not fully understood.

However, curcumin does have apoptotic effect on cancerous cells through mitochondria and the process occurs through activation of different caspases. Here, we have assessed the anticancerous properties of curcumin bioconjugates against human cancer cell lines, HeLa (Fig. 1) and KB (Fig. 2).

In the present study curcumin diglutamoyl derivative was found to be more potent against cancer cell lines, HeLa and KB, than other derivatives, more over the monoester of curcumin with valine and the corresponding diester showed almost similar results in HeLa cells. The demethylenated piperic acid shows better results than the curcumin–piperic acid conjugate at higher concentration in HeLa cells.

In the case of KB cells similar pattern was observed showing highest activity in the case of curcumin diglutamoyl derivative while monoester of curcumin with valine shows less activity than the corresponding diester of valine with curcumin.

Generation of free radicals has been shown to be responsible for cell death. In several apoptotic models, increased generation of ROS was described as an early event; in addition enhanced ROS formation and impairment of the cellular antioxidant mechanism may also lead to cellular apoptosis.

We have also found that diesters show better anticancerous property than their corresponding monoesters, and best result was seen in diglutamoyl curcumin (**III**), divalinoyl curcumin (**IV**) and diglycinoyl curcumin (**V**) esters. However, it may be that apoptosis induction is due to the activation of caspases which is facilitated due to accumulation and better stability of diglutamic curcumin and other diesters of curcumin. However, further experiments are needed to confirm the hypothesis.

4. Conclusion

The advantage with herbal drugs is that their target proteins are already known; moreover, these are not toxic to living systems. Therefore, it is advantageous to start with a food component, which can well internalize with the cellular environment, and subsequently modify it chemically to suit the requirement. This is what we have achieved in the present work, i.e., assessment of bioconjugates of curcumin with appropriate biomolecules which can help in transporting these molecules to their specific targets for their therapeutic importance. Chemical modification of the herbal products, specially their attachment to suitable ligands which get internalized with the cellular environment can enhance their therapeutic activity multiple times. Use of biodegradable linkages for attaching the ligands makes these conjugate ‘‘prodrugs’’, i.e., a significant concentration can be built up inside the infected cells and then can get hydrolysed to release the active component at the target site. However, still the toxicity of these bioconjugates has to be tested *in vivo*.

How curcumin conjugates produce therapeutic effects is not fully understood, but they are probably mediated in part through the antioxidant and anti-inflammatory actions of curcumin. It is quite likely that curcumin mediates its effects through other mechanisms as well. Over a dozen different cellular proteins and enzymes have been identified to which curcumin binds.

The better results in the case of curcumin bioconjugates are probably due to their better solubility, enhanced cellular uptake through carrier proteins and decreased metabolic degradation. Though our work is still in its infancy our results cannot be ignored. It opens a new era for exploring suitably designed curcumin bioconjugates as potential antibacterial, antifungal and antiproliferative drugs. Exhaustive work based on the designing and testing of the conjugates according to the molecular organization of several pathogens and cell lines is required before any definite conclusion can be reached. However, further studies are needed to evaluate the *in vivo* activity and selectivity of the compounds presented.

A large number of studies unequivocally identified the numerous pharmaceutical actions of curcumin, its acceptance as a ‘wonder compound’ is slowly forthcoming. Curcumin has a plethora of beneficial effects and certainly qualifies for serious consideration as a pharmaceutical/nutraceutical/phytoceutical agent.

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