Chemical Approaches Toward Preparation of Water-soluble Curcumin Derivatives

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DECLARATION

I hereby declare that the thesis entitled **"CHEMICAL APPROACHES TOWARD PREPARATION OF WATER-SOLUBLE CURCUMIN DERIVATIVES**" submitted to **UNIVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY** is the work carried out by me at laboratories of Plantation Products Spices & Flavor Technology department under the guidance of Dr. P. Srinivas, Head, Department of Plantation Products Spices & Flavor Technology, CFTRI, Mysore – 570020, India, during the period 2006 – 2009. I further declare that the results are not submitted for the award of any other degree or fellowship.

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CERTIFICATE

I hereby certify that the thesis entitled *"***CHEMICAL APPROACHES TOWARD PREPARATION OF WATER-SOLUBLE CURCUMIN DERIVATIVES***"* submitted by **Ms. K. S. Parvathy** for the degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY** to the **UNIVERSITY OF MYSORE** for the result research work carried out by her at laboratories of Plantation Products Spices & Flavor Technology department, CFTRI, Mysore – 570020, India, under my guidance and supervision during the period 2006 – 2009.

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ACKNOWLEDGEMENTS

CONTENTS

SYNOPSIS

Curcumin, the major coloring principle of turmeric (*Curcuma longa* L., *Zingiberaceae*), has a wide array of biological activities. It is soluble in organic solvents like acetone and dioxane. But, it is insoluble in water at acidic or neutral pH, which is a disadvantage for its use in water-based food products. Also, the molecule undergoes degradation on exposure to light. Hence, newer strategies to prepare simple and safe adducts/derivatives are needed to overcome these drawbacks. In order to make curcumin water-soluble, it is essential to attach to it a polar group or molecule that can impart hydrophilic nature to it. The major objectives of the present investigation were development of alternative, efficient and stereo-specific methods for obtaining specific derivatives of curcumin, their characterization, analysis and study of their bioactive attributes. Approaches delineated in literature on curcumin derivatization are wide-ranging in nature. In the present study, particular emphasis was laid on preparation of curcumin derivatives with a sugar or an amino acid, as the resultant compounds would on hydrolysis afford natural molecules. Also, keeping in view the labile nature of curcumin, strategies evolved needed bringing about the derivatization under milder conditions and in higher yields. Thesis, describing the research results, includes five chapters. Chapter one covers literature on curcuminoids and their derivatives. Chapters 2, 3 and 4, describe the several synthetic methodologies developed in this study for selected curcumin derivatives. In Chapter 5, the *in vitro* antioxidant, antibacterial and antimutagenic properties of all the curcumin derivatives prepared in the current work are presented. The salient findings of the investigation are summarized.

CHAPTER 1. CURCUMINOIDS AND THEIR DERIVATIVES

This introductory chapter describes the physico-chemical, spectral and stability aspects of curcuminoids. Analytical protocols for curcuminoids are also briefly discussed. Biological attributes of curcumin like antioxidant, antimicrobial and antimutagenic effects in several *in vitro* as well as animal models along with several other biological activities of curcumin are detailed in this chapter. This chapter also elaborates several known synthetic approaches to preparation of selected derivatives of curcumin to

improve its solubility and photo-stability. Few important examples of curcumin derivatives - synthesis and their biological properties - are discussed.

CHAPTER 2. SYNTHESIS OF CURCUMIN GLUCOSIDES

Glycosidation of curcumin is one of several approaches to render curcumin water-soluble. Reaction of α-acetobromoglucose with potassium salt of curcumin under either thermal or high pressure conditions results in drastic reduction in the yields of the glucosides. In the present study, this drawback was effectively overcome by carrying out reaction of 2, 3, 4, 6-Tetra–*O*-Acetyl-α–D-glucopyranosyl bromide with potassium salt of curcumin under biphasic conditions in the presence of phase transfer catalysts and under the influence of ultrasound as a new approach to Koenigs-Knorr reaction. The reaction under the sonochemical conditions was faster and resulted in the increased yield of the glucoside products (> 65%). The reaction, when investigated in detail, clearly underscored the significance of the nature and quantity of the phase transfer catalyst employed in the reaction. The amount of potassium hydroxide, employed for the formation of the salt of curcumin too played a critical role in the reaction. Optimum yield and selectivity towards preponderant formation of the curcumin di-β-glucoside tetracetate was obtained when benzyltributylammonium chloride was used as PTC and the base was used in 10 mmol, wherein the reaction was complete in 4 h. The reaction was also investigated under mono-phasic conditions with the benzyltributylammonium salt of curcumin, produced *in situ* from the potassium salt of curcumin and PTC, in DMSO and alcoholic solvents. Among these, the reaction in DMSO afforded the curcumin mono-β-glucoside tetracetate in 50 % yield. The curcumin β-glucoside tetraacetates were deacetylated to afford pure curcumin β-glucosides. Thus, the study established a simple synthetic protocol for the glucoside derivatives of curcumin in high yields and selectivity using ultrasound.

CHAPTER 3. SYNTHESIS OF CURCUMIN AMINO ACID CONJUGATES

Another approach to solublize curcumin is the preparation of its amino acid conjugates. Recent literature reports preparation of di-*O*-glycinoyl curcumin and di-*O*-glycinoyl-C^{I4}-glycylcurcumin by condensation of curcumin with N-phthaloyl glycinoyl

chloride in anhydrous pyridine. Also, preparation of derivatives of curcumin viz., 4,4'-di-(O-glycinoyl) curcumin, 4,4'-di-(O-glycinoyl-di-N-piperoyl) curcumin, 4,4'-di-(O-piperoyl)curcumin and 4,4'-(O,O-cysteinoyl)-3,3'-dimethoxydi-phenyl-1,6-heptadiene-3,5-dione has been described. A multi-step synthesis of tetraglycine conjugate of curcumin too is reported. However, applicability of these methods, to general synthesis of other amino acid derivatives of curcumin under mild conditions is not demonstrated.

In the present investigation, a general synthetic method with superior yields of amino-acid conjugates of curcumin was developed. The protocol involved reaction of curcumin in dry dioxane with t-Boc-amino acids in the presence of a dehydrating agent like dicyclohexylcarbodiimide with 4-dimethylaminopyidine and triethylamine as catalysts. Under an inert atmosphere (N_2) and at $25 - 30$ ^{0}C, the reaction afforded curcumin t-Boc-amino acid conjugates in high yields (50 – 84 %). The reaction was demonstrated with several t-Boc protected amino acids viz., i) an un-substituted one like glycine, ii) alkyl substituted ones like alanine, leucine, isoleucine and valine, iii) an amino acid that is an aryl substituted one like phenylalanine and phenylglycine, iv) an amino acid containing sulphur like cysteine, v) an amino acid present as an imino acid like in proline, and vi) an amino acid that contains a hydroxyl group like serine. Also, in this study, an efficient protocol was developed under milder conditions for the rapid removal of the t-Boc group with TFA (10 %) using ultrasound. Several new amino-acid conjugates of curcumin are reported in this study.

CHAPTER 4. GLUCURONIDE AND GLUCAL DERIVATIVES OF CURCUMIN

Glucuronide derivative of curcumin and its reduced forms are reported as major metabolites of curcumin in the experiments on its metabolism in rat models. In the present study, a synthetic pathway for this derivative of curcumin was delineated. In this approach, glucuronolactone was stirred with NaOMe in methanol to cleave the lactone ring followed by methylation of free carboxyl group. The product formed was acetylated with acetic anhydride / perchloric acid reagent. The resultant acetylated compound was brominated at the anomeric position. Reaction of the methyl α-bromoglucuronide triacetate with curcumin was carried out under biphasic conditions with phase transfer

catalyst (PTC) using ultrasound to afford curcumin methyl mono-β-glucuronide triacetate. The product was deacetylated to yield curcumin carboxymethyl mono-β-glucuronide. 2,3-Unsaturated glycosides are versatile synthetic intermediates and also constitute the structural units of several natural products. Allylic rearrangement of glycals, known as the Ferrier rearrangement, in the presence of a nucleophile leads to the formation of 2,3-unsaturated glycosides. Ferrier reaction of 3,4,6-tri-O-acetyl-D-glucal with curcumin in presence of ferric sulfate catalyst at 40 ^{o}C in acetonitrile afforded the mono glucal derivative of curcumin. Deacetylation of the glucal derivative afforded the 2, 3- dideoxysugar derivative of curcumin in 50 % yield.

CHAPTER 5. *IN-VITRO* ANTIOXIDANT, ANTIBACTERIAL AND ANTIMUTAGENIC ATTRIBUTES OF CURCUMIN DERIVATIVES

Curcumin derivatives - glucosides, amino acid conjugates, glucuronide and 2,3–dideoxyglucose compounds of curcumin - synthesized in the present study were soluble in water. The solubility of curcumin glucoside and glucuronide was 10 mg/ml in water. The curcumin glucal derivative was soluble at 1 mg/ml concentration. While all curcumin amino acid conjugates were soluble in water at 1 mg/ml concentration at pH 6, the conjugates of curcumin with glycine and proline exhibited higher solubility in water (10 mg/ml).

It was important to determine the effect of derivatization of curcumin on its' vital innate biological attributes for which this important nutraceutical molecule is highly valued. Hence, the *in vitro* antioxidant, antibacterial and antimutagenic properties of the various derivatives of curcumin prepared in this study were assessed and compared with those of curcumin.

The radical scavenging activity by DPPH method as well as antioxidant activity by *β*–carotene-bleaching assay showed 67-68% and 100 % activity at 0.15 μM levels for curcumin and curcumin glucoside respectively. Both curcumin and its glucoside showed strong antimutagenic activity with *Salmonella Typhimurium* TA 98 against sodium azide at all the concentrations tested. In case of TA 1531, curcumin had moderate antimutagenic activity but its glucoside showed significantly higher activity at 625

µg/plate. These results indicated higher antimutagenic potential of curcumin glucoside compared to curcumin. Curcumin and its glucoside showed 27% and 70% inhibition against *E. coli* at 0.14 and 0.15 µM concentrations respectively. In case of *Y. enterocolitica,* curcumin and its glucoside showed 100% inhibition at 0.68 and 0.87 µM respectively. Against *B. cereus,* curcumin and its glucoside showed 30% and 66% inhibition at 0.07 µM. In the case of *S. aureus* curcumin and its glucoside showed 68% and 100% inhibition at 0.07 and 0.05 µM concentrations. These results demonstrated that curcumin glucoside possessed superior antibacterial activity against the microorganisms tested.

Curcumin amino acid conjugates showed lower IC_{50} values than curcumin in DPPH method as well as β–carotene-bleaching assay. Conjugates of curcumin with amino acids like alanine, valine, serine, cysteine and isoleucine were effective even at ~50 % of the concentration of curcumin.

Amino acid conjugates of curcumin showed strong antimutagenic activity against *Salmonella Typhimurium* TA 98, with sodium azide and methyl methanesulphonate as mutagens at 100 as well as 250 µg/plate concentrations. With *Salmonella Typhimurium* TA 1531 at 100 µg/plate concentration, against sodium azide as mutagen, conjugates of curcumin with proline, leucine, serine and valine showed activity similar to curcumin; other derivatives showed higher activity than curcumin. In the case of MMS as mutagen, cysteine, glycine and proline conjugates of curcumin showed lower activity than curcumin while other derivatives showed higher activity than curcumin. However, all the amino acid conjugates showed higher activity than curcumin against TA 1531 at 250 µg/plate concentration.

Curcumin amino acid conjugates showed lower MIC values compared to curcumin against the Gram - negative bacteria, E. c*oli* and Y. *enterocolitica*. Against *S.aureus,* all derivatives, except alanine, phenylalanine and alanine conjugates of curcumin, exhibited higher inhibition. Against *B. cereus,* isoleucine, phenylalanine, valine, proline and glycine conjugates of curcumin showed lower inhibition compared to curcumin. IC_{50} values of glucuronide and glucal derivatives were lower than the parent

molecule in antioxidant assays. Both the derivatives showed strong antimutagenicity as the parent molecule against sodium azide and MMS at 100 and 250 μg/plate concentrations. Inhibition against *E. coli* and *Y. enterocolitica* was higher compared to curcumin in the case of glucuronide derivative. Glucal derivative of curcumin also showed good inhibition against *E. Coli* but comparable to curcumin in the case of *Y. enterocolitica.*

SUMMARY

In the present research study, several curcumin derivatives viz., glucosides, amino acid conjugates, glucuronide and 2,3-dideoxyglucose derivatives have been synthesized in moderate to high yields. The binding of the phenolic hydroxyl moiety in curcumin to various groups rendered the curcumin derivatives soluble in water at 1 to 10 mg/ml concentrations. The water-soluble derivatives, thus prepared, exhibited more pronounced *in vitro* antioxidant, antibacterial and antimutagenic properties compared to curcumin.

Signature of the candidate   **Signature of the guide**
(Parvathy K.S)   **(P. Srinivas)**
Date: **Date:**

CHAPTER 1

INTRODUCTION

1.1 Turmeric

Turmeric, a very important spice in India, comes from the root of *Curcuma longa*, a leafy plant of the ginger family. The plant is an herbaceous perennial, 60 - 90 cm high, with a short stem and tufted leaf. The yellow turmeric tuberous root belongs to the group of aromatic spices. The root or rhizome has a tough brown skin and bright orange flesh. In fresh state, it has a strong and spicy fragrance, which on drying gives way to a more mellowed aroma. The spice is sometimes called the 'Indian saffron' due to its brilliant yellow color. Turmeric forms part of most Indian spice powders. It is widely consumed as a dietary spice and a natural food colorant. It is a natural antiseptic that finds extensive use in traditional Indian medicine for the treatment of various ailments. It is also used in the textile and pharmaceutical industries.

1.1.1 Turmeric Production

Curcuma, a genus in the plant family of *Zingiberacea*, is the biological source for curcuminoids with curcumin as the major constituent (Aggarwal, et al., 2005; Govindarajan, 1980**)**. It is grown in Southeast Asia, China, North Australia, West Indies and South America and of late also grown in few African countries. Production of turmeric in India for the year 2005-2006 was estimated to be about 8, 92,180 tons (Spices Board of India, 2006). Around 42,750 tons of turmeric has been exported from India in the period April 2006 - January 2007. The genus Curcuma (family of *Zingiberacea)* contains 49 genera. Among these, *Curcuma Longa L,* is of commercial importance. Turmeric is commercialized as whole spice, powder and oleoresin. Oleoresin contains mixture of volatile oil along with

non-fatty and resinous material. Solvents like hexane, acetone and alcohol are often employed for the extraction of curcumin from turmeric. The high solubility of curcumin in acetone renders it the solvent of choice for the extraction. The extraction time is variable but in a soxhlet equipment the yield is about 8-10 %, containing 42–50 % curcuminoids in 4-5 h (Delgado–Vergas & Paredes – Lopez, 2003).

1.1.2 Varieties of Turmeric and its uses

The *Curcuma* genus has an estimated 1,000 species. *Curcuma alismatifolia*, *C. Cordata, C. gracillima, C. longa 'O'Lena* & *C. Parviflora* are few to mention. More than 50 cultivars are known in India and are known mostly by the name of location where they are cultivated. Madras variety, named Alleppy, has higher curcuminoid content of 5–7 %. Some of the other popular cultivars are Duggirala, Tekurpeta, Sugandham, Amalapuram, Erode local, Moovattupuzha, and Lakadong. Turmeric is used for various purposes such as a coloring agent and a condiment. It is a principal ingredient in Indian curry powder. Turmeric oleoresin is used in brine pickles, to some extent in mayonnaise, relish formulations, in nonalcoholic beverages, gelatins, butter and cheese. Turmeric is also used as a dye in textile industry, in cosmetics, preparation of medicinal oils, ointments and poultice. It possesses stomachic, carminative, tonic, blood purifier and antiseptic properties. The aqueous extracts also have bio-pesticide properties (Delgado–Vergas & Paredes – Lopez, 2003**)**.

1.1.3 Constituents of turmeric

Total extracts of turmeric, curcumin and turmeric oil, are all credited with medicinal properties. Biological activities of these components and their constituents however, differ considerably. It is reported that the proportions of curcuminoids play a considerable role in optimum bio-protective activity of turmeric. The yellow-pigmented fraction of *Curcuma longa* contains curcuminoids, which are chemically related to its principal ingredient, curcumin. Curcuminoids are generally present in the range of 3–5% in turmeric. Curcumin and its analogs are present in turmeric along with other components like essential and fixed oils, starch, protein, fiber, bitter principles and moisture.

1.2 Curcuminoids: Analogs, isolation techniques and stability

Curcumin, a phenolic component present in turmeric along with two other analogs, is responsible for its bioactive properties. Curcumin, an orange yellow pigment, has major role as colorant and as flavoring for the Indian food industries. Curcuminoids are extracted from turmeric by extraction with selected organic solvents. The three main curcuminoids isolated from turmeric are curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Fig. 1.1 a - c). As the curcuminoid pigments vary in chemical structures, their chemical and color characteristics as well as the functional properties (Table 1.1) could be different. Curcumin of very high purity (> 99%), obtained by selective crystallization process, is highly valued as a nutraceutical. As demethoxycurcumin and bis-demethoxycurcumin, being minor constituents, are not commercially available, their separation and characterization assumes significance.

1.2.1 Isolation, purification, separation and characterization of curcuminoids

Curcumin was first isolated in 1815 by Vogel, obtained in crystalline form in 1870 by Daube and identified as (*1E, 6E*)-1,7-bis(4-hydroxy-3-methoxyphenyl), 1,6-heptadiene-3,5-dione). In 1910, the feruloylmethane skeleton of curcumin was confirmed. Curcumin is a yellow-orange powder that is insoluble in water, sparingly soluble in ether and soluble in acetone, dioxane, ethanol and dimethyl sulfoxide. It has a melting point of 182-183 ^{0}C, a molecular formula of $C_{21}H_{20}O_6$, and a molecular mass of 368.38. The compound exhibits maximum absorption (λ_{max}) at 430 nm in methanol and at 415–420 nm in acetone (Boong, 2000). A 1 % solution of curcumin contains 1650 absorbance units (Delgado–Vergas & Paredes – Lopez, 2003). Curcumin appears in brilliant yellow hue at pH 2.5–7 and red at pH > 7. Curcumin exists in enolic and diketonic forms.

Table 1.1 Physico chemical properties of curcuminoids

Trivial name	Curcumin	Demethoxy curcumin	Bis-Demethoxy curcumin
Chemical Name	Diferuloyl methane	4– Hydroxycinnamoyl feruloyl methane	Bis-4-Hydroxy-cinnamoylmethane
Molecular weight	368.38	338.36	308.33
Chemical formula	$C_{21}H_{20}O_6$	$C_{20}H_{18}O_5$	$C_{19}H_{16}O_4$
Melting point (^{o}C)	182-183	172-174	223-224
Absorption maxima (λ_{max}) in ethanol	427	424	418
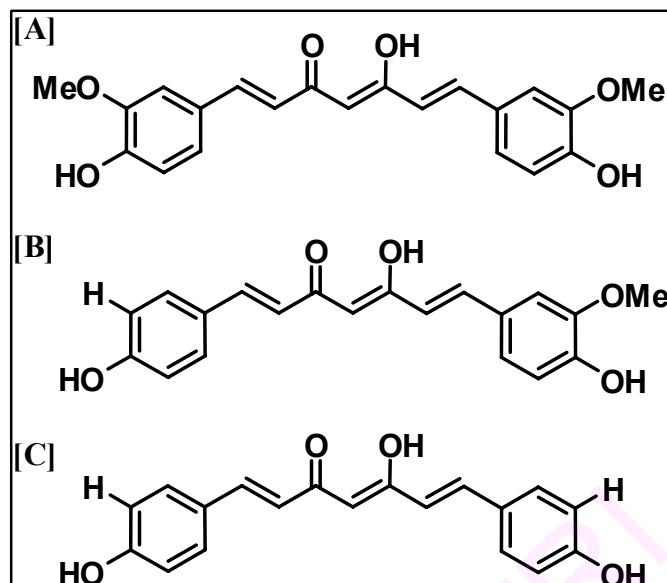
Fig.1.1 Structures of [A] Curcumin, [B] demethoxycurcumin & [C] bis demethoxycurcumin
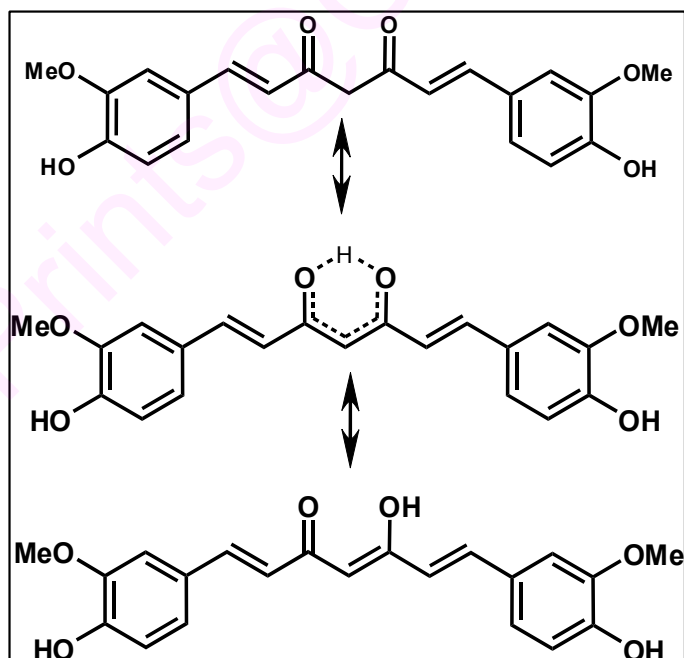
Fig. 1.2 Keto – enol tautomerism Curcumin

Curcuminoids are separated by column chromatography and HPLC. Many methods are reported for the separation of curcuminoids by HPLC (Jayaprakasha, et al., 2002; Hiserodt, et al., 1996; Braga, et al., 2003; Peret-Almeida, et al., 2005), Reports are available on rapid quantitation of curcumin by NMR and LC-tandem mass spectrometry (Goren, et al., 2009) and HPTLC (Paramasivam, et al., 2009). Curcumin is a bis-α, β-unsaturated 1,3-diketone. As such, curcumin exists in equilibrium with its enol tautomer (Fig. 1.2) (Sharma, et al., 2005).

1.2.2 Stability aspects of curcumin

The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices have been established (Wang, et al., 1997). When curcumin is incubated in 0.1 *M* phosphate buffer and serum-free medium (pH 7.2 at 37 ^{0}C), about 90% decomposes within 30 min. A series of pH conditions ranging from 3 to 10 are tested and the results show that decomposition is pH-dependent and occurs faster at neutral or basic conditions. It is more stable in cell culture medium containing 10% fetal calf serum and in human blood. Less than 20% of curcumin decomposes within 1 h, and after incubation for 8 h, about 50% of curcumin still remains. (*2Z,5E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal is suggested to be the major degradation product (Fig. 1.3). Vanillin, ferulic acid, and feruloyl methane are identified as minor degradation products (Wang, et al., 1997). The amount of vanillin increases with incubation time. Curcumin is insoluble in water and at acidic pH but soluble under alkaline conditions. Curcumin hue progressively

transforms from yellow to intense red above pH 7. Tonnesen, et al., (1985) has first reported the complex kinetics of pH-dependent degradation of curcumin in aqueous solution. These studies reveal that curcumin degrades rapidly under alkaline pH (Tonnsen, et al., 1986) and is extremely unstable at pH above 7 in aqueous systems.

1.3 Metabolism of curcumin

Water-insolubility and photo-instability of curcumin render it less suitable for food / pharmacological applications. The enol form ensures its stability to resonance structures, which give it pseudo-aromatic character and helps curcumin to form hydrogen bonds with metals to form complexes. Although the exact mechanism of degradation is not completely understood, curcumin should be stable in the stomach and small intestines because the pH is between 1 and 6, and degradation of curcumin is slow under these conditions (Wang, et al., 1997). Biotransformation of curcumin has been studied, its metabolites isolated after oral i. p. administration in mice and identified. THC is a major metabolite of curcumin and its stability at different pH has been studied (Pan, et al., 1999). *In - vitro* studies show the metabolic steps involve the glucuronidation of curcumin and its major reduced forms viz., tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin (Fig. 1.4). Glucuronides of curcumin, hexahydrocurcumin, and other analogs have been synthesized using rat and human liver microsomes and characterized by LC-MS/MS analysis (Pfeiffer, et al., 2007).
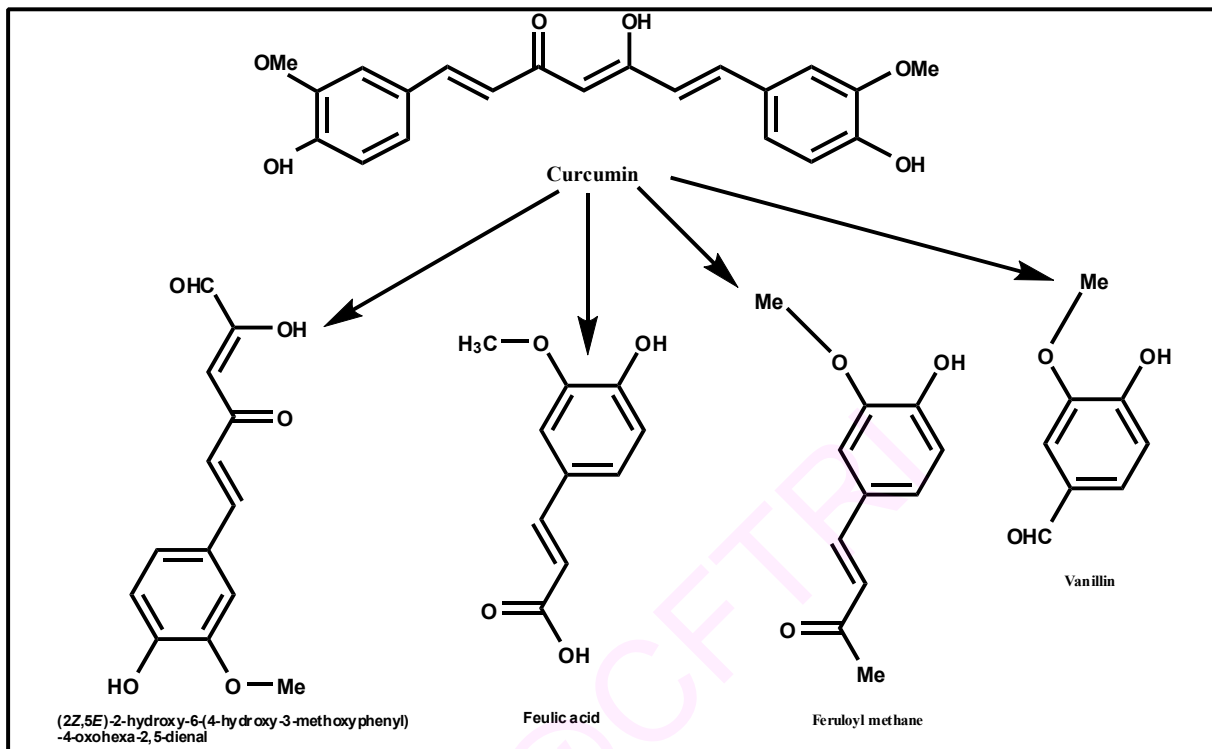
Fig. 1.3 pH dependent degradation of curcumin
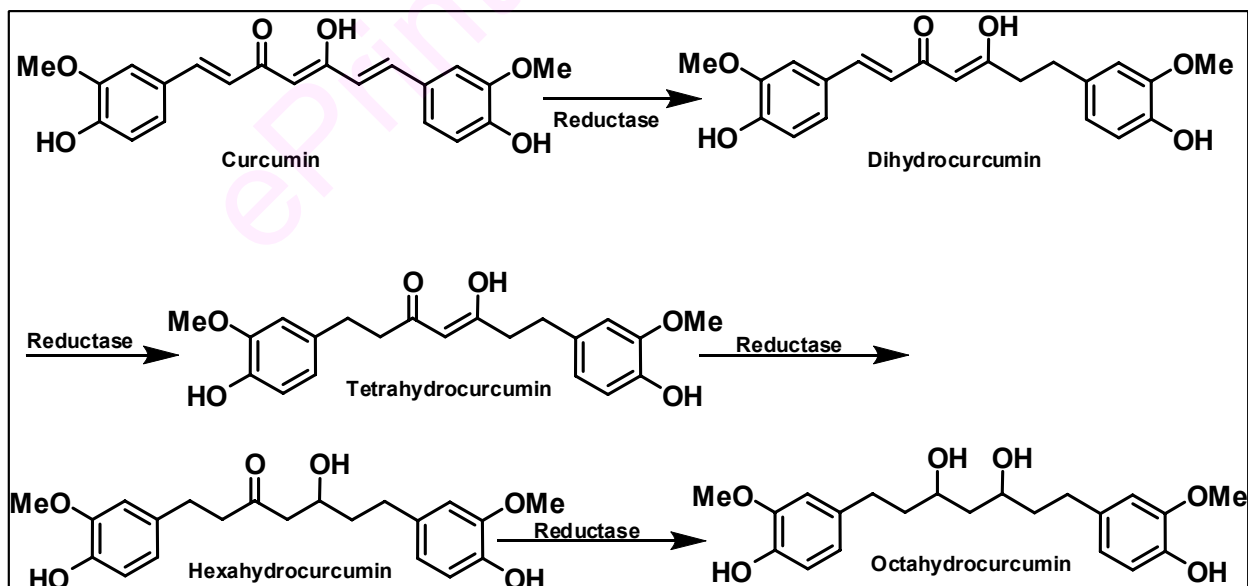
Fig. 1.4 Curcumin metabolism

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1.4 Biological attributes of curcumin

Curcumin shows many pharmacological applications from ancient ayurveda to modern homeopathy because of its high biological activity (Singh, 2007). Curcumin, a constituent of turmeric, has anti-inflammatory, anti-carcinogenic, and chemo-preventive effects as evidenced in several animal tumor models. Curcumin has potent antioxidant, antitumor and anticancer properties (Goel, et al., 2008). The molecule is known to possess multiple biological activities like wound healing, antifertility, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic and antivenom (Aggarwal et al. 2007). Curcumin possesses diverse effects on cellular enzymes and on angiogenesis and cell adhesion. In particular, ability of curcumin to affect gene transcription and induce apoptosis in pre-clinical models advocates its potential utility in cancer chemoprevention and chemotherapy. Although curcumin's low systemic bio-availability following oral dosing seems to limit the number of tissues that it can reach at efficacious concentrations to exert beneficial elects, the attainment of such levels in the gastrointestinal tract, particularly the colon and rectum, has been demonstrated in animals and humans (Alpers, 2008). In view of the peer-reviewed reports of the pharmacological properties of curcumin, its phase II clinical evaluation in individuals at risk of developing cancer, especially of the gastrointestinal tract, appears opportune (Kunnumakkara & Aggarwal, 2008)

Curcumin inhibits tumorogenesis and also suppresses mammary carcinogenesis. Curcumin exhibits antiproliferative effects against cancer cells. Curcumin has been shown to inhibit the proliferation of a wide variety of tumor

cells, including B-cell and T-cell leukemia, colon carcinoma, and epidermoid carcinoma cells. It has also been shown to suppress the proliferation of various breast carcinoma cell lines in culture. Curcumin administration in initiation and post initiation period shows inhibition of colon tumorogenesis, curcumin with low chronic toxicity will be advantageous to combat against cancer. The molecular basis of anticarcinogenic and chemopreventive effects of curcumin is attributed to its effect on specific targets which include transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and signaling molecules (Maheshwari,, et al., 2006; Anand, et al., 2008; Aggarwal, et al., 2005; Anand, et al., 2008a). It has attracted special attention due to its pharmacological activities such as protection of cells from β-amyloid insult in Alzheimer's disease (Park & Kim, 2002). Antioxidant property of curcumin, clinical evaluation of curcumin as an anticancer agent in humans, effect of curcumin on lymphocytes, platelet aggregation, detoxification mechanisms, cell cycle and apoptosis, diabetes, wound healing, stress responses and antiviral effects are well established (Joe, et al., 2004).

Few important examples from recent literature that underscore the importance of curcumin as a therapeutic and nutraceutical compound are enlisted below.

- Curcumin is a potent antioxidant by virtue of which it has anti-inflammatory and anti-carcinogenic potential (Srinivasan, 2005).

- Curcumin is also an effective hypolipidemic agent (Srinivasan et al., 2004). It is useful as an anti-diabetic food adjunct (Srinivasan, 2005a).

- Curcumin possesses antilithogenic property (Hussain and Chandrasekhara, 1993, 1994).

- The pharmacodynamics and pharmacokinetics of curcumin have been examined in animals and in humans. Curcumin protects against various forms of stress, curcumin modulates multiple sclerosis, reduces the formation of cholesterol gallstone formation, suppresses the symptoms of arthritis, regulates myocardial infarction, inhibits platelet aggregation and lowers the serum cholesterol levels (Chattopadhyay, et al., 2004).

- Curcumin has various pharmacological and therapeutic properties as demonstrated in *in vitro* and *in vivo* studies (Aggarwal, et al., 2009).

- Curcumin is a good scavenger of reactive oxygen species (Elizabeth and Rao, 1990).

- Curcumin is a potent singlet oxygen quencher at physiological or pharmacological concentrations (Das & Das, 2002).

- Curcumin is shown to penetrate the blood - brain barrier and bind to A-beta in brain cells both in *in vivo* and *in vitro* studies (Balasubramanian, 2006).

- Curcumin inhibits proliferation of cancer cells by perturbing microtubule assembly dynamics and efficacious curcumin analogs have been developed for trials in cancer chemotherapy (Gupta & Balasubrahmanyam, 1998).

- Curcumin down-regulates the multi drug-resistance mdr1b gene by inhibiting the PI3K/Akt/NFkB pathway（Choi, et al., 2008）.

- Curcumin and its decomposition products vanillin, vanillic acid, ferulic aldehyde and ferulic acid inhibit human recombinant cytochrome P450s. (Appiah – Opang & Commandeur, 2007).

- Curcumin induces changes in the expression of genes in cholesterol homeostasis (Peschel, et al., 2007).

- Curcumin reduces lead-induced neurotoxicity in male wistar rats (Dairam, et al., 2007).

- Curcumin prevents Fos-Jun-DNA complex formation (Kim & Yang, 2004).

- Tetrahydrocurcumin (THC), a major colorless metabolite of curcumin, is shown to exhibit a more prominent anti-diabetic and plasma glycoprotein controlling ability than curcumin (Pari & Muruan, 2007).

- Curcumin has antidepressant effect, which is related to serotonergic system and mediated by interactions with 5-HT1 and 5-HT2 receptors (Wang, et al., 2008).

- Curcumin exhibits anti-lymphangiogenic property as shown by its ability to inhibit the formation of capillary-like tubes by rat lymphatic endothelial cells (Matsuo, et al., 2007).

- Curcumin attenuates haloperidol-induced oxidative damage in all the regions of the brain especially in the sub-cortical region containing striatum, of possible therapeutic relevance for the treatment of tardive dyskinesia (TD), a hyper-kinetic movement disorder (Bishnoi, et al., 2008).

- Active site binding modes of curcumin in HIV-1 protease and integrase are reported wherein, symmetrical structure of curcumin seems to play an

important role for binding to the PR protein, wherein the keto–enol and only one side of the terminal O-hydroxyl show tight binding to the IN active site (Vajragupta, et al., 2005).

- Curcumin prevents and reverses murine cardiac hypertrophy (Li, et al., 2008).

- Curcumin inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy, et al., 1994).

- Studies show curcumin in aqueous preparations consisting of cylcodextrin, DMSO, surfactant like triton, sodium aliginate and liposomes in combination with light affected salivary gland acinar cells. The study points out to the possible use of curcumin as a photosensitizer in the treatment of oral lesions (Bruzell, et al., 2005).

- Curcumin displays differential effects on vasoactive factor expression in the heart and that the tissue microenvironment is of importance in treatment of diabetic complications as demonstrated in experiments with Streptozotocin-induced diabetic rats (Farhangkhoee, et al., 2006).

- Curcumin together with EGF-R related protein causes a greater inhibition of growth of colon cancer cells (Sudha Reddy, et al., 2006).

- Effect of antioxidant activity of turmeric, turmerin and curcumin as anti HIV drugs has been studied. Water-soluble extract, turmerin, inhibits HIV infected T–cell proliferation and combination with 3' azido-3'-deoxythymidine decreases T-cell infection and increases cell viability (Cohly, et al., 2003).

- Curcumin is an effective chemo-preventive compound. The mechanism of action of curcumin is complex and likely multi-factorial. It modulates proteins involved in iron metabolism in cells and tissues and acts as a good chelator for iron (Jiao, et al., 2006).

- Curcumin and its analogs are shown to induce Phase 2 detoxification enzymes in murine hepatoma cells underlining the chemo-protective potential of these compounds. The activities of molecules indicate Keto – enol moiety plays an important role for the biological activities (Dinkova & Talalay, 1999).

1.4.1 Antioxidant activity of curcumin

Antioxidant activity of curcumin has been assayed by various *in vitro* antioxidant assays and compared against BHA, BHT, α-tocopherol and trolox standards (Tuba Ak & Gulcin, 2008). These include 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging activity, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, *N,N*-dimethyl-*p*-phenylenediamine (DMPD) radical scavenging activity, total antioxidant activity determination by ferric thiocyanate, total reducing ability determination by the Fe^{3+}–Fe^{2+} transformation method, superoxide anion radical scavenging by the riboflavin/methionine/illuminate system, hydrogen peroxide scavenging and ferrous ions (Fe^{2+}) chelating activity. Curcumin, bisdemethoxycurcumin and demethoxycurcumin are tested for their antioxidant activities by *in vitro* model systems, such as the phosphomolybdenum and

linoleic acid peroxidation methods. Curcumin shows stronger activity compared to its analogs (Sandur, et al., 2007, Jayaprakasha, et al., 2006).

1.4.2 Antioxidant mechanisms of curcumin

Curcumin molecule possesses high antioxidant activity. Several mechanisms are postulated for proton transfer during radical scavenging action. The phenolic hydroxyls of curcumin are shown to be involved in the route of HAT (Hydrogen Atom Transfer) mechanism of antioxidant activity. Alternatively sequential proton loss electron transfer (SPLET) postulates that keto – enol moiety of curcumin has a more easily dissociable proton, which is supposed to be faster than the other mechanism, as the enolic proton is more acidic than the other two hydroxyls of phenol rings in curcumin (Litwinienko & Ingold, 2004). This hypothesis is also supported by theoretical calculations like Density Functional Theory (DFT) and Time Dependent Density Functional Theory (TD-DFT), which also support sequential proton loss electron transfer (SPLET) in curcumin (Shen & Ji, 2007). Studies reveal that curcumin is significantly more stable in keto-enol form. This is due to strong internal hydrogen bonding formed in the enol along with the extended conjugation in the enol chain (Wright, 2002). The studies by Sun et al., 2004, also show the computational results indicating enol form of curcumin is more stable than diketo form because of the intra-molecular hydrogen bonding.

1.5 Curcumin Derivatives

Curcumin's vast array of biological properties and their molecular mechanisms has been the subject of several scientific investigations.

Derivatization of curcumin is made in order to enhance its biological activity, to make it hydrophilic or in certain cases to increase lipophilicity. Accordingly, several curcumin derivatives with specific biological activities as targets have been prepared and their bio-attributes evaluated. Chemical modifications of this natural phytochemical are carried out either at its phenolic position or at the keto-enolic position. The physical modifications are also carried out as a parallel approach. Methods like glycosylation allow the conversion of water-insoluble compounds into corresponding water-soluble ones, which improve their bioavailability, pharmacological properties and assist targeted delivery of drugs. A brief discussion on these methodologies is presented with relevant examples from literature.

1.5.1 Physical modifications to make curcumin water-soluble

Curcumin is water-insoluble which limits its usage in water based foods and pharmaceutical applications. The oral bioavailability of curcumin is reported to be 1% in rat (Yang, et al., 2007) wherein fast excretion of curcumin is observed from the living system due to poor absorption after oral administration.

Attempts have been made to enhance the solubility of curcumin using oil-in-water (O/W) nanoemulsions of different sizes to encapsulate curcumin. Such preparations also exhibit improved anti-inflammatory activity, as substantiated by using a mouse-ear inflammation model (Wang, et al., 2008a).

Curcumin forms inclusion complex with β-cyclodextrin. The binding to β-cyclodextrin is confirmed by stop flow technique. Curcumin - β-cyclodextrin

complex renders curcumin water-soluble and shows increased radical scavenging activity compared to curcumin (Swaroop, et al., 2007).

Curcumin has been rendered water-soluble by use of an emulsifier like Tween-60. Curcumin formulation thus prepared has been incorporated in extruded food formulations and its stability studied in comparison with tartrazine (synthetic yellow color) (Sowbhagya, et al., 2005).

Solid dispersion of curcumin with PVP has been prepared in different ratios with a view to enhancing the solubility of curcumin. Such a preparation shows complete dissolution within 30 min, whereas curcumin does not show any dissolution even after 90 min (Paradkar, et al., 2004).

Synthesis of biodegradable and self-assembling methoxy poly (ethylene glycol)–palmitate nanocarrier for curcumin delivery to cancer cells is reported. The encapsulated derivative has been shown to assist in the target delivery of the drug (Sahu, et al., 2008).

Efforts are made to improve the water solubility, phase distribution, hydrolytic stability and photo stability of curcumin in cyclodextrin solutions. While these derivatives prepared show higher solubility in aqueous systems and superior hydrolytic stability than curcumin, limitations still remain with respect to its photo stability. This study also indicates that the two halves of the symmetric curcumin molecule act as two separate units and scavenge one radical each and underscores the importance of both enol group as well as phenolic moiety for its antioxidant behavior (Tomren, et al., 2007).

1.5.2 Derivatization at keto-enolic position of curcumin

Attempts have been made to synthesize derivatives of curcumin by reaction at the diketo / keto-enolic group and the α-methylene position as shown in the examples below.

❖ Copper (II) conjugates (Fig. 1.5) of curcumin and its derivatives are prepared, structurally characterized and evaluated for their potential of inhibiting TNF induced NF-kB activation and proliferation in human leukemic KBM-5 cells (Zambre, et al., 2006).

❖ Binding of Cu (II) to various sites in curcumin and its analogs, demethoxycurcummin and bisdemethoxycurcumin, have been studied. The keto-enolic proton has been shown to be one of the sites for the Cu (II) binding. These derivatives have been shown to exhibit antioxidant and DNA cleavage potential (Ahsan, et al., 1999).

❖ The copper (II) complexes of two bis-curcuminoids having diphenylmethane linkage have been synthesized and characterized by mass and NMR spectroscopy (Sundaryono, et al., 2003).

❖ Electron-rich pyrazole and isoxazole analogs have been prepared by Michel addition to α, β-unsaturated 1,3–diketone moiety of curcumin. These derivatives show antiproliferative activity (Amolins, et al., 2009).

❖ Rare earth metal complexes of curcumin are prepared by the reaction of curcumin with rare earth (Sm, Eu, Dy) (III) nitrates by coordination at the β-diketone moiety and tested for their antibacterial properties (Song, et al., 2009).

- ❖ Selective hydrogenation of α, β-unsaturated olefinic bonds in curcumin, affords tetrahydrocurcumin (Fig. 1.6), a colorless derivative of curcumin (Pattekhan, et al. 2005).

- ❖ Reaction of tetrahydrocurcumin with chitosan is also reported. The product exhibits good antioxidant and antibacterial properties (Portes, et al., 2008).
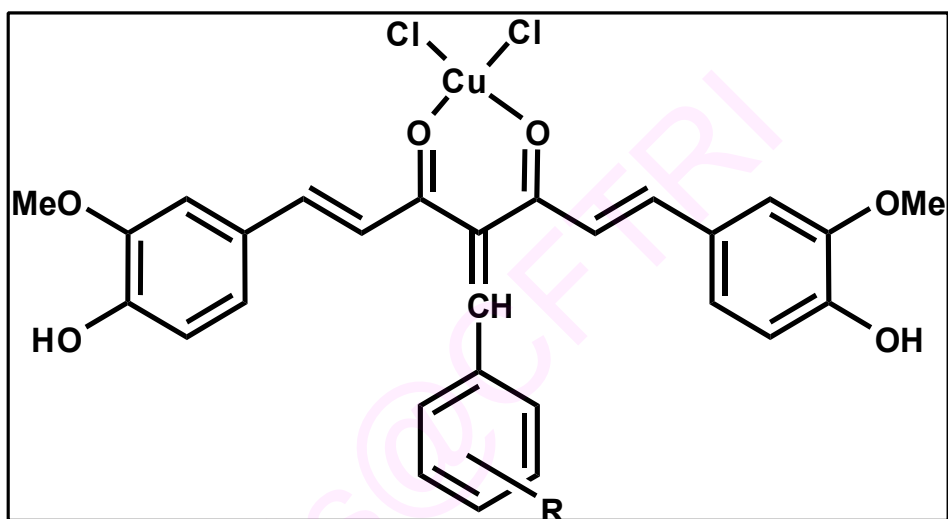
Fig. 1.5 Copper (II) conjugates of curcumin
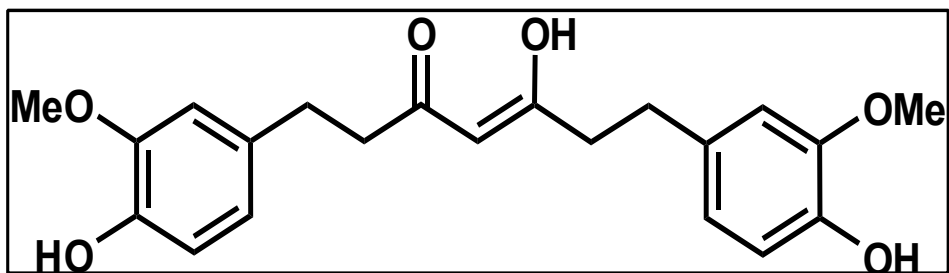
Fig. 1.6 Tetrahydrocurcumin

❖ Isoxazolcurcumin (IOC) is synthesized and its interactions with calf thymus DNA investigated by UV–Visible spectroscopy, fluorescence, circular dichroism, viscosity measurements and docking studies. The binding studies indicate the derivative has the capacity to bind to the minor grooves of nucleic acids unlike the parent molecule (Bera, et al., 2008).

❖ Binding interactions of bovine serum albumin (BSA) with isoxazolcurcumin and diacetylcurcumin (Fig.1.7 [A] & [B]) have been studied using FT –IR and circular dichroism. The results indicate isoxazolcurcumin shows strong binding towards BSA (Sahoo, et al., 2008).

❖ Cyclic analogs of curcumin are synthesized by using boron trioxide-mediated aldol condensation using conventional heating and microwave irradiation. Microwave synthesis provides increased yield of 2-arylidene-6-(3-arylacryoyl)-cycloalkanone derivatives of curcumin. The derivatives are found to have significant anticancer activity against representative murine and human cancer cell lines (Youssef, et al., 2007).

❖ Binuclear and mononuclear ortho-palladated complexes based on a functionalized 2-phenylquinoline ligand have been synthesized and tested for their *in vitro* cytotoxic activity (Pucci, et al., 2007). Conjugating cyclopalladated fragments to curcumin β-diketone moiety gives two different functionalities in one single molecule.

❖ Mono-carbonyl analogs of curcumin are synthesized and their structures confirmed by NMR analysis and tested for anti-inflammatory activities (Liang, et al., 2007).

- ❖ Curcumin effectively chelates to Cu (II). Cu (II)–curcumin complexes are shown to prevent A-beta aggregation (Shen, et al., 2005).

- ❖ Three manganese complexes of curcumin and related compounds, diacetylcurcumin (AcylCp) and ethylenediamine derivative (CpED), are synthesized and evaluated *in vitro* for antilipid peroxidation and superoxide dismutase activity (Virajagupta, et al., 2003).

- ❖ Hydrazinocurcumin (Fig. 1.8) is synthesized which is shown to be a potent inhibitor of endothelial cell proliferation (Shim, et al., 2002).

- ❖ Hydrazinocurcumin is a very potent multi-activity compound found to have several pharmaceutical applications (Rathore, et al., 2007).

- ❖ Curcumin chelated with Mg – Al layered double hydroxides are prepared. These complexes show enhanced thermal stability and exhibit slow drug release at pH 6.5 (Ni, et al., 2008).

- ❖ A large number of curcumin analogs have been synthesized with different substituents at the α-methylene carbon in the 1,3-diketo systems in curcumin molecule. These derivatives have been shown to possess anti-androgen activity with relation to treatment of prostrate cancer (Ohtsu, et al., 2002), anti tumor activity (Ohtsu, et al., 2003) and inhibition of cytochrome – P450 (Appiah-Opong, et al., 2007 & 2008).
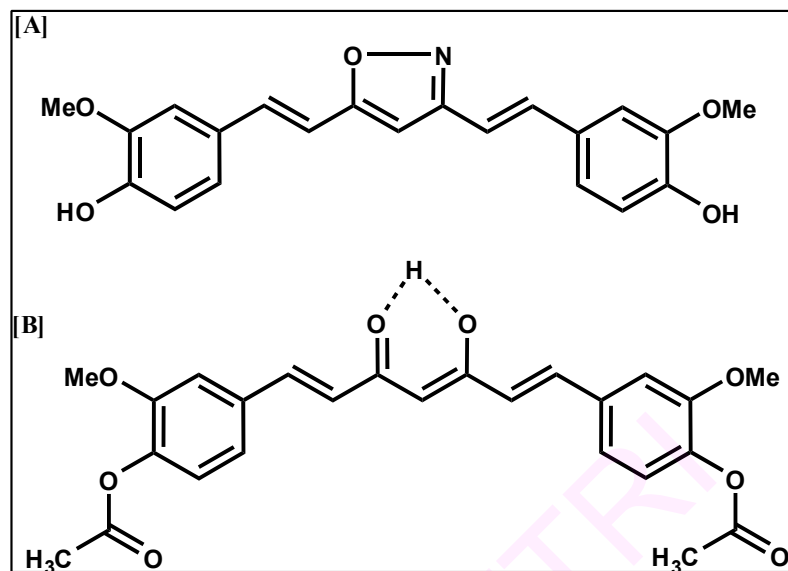
Fig. 1.7 [A] Isoxazolcurcumin [B] Diacetyl curcumin derivatives
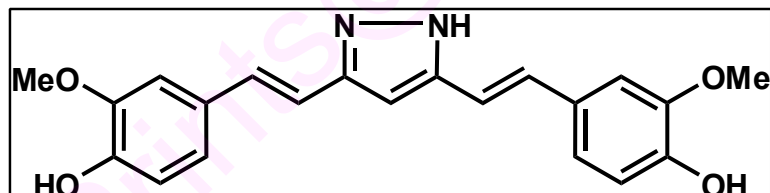
Fig. 1.8 Hydrazinocurcumin
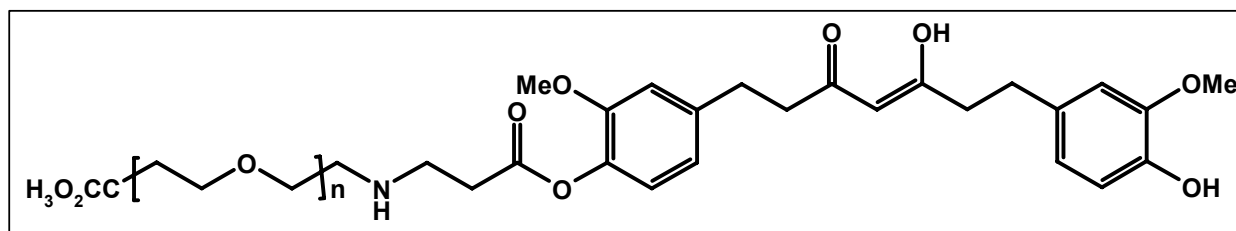
Fig. 1.9 Curcumin – polyethylene glycol conjugate

1.5.3 Derivatizations at phenolic position of curcumin

➢ Curcumin analogs are prepared by replacing the –OH group of the phenolic group with methoxyls and few derivatives are synthesized by replacing methoxyl with all -OH. In this case the derivatives bearing multiple – OH groups on the phenolic rings of curcumin show anti–hemolytic activity. These derivatives inhibit free radical induced oxidative hemolysis of human RBC (Deng, et al., 2006).

➢ The monoesters of curcumin, a symmetric diphenol with valine, glycine glutamic acid and demethylenated piperic acid have been prepared by a novel solid phase synthesis and their antimicrobial and antiproliferative effects demonstrated (Dubey, et al., 2008).

➢ Pyrazole derivative of curcumin is synthesized and its effects on memory tested (Maher, et al., 2008).

➢ In an attempt to make curcumin water-soluble, it is conjugated to *N-*acetylamino PEG-carboxylic acid by a carbodiimide-mediated esterification at the phenolic hydroxyl group. The reaction is standardized with different sized polyethylene glycols (Fig. 1.9). With the modification, enhancement in the cytotoxicity is found due to its aqueous solubility (Safavy, et al., 2007).

➢ Curcumin boron complexes with phenolic group substituted with nitro, methyl and hydroxyls (Fig. 1.10) show better inhibition of Jun – Fos – DNA complexation than curcumin (Kim & Yang, 2004).

- Catharanthus roseus cell suspension cultures are capable of converting exogenously supplied curcumin to glucosides (Kaminaga, et al., 2003). Sugar derivative of curcumin would increase its water solubility as well as reduces toxicity (Kaminaga, et al., 2004).

- Reactions are catalyzed using an amyloglucosidase (glucan 1,4-alpha-glucosidase) from Rhizopus and a beta-glucosidase with several carbohydrate moieties (D-glucose, D-mannose, maltose, sucrose and D-mannitol) in di-isopropyl ether (Vijay Kumar & Divakar, 2007).

- Known methods of preparation of curcumin sugar derivatives employ the reaction of α-D-tetraacetohaloglucose with curcumin under biphasic conditions in the presence of a phase transfer catalyst at higher temperatures but give very low yields (Hergenhahn, et al., 2003; Mishra, et al., 2005).

- Condensation of glycosylated arylaldehyde with acetylacetone–B_2O_3 complex gives a corresponding diglycosyl curcuminoid, and a similar reaction using a mixture of arylaldehyde and glycosylarylaldehyde affords an unsymmetrical monoglycosyl curcuminoid, both as boron-complexes. The boron is removed from the complexes by heating in methanol, thus achieving the synthesis of di- and mono-glycosyl curcuminoids (Mohri, et al., 2003).
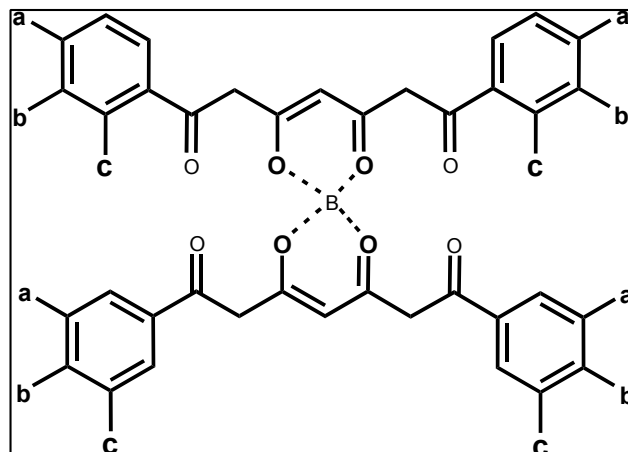
Derivative 1 a,b,c ; -H, -CH$_3$, -H

Derivative 2 a,b,c ; -NO$_2$, -CH$_3$, -H

Derivative 3 a,b,c ; -NO$_2$, -OH, -

Fig. 1.10 Curcumin boron complexes
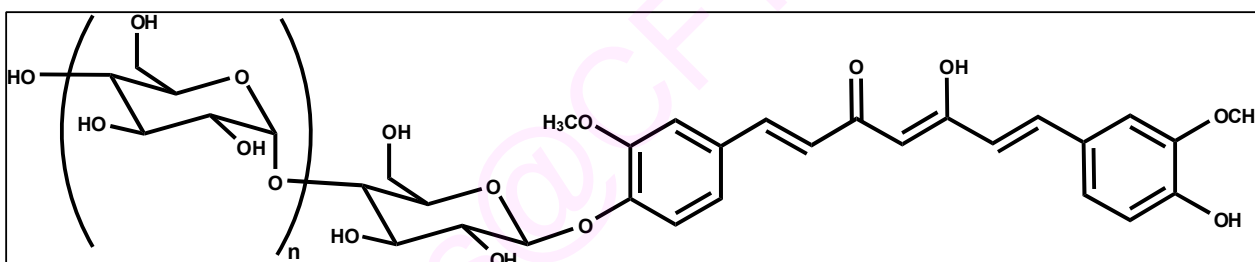
Fig. 1.11 Curcumin β-maltooligosaccharide derivatives

1. [VO(cur)$_2$], VO(DAC)$_2$] n = 2; M=VO, R1 = H, Ac; R2 = OCH$_3$

2. [VO(BDC)$_2$], VO(DABC)$_2$] n = 2; M=VO, R1 = H, Ac; R2 = H

3. Ga(cur)$_3$], [Ga(DAC)$_3$] n = 3; M = Ga, R1 = H, Ac; R2 = OCH$_3$

4. [In(cur)$_3$], [In(DAC)$_3$] n = 3; M = In, R1 = H, Ac; R2 = OCH$_3$
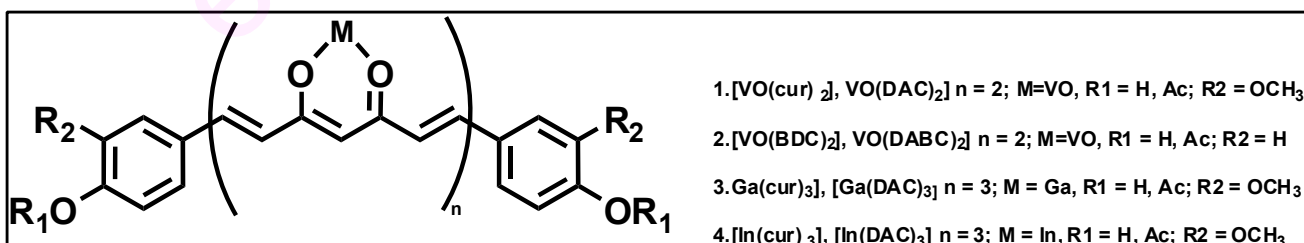
Fig. 1.12 Curcumin VO^{2+}, Ga(III) and In(III) complexes

- 4,4'-di-(O-acetyl) curcumin, 4,4'-di-(O-glycinoyl) curcumin, 4,4'-di-(O-glycinoyl-di-N-piperoyl) curcumin, 4,4'-di-(O-piperoyl) curcumin, and 4,4'-(O,O-cysteinoyl)-3,3'-dimethoxydiphenyl-1,6-heptadiene-3,5-dione are synthesized by reacting the phenolic hydroxyl of curcumin with N-phthaloylglycinoyl chloride, *p*-Nitrophenyl ester of piperic acid and Fmoc-protected cysteine respectively. These conjugates prepared are tested for their apoptotic potential on tumor cells. (Mishra, et al., 2005a).

- Curcumin β-maltooligosaccharides (Fig. 1.11) are synthesized by sequential biocatalytic glycosylation of using curcumin β-D-glucoside by Strophanthus gratus cell culture and cyclodextrin glucanotransferase (CGTase) (Shimoda, et al., 2007).

- Metal complexes of curcumin diacetyl derivatives with VO^{2+}, Ga(III), and In(III) (Fig.1.12) have been synthesized and characterized. Gallium and indium tris complexes of curcumin have much lower IC_{50} (Mohammadi, et al., 2005).

1.6 Present Study: Objectives and Outline

The solubility of curcumin in aqueous phase is a serious drawback for its use in water-based food matrices. The different strategies for derivatization made in order to achieve the solubility of curcumin in water have been discussed in previous sections. The present investigation deals with achieving the aqueous solubility of curcumin through the approach of attachment of sugar or amino acid moieties and study of the *in vitro* antioxidant, antibacterial and antimutagenic properties of such derivatives.

Results of the present investigation are described in chapters two to five. In chapter 2 (Sections 2.1 to 2.4) synthesis of curcumin glucosides and the standardization of the reaction conditions for optimization of yields have been described. Chapter 3 (Sections 3.1 to 3.4), deals with the development of a general protocol for the synthesis of curcumin amino acid conjugates. Chapter 4 includes the synthesis of curcumin glucuronide (Sections 4.1.1 to 4.1.3) and curcumin glucal derivatives (Sections 4.2.1 to 4.2.3). Chapter 5 describes the *in vitro* antioxidant, antibacterial and antimutagenic properties of curcumin and its derivatives mentioned in chapters 2, 3, and 4. The salient findings of this study are summarized.

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CHAPTER 2

SYNTHESIS OF GLUCOSIDES OF CURCUMIN

2.1 INTRODUCTION
2.1.1 Phase transfer catalysts (PTC) in organic synthesis

Phase transfer catalysis is an efficient methodology often employed for biphasic reactions in organic synthesis. Selectivity and increased reaction rates with concomitant rise in yields are its advantages. The rate enhancement of a reaction between chemical substances of preferential solubility in heterogeneous phases is effected by the action of a catalyst across the interface between the two phases. The efficiency of phase transfer catalysis is influenced by the nature of the groups in the catalyst. The advantage with use of phase transfer catalysts is their ability to form desired products in good yields, with reduction in the formation of side products. Examples of phase transfer catalysts are salts of 'onium ions' (e.g. tetraalkylammonium salts) or agents that complex inorganic cations (e.g. crown ethers). The catalyst cation soluble in aqueous phase is not consumed in the reaction but an anion exchange does occur. The attached groups are lipophilic and soluble in organic phase, which help to transport the anion from aqueous to organic phase, where the reaction takes place with the reactant (Makosza, 2000). The choice of PTC and the careful monitoring of the reaction conditions are essential features that govern the course of the reaction and determine the yields of the products. Specific protocols for the standardization of PTC methodology have been elucidated (Fiamegos & Stalikas, 2005). There are several advantages of the phase transfer catalysis systems over single-phase systems, such as an increased reaction rate, greater mass transfer between the two phases, lower reaction temperatures, lesser need for expensive anhydrous or aprotic solvents and the use of water along with an

organic solvent as the reaction medium. Also, elimination of hazardous reactants (NaH, NaNH$_2$, etc.,), simplicity of the procedures, low energy consumption and minimization of industrial wastes are few other useful attributes. The advantages of PTC for industrial processes are discussed and progress in catalysts, methodology and applications is critically reviewed (Freedman, 1986). Various trends and applications of commercial interest where PTC is involved are emphasized. The reactions of aza anions, 2-bromocarboxylic esters and epoxides have been tried under PTC conditions to yield protected α - amino acids. The standardization of reduction of allylic nitro derivatives with CS$_2$ to oximes under solid – liquid and liquid – liquid PTC conditions are reported along with the synthesis of hydrofluorinating agents for solid – liquid PTC reactions (Albanese, et al., 1999). The kinetics and mechanism of dichlorocarbene addition across the double bond under solid – liquid / liquid – liquid PTC conditions and the effect of physical factors on the reaction have been studied (Sirovski, et al., 2003)

Polyethylene glycol of different molecular weights are used as environmentally friendly reaction solvents for the synthesis of dibenz[b,*f*]-1,4-oxazepine in the presence of PTC to enhance the yields (Jorapur, et al., 2008). Kinetic model for phase-transfer catalyzed reactions involving two liquid phases and a homogeneous catalyst has been developed. The proposed new model introduces the separation of the contributions of the phase-transfer catalysis (PTC) enhanced reaction and the non-PTC reaction toward the overall conversion. The industrially important reaction, synthesis of benzaldehyde from

benzyl chloride, has been used to validate the model (Satrio & Doraiswamy, 2002). Reaction of alkyl bromides with aqueous sodium sulfide under phase-transfer catalysts (PTC) employing ultrasound affords sulfide derivatives. Synthesis of chiral polyamine based phase transfer catalysts and its role in synthesizing phenylalanine has been reported (Kano, et al., 2004). The low power microwave irradiated solid–liquid phase transfer catalysis, (MISL-PTC), has brought out enhancements of the rates of selective *O*-alkylation of sodium salt of *O*-hydroxyacetophenone with benzyl chloride by using tetra-*n*-butylammonium bromide as a catalyst for the synthesis of benzyloxyacetophenone, a pharmaceutical intermediate in the manufacture of related drug molecules. Synergistic combination of solid – liquid phase transfer catalysis in microwave irradiation showed 100% conversion of desired product (Yadav & Bisht, 2004). Microwave activation is used in most to the organic reactions where slow reacting phase transfer catalysts are involved. N-alkylations, C- alkylations, deprotonations and nuclephilic substitution reactions are also reported under microwave conditions (Deshayes, et al., 1999). Synthesis of phenylacetyl arylthioureas under microwave irradiation is reported. The effects of microwave irradiation power, catalyst and solvent been studied (Bai, et al., 2002).

2.1.2 Ultrasound in organic reactions

The first report of use of ultrasound to describe its influence on reaction rates was by Richards and Loomis using high frequencies in range of 100 - 500 KHz. High-power ultrasound can generate cavitation in which the formation,

growth and implosions of the bubbles occur within a liquid. Cavitation bubbles are vacuum bubbles created by fast moving surface on one side and an inert liquid on the other side. Cavitational collapse of the bubbles produces intense local heating. Such uses of ultrasound for chemical reactions have been grouped under the general name sonochemistry. Ultrasound does provide a distinct alternative to other, more traditional, techniques of improving reaction rates and product yields. Ultrasonic reactors normally employed in organic synthesis, are bath or probe type. In 1989, Einhorn et al., summarized many reactions irradiated by ultrasonic waves. Work on non-aqueous reaction systems has been described by Miethchen, 1992, where Friedel–crafts reactions, functionalization of carbohydrates, reverse micelle formations demonstrate the applicability of ultrasonic waves in homogeneous and heterogeneous reactions. Some other important applications of ultrasonic waves include its use in biomedical applications, manufacture of protein micro – spheres used for time released drug delivery and in making amorphous nanoparticles (Suslick, et al., 1999). Ultrasound accelerated chemical reactions, in fact, are well known and documented in literature (Mason, 1997). It is demonstrated as an alternative energy source for organic reactions normally accomplished by employing higher temperatures. Improved yields and increased selectivity are reported when ultrasound is employed in reactions of S_N1-active alkyl halides with metal thiocyanates (Bettadaiah, 2003). The use of power ultrasound in synthesis is reviewed and the cavitational chemistry, factors affecting cavitation and different

types of organic reactions under sonic conditions are well documented (Cravotto & Cintas, 2006).

2.1.3 Effect of ultrasound on phase transfer catalysis

Ultrasound as a technique of synthesis is extensively used in synthesis of organometallics, in enzyme-catalyzed reactions and many other synthetic reactions involving heterogeneous reaction mixtures. Ultrasound is employed increasingly in organic chemistry in recent times in both homogeneous as well as heterogeneous reactions. Use of ultrasound in biphasic reaction systems helps in facilitating efficient mass transfer between the two phases. Ultrasound helps in interfacial mixing of the two phases than the conventional forms of energy. This form of energy is very suitable for the reactions especially those involving heat-labile substrates. Also, application of ultrasound accelerates PTC reactions, which being biphasic can be slow. Kinetics for dichlorocyclopropanation of 1, 7-octadiene under the influence of ultrasound and phase-transfer catalysis conditions have been reported (Wang & Rajendran, 2007). Ultrasound is used in electro - organic synthesis, as it helps in the cleaning of the electrodes by enhancing mass transfer. Electro oxidation of L- di-ketone-L-sorbose into the corresponding ketogulonic acid and the direct electroreduction of acetophenone into pinacol are illustrated under sonic and PTC conditions (Cognet, et al., 2000). Reactions under ultrasonic conditions are reported to enhance the rate of PTC reactions. Canizzaro reaction under sonic conditions along with PTC leads to the greater interfacial mixing and enhanced mass transfer between the phases as showed by the accelerated reaction rates (Entezari, et al., 2000). The reaction of

oximes with dichloromethane is carried out in the presence of sodium hydroxide by combination of benzyldimethyltetradecylammonium chloride and application of ultrasonication results in considerable reduction in the reaction time compared to classical methods (Li & Li, 2007). Ultrasound and phase transfer catalysts enhances the conversion of benzamide to benzonitrile (Sivakumar, 2001).

2.1.4 Koenigs - Knorr glycosylation

Koenigs-Knorr method for the synthesis of glycosides and alkyl glycosides probably represents the most widely used procedure in the chemistry of carbohydrate derivatives. Koenigs-Knorr synthesis of glycosides under classical conditions involves formation of glycosyl halides followed by the glycosyl transfer in the presence of heavy metal salts to afford the glycoside. (Koenigs & Knorr, 1901; Goldschmid & Perlin, 1961; Sinay, 1998). Application of the Koenigs-Knorr method confers a strong glycosyl donor character to the activated species by exchange of the anomeric hydroxyl functionality by a bromine or chlorine atom in the activation step. The α-halo-substituted sugar generated in the activation step, can be readily further activated in the glycosylation step by halophilic promoters, i.e. heavy metal salts, resulting in an irreversible glycosyl transfer to the acceptor. This method is the basis of a valuable technique for the synthesis of complex alkyl glycosides, oligosaccharides or glycoconjugates.

2.1.5 Glycosylation of curcumin

Curcumin is insoluble in water, which limits its usage in many food products as well as in pharmaceuticals. The oral bioavailability of curcumin is reported to be only 1% in rat as fast excretion of curcumin is observed from the

living systems (Yang, et al., 2007). Higher plants accumulate wide range of glycosides, as secondary metabolites and are capable of conjugating sugar residues to the targeted sites. The biological activities of glycosylated components have become more important because these are widely distributed in plants and they play a key role in the detoxification and stabilization pathways. Glycosylation allows the conversion of water-insoluble compounds into corresponding water-soluble derivatives, which could improve their bioavailability and pharmacological properties. Although curcumin is insoluble in water at acidic and neutral pH, it can be made soluble under alkaline conditions, but the color of the chromophore changes to deep red and it also undergoes degradation. Its low solubility in aqueous systems is a disadvantage. The alkyl and aryl portion of the molecule makes it lipophilic and hence, soluble only in fats and organic media. In order to make it water-soluble, it was envisaged that attachment of a polar group or molecule would enhance the hydrophilicity of the molecule. This can be achieved, for example, by making suitable sugar derivatives. Known methods of preparation of curcumin sugar derivatives employ the reaction of α-D-halotetraacetoglucose with curcumin under biphasic conditions in the presence of a phase transfer catalyst at higher temperatures but these give very low yields (Hergenhahn, et al., 2003; Mishra, et al., 2005). Condensation reaction of arylaldehyde with acetyl acetone–B_2O_3 complex also gives curcumin glycoside boron complex (Mohri, et al., 2003). Attempts are also made to synthesize curcumin glucoside by enzymatic means using amyloglucosidase (Vijay kumar & Divakar, 2007) and *Catharanthus roseus* cell cultures by supplying curcumin

exogenously (Kaminaga, et al., 2003). Lower yields, higher temperatures and longer reaction times are the drawbacks of chemical and enzymatic synthetic methods reported hitherto. Recent reports are available on synthesis of oligosaccharide conjugates of curcumin (Shimoda, et al., 2007) and synthesis of PEG curcumin (Safavy, et al., 2007).

The present study aimed at synthesis of glucoside derivatives of curcumin with improved yields by addressing to the limitations of methods available in literature and looking at means to overcome these drawbacks.

2.2 EXPERIMENTAL

2.2.1 Apparatus and materials

Acetic anhydride, methanol and dimethylsufoxide were procured from s. d. fine chemicals Mumbai, India and dried as per the standard protocol (Armarego & Chai, 2003). Perchloric acid (70%), Benzyltributylammonium chloride, Cetyltributylammonium bromide, Dowex (IR-120) and Tetrabutylammonium bromide were procured from Sigma – Aldrich chemical company, USA. Alumina coated TLC plates were procured from E - Merck India Ltd. Curcumin (97 %) was procured from Spicex Pvt. Ltd., Mysore, India. Glucose, red phosphorous, bromine, dichloromethane, sodium bicarbonate, diethylether, hexane, sodium sulfate, chloroform, potassium hydroxide were procured Qualigens India Ltd. Mumbai, India. Silica gel was obtained from s. d fine chemicals, Mumbai.

^{1}H NMR spectra for the compounds were recorded on a Bruker Avance 500 MHz spectrometer using deuterated solvents, $CDCl_3$ and DMSO-d_6. Coupling constants (*J* values) are given in Hz. Mass spectral analyses of the synthetic compounds were carried out using MS (Waters Q-Tof Ultima) in the ES positive mode. Ultrasound device used for the reaction was Vibracell with a high intensity tapered probe of tip diameter - 6.5 mm from Sonics and Materials Inc., Newtown, USA. For the reactions, the ultrasound reactor was set at 25% amplitude and pulse cycle of 25 sec. (on) and 5 sec. (off) with frequency of 40 KHz and an output power of 750 W. Thin-layer chromatographic (TLC) analysis was performed on silica gel 60 F_{254} (Merck KgaA, 64271 Darmstadt, Germany) coated on alumina sheet and 3% methanol in chloroform was used as the

developing solvent. Isolation of the products was done by column chromatography on silica gel (100-200 mesh) with chloroform as the eluent. High performance liquid chromatography of these samples was carried out on reverse phase C-18 column with methanol: water (70:30) containing trifluroacetic acid (0.1%) as the mobile phase at flow rate of 1 ml/min and monochromatic detection at 423 nm.

2.2.2 Preparation of 2, 3, 4, 6-Tetra–*O*-Acetyl-α–D-Glucopyranosyl Bromide (Furaniss, 1989)

To acetic anhydride (145 g, 1.42 mol), cooled to 4 ^{0}C, perchloric acid (1 ml) was added drop-wise maintaining the temperature below 4 ^{0}C. Glucose (35 g, 0.194 mol) was then added slowly maintaining the temperature between 30-40 ^{0}C. After cooling the reaction mixture to 20 ^{0}C, red phosphorus (10.25 g) was added followed by drop-wise addition of bromine (20 ml) maintaining the temperature below 20 ^{0}C. Water (12 ml) was then added over a period of 1 h maintaining temperature below 20 ^{0}C. The reaction mixture was stirred for 2 h, diluted with dichloromethane (100 ml) and then filtered through glass wool. The organic layer was washed with a cold saturated solution of sodium bicarbonate followed by chilled water. The organic phase was filtered through activated silica gel and the solvent distilled under reduced pressure. The product was crystallized from diethyl ether - hexane (1:2) mixture. The product was mixed with 0.1 % of potassium carbonate and stored in an airtight amber colored glass bottle at 4 ^{0}C in a refrigerator. [65 g, 81 %, m. p. 87 ^{0}C, ^{1}H NMR (CDCl$_3$): 2.04 (s, 3H); 2.06 (s, 3H); 2.10 (s, 3H); 2.11 (s, 3H); 4.13(dd, 1H, *J* = 1.5 Hz and 12.5 Hz);

4.29-4.35 (m, 2H); 4.85(1H, dd, J = 4 Hz and 10 Hz); 5.17 (t, 1H, J = 9.5 Hz); 5.56 (t, 1H, J = 9.5 Hz); 6.62 (d, 1H, J = 4 Hz).

2.2.3 General procedure for the preparation of Curcumin glucoside tetraacetate (CGTA)

i) Biphasic reaction conditions

To a solution of curcumin (**1**, Fig. 2.1, 1 g, 2.7 mmol) in chloroform (40 ml), aqueous KOH (0.6 g, 10.8 mmol in 20 ml water) was added and stirred for 5- 10 min. Transfer of the yellow curcumin compound from organic to the aqueous phase indicated the formation of the potassium salt of curcumin. A solution of 2, 3, 4, 6-tetra–O-acetyl-α–D-glucopyranosyl bromide (2.2 g, 5.35 mmol) in chloroform (40 ml) was then added. To the heterogeneous reaction mixture, an aqueous solution of benzyltributylammonium chloride (0.5 g, 1.6 mmol in 20 ml water) was added. On stirring for 5-10 min, a homogeneous solution was obtained. The mixture was sonicated until the completion of the reaction (4 h), which was indicated, by phase separation, discoloration of the aqueous layer and TLC analysis of the product, which was devoid of the spot corresponding to curcumin. The organic layer was washed with water, dried over anhydrous sodium sulfate and distilled under reduced pressure. The crude sample was loaded on to silica gel (100-200 mesh) column and eluted out with $CHCl_3$. Pure fractions were pooled and evaporated to afford curcumin mono and di-β-glucoside tetraacetates (**2′ and 3′**) in 78% yield.

ii) Mono-phasic reaction conditions

Potassium salt of curcumin was prepared by the reaction of curcumin (1 g) with KOH (0.056 g) in ethanol (50 ml) under stirring for 1 h. The solvent was

distilled under reduced pressure and the salt was dried over KOH in a vacuum desiccator (1 Torr). The potassium salt of curcumin (1.1 g, 2.7 mmol) was taken along with benzyltributylammonium chloride (0.5 g, 1.6 mmol) and 2, 3, 4, 6-tetra–O-acetyl-α–D-glucopyranosyl bromide (2.2 g, 5.35 mmol) in DMSO (30 ml) and was sonicated until the completion of the reaction (20 h). Then, solvent in the reaction mixture was evaporated under reduced pressure. The residue was dissolved in chloroform and the organic layer washed with water, dried over anhydrous sodium sulfate and distilled under vacuum. The crude sample was loaded on to silica gel (100-200 mesh) column and eluted out with distilled CHCl$_3$. Pure fractions were pooled and evaporated to afford curcumin mono-β-glucoside tetraacetate in 50% yield (**2´**).

2.2.4 General procedure for the preparation of curcumin glucoside (3)

To a solution of curcumin di-β-glucoside tetraacetate *(3´,* 1 g) in dry methanol (10 ml), sodium methoxide, prepared by dissolving sodium (45 mg) in dry methanol (6 ml), was added drop-wise and the mixture was stirred. The deacetylation reaction was monitored on TLC. At the end of the reaction (0.5 h), the solution was neutralized by the addition of freshly prepared Dowex (IR-120) H$^+$ resin. The resin was filtered and the solvent distilled under reduced pressure to afford pure curcumin di-β-glucoside in 95% yield (**3**).

2.3 RESULTS AND DISCUSSION

2.3.1 Reaction of curcumin with 2, 3, 4, 6-Tetra–*O*-Acetyl-α–D-glucopyranosyl bromide

Reaction of a solution of curcumin in chloroform with aqueous KOH led to facile formation of potassium salt of curcumin indicated by the decolorization of the organic layer with concomitant transfer of the pigment to the aqueous phase. A solution of 2, 3, 4, 6-tetra–O-acetyl-α–D-glucopyranosyl bromide in chloroform was then added, which constituted a biphasic system with the glycosyl halide in the organic phase and the potassium salt of the phenolic substrate, curcumin, in the aqueous phase. Aqueous solution of benzyltributylammonium chloride was then added and the contents were stirred which resulted in the formation of a homogeneous mixture. The reaction mixture when refluxed for 12 h under stirring resulted in the separation of aqueous and organic phases with concomitant discoloration of the aqueous layer. The organic layer after work up and chromatographic separation afforded mixture of mono- and diglucoside tetracetates of curcumin in an isolated yield of 15 %. The reaction was also carried out under pressure of 10 bar and with stirring in a pressure vessel for 4 h and the reaction product purified by chromatography afforded curcumin glucoside tetracetates in 10 % yield. The results indicated that the reactions employing higher temperatures and pressures mainly led to formation of intractable products, apparently due to the labile nature of curcumin, which is prone to degradation under high temperatures and alkaline pH. This apparently affected the overall yield of the glucoside products. The use of ultrasound as an alternative source for providing the activation energy for this reaction was thus

envisaged. Also, in our earlier work on reaction of S_N1-active halides with zinc thiocyanate (Bettadaiah, et al., 2003), we showed that ultrasound accelerated the nucleophilic substitution reaction with the ambident thiocyanate nucleophile and afforded superior selectivity with respect to preponderant formation of alkyl thiocyanates. In the present work, reaction of 2, 3, 4, 6-Tetra–O-Acetyl-α–D-glucopyranosyl bromide with potassium salt of curcumin under biphasic conditions using phase transfer catalysts was explored under the influence of ultrasound for the synthesis of curcumin glucosides (Fig. 2.1), as a new approach to Koenigs-Knorr reaction. The reaction of potassium salt of curcumin with 2, 3, 4, 6-tetra–O-acetyl-α–D-glucopyranosyl bromide, accordingly, when carried out under sonochemical conditions afforded enhanced yield of the glucosides (> 65 %). This interesting finding prompted us to further probe the reaction in detail with a view to optimizing the yield and studying the selectivity of the formation of glucosides.

2.3.2 Reaction employing different phase transfer catalysts

To study the effect of role of the phase transfer catalyst in the reaction and optimize the reaction conditions, the reaction was carried out with three different phase transfer catalysts, tetra-substituted ammonium halides. The reaction with benzyltributylammonium chloride afforded higher yield of the glucosides in a reaction time of 4 h (Table 2.1, entry 1). In this reaction, the selectivity is also reflected in the preponderant formation of the diglucoside. Cetyltributylammonium bromide facilitated the completion of the reaction in 4 h but the yield of the product was less (Table 2.1, entry 2). Interestingly, here the

formation of the mono-β-glucoside was observed. However, tetrabutylammonium bromide did not catalyze the reaction. The results clearly indicated the vital role in the catalysis played by the nature of the alkyl or aryl nature of the substituents on the ternary ammonium salt. The critical requirement of an aryl moiety in the phase transfer catalyst can also be related to the structural features of curcumin.

Fig. 2.1   Curcumin and its glucoside derivatives
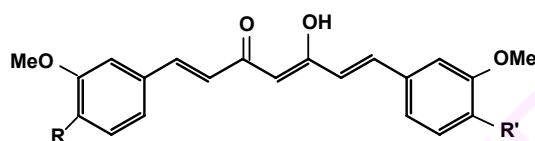
Compound	Structure number	R	R'
Curcumin	**1**	OH	OH
Curcumin mono-β-glucoside tetraacetate	**2´**		OH
Curcumin mono-β-glucoside	**2**		OH
Curcumin di -β-glucoside tetraacetate	**3´**		
Curcumin di-β-glucoside	**3**		

Table 2.1 The effect of phase transfer catalyst on the yields of ultrasound assisted reaction of potassium salt of curcumin with α-2, 3, 4, 6-tetra-*O*-acetyl-1-bromoglucose.

Entry	Catalyst[a]	Time (h)	Yield (%)	X: Y[b]
1	Benzyltributylammonium chloride	4	78	0.4: 1
2	Cetyltributylammonium bromide	4	50	1:0
3	Tetrabutylammonium bromide	10	No reaction	

[a] Salt of curcumin to catalyst ratio: 1: 0.59; [b] X and Y - Compounds **2′** and **3′** (Fig. 2.1)

Probe head

Probe Tip
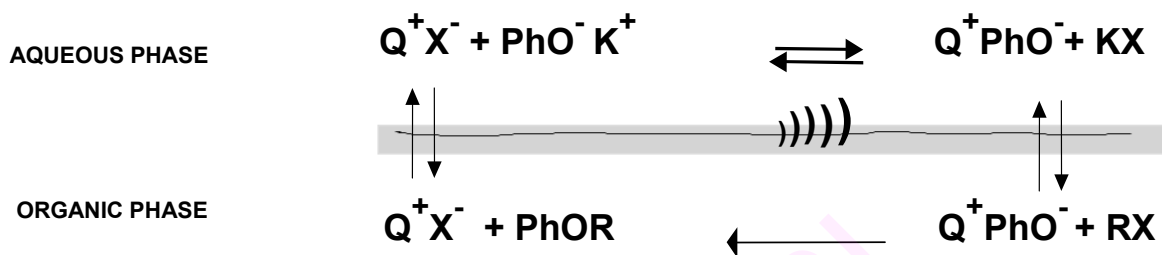
Reaction vessel

Reaction mixture

Fig. 2.2 Ultrasonic reactor used for our studies

2.3.3 Mechanism of the reaction

In heterogeneous reactions containing two immiscible liquid phases, the limited mass transfer of the reactants between the phases due to their solubility characteristics is the cause for slow reaction rates. While, phase transfer catalyst (PTC) causes homogenization of the two phases, ultrasound is known to effect cavitation and facilitate the mixing of two phases, thereby, providing high interfacial area with the formation of fine emulsion and enhancing interfacial contact between the reactants. The probe type ultrasound reactor used in the present study is depicted in Fig 2.2 and the reaction sequence envisaged is presented in Fig. 2.3.

Reaction of potassium salt of curcumin (PhO^-K^+) with the trialkylarylammonium halide ($Q^+ X^-$, PTC) in the aqueous phase results in the formation of the trialkylarylammonium salt of curcumin ($Q^+ PhO^-$). Migration of the latter to organic phase was indicated by the clarification of the aqueous phase, which almost turns colorless. In the organic layer it reacts with the halosugar (RX) to afford curcumin glucoside (PhOR). The trialkylarylammonium halide is regenerated and the reaction, which is accelerated by ultrasound, proceeds further along the above pathway. In fact, the reaction at ambient temperature under stirring without ultrasonic irradiation showed negligible product formation even after 48 h. Therefore, it is now demonstrated that Koenigs – Knorr type reaction of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-1-bromoglucose with curcumin is facilitated by combined effect of PTC and ultrasound which

promote efficient mixing along with superior mass transfer of reactants between the two phases.

$$Q^+X^- + PhO^-K^+ \rightleftharpoons Q^+PhO^- + KX$$ (AQUEOUS PHASE)

$$Q^+X^- + PhOR \longleftarrow Q^+PhO^- + RX$$ (ORGANIC PHASE)

Q   **- Quaternary ammonium salt (Phase transfer catalyst)**

PhOH   **- Curcumin**

RX   **- 2, 3, 4, 6-Tetra–*O*-acetyl-α–D-glucopyranosyl bromide**

Fig. 2.3 Mechanism of the reaction of potassium curcuminate with α-2, 3, 4, 6-tetra-*O*-acetyl-1-bromoglucose under the combined effect of ultrasound and PTC.

2.3.4 Spectral Characterization of products

Completion of the reaction in these experiments was indicated by separation of the layers and the discoloration of the aqueous phase and also confirmed by analysis of the product by TLC. The product was easily isolable from the reaction mixture by separation of the organic phase, its treatment with water, followed by drying of the organic layer and distillation of the solvent. The crude product was purified by column chromatography on silica gel. TLC of the product showed two spots with RF values of 0.75 and 0.60 respectively. The compounds were separated by column chromatography of the product on silica gel with chloroform for elution. HPLC of the products showed (Fig. 2.4) presence of two compounds with retention times of 11.7 and 15.62. Pure curcumin showed retention of 14.66 min under similar conditions. Mass spectral analysis of the product [ESI - MS] exhibited two molecular mass ions at 721.37 $[M + Na]^+$ and m/z 1051.56 $[M + Na]^+$ corresponding to mono- and diglucoside tetracetates of curcumin (Fig. 2.5).

They were characterized as the tetracetates of mono- and di-β-glucosides of curcumin by the ^{1}H NMR spectral data (**2′** and **3′,** Table 2.2, Fig. 2.6). The tetracetate of curcumin di-β-glucoside (**3′**) showed signals at 3.97 corresponding to the methoxyl and those at 6.95, 7.07 and 7.14 to aromatic protons of the curcumin moiety. While signals corresponding to the olefinic protons appear as doublets at 6.50 and 7.61 with *J* value of 16 Hz, the enolic proton appears at 5.82. The four singlets at 2.09 - 2.13 corresponded to the acetyl groups of the glucoside moiety. Protons on carbons 1 to 6 in the glucose portion of the molecule appeared at characteristic positions in the 4.25-6.66 range. 1H NMR

spectrum of mono β-glucoside tetraacetate (**2′**) exhibited similar structural features. However, the integrals for the signals of methoxyl to acetyl groups in 1:2 and 1:4 ratio and the number of C1-C6 protons of the glucose moieties characterized **2′** and **3′** as the mono and the di-β-glucoside tetraacetate derivatives of curcumin respectively.

Table 2.2 ^{1}H NMR spectral data for the curcumin glucoside tetraacetates

Compound	^{1}H NMR signals from aromatic nucleus and substituents on the aromatic moiety of aglycone	^{1}H NMR signals from hepta-3, 5-dienone moiety of aglycone	^{1}H NMR signals from glucoside moiety
Curcumin **(1)**	3.90 (s, 6H, OCH$_3$), 6.88 (d, 2H, J = 8 Hz), 7.00(s, 2H), 7. 07 (d, 2 H, J = 8.5 Hz),	6.42 (d, 2H, J = 16 Hz), 7.54 (d, 2H, J = 16 Hz), 5.75 (s, 1H),	-
Curcumin mono-β-glucoside tetraacetate **(2′)**	3.95 (s, 6H, OCH$_3$), 6.94 (d, 2H, J = 8.5 Hz), 7.06 (d, 2H, J= 1.5 Hz), 7.13 (dd, 2H, J = 1.5 and 8.25Hz).	6.49 (d, 2H, J = 16 Hz), 7.60 (d, 2H, J = 16 Hz), 5.81 (s, 1H)	2.02 (s, 3H, COCH$_3$), 2.03 (s, 3H, COCH$_3$), 2.07 (s, 3H, COCH$_3$), 2.11 (s, 3H, COCH$_3$), 4.17(dd, 1H, J= 2.5 Hz), 4.3 (dd, 1H, J= 5.5 Hz), 4.45 (d, 1H, J= 8 Hz), 5.01,(t, 1H, J= 8 Hz), 5.18 (t, 1H, J= 9.5Hz), 5.23 (t, 1H, J= 9.5 Hz), 5.97(m, 1H)
Curcumin di -β-glucoside tetraacetate **(3′)**	3.97 (s, 6H, OCH$_3$), 6.95 (d, 2H, J = 8 Hz), 7. 07 (d, 2 H, J = 2 Hz), 7.14 (dd, 2H, J = 2 and 8 Hz).	6.50 (d, 2H, J = 16 Hz), 7.61 (d, 2H, J = 16 Hz), 5.82 (s, 1H).	2.09 (s, 6H, COCH$_3$), 2.12 (s, 6H, COCH$_3$), 2.13 (s, 6H, COCH$_3$), 2.13 (s, 6H, COCH$_3$),4.25 (dd, 2H, J = 3 Hz and 7Hz), 4.40 (dd, 2H, J = 3 Hz and 6 Hz), 4.45 (dd, 2H, J = 7 Hz and 12 Hz), 5.25 (dd, 2H, J = 4.5 Hz and 5Hz), 5.58 (d, 2H, J = 4 Hz), 5.92 (br, s, 2H), 6.66 (s, 2H).
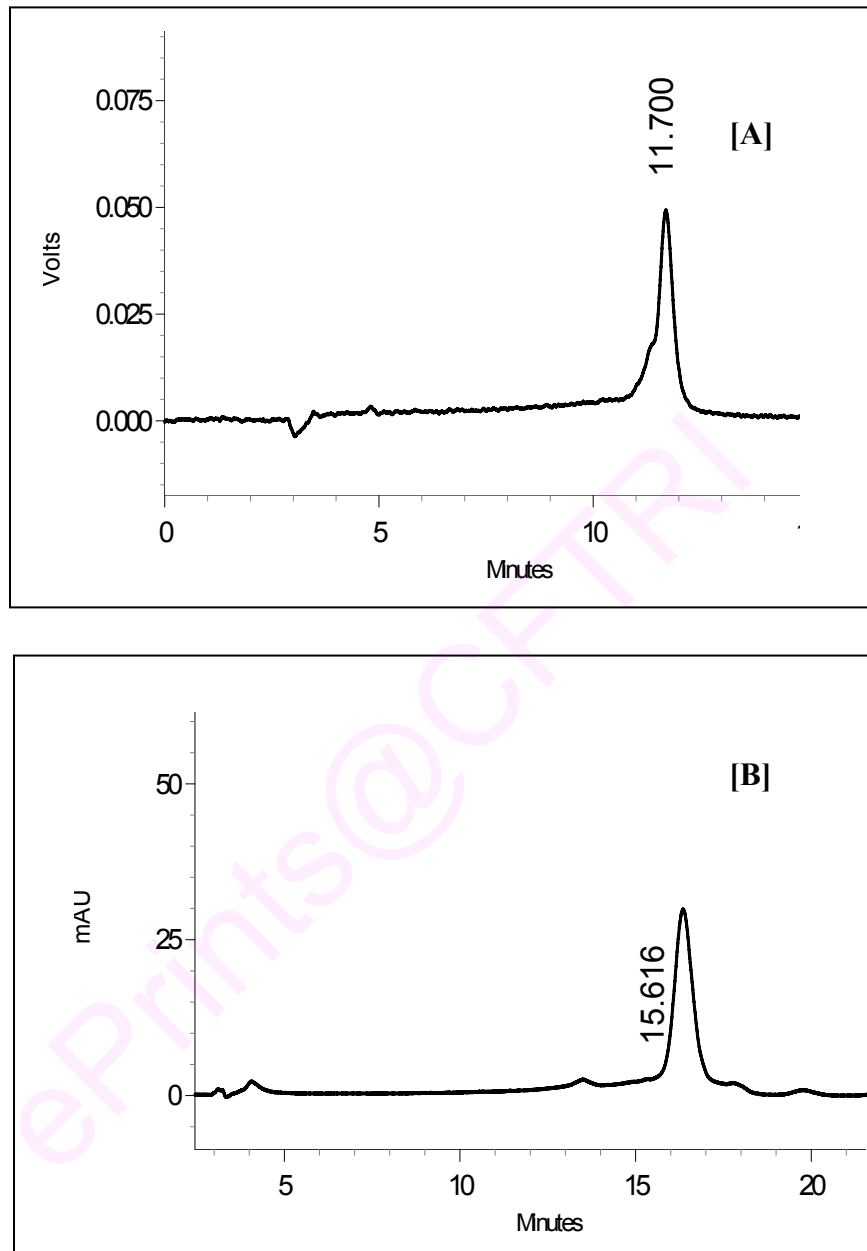
Fig. 2.4 HPLC profile of curcumin [A] mono- and [B] di-β-glucoside tetraacetates

Fig. 2.5 ESI–MS ($^+$ve) of curcumin [A] mono- and [B] di-β-glucoside tetraacetates

Aromatic region of curcumin

6.95 (d, 2H, J = 8 Hz)

7. 07 (d, 2 H, J = 2 Hz),

7.14 (dd, 2H, J = 2 and 8 Hz).

Olefinic region of curcumin

6.50 (d, 2H, J = 16 Hz),
7.61 (d, 2H, J = 16 Hz),
5.82 (s, 1H).

Sugar region of glucose
4.25 (dd, 2H, J = 3 Hz and 7Hz),
4.40 (dd, 2H, J = 3 Hz and 6 Hz),
4.45 (dd, 2H, J = 7 Hz and 12 Hz),
5.25 (dd, 2H, J = 4.5 Hz and 5Hz),
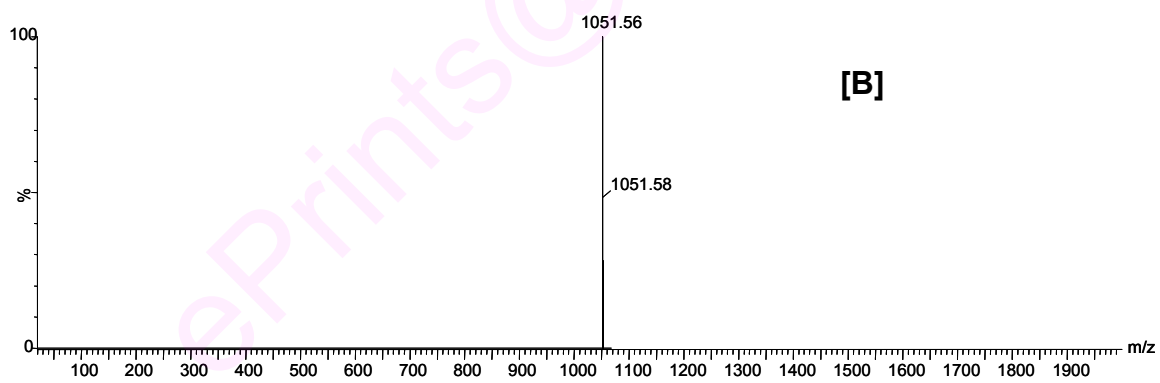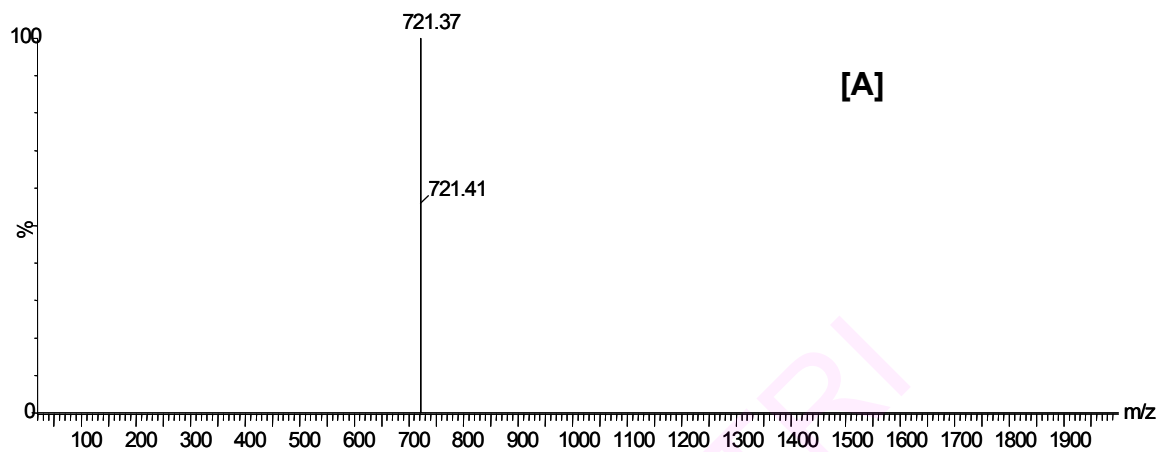5.58 (d, 2H, J = 4 Hz),
5.92 (br, s, 2H),
6.66 (s, 2H).

Acetyl region of glucose
2.09 (s, 6H, COCH3),
2.124 (s, 6H, COCH3),
2.128 (s, 6H, COCH3),
2.13 (s, 6H, COCH3),

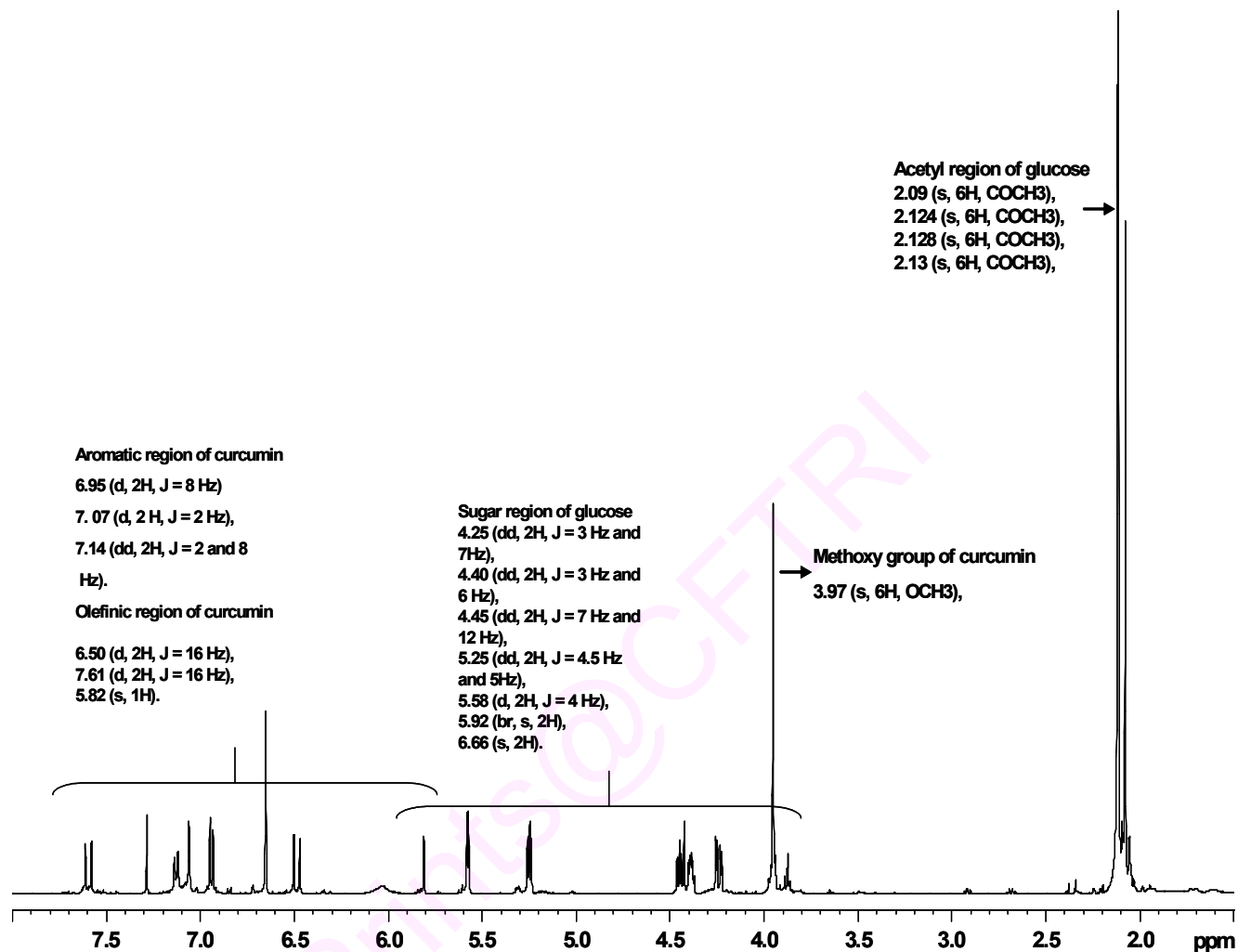**Methoxy group of curcumin**

3.97 (s, 6H, OCH3),

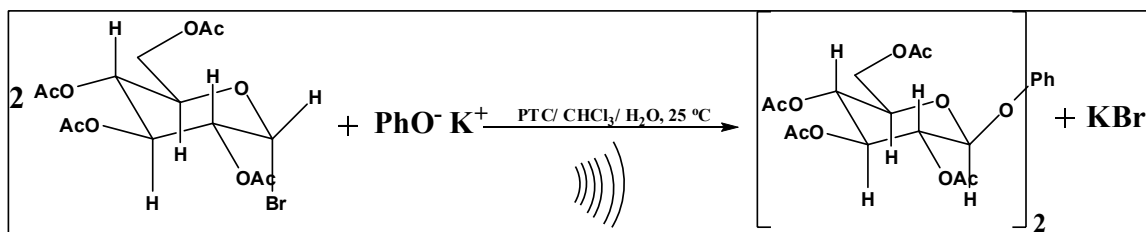**Fig. 2.6 ^{1}H NMR spectra of curcumin di β-glucoside tetraacetate**

Fig. 2.7 Ultrasound-assisted reaction of potassium salt of curcumin with α-2, 3, 4,6-tetra-*O*-acetyl-1-bromoglucose under PTC conditions

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2.3.5 Optimization of quantities of catalyst and base in the reaction

Experiments were carried out to determine the optimum quantity of the catalyst in the reaction of 2, 3, 4, 6-tetra–O-acetyl-α–D-glucopyranosyl bromide with potassium salt of curcumin. When benzyltributylammonium chloride was employed in 0.8 mmol, the reaction did not occur (Table 2.3, entry 1). Only when used in 1.6 mmol, the reaction did progress smoothly and, afforded the glucoside tetracetates in 4 h and in more than 75 % yield. Higher quantities of the catalyst neither facilitated the reaction nor improved the selectivity of the reaction. These findings suggest the amount of catalyst also is an important aspect of the reaction conditions.

Interestingly, the amount of the base, potassium hydroxide, employed for the formation of the salt of curcumin played a critical role in the reaction. Surprisingly, when the base was employed in 5 mmol (entry 1, Table 2.4), the reaction did not occur. Use of base in more than 15 mmol (entry 3, Table 2.4) led to obtaining good yields of the glucosides but the selectivity shifted more towards the formation of the mono-glucoside. Further increase of base to 21 mmol (entry 4, Table 2.4) slowed the reaction and also decreased the yield. Optimum yield and selectivity towards preponderant formation of the di-glucoside was obtained when the base was used in 10 mmol (entry 2, Table 2.4), wherein the reaction was complete in 4 h.

Table 2.3 **The effect of amount of catalyst on product yields of ultrasound assisted reaction of potassium salt of curcumin (2.7 mmol) with α- 2, 3, 4, 6-tetra-O-acetyl- 1-bromoglucose (5.35 mmol)**

Entry	Catalyst (mmol)	Time (h)	Yield (%)	X: Y
1	0.8		**NO REACTION**	
2	1.6	4	78	0.4:1
3	3.2	4	50	1:0.85
4	3.8	12	43	0.3:1

Table 2.4 Ultrasound-assisted reaction of curcumin (2.7 mmol) with α-2, 3, 4, 6-tetra-O-acetyl-1-bromoglucose (5.35 mmol) in presence of benzyltributylammonium chloride (1.6 mmol) and KOH in chloroform (80 ml) and water (40 ml)

Entry	KOH (mmol)	Reaction time (h)	Product (X: Y)	Isolated yield (%)
1	5.4	10	**No reaction**	
2	10.8	4	0.4:1	78
3	16.2	10	0.76:1	74.9
4	21.6	12	0.3:1	72.3

Table 2.5 Ultrasound-assisted reaction of potassium salt of curcumin (2.7 mmol) with α- 2, 3, 4, 6-tetra-O-acetyl 1-bromoglucose (5.35 mmol) and benzyltributylammonium chloride (1.6 mmol) in organic solvents (30 ml)

Entry	Solvent	Reaction time (h)	Product (X: Y)	Isolated yield (%)
1	Ethanol	2	1:0	21.7
2	DMSO	20	1:0.3	50.6
3	Methanol	2	1:0	36.2

X and Y - Compounds **2′** and **3′** (Fig. 2.1)

2.3.6 Reaction under mono-phasic conditions

The reaction was also investigated under mono-phasic conditions with the aryltrialkylammonium salt of curcumin produced in situ from the potassium salt of curcumin and the phase transfer catalyst. The reaction was carried out under the influence of ultrasound in alcoholic solvents and DMSO. Among these, the reaction in DMSO afforded the monoglucoside tetraacetate in twenty hours in 50 % yield (Table 2.5, entry 2). Interestingly, the selective formation of monoglucoside tetraacetate was obtained in these reactions.

2.3.7 Deacetylation of tetracetates to glucoside derivatives

Tetraacetates of mono- and di-β-glucosides of curcumin could be conveniently deacetylated using sodium methoxide in dry methanol at room temperature. HPLC analysis showed two peaks of retention time 6.25 and 5.54 (Fig. 2.8 (a) & (b)). The MS analysis of the products [ESI-MS] exhibited two molecular mass ions (Fig. 2.9) at 553.28 $[M + Na]^+$ and m/z 715.34 $[M + Na]^+$, which were further characterized as mono- and di-β-glucosides of curcumin by NMR spectral data (**2** and **3**, Table 2.6, Fig. 2.10).

The di-β-glucoside of curcumin (**3**) showed signals at 3.77 corresponding to the methoxyl and those at 7.05, 7.18 and 7.32 to aromatic protons of the curcumin moiety. While signals corresponding to the olefinic protons appear as doublets at 6.81 and 7.52 with *J* value of 16 Hz, the enolic proton appears at 6.04. The sugar protons appeared in the range 3.10-5.25 accounting for protons from two glucose moieties. Deacetylation was indicated by the absence of the four singlets at 2.09 - 2.13 corresponding to the acetyl groups seen in the

spectrum for the corresponding tetraacetate. Also, the chemical shift value of anomeric proton at 5.06, compared well with the literature value of 5.09 in curcumin β-glucoside (Kaminaga et al., 2003). The nucleophilic substitution takes place by inversion of the configuration at the anomeric position of the sugar moiety resulting in the formation of β-glucoside as shown in the reaction in Fig. 2.7.

Table 2.6 ^{1}H NMR spectral data for the glucoside derivatives of curcumin

| Compound	^{1}H NMR signals from aromatic nucleus and substituents on the aromatic moiety of aglycone	^{1}H NMR signals from hepta-3, 5- dienone moiety of aglycone	^{1}H NMR signals from glucoside moiety
Curcumin **(1)**	3.90 (s, 6H, OCH$_3$), 6.88 (d, 2H, J = 8 Hz), 7.00(s, 2H), 7. 07 (d, 2 H, J = 8.5 Hz).	6.42 (d, 2H, J = 16 Hz), 7.54 (d, 2H, J = 16 Hz), 5.75 (s, 1H).	-
Curcumin mono-β-glucoside **(2)**	3.77 (s, 6H, OCH$_3$), 7.05 (d, 2H, J= 8.5 Hz), 7.18 (d, 2H, J=8Hz), 7.32 (s, 2H).	6.81(d, 2H, J=16 Hz), 7.52 (d, 2H, J= 16 Hz), 6.04 (s, 1H).	3.6 (m, 1H), 4.31 (br, s, 1H), 4.5 (t, 1H, J = 5.5 Hz), 4.93 (d, 1H, J = 7 Hz), 4.98 (d, 1 H, J = 5 Hz), 5.06 (br, s, 1H), 5.25 (d, 1H, J = 4 Hz), 3.1-3.4 (m, 4H).
Curcumin di-β-glucoside **(3)**	3.77 (s, 6H, OCH$_3$), 7.05 (d, 2H, J= 8.5 Hz), 7.18 (d, 2H, J=8Hz), 7.32 (s, 2H).	6.81(d, 2H, J=16 Hz), 7.52 (d, 2H, J= 16 Hz), 6.04 (s, 1H).	3.6 (m, 3H), 4.31 (br, s, 1H), 4.5 (t, 2H, J = 5.5 Hz), 4.93 (d, 2H, J = 7 Hz), 4.98 (d, 2 H, J = 5 Hz), 5.06 (br, s, 2H), 5.25 (d, 2H, J = 4 Hz), 3.1-3.4 (m, 8H).
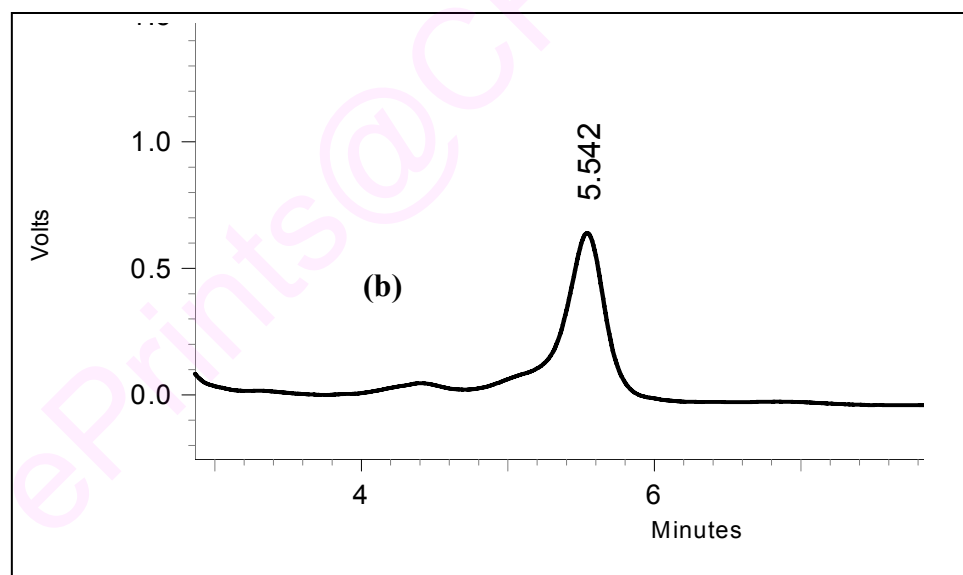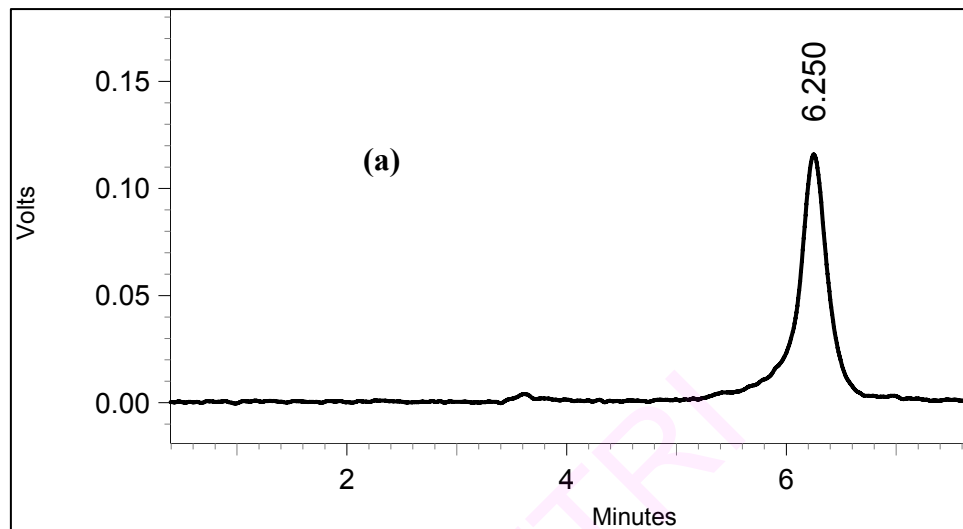
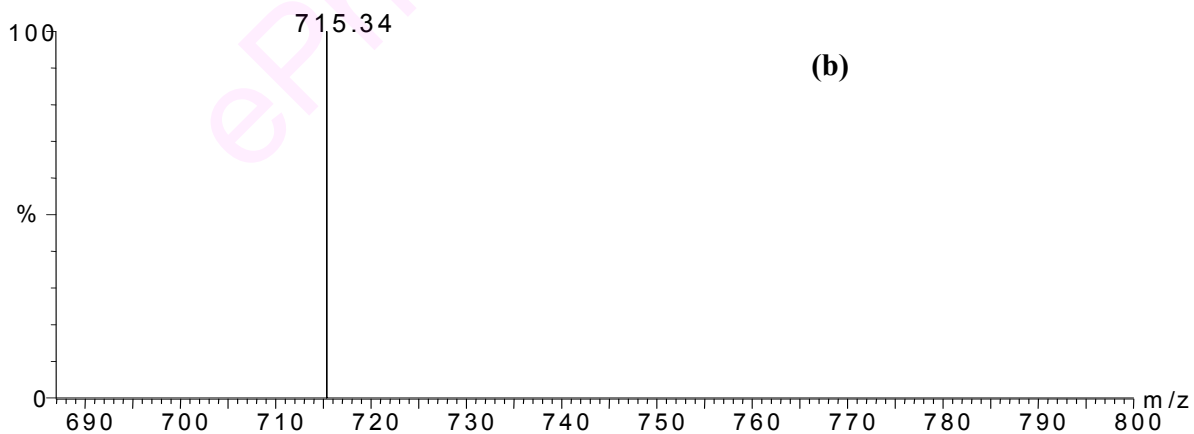**Fig. 2.8 HPLC profile of curcumin mono- and di-β-glucosides**
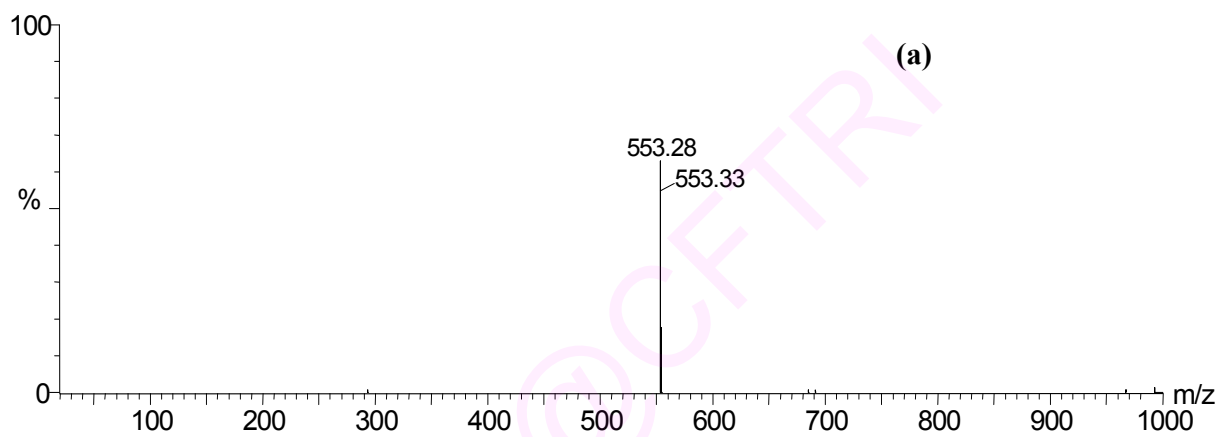
Fig. 2.9 ESI-MS of curcumin mono- and di-β-glucosides

Methoxy group of curcumin
3.77 (s, 6H, OCH3)

Sugar protons of glucose

3.6 (m, 3H)

4.31 (br, s, 1H)

4.5 (t, 2H, J = 5.5Hz)

4.93 (d, 2H, J = 7Hz)

4.98 (d, 2 H, J = 5 Hz),

5.06 (br, s, 2H)

5.25 (d, 2H, J = 4 Hz)

3.1-3.4 (m, 8H)

Aromatic region of curcumin

7.05 (d, 2H, J= 8.5 Hz),
7.18 (d, 2H, J= 8Hz),
7.32 (s, 2H)

Olefinic region of curcumin

6.81(d, 2H, J=16 Hz),
7.52 (d, 2H, J= 16 Hz),
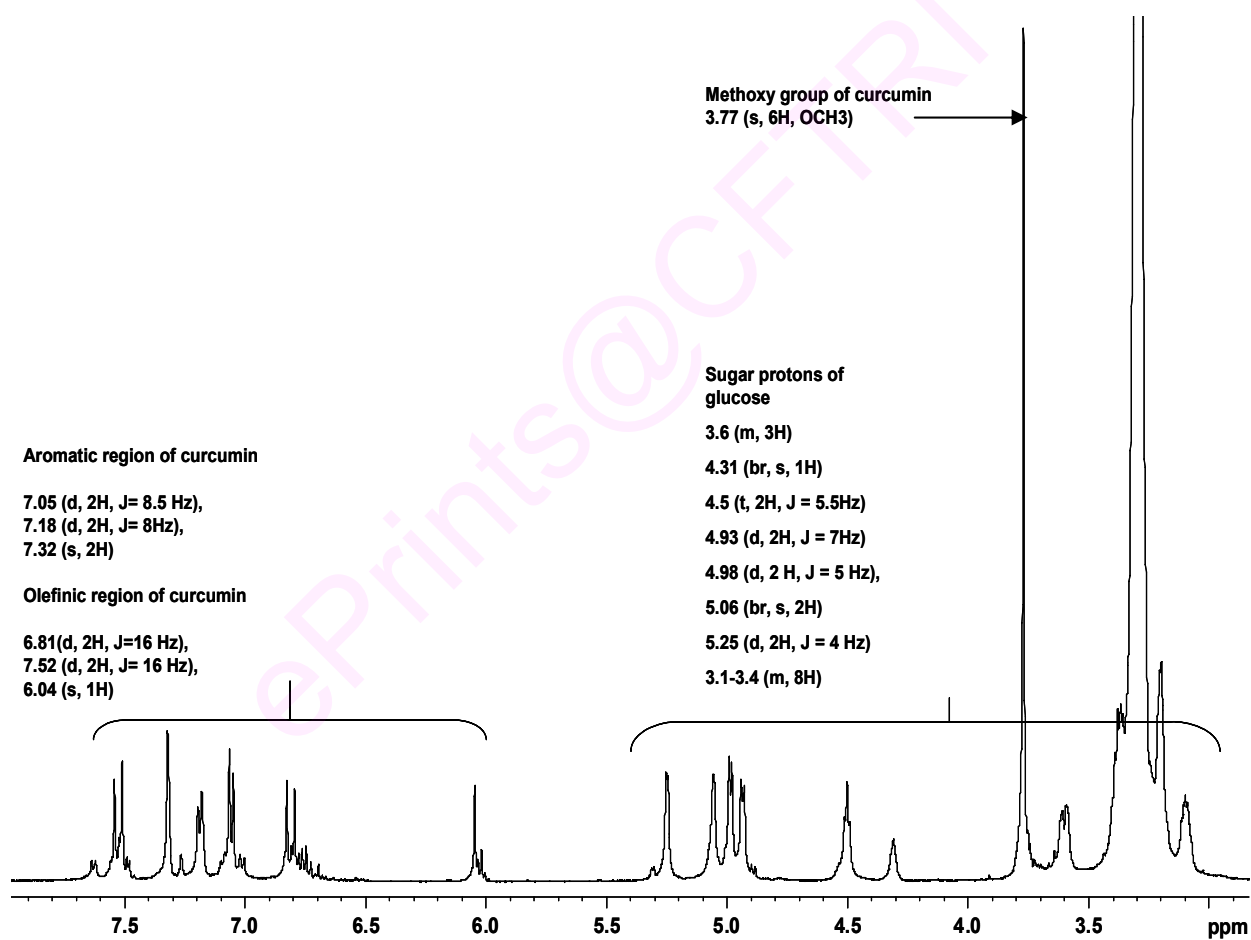6.04 (s, 1H)

Fig. 2.10 ^{1}H NMR spectra of curcumin di-β-glucoside

2.4 CONCLUSIONS

Ultrasound brought about acceleration and increase in yields of the curcumin glucosides in the Koenigs – Knorr type reaction of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-1-bromoglucose with the potassium salt of curcumin [bis-1, 7-(3'-methoxy-4'-hydroxy)phenyl-5-hydroxy-1,4,6-heptatrien-3-one] under the biphasic reaction conditions in the presence of benzyltributylammonium chloride as a phase transfer catalyst. The reaction was carried out conveniently at ambient temperature overcoming the drawbacks of the earlier methods, employing heat in presence of a base, which led to decomposition of the labile curcumin substrate and resultant lower yields. The importance of the role of the nature and quantity of the phase transfer catalyst in the reaction has been underscored in this study. The reaction was stereo-selective leading to the preponderant formation of either mono- or di-β-glucoside tetracetates of curcumin under controlled conditions in mono- and biphasic reactions respectively.   This work establishes a simple synthetic methodology for the glucoside derivatives of curcumin in high yields and selectivity using ultrasonic waves.

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CHAPTER 3

SYNTHESIS OF AMINO ACID

CONJUGATES OF CURCUMIN

3.1 INTRODUCTION

Curcumin (diferuloyl methane), the natural yellow pigment in turmeric, is isolated from the rhizomes of the plant *Curcuma longa*. It constitutes about 3–4% of the composition of turmeric, used in Indian cuisine mainly for its coloring and flavoring characteristics. Curcumin is the major coloring constituent with several important biological attributes. It is soluble in organic solvents like acetone and dioxane but is practically insoluble in water at acidic or neutral pH. The pK_a values for the dissociation of the three acidic protons in curcumin are 7.8, 8.5 and 9.0. Curcumin is used worldwide for food, medicinal and nutraceutical purposes (Aggarwal, et al., 2005; Joe, et al., 2004; Sharma, et al., 2005). It has also potent pharmacological activities such as to protect cells from β-amyloid insult in Alzheimer's disease and cancer preventive properties (Park & Kim, 2002; Ishida, et al., 2004). Biological activities of curcumin chelated to metal ions as well as antioxidant effects of curcumin are also reported (Jiao, et al., 2006). Anti-inflammatory properties of curcumin are reported in literature (Chainani, 2003). The antioxidant and anti-inflammatory properties of curcumin and their mode of action are described (Menon & Sudheer, 2007). Curcumin exhibits great promise as a therapeutic agent, and is currently used in human clinical trials for a variety of conditions, including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, colon cancer, psoriasis and Alzheimer's disease (Hatcher, et al., 2008). Pharmacological attributes of curcumin in animal models are discussed (Ammon & Wahl., 1991).

Curcumin and its derivatives have been shown to possess a number of bioactive attributes, especially antioxidant, anti-inflammatory and anti-carcinogenic properties (Srimal, 1997). Antioxidant properties of a number of ring-substituted analogs of curcumin show that the highest antioxidant activity is obtained when the phenolic group is hindered sterically viz., by the introduction of two methyl groups at the *ortho* position. This and several other compounds are more active than the standard antioxidants, α-tocopherol and "trolox" (Venkatesan & Rao, 2000). Curcumin analogs prepared by replacing the –OH group of the phenolic group with methoxyls and few are synthesized by replacing methoxyl with -OH. Derivatives bearing multiple – OH groups on the phenolic rings of curcumin show high anti – hemolysis activity (Deng, et al., 2006). A study also reports comparison of the relative electrophilicity of diketone, pyrazole and isooxazole derivatives of curcumin (Amolins, et al., 2009).

The drawback of curcumin is related to its poor solubility in aqueous systems. Though it is soluble under aqueous alkaline conditions, the color of the chromophore not only changes to deep red but it also undergoes degradation. The alkyl and aryl portion of the molecule renders it lipophilic and hence, soluble only in fats and organic media. Attachment of a polar group or molecule, for example by making suitable sugar derivatives, would enhance the hydrophilic nature of the molecule. Basically, the Koenigs-Knorr synthesis of phenolic glycosides under classical conditions involves formation of glycosyl halides followed by the glycosyl transfer to a phenol in the presence of heavy metal salts. Reaction of α-D-tetraacetohaloglucose with curcumin under biphasic conditions

in the presence of a phase transfer catalyst at higher temperatures is reported, but these reactions afford very low yields (Hergenhahn, et al., 2003; Mishra, et al., 2005). Synthesis of mono- and di-β-glucosides of curcumin in high yields by ultrasound-assisted reaction of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide with potassium salt of curcumin under phase transfer catalytic conditions is reported (Parvathy & Srinivas, 2008).

Synthesis of selected amino acid conjugates of curcumin are reported in literature. Curcumin bioconjugates, viz. di-*O*-glycinoylcurcumin, di-*O*-glycinoyl-*C*[4]-glycylcurcumin, 5'-deoxy-5'-curcuminylthymidine and 2'-deoxy-2'-curcuminyluridine are synthesized and characterized by ^{1}H NMR. Derivatives are tested in comparison with curcumin particularly against β-lactamase-producing microorganisms. Study proves the efficacy of curcumin and its bioconjugates and as antibacterial compounds. (Kumar, et al., 2001; Kumar, et al., 2000).

Curcumin bioconjugates viz. 4,4'-di-O-glycinoyl-curcumin, 4,4'-di-O-D-alaninoylcurcumin, 4,4'-di-O-(glycinoyl-di-N-piperoyl)-curcumin, 4,4'-di-O-piperoyl curcumin, curcumin-4,4'-di-O-β-D-glucopyranoside, 4,4'-di-O-acetyl-curcumin along with piperoyl glycine, have been synthesized by condensation of curcumin with *N*-phthaloyl glycinoyl chloride in anhydrous pyridine and characterized by ^{1}H NMR. The enhanced activity of these bioconjugates against different bacteria and fungi compared to curcumin may be due to improved cellular uptake or reduced metabolism of these bioconjugates resulting in building up of concentration inside the infected cells (Mishra, et al., 2005). Formation of an

active intermediate like *p*-nitrophenyl ester has been envisaged for preparation of the mixed conjugates.

Derivatives of curcumin, i.e., 4,4'-di-(*O*-glycinoyl) curcumin, 4,4'-di-(*O*-glycinoyl-di-*N*-piperoyl) curcumin, 4,4'-di-(*O*-piperoyl) curcumin, and 4,4'-(*O*,*O*-cysteinoyl)-3,3'-dimethoxydiphenyl-1,6-heptadiene-3,5-dione have been described and their apoptotic potential on tumor cells demonstrated. The results indicate the activities are attributed to the cellular uptake and enzymatic degradation of curcumin derivatives to yield free curcumin and also to the chemical nature of the derivative and length of the attached moiety (Mishra, et al., 2005a). These conjugates with ester, peptide, thiol and disulfide links show enhanced bioactivities (Kapoor, et al., 2007). Synthesis of telomerase targeted anticancer bioactive pro-drug DNA-curcumin-tetraglycine by antisense-based approach is reported (Kapoor, et al., 2007a).

However, applicability of these methods, to general synthesis of other amino acid conjugates of curcumin are not demonstrated. In the present investigation, synthesis of several amino acid conjugates was studied with emphasis on the development of a general method with superior yields.

3.2 EXPERIMENTAL

3.2.1 Materials and equipment

Dimethylsulfoxide (DMSO), procured from s. d. fine chemicals Mumbai, India, was dried as per the standard protocol (Armarego & Chai, 2003). All the t-Boc amino acids, dicyclohexylcarbodiimide, dimethylaminopyridine and trifluoroacetic acid were procured from Sigma –Aldrich chemical company, USA. Alumina coated TLC plates were procured from E - Merck India Ltd. Curcumin (97 %) was procured from Spicex Pvt. Ltd., Mysore, India. Dichloromethane, triethylamine, hexane, sodium sulfate, chloroform, potassium hydroxide and potassium carbonate were procured from Qualigens India Ltd. Mumbai, India. Silica gel was obtained from s. d. Fine chemicals, Mumbai, India.

Ultrasound reactor used was Vibracell-750 with tapered probe of tip diameter 6.5 mm from Sonics and Materials Inc., Newtown, USA and was set at 25% amplitude and pulse cycle of 25 s (on) and 5 s (off) with frequency of 40 KHz and an output power of 750 W. Ultrasound bath used was Bransonics-1510E-DTH from Branson Ultrasonics Corporation, Daburry, USA and with frequency of 20 KHz. ^{1}H NMR spectra for the compounds were recorded on a 500 MHz NMR spectrometer (Bruker Avance, Reinstetten, Germany) using, $CDCl_3$ and CD_3OD solvents. Chemical shift values and coupling constants are given in δ and Hz respectively. Mass spectral analyses of compounds were carried out using MS (Waters Q-Tof Ultima, Manchester, UK) in the ESI positive mode. Thin-layer chromatographic (TLC) analysis was performed on silica gel 60 F_{254} (Merck, Germany) coated on alumina sheet with 3% methanol in chloroform as the developing solvent. Samples were analyzed on HPLC (Waters, LC 10 A,

USA) using a reverse phase C-18 column with methanol : water (70:30) containing trifluroacetic acid (0.1%) as the mobile phase at flow rate of 1 ml/min and monochromatic detection at 423 nm.

3.2.2 General method for the synthesis of curcumin *t* -Boc amino acid Conjugates

To the solution of curcumin (2.71 mmol) in dry dioxane (10 ml), a *t* -Boc amino acid (Table 3.1) (5.41 mmol) was added followed by the addition of 4-dimethylaminopyridine (2.70 mmol) and triethylamine (1 ml) and stirred under nitrogen atmosphere for 5 min; to the reaction mixture *N,N'*-dicyclohexylcarbodiimide (4.85 mmol) was added. The mixture was stirred at 25 ^{0}C-30 ^{0}C until the completion of the reaction (8-12 h) as indicated by TLC, where the disappearance of curcumin was observed. The organic layer was filtered to remove dicyclohexylurea formed as a byproduct of the reaction. The filter paper was washed with chloroform and the filtrate was diluted with chloroform (50 ml) followed by washing with water (100 ml). The organic layer thus collected was dried over anhydrous sodium sulfate and evaporated to dryness under vacuum. The residue was dissolved in chloroform (10 ml), to this hexane (20 ml) was added to remove residual dicyclohexylurea (DHU) by decantation. The compound, separated as a precipitate during the process, was purified on column chromatography on silica gel (200-400 mesh) with chloroform as the eluent. Initially, TLC checked the isolated product for its purity. The pure fractions were pooled. Yields of the various curcumin *t* -Boc amino acid conjugates synthesized are presented in Table 3.1.

3.2.3. General method for deprotection of curcumin-*t*-Boc amino acid conjugates

The deprotection of curcumin *t*-Boc amino acid conjugate (1 g) was done with 10% TFA in dichloromethane (5 ml) under ultrasound (10 min). The mixture in dichloromethane was neutralized by potassium carbonate. The product separated as a precipitate as it was insoluble in dichloromethane. It was filtered and dried over KOH in a vacuum desiccator under reduced pressure (1 torr).

3.2.4 Synthesis of amino acid conjugates- Representative examples

(i) 1,7-Bis(4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4)

To a solution of **1** (1 g, 2.71 mmol) in dioxane (10 ml), *t*-Boc leucine (1.25 g, 5.41 mmol) was added followed by the addition of dimethylaminopyridine (0.33 g, 2.70 mmol) and triethylamine (1 ml). The mixture was stirred under nitrogen atmosphere for 5 min; dicyclohexylcarbodiimide (1 g, 4.85 mmol) was then added. The reactants were stirred until the completion of the reaction (8 h), which was checked by TLC for the disappearance of curcumin. The organic layer was filtered to remove dicyclohexylurea (DHU) formed as a byproduct in the reaction. The filter paper was washed with chloroform (10 ml) and the filtrate was diluted with chloroform (50 ml) followed by washing with water. The organic layer thus collected was evaporated to dryness under vacuum. The residue (2.5 g) after drying was redissolved in chloroform (10 ml). To this hexane (20 ml) was added to remove residual DHU in the product by decantation. The compound that precipitated out (2 g) during the process was loaded on to the column chromatography on silica gel (200-400 mesh) with chloroform as the eluent. TLC checked the isolated product for its purity. The pure fractions were pooled and

evaporated to dryness. Isolated product yield was 1.7 g (79 %), R_f 0.58 (CHCl$_3$ – MeOH: 95:5), m. p. 83 – 85°C, HPLC retention time -13.16.

Curcumin *t*-Boc lecuine conjugate (1 g) was dissolved in dry dichloromethane (10 ml). To it trifluoroacetic acid (10 %) in dichloromethane (5 ml) was added. The solution was sonicated until the completion of the reaction (10 min) as indicated by the TLC analysis of the product. The product (**4**, Table 3.3), being insoluble in dichloromethane, precipitated out of the solution of dichloromethane after the removal of *t*-Boc group. Isolated product yield was 0.9 g, R_f 0.5 (CHCl$_3$ – MeOH - 50:50), m. p. 161 – 163 ^{o}C. HPLC retention time - 6.33.

ii) 1,7-Bis(4-*O*-L-alaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (6)

To curcumin (1 g, 2.71 mmol) in dry dioxane (10 ml), *t*-Boc alanine (1.026 g, 5.42 mmol) was added followed by the addition of dimethylaminopyridine (0.33 g, 2.70 mmol) and triethylamine (1 ml). The contents were stirred under nitrogen atmosphere for 5 min; to the reaction mixture dicyclohexylcarbodiimide (1 g, 4.85 mmol) was added. The mixture was stirred until the completion of the reaction (8 h), which was indicated by TLC. The organic layer was filtered to remove dicyclohexylurea formed as a byproduct of the reaction. The filter paper was washed with chloroform and the filtrate was diluted with chloroform followed by washing with water. The organic layer thus collected was evaporated to dryness under vacuum. The residue (2.56 g) after drying was re-dissolved in chloroform (10 ml). To this hexane (20 ml) was added to remove residual DHU in the product by decantation. The compound that precipitated out (2.16 g) during the

process was loaded on to the column chromatography on silica gel (200-400 mesh) with chloroform as the eluent. TLC checked the isolated product for its purity. The pure fractions were pooled and evaporated to dryness. The isolated yield was 1.6 g (84 %), R_f 0.61 (CHCl$_3$ – MeOH: 95:5), m. p. 78 – 80 ^{o}C. HPLC retention time- 8.05.

The *t* -Boc-alanine conjugate of curcumin (**6´**, Table 3.1, 1g) was dissolved in dry dichloromethane (10 ml) and to this 10% trifluoroacetic acid in dichloromethane was added (5 ml). The solution was sonicated and the reaction was monitored on TLC for the completion. After the deprotection of *t* -Boc group, the conjugate (**6**) exhibited insolubility in dichloromethane and precipitated out. The product was filtered and washed with dichloromethane (20 ml). Product yield was 0.9 g, m.p. 133 – 135°C. HPLC retention time-5.06.

3.3 RESULTS AND DISCUSSION

3.3.1 Reaction of curcumin with *t*-Boc amino acids

Protection of amino group in amino acids by *t*-Boc moiety was envisaged in their reaction with curcumin. The *t*-butoxycarbonyl (Boc) is one of the most frequently used protective groups used in organic synthesis due to its chemical stability to basic and mildly acidic conditions and its ease of removal under specific conditions. The α-amino group of the amino acid, after protection by a *t*-butoxycarbonyl (BOC) group, reduces the nucleophilicity of the nitrogen (Green & Wuts, 1999). In case of serine, it was taken with *t*-Boc protection of the amino moiety, but the –OH group was unprotected. Reaction of curcumin with *t*-Boc protected amino acids was carried out in dry dioxane in the presence of a dehydrating agent like *N,N'*-dicyclohexylcarbodiimide with 4-dimethylaminopyridine (DMAP) and triethylamine (TEA) as catalysts. The reaction of –COOH with Ph-OH group assisted by dicyclohexylcarbodiimide (DCC) is shown in Fig. 3.1

The reaction was tried in methanol, DMSO and dichloromethane solvents but the product formation was very low. This was apparently due to low solubility of curcumin in these solvents. The high solubility of curcumin in dry dioxane helped in overcoming this limitation. The reaction was carried out in presence of individual catalysts DMAP and triethylamine, but was found to be very slow. However, when the reaction was carried out in presence of both DMAP and triethylamine, the reaction was found to be faster and could go to completion. The reaction was carried out under nitrogen atmosphere at ambient temperature

as employing higher reaction temperatures resulted in very low product yields. Accordingly, the reaction of curcumin with different *N*–protected amino acids was carried out at 25 ^{0}C –30 ^{0}C under stirring until completion of the reaction (8-12 h). Facile reaction work up protocol included initial removal of dicyclohexylurea (DHU) formed in the reaction by filtration. The reaction mixture was washed with water and the compounds were extracted in chloroform. The chloroform layer was washed with water (2-3 times), dried and distilled under reduced pressure. The crude conjugates thus obtained were purified by column chromatography on silica gel of 200-400 mesh with $CHCl_3$ as eluent. The pure fractions were collected and dried.

3.3.2 Curcumin-*t*-Boc amino acid conjugates

HPLC and MS analyzed all the curcumin-amino acid conjugates synthesized with *t*-Boc *N*-protected amino acids. Isolated yields, their melting points, HPLC retention times and molecular masses are presented in Table 3.1. The product yields were in the range of 70-84% in case of several amino acids (**4′**, **5′**, **6′**, **7′**, **8′**, **10′** & **13′**, Table 3.1). Only in case of serine, glycine and phenylglycine (**9′**, **11′** & **12′**, Table 3.1) the yields of the conjugates were in 50–66% range.

Further, NMR characterized the compounds and the ^{1}H spectral data is presented in Table 3.2. The *t*-Boc group showed characteristic –C$\underline{H}_3$ chemical shifts between 1.4-1.5 corresponding to eighteen protons of tertiary methyl groups of two *t*-Boc moieties indicating the coupling at both the phenolic –OH positions of curcumin. In the case of conjugates of curcumin with amino acids

such as leucine (**4´**), isoleucine (**7´**) and valine (**8´**) signals corresponding to twelve protons were seen for four methyl groups. In the spectrum of curcumin-alanine conjugate, signals for six protons corresponding to two methyl groups were present. The compounds showed characteristic signals for two –OCH$_3$ groups of curcumin between 3.7 - 3.9. The conjugates showed distinct signals pertaining to aromatic region of curcumin at 6.8 – 7.64. The enolic proton of curcumin was seen in the region from 5.75 – 6.22. All amino acids exhibited characteristic signals for other protons related to the different functional groups. In the case of **5´** and **11´**, the conjugates of curcumin with amino acids having phenyl substitution, the aromatic protons were seen in the 7.26 – 7.55 range (Table 3.4).
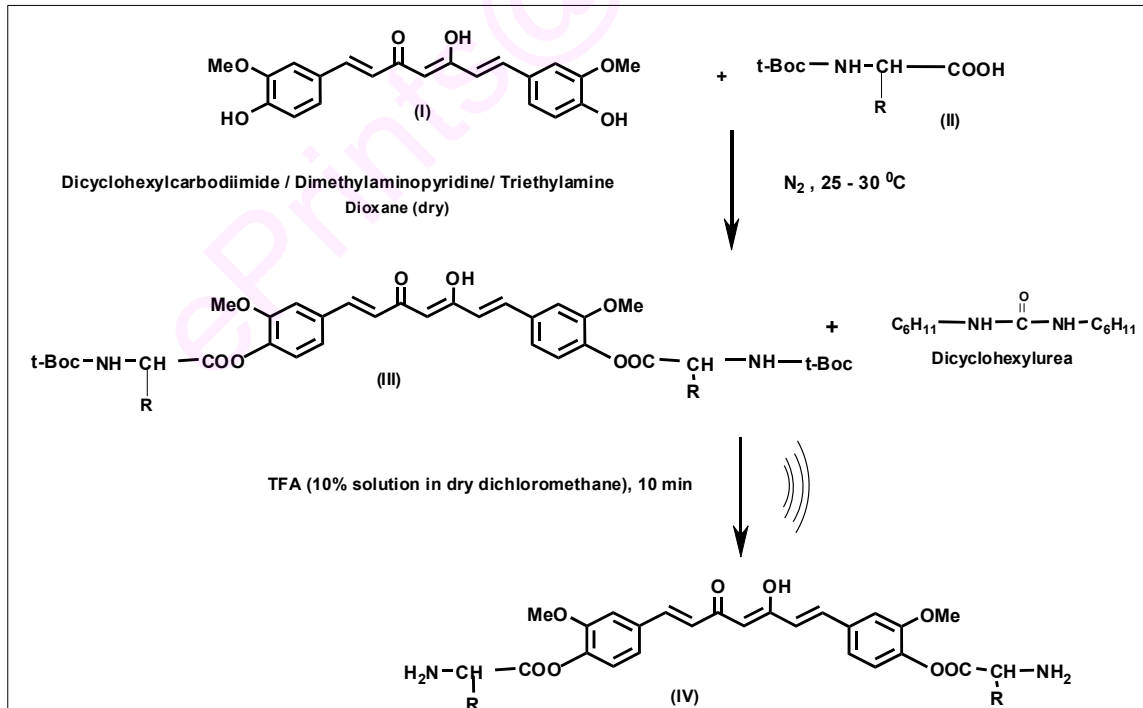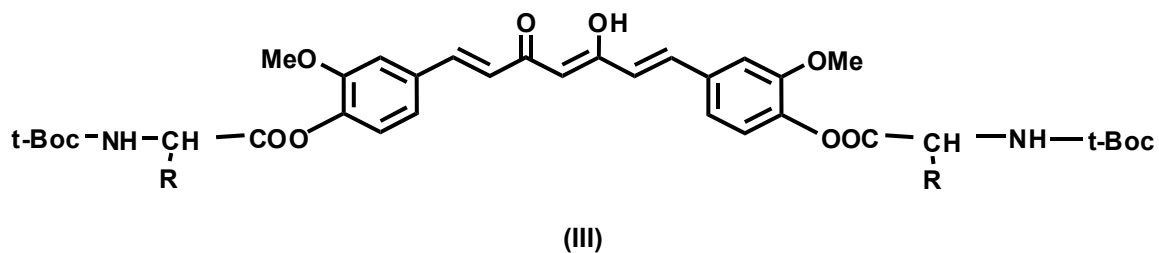

Fig. 3.1 Synthesis of curcumin amino acid conjugates

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Table 3.1 *t*-Boc Amino acid conjugates of curcumin

(III)

| Compound	Structure III, R =	Isolated Yield (%)	Retention time (Min)	Mass (M^++23)	m p. $^\circ$C
4´	H₃C–CH–CH₂— / H₃C	79	13.16	817	83-85
5´	(benzyl) CH₂—	75	7.59	885	69-70
6´	—CH₃	84	8.05	733	78-80
7´	CH₃ / H₃C—CH₂—CH—	70	18.38	817	79-80
8´	H₃C / C— / H₃C H	72	13.25	789	66-68
9´	HO—CH₂—	50	10.57	765	58-60
10´	HS—CH₂—	78	6.30	973	72–74
11´	(phenyl)	66	16.26	857	90–92
12´	—H	63	13.08	705	82–85
13´	—CH₂—CH₂—CH₂— (as part of a pyrolidine ring)	78	8.06	786	85-87

Table 3.2. ^{1}H NMR spectral data for the curcumin *t*-Boc amino acid conjugates

Compound	^{1}H NMR signals from aromatic nucleus and substituents on the aromatic moiety of aglycone	^{1}H NMR signals from hepta-3, 5- dienone moiety of aglycone	^{1}H NMR signals from Boc amino acid moiety
1	3.90 (s, 6H, OCH$_3$), 6.88 (d, 2H, J = 8 Hz), 7.00 (s, 2H), 7. 07 (d, 2 H, J = 8.5 Hz),	6.42 (d, 2H, J = 16 Hz), 7.54 (d, 2H, J = 16 Hz), 5.75 (s, 1H),	-
4′	3.88 (s, -OCH$_3$, 6H), 7.08 –7.19 (m, 6H)	5.88 (s, 1H), 6.59 (d, 2H, J = 16 Hz), 7.64 (d, 2H, J = 16 Hz).	1.05 (d, 12 H, J = 6 Hz), 1.48 (s, 18 H), 1.68 (d, 2 H, J = 8.5 Hz), 1.9 (d, 4H, J = 7 Hz), 4.60 (dd, 2H, J = 9.5 Hz),
5′	3.89 (s, 6H, -OCH$_3$), 7.03 -7.08 (m, 2H), 7.14 - 7.19 (m, 4H),	5.87 (s, 1H), 6.58 (d, 2H, J = 16 Hz), 7.64 (d, 2H, J = 16 Hz).	1.41 (s, 3H, -CH$_3$), 1.44 (s, 12 H, -CH$_3$), 1.47 (s, 3H, -CH$_3$), 3.24 (dd, 1H, J = 6 and 14 Hz), 3.36 (dd, 1H, J = 6 and 14 Hz), 3.72 (br, 2H), 4.90 (d, 1H, J = 6 Hz), 5.04 (d, 1H, J = 6 Hz), 7.26 –7.37 (m, 10 H)
6′	3.88 (s, 6H, -OCH$_3$), 7.02 –7.20 (m, 6H)	5.88 (s, 1H), 6.59 (d, 2H J = 16 Hz), 7.64 (d, 2H, J = 16 Hz).	1.60 (d, 6H, J = 7.5 Hz, -CH$_3$), 1.49 (s, 18H, -CH$_3$), 4.62 (br, 2H)
7′	3.87 (s, 6H, -OCH$_3$), 7.21 (d, 2H, J = 8 Hz), 7.40 (d, 2H, J = 8 Hz), 7.59 (br, 2H),	6.22 (s, 1H), 6.59 (d, 2H, J = 16 Hz), 7.63 (d, 2H, J = 16 Hz)	1.49 (s, 18H, -CH$_3$), 1.21 (d, 6H, -CH$_3$, J = 6 Hz), 1.25 (d, 6H, -CH$_3$, J = 6 Hz), 3.72 (s, 1H), 4.13 (s, 1H), 4.23 (s, 2H), 4.58 (dd, 1H, J = 4 and 9 Hz), 4.98 (d, 1H, J = 7.5 Hz), 5.12 (d, 2H, J = 9.5 Hz).
8′	3.86 (s, 6H, -OCH$_3$) 7.06 – 7.09 (m, 2H), 7.10 – 7.17 (m, 4H),	5.85, (br, 1H), 6.58 (d, 2H, J = 16 Hz),	1.06 (d, 6H, J = 6.5 Hz, –CH$_3$), 1.11 (d, 6H, J = 6.5 Hz, -CH$_3$), 1.49 (s, 18H –CH$_3$), 3.72 (s, 2H), 3.94 (s, 2H).
9′	3.72 (s, 6H –OCH$_3$), 6.81-6.86 (m, 2H), 7.12 – 7.15 (m, 2H), 7.24-7.39 (m, 2H),	6.05 (s, 1H), 6.63 – 6.70 (m, 2H), 7.58 – 7.65 (m, 2H)	1.44 –1.52 (m, 18 H, – CH$_3$), 3.21 (d, 2H, J = 3.5 Hz), 3.88 – 3.89 (m, 4H).
10′	3.88 (s, 6H, -OCH$_3$), 7.13-7.15 (m, 2H), 7.17-7.20 (d, 4H, J = 3 Hz),	5.88 (s, 1H), 6.59 (d, 2H, J = 16 Hz), 7.63 (d, 2H, J = 16 Hz)	1.49 (br, 18H, -CH$_3$), 1.38 (s, 18H), 3.3-3.43 (m, 4H), 4.92 (d, 2H, J = 5 Hz).
11′	3.72 (s, 6H, -OCH$_3$), 6.94 (d, 1H, J = 8 Hz), 7.02 (d, 1H, J = 8 Hz), 7.06 (d, 2H, J = 1.5 Hz), 7.12 (d, 2H, J = 8.5 Hz).	7.58 (d, 2H, J = 16 Hz), 5.84 (br, 1H), 6.55 (d, 2H, J = 16 Hz).	1.48 (s, 18H, –CH$_3$), 7.38–7.55 (m, 10H), 4.10 (m, 2H).
12′	3.88 (s, 6H, -OCH$_3$), 7.11 – 7.17 (m, 6H).	5.82 (s, 1H, J = 9.5 Hz), 6.59 (d, 2H, J = 16 Hz), 7.63 (d, 2H, J = 16 Hz).	1.49 (s, 18H, –CH$_3$), 4.25 (d, 4H, J = 5.5 Hz),
13′	3.97 (s, 6H, -OCH$_3$), 6.96 (d, 2H, J = 8 Hz), 7.07 (s, 2H), 7.14 (d, 2H, J = 6.5 Hz),	5.84 (br, 1H), 6.48 – 6.6 (m, 2H), 7.61 (d, 2H, J = 16 Hz).	1.28 – 1.44 (m, 2H), 1.46 (s, 9H –CH$_3$), 1.5 (s, 9H –CH$_3$), 1.73 (s, 2H), 1.78 – 1.84 (m, 2H), 1.97 (s, 2H), 2.07 – 2.14 (m, 2H), 2.31- 2.39 (m, 2H), 3.43 – 3.66 (m, 2H).

3.3.3 Reaction mechanism

The reaction of carboxylic acid of *t*-Boc protected amino acid with phenolic hydroxyl of curcumin was carried out in the presence of dehydrating agent like dicyclohexylcarbodiimide (DCC) in the presence of dimethylaminopyridine and triethylamine as catalysts. The reaction mechanism envisaged is presented in Fig. 3.2. Carboxylic acids capable of forming ketene intermediates undergo esterification upon treatment with carbodiimides (Steps 1 & 2, Fig. 3.2). Accordingly, protection of NH_2 group in amino acids by *t*-Boc moiety led to smooth reaction of the carboxyl group of amino acid with DCC for the formation of reactive intermediates.

Further, selective acylation of phenols, lacking strong electron-withdrawing groups, need acylation catalysts, mainly pyridine derivatives, to increase the reactivity of electrophilic species for the preparation of esters through carbodiimide couplings (Shelkov, et al., 2004). In the present reaction, the relatively less reactive nature of phenolic group in curcumin warranted the use of triethylamine along with DMAP for the smooth progress of the reaction. The reaction of phenolic group with activated carboxyl moiety leading to the formation of curcumin *t*-Boc amino acid conjugate and dicyclohexylurea is shown in Step 3. Also, it was observed that in case of amino acids containing other groups, such as –OH and –SH as in serine and cysteine respectively, no interference was observed from these moieties.

STEP 1

STEP 2

STEP 3

R =
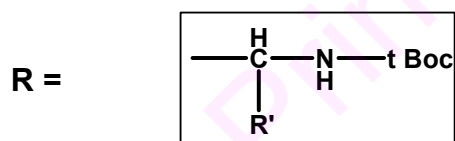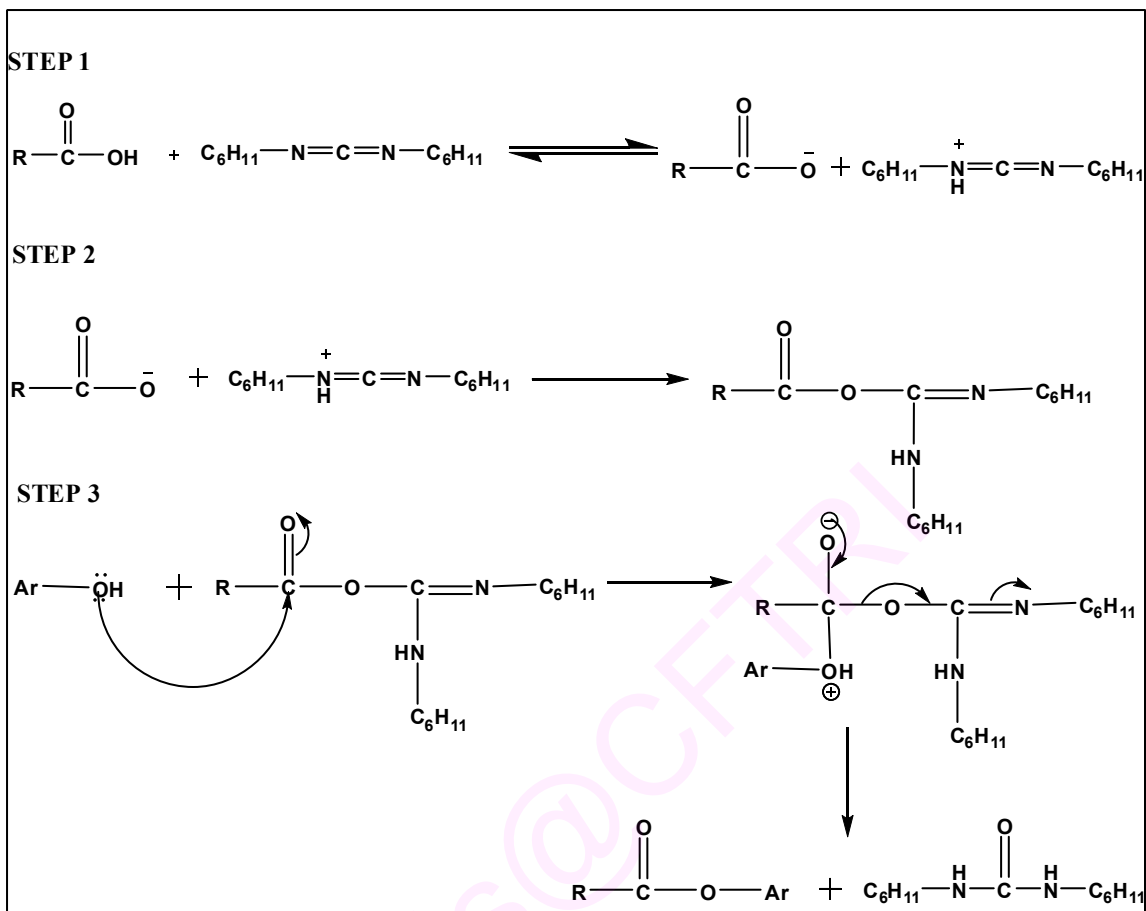
R′ = alkyl / aryl / imino / substituted alkyl groups from amino acids
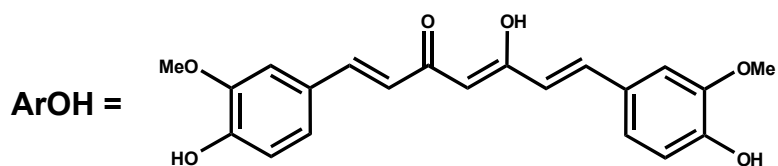
ArOH =

Fig. 3.2 Mechanism of *t*-BOC amino acids with curcumin

3.3.4 Curcumin-amino acid conjugates

The deprotection of *t*-Boc group was carried out using the conventional method, where 50% TFA was used at RT for 1-2 h. The low product yield observed was apparently due to exposure of the compounds to highly acidic environment for long periods. An improvement in the protocol for the deprotection of *t*-Boc amino acid-curcumin conjugates was envisaged with TFA in dry dichloromethane under ultrasonic conditions. Use of ultrasound for the deprotection was studied at different concentrations of trifluoroacetic acid (Table 3.3). The deprotection reaction was carried out with 5, 10, 15 and 20% TFA in dichloromethane at 20 and 40 KHz frequencies. The reactions at 5 % level of TFA (entry 1, Table 3.3) showed complete deprotection in 40 and 30 min under 20 and 40 KHz frequencies respectively, whereas the same reaction under stirring took 48 h for completion of the reaction. Similarly reactions with 10 % TFA in dichloromethane (entry 1, Table 3.3) took 30 and 15 min for the completion. The deprotection rate increased as the TFA level was increased (entries 3 and 4, Table 3.3); the time taken for the deprotection was as low as 5 min.

**Table 3.3 Deprotection of *t*-Boc group under ultrasonic and normal conditions**

		Reaction time		
Entry	Concentration of TFA	Ultrasonic bath (20KHz), min	Ultrasonic reactor (40KHz), min	Stirring at RT, h
1	5% TFA in DCM	40	30	48
2	10% TFA in DCM	30	15	36
3	15% TFA in DCM	25	10	24
4	20% TFA in DCM	15	5	20

For the deprotection of all the conjugates of curcumin, 10% TFA in dichloromethane was the reagent of choice and sonication (40 KHz) was employed for removal of *t*-Boc group. The curcumin amino acid conjugates formed were insoluble in dichloromethane as the removal of *t*-Boc moiety made the compounds less lipophilic due to the free –NH$_2$ group in the final product. This facilitated the easy isolation of the curcumin-amino acid conjugates.

The method developed was milder and faster than the general protocol for *t*-Boc deprotection. The compounds thus prepared were obtained in pure form with an overall yield of 45-76%. The purity of the samples was assessed by HPLC (Table 3.4). The removal of the *t*-Boc groups made these derivatives hydrophilic. The retention time of the all the derivatives were lesser compared to the lipophilic curcumin (14.66 min, retention time). The *t*-Boc derivatives showed the retention times ranging from 6.3 – 18.38, while the deprotected derivatives showed retention times ranging from 4.04 – 6.33. Deprotection of *t*-Boc group was confirmed by the absence of eighteen protons of tertiary methyl groups of two *t*-Boc moieties in the ^{1}H NMR spectra of curcumin- amino acid conjugate (Table 3.5). The deprotection of *t*-Boc group indicated shift in the positions of signals pertaining to α– methine protons in amino acids. However, the methoxy protons of curcumin showed very slight shift. Earlier studies reported glycine and alanine conjugates of curcumin using corresponding *N*-phthaloyl amino acids wherein carboxyl group was in activated acid chloride or *p*-nitrophenyl ester form. In the present study, a simple and general reaction protocol has been delineated

for synthesis of curcumin-amino acid conjugates, several of them being reported

for the first time, in high yields.

Table 3.4 Curcumin amino acid conjugates

(IV)
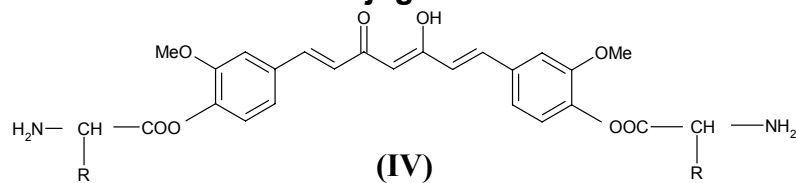
Structure No.	Compound	Structure IV, R =	Overall Yield (%)	Retention time (Min)
4	1,7-Bis (4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	H_3C—CH—CH_2— ($(CH_3)_2CH-CH_2-$)	71	6.33
5	1,7-Bis (4-*O*-L-phenylalaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	(benzyl) $C_6H_5-CH_2-$	68	6.01
6	1,7-Bis (4-*O*-L-alaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	—CH_3	76	5.06
7	1,7-Bis (4-*O*-L-isoleucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	H_3C—CH_2—CH— with CH_3	63	6.14
8	1,7-Bis (4-*O*-L-valinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	$(H_3C)_2CH-$	65	4.86
9	1,7-Bis (4-*O*-L-serinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	$HO-CH_2-$	45	5.06
10	1,7-Bis (4-*O*-L-cysteinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	$HS-CH_2-$	70	4.76
11	1,7-Bis (4-*O*-L-phenylglycinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	(phenyl) C_6H_5-	59	4.78
12	1,7-Bis (4-*O*-L-glycinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	—H	57	4.04
13	1,7-Bis (4-*O*-L-prolinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	—CH_2—CH_2—CH_2— (as part of a pyrolidine ring)	70	5.00

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Table 3.5 ^{1}H NMR spectral data for the curcumin amino acid conjugates

Compound	^{1}H NMR signals from aromatic nucleus and substituents on the aromatic moiety of aglycone	^{1}H NMR signals from hepta-3, 5- dienone moiety of aglycone	^{1}H NMR signals from amino acid moiety
1	3.90 (s, 6H, OCH$_3$), 6.88 (d, 2H, J = 8 Hz), 7.00(s, 2H), 7. 07 (d, 2 H, J = 8.5 Hz),	6.42 (d, 2H, J = 16 Hz), 7.54 (d, 2H, J = 16 Hz), 5.75 (s, 1H),	-
4	3.89 (s, 6H, -OCH$_3$), 7.20 (d, 2H, J = 8 Hz), 7.27-7.33(m, 2H), 7.44 (d, 2H, J = 1.5 Hz).	6.12 (s, 1H), 6.88 (d, 2H, J = 16 Hz), 7.7 (d, 2H, J = 16 Hz).	1.12 (d, 6H, J = 6.5 Hz), 1.11 (d, 6H, J = 6.5 Hz), 1.82-1.90 (m, 2H), 1.98 –2.10 (m, 4H), 4.37 (dd, 2H, J = 7.5 and 14 Hz),
5	3.94 (s, 6H, -OCH$_3$), 7.12 (d, 2H, J = 8 Hz), 7.29 (d, 2H, J = 8 Hz), 7.39 (m, 2H).	6.12 (s, 1H), 6.88 (d, 2H, J = 16 Hz), 7.69 (d, 2H, J = 16 Hz).	3.36 (d, 1H, J = 7.5 Hz), 3.41 (d, 1H, J= 7.5 Hz), 3.52 (dd, 2H, J = 6 and 14.5 Hz), 4.68 (dd, 2H, J = 6 and 7.5 Hz), 7.44 (m, 10H).
6	3.92 (s, 6H, -OCH$_3$), 7.19 (d, 2H, J = 8 Hz), 7.31 (d, 2H J = 8.5 Hz), 7.43 (s, 2H),	6.11 (s, 1H), 6.59 (d, 2H J = 16 Hz), 7.64 (d, 2H, J = 16 Hz)	1.76 (d, 6H, J = 7.5 Hz, -CH$_3$) 4.44 (dd, 2H, J =7.5 and 14.5 Hz),
7	3.86 (s, 6H, -OCH$_3$), 7.21 (d, 2H, 8 Hz) 7.40 (d, 2H, J = 8 Hz), 7.59 (br, 2H),	6.22 (s, 1H), 7.05 (d, 2H, J = 16 Hz), 7.68 (d, 2H, J = 16 Hz).	0.97 (t, 6 H, J = 7 Hz, -CH$_3$), 1.08 (d, 6H, J = 7 Hz, -CH$_3$), 1.40-1.45 (m, 2H), 1.60-1.67 (m, 2H), 2.06 (d, 2H, J = 3.5 Hz), 4.35 (s, 2H),
8	3.89 (s, 6H, -OCH$_3$), 7.11 –7.17 (m, 2H), 7.23 – 7.29 (m, 2H), 7.42 (d, 2H, J = 8.5 Hz),	6.09 (s, 1H), 6.8 –6.87 (m, 2H), 7.69 (d, 2H, J = 16 Hz).	1.22 (d, 6H, J = 6. 5 Hz Hz), 1.20 (d, 6H, J = 6.5 Hz), 1.46-2.53 (m, 2H), 4.25 (d, 2H, J = 4.5 Hz),
9	3.93 (s, 6H, – OCH$_3$), 6.81 – 6.86 (m, 2H), 7.12 – 7.15 (m, 2H), 7.24 – 7.28 (m, 2H),	6.05 (s, 1H), 6.64 – 6.70 (m, 2H), 7.58 – 7.65 (m, 2H)	1.69 (s, 2H), 3.91 (s, 4H)
10	3.93 (s, 6H, -OCH$_3$), 6.84 (dd, 4H, J = 3 and 8 Hz), 7.13 (d, 2H, J = 8 Hz),	6.01 (s, 1H), 6.66 (d, 2H, J = 16 Hz), 7.59 (d, 2H, J = 16 Hz).	1.32 (t, 2H, J = 8 Hz), 1.37-1.39 (m, 2H), 4.47 (d, 2H, J = 8 Hz).
11	3.76 (s, 6H, -OCH$_3$), 7.06 (dd, 2H, J = 3.5 and 8 Hz), 7.22 (d, 2H, J= 6 Hz).	6.67 (s, 1H), 6.83 (d, 2H, J = 9 Hz), 7.33 (d, 2H, J = 5 Hz).	3.90 (s, 2H), 7.52-7.68 (m, 12H).
12	3.92 (s, 6H, -OCH$_3$), 7.20 (d, 2H, J = 8 Hz), 7.28 – 7.32 (m, 2H), 7.43 (br, 2H),	6.11 (s, 1H), 6.88 (d, 2H, J = 16 Hz), 7.69 (d, 2H, J = 16 Hz).	4.19 (s, 4H)
13	3.93 (s, 6H, -OCH$_3$), 7.34 (m, 2H), 7.23 (d, 2H, J = 8 Hz), 7.44 (d, 2H, J = 1.5 Hz),	6.31 (s, 1H), 6.89 (d, 2 H, J = 16 Hz), 7.69 (d, 2H, J = 16 Hz).	2.17-2.2 (m, 4H), 2.44 – 2.49 (m, 2H), 2.6-2.66 (m, 2H), 3.4-3.55(m, 5H), 4.35-4.45 (m, 1H),

3.3.5 Characterization of amino acid conjugates- Representative examples

(i) 1,7-Bis(4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4)

The purity of the sample was assessed by HPLC where a single peak at 13.16 min was seen for **4′** (Table 3.1, Fig. 3.3). Pure curcumin showed retention of 14.66 min under similar conditions.

The mass spectral analysis showed that **4′** had a M+Na adduct at 817 corresponding to the di-Boc leucinoyl derivative of curcumin (Table 3.1, Fig. 3.4). The ^{1}H NMR of **4′** showed characteristic tert-butylcarbamate associated –C$\underline{H}_3$ signals for eighteen protons at a chemical shift of 1.48. The two –C$\underline{H}_3$ group protons of the leucine moiety attached to curcumin were seen at 1.05 as a doublet corresponding to twelve protons indicating the two Boc–leucine groups were attached to the two phenolic hydroxyls of curcumin (Table 3.2, Fig. 3.5). The –OC$\underline{H}_3$ signals of curcumin were seen at 3.88 corresponding to 6H. The characteristic –CH proton of the *t*-Boc leucine linked to curcumin signals were seen at 4.60 corresponding to 2H compared to *t*-Boc leucine standard which shows the –CH linked to carboxyl at 4.29. This indicated the conjugation of two moieties of *t*-Boc leucine to curcumin. The methylene group of the amino acid moiety showed signal at 1.9 corresponding to 4H. The methine moiety α to two methyls showed signal at 1.68 (2H).
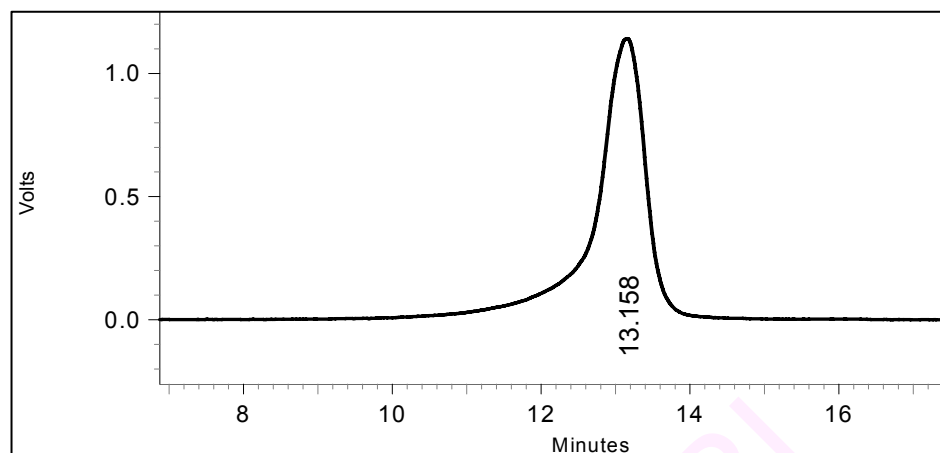
Fig. 3.3 HPLC profile of 1,7-Bis (4-*O*-L-*t*-Boc-leucinoyl- 3-methoxyphenyl)-1,4,6- heptatriene-5-ol-3-one (4´)
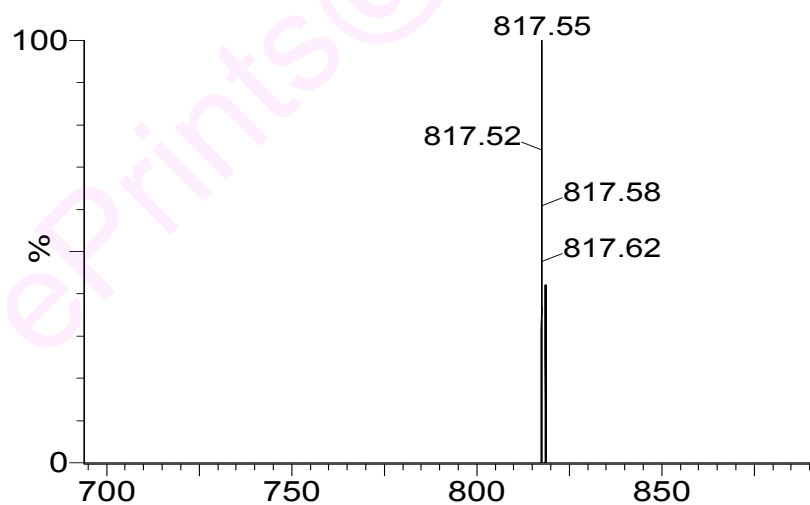

Fig. 3.4 ESI-MS of 1,7-Bis (4-*O*-L-*t*-Boc-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4´)
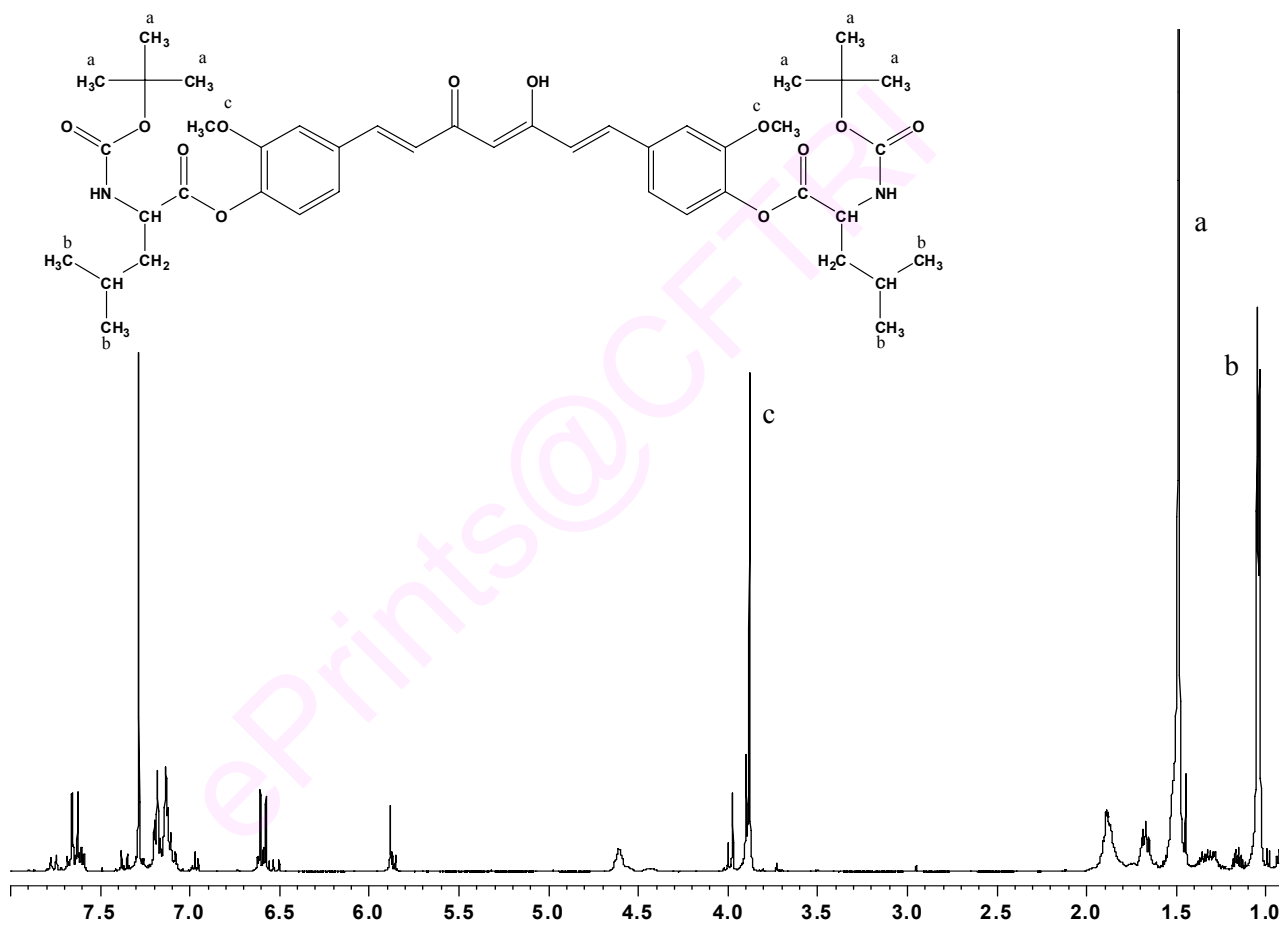
Fig.3.5 ^{1}H NMR spectra of 1,7-Bis (4-*O*-L-Boc-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4´)

After deacetylation the product was checked for its purity by HPLC. The compound showed retention at 6.33 (Fig. 3.6). The removal of *t*-Boc groups showed shift in the retention time from 13.16 for curcumin-*t*-Boc leucine conjugate to 6.33 for curcumin-leucine conjugate. Further, the compound was characterized by ^{1}H NMR (Table 3.5). Disappearance of –CH$_3$ (18H) signals from the *t*-Boc groups in the conjugate showed completion of deprotection (Fig. 3.7). The -CH$_3$ group signals of the leucine moiety were seen at a chemical shift value of 1.11-1.12 (12 H). The –OCH$_3$ of curcumin corresponding to six protons was seen at 3.89. The α–methine protons showed signal at 4.37. Signals for the other methine group of leucine linked to –CH$_3$ was observed at 1.82-1.90 (m, 2H) and the methylene group was observed at 1.98 - 2.1 (m, 4H). The aromatic protons of curcumin in the curcumin – leucine conjugate now appeared at 7.20 (2H), 7.27-7.33 (2H) and 7.44 (2H).

(ii) 1,7-Bis (4-*O*-L-alaninoyl-3-methoxyphenyl)-1,4,6- heptatriene-5-ol-3-one (6)

The purity of the compound was assessed by HPLC where the single peak at 8.06 for **6´** (Table 3.1, Fig 3.8). NMR and ESI MS characterized the compound **6´**. The ESI MS (+ve mode) spectral analysis showed that **6´** had a M+Na adduct at 733 corresponding to di-Boc alaninoyl derivative curcumin the of (Table 3.1, Fig. 3.9). ^{1}H NMR further confirmed the *t*-Boc derivative of curcumin alanine conjugate. The spectra showed chemical shift values at 1.66 and 1.59 corresponding to the six protons of two –CH$_3$ groups of alanine. The chemical shift values of 1.49 were shown for *t*-Boc moiety corresponding to eighteen protons. The –OCH$_3$ signal of curcumin was at 3.88 a singlet showing integration

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for 6 protons. The α–methine protons of Boc alanine linked to curcumin showed signal at 4.62 corresponding to 2H, while the standard *t*-Boc alanine showed signal at 4.37 for the same. The spectra confirmed the formation of *t*-Boc alanine linked at both the phenolic hydroxyls of curcumin (Fig. 3.10).

After deprotection of *t*-Boc group, the product was checked for its purity by HPLC. The compound **6** showed retention time of 5.06 as a single peak confirming its purity (Fig. 3.11). ^{1}H NMR using CD_3OD as a solvent further characterized the product **6**. The spectra showed the absence of *t*-Boc methyl groups (18H) confirming the deprotection (Fig. 3.12). Chemical shift value of 1.76 (d, 6H, J = 7.5 Hz, $-CH_3$) indicated the presence of the two methyl groups of alanine moiety, the $-OCH_3$ showed singlet at 3.92 from curcumin moiety of **6**. The α–methine protons of curcumin-alanine conjugate was seen at 4.44 (2H). The aromatic protons of curcumin in the curcumin–alanine conjugate now appeared at 7.19 (2H), 7.31 (2H) and 7.43 (2H).
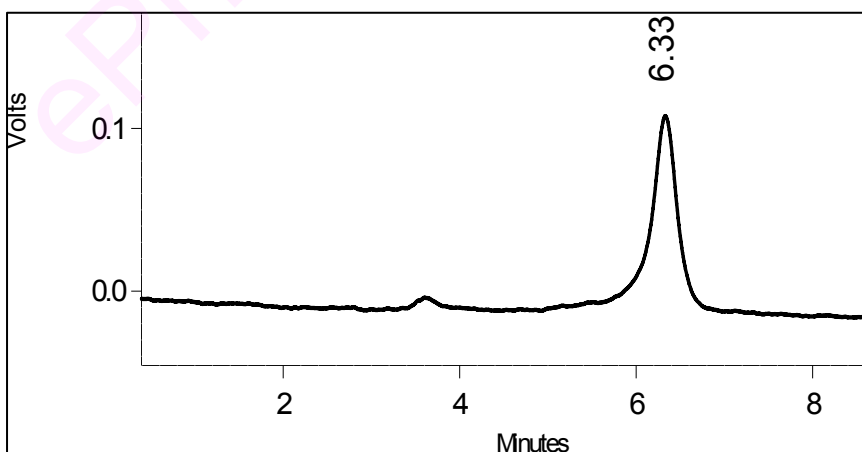
Fig. 3.6 HPLC profile of 1,7-Bis (4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4)
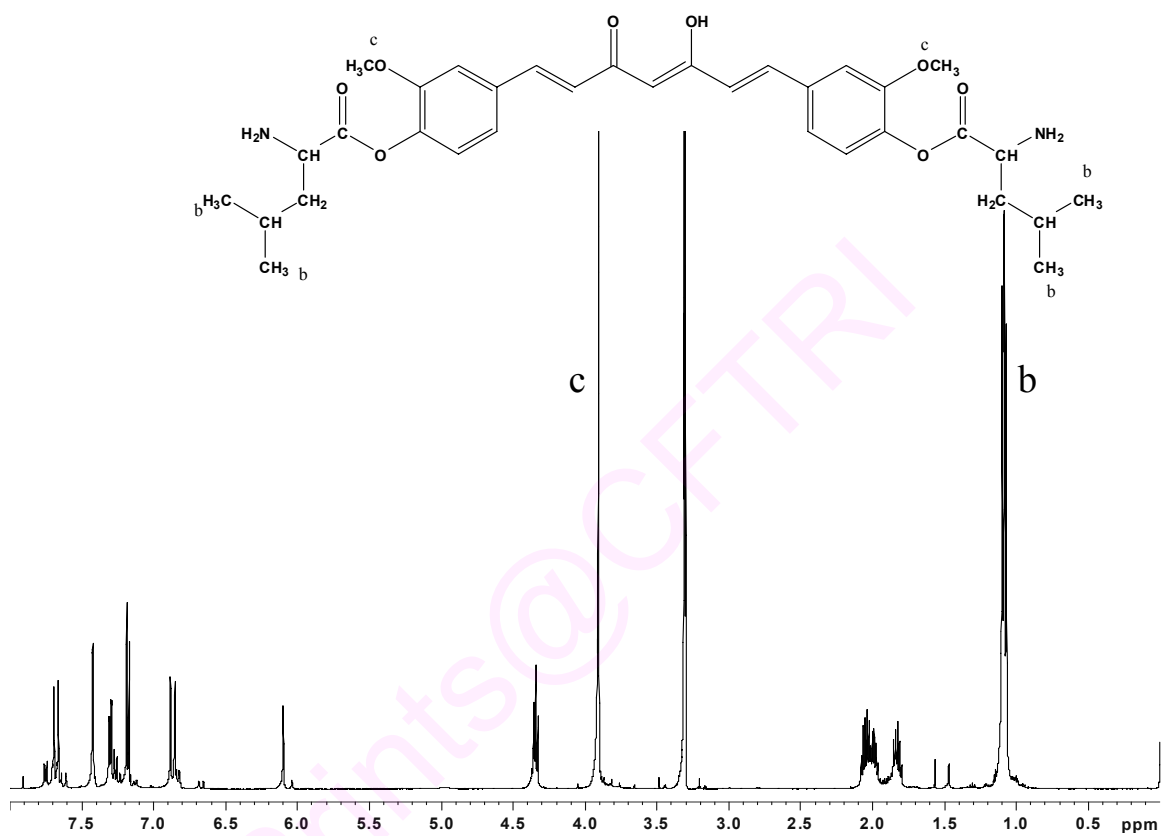
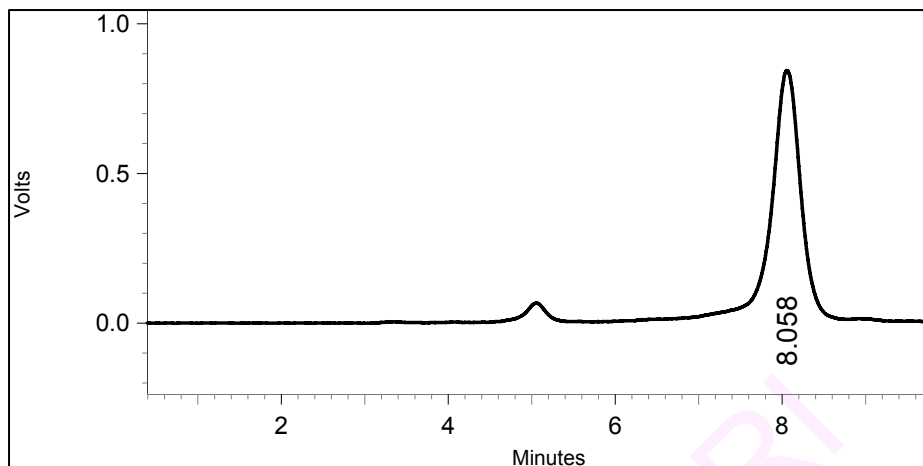**Fig. 3.7 ¹H NMR spectra of 1,7-Bis (4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (4)**

Fig. 3.8 HPLC profile of 1,7-Bis (4-*O*-L-*t*-Boc alaninoyl -3-methoxyphenyl)- 1,4,6- heptatriene-5-ol-3-one (6′)
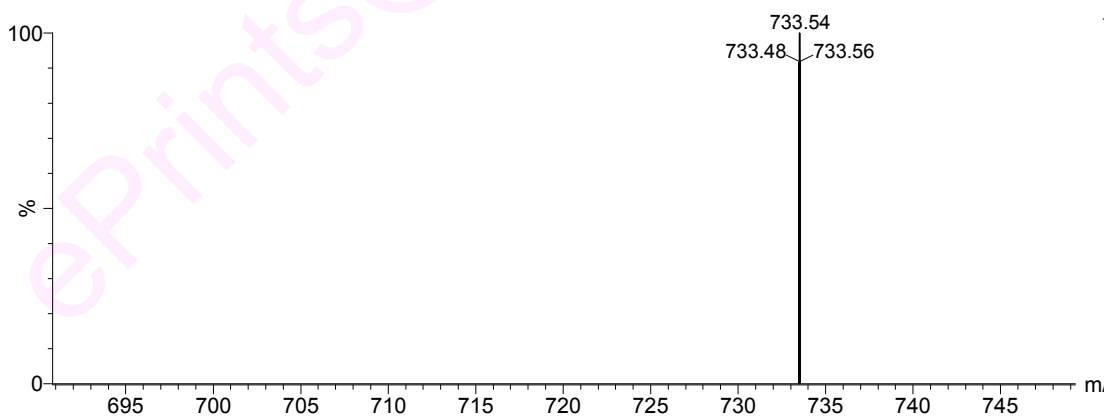

Fig. 3.9 ESI-MS of 1,7-Bis (4-*O*-L-Boc-alaninoyl-3-methoxyphenyl)-1,4,6- heptatriene-5-ol-3-one (6′)
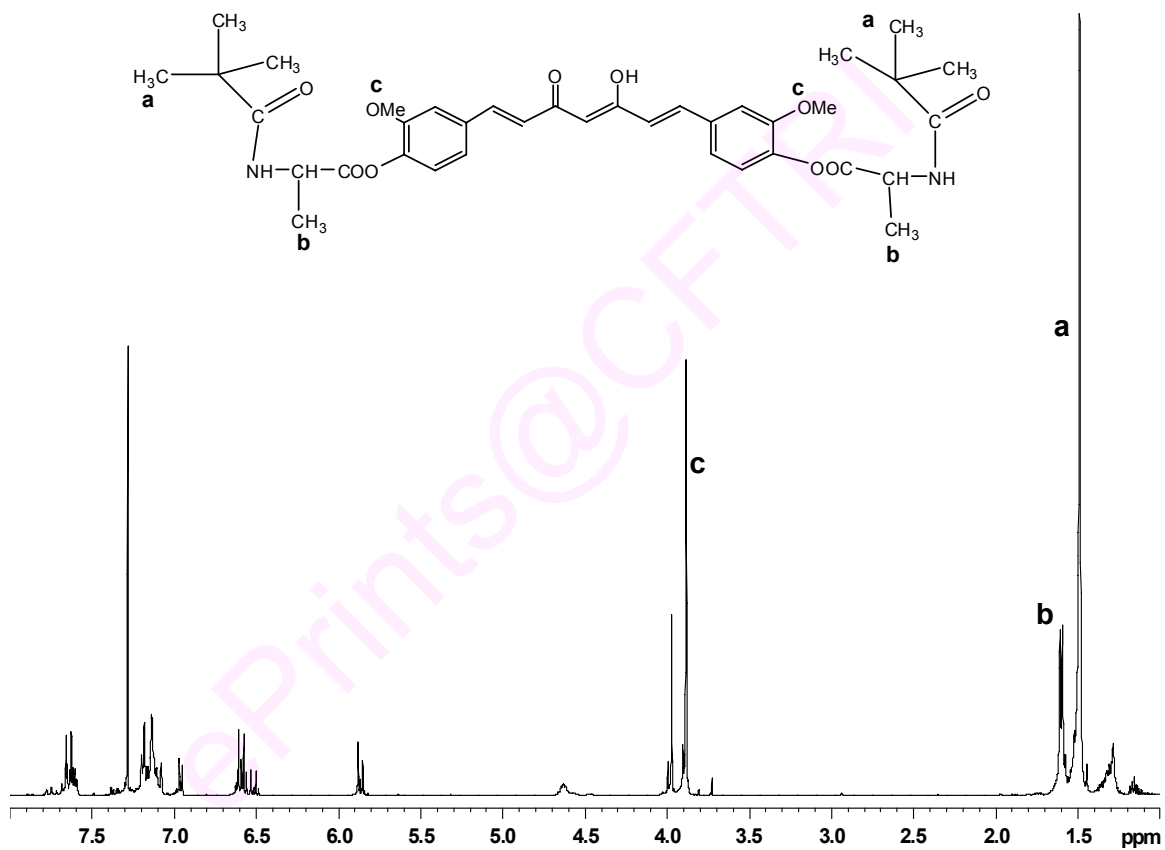
Fig. 3.10 ¹H NMR spectra of 1,7-Bis (4-*O*-L-Boc-alaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (6´)
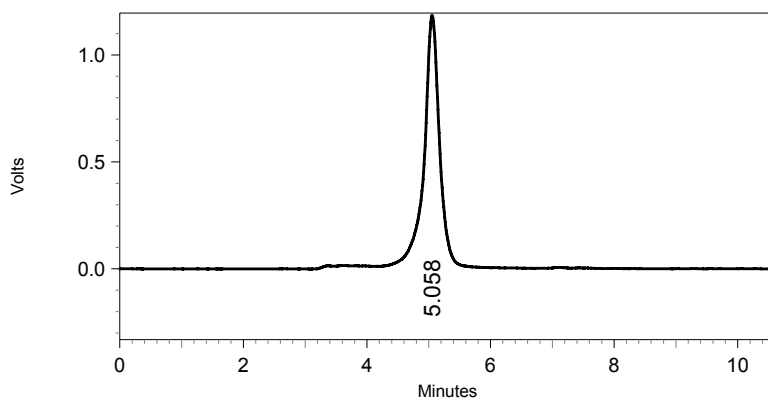
Fig. 3.11 HPLC profile of 1,7-Bis (4-*O*-L- alaninoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (6)

Fig. 3.12 ^{1}H NMR spectra of 1,7-Bis (4-*O*-L- alaninoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one (6)

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3.4 CONCLUSIONS

In this present study, a synthetic protocol was developed for the preparation of several curcumin-amino acid conjugates in high yields. In the reaction of curcumin with amino acids, the amino functionality in amino acids was protected by a N-*tert*-butoxycarbonyl (*t*-Boc) group. Selective dehydration reaction between the phenolic groups in curcumin and the carboxyl functional group of the amino acids as effected by N, N'-dicylohexylcarbodiimide (DCC) and catalyzed by dimethylaminopyridine and triethylamine led to the formation of the *t*-Boc protected amino acid conjugates of curcumin. The synthetic methodology developed envisaged facile reaction of the *N*-protected (*t*-Boc) amino acids with curcumin and included an improved accelerated protocol for the deprotection of *t*-Boc group to afford the formation of amino-acid conjugates of curcumin in high yields. Accordingly, several amino acid conjugates of curcumin were synthesized in 45–76% yields. Covalent binding of amino acids to curcumin at phenolic positions rendered the curcumin derivatives water-soluble.

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CHAPTER 4

SYNTHESIS OF GLUCURONIC ACID &

GLUCAL DERIVATIVES OF CURCUMIN

4.1 GLUCURONIC ACID DERIVATIVE OF CURCUMIN

4.1.1 INTRODUCTION

Biotransformation of curcumin has been studied and the structure of its metabolites after oral or i. p. administration in mice characterized. THC is a major metabolite of curcumin and its stability at different pH is also studied (Pan, et al., 1999). These studies show the metabolic steps of curcumin involve the glucuronidation of curcumin and its major reduced forms viz., tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin. Glucuronides of curcumin, hexahydrocurcumin, and other analogs have been synthesized using rat and human liver microsomes and characterized by LC-MS/MS analysis (Pfeiffer, et al., 2007). Biotransformation of curcumin shows that curcumin is first bio-transformed to dihydrocurcumin and tetrahydrocurcumin and that these compounds subsequently are converted to monoglucuronide conjugates. Thus, curcumin glucuronide, dihydrocurcumin glucuronide, tetrahydrocurcumin glucuronide and tetrahydrocurcumin are major metabolites of curcumin in mice (Lin, et al., 2000). As the glucuronidation is a part of metabolic pathway of curcumin, the synthesis of curcumin glucuronide has been taken up in this present work, as this derivative would be water-soluble.

Curcumin metabolism and its absorption are studied after its oral administration in rats at different dosages of [^{3}H] curcumin. The radioactivity studies have shown that curcumin gets poorly absorbed in gut and transforms into more polar and colorless compounds. These experiments indicate that curcumin undergoes transformation during absorption from the intestine and the

studies on the tissue distribution are also studied. Rat intestines when incubated with 50-750 µg of curcumin in incubation medium, 30-80% of the added curcumin disappears from the mucosal side. Curcumin is not detectable in the serosal fluid. Less than 3% of the added curcumin is found in the tissue at the highest concentration, which shows curcumin gets transformed in the tissues (Ravindranath & Chandrasekhara, 1980; 1981; 1982). Curcumin metabolism is studied in human and rat hepatocytes and also in rats *in vivo*. Analysis by HPLC with detection at 420 and 280 nm show metabolites with both intact diferuoylmethane and increased saturation of the heptatrienone chain. Results are confirmed by mass spectral analysis where, hexahydrocurcumin and hexahydrocurcuminol are identified as major metabolites of curcumin. In rats, *in vivo*, curcumin administered (40 mg/kg) disappears from the plasma within 1 h of dosing. After administration (500 mg/kg), parent drug is present in plasma at levels near the detection limit. The major products of curcumin biotransformation identified in rat plasma are curcumin glucuronide and curcumin sulfate, whereas hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide are present in small amounts. (Iresson, et al., 2002). The effect of curcumin on lipid metabolism in rats fed on a control, moderately high-fat diet and those given supplements of 0.2 g curcuminoids/100 g diet is studied. Triacylglycerol and cholesterol contents are lower in rats fed curcumin than in control rats. Results indicate that dietary curcuminoids have lipid-lowering potency *in vivo*, probably due to alterations in fatty acid metabolism (Asai & Miyazawa, 2001).

Methods are reported in literature where various strategies are adopted for the covalent linking of glucuronic acid to other molecules. Acetylated glucuronic acid moiety is made to react with alcohols to give glycosides (Tosin & Murphy, 2004). The glycosidation of glucuronic acid with 4-hydroxycinnamic acid has been reported with the anomeric activation (Pearson, et al., 2005). 2,3,4-Tri-O-acetyl-D-glucurono-6,1-lactone is obtained in 'one-pot' synthesis from D-glucuronic acid. The lactone opening with different alcohols in the presence of a variety of catalysts has been studied. All reactions have been performed under microwave irradiation in solvent-free conditions (Rat, et al., 2007). Different synthetic routes to differentially protected D-glucuronic acid glycols that may serve as intermediates en route to a variety of natural products are reported (Schell, et al., 2001). Glucuronidation of opium drug like thienorphine is carried out and its synthetic protocol developed (Liu, et al., 2005). Synthesis of some phenolic glycosides (urine drug metabolites) is reported (Arewang, et al., 2007). Glucuronide prodrugs of 9-aminocamptothecin have been synthesized and shown to possess the necessary prerequisites to be considered as candidates for cancer prodrug monotherapy and antibody directed enzyme prodrug therapy of cancer (Leu, et al., 1999).

In the present study, a synthetic pathway to curcumin glucuronide was envisaged starting from glucuronolactone, a readily available substrate. The synthetic route involves opening of the lactone ring with concomitant protection of the carboxyl group and activation at the anomeric carbon for facile bonding of the phenolic groups of curcumin with the sugar moiety.

4.1.2 EXPERIMENTAL

(a) Materials and equipment

Acetic anhydride, acetonitrile and methanol were procured from s. d. fine chemicals Mumbai, India and dried as per the standard protocol (Armarego & Chai, 2003). Glucuronic acid γ-lactone, Titanium tetrabromide, Perchloric acid (70%), Benzyltributylammonium chloride, Cetyltributylammonium bromide, Dowex (IR-120), Tetrabutylammonium bromide and 3,4,6-tri-O-acetyl-D-glucal were procured from Sigma–Aldrich chemical company, USA. Alumina coated TLC plates were procured from E-Merck India Ltd. Curcumin (97 %) was procured from Spicex Pvt. Ltd., Mysore, India. $Fe_2(SO_4)_3 \cdot xH_2O$, Dichloromethane, triethylamine, hexane, sodium sulfate, chloroform, potassium hydroxide and potassium carbonate were procured from Qualigens India Ltd. Mumbai, India. Silica gel was obtained from s. d. Fine chemicals, Mumbai, India.

Ultrasound reactor used was Vibracell-750 with tapered probe of tip diameter 6.5 mm from Sonics and Materials Inc., Newtown, USA and was set at 25% amplitude and pulse cycle of 25 s (on) and 5 s (off) with frequency of 40 KHz and an output power of 750 W. ^{1}H NMR spectra for the compounds were recorded on a 500 MHz NMR spectrometer (Bruker Avance, Reinstetten, Germany) using, $CDCl_3$ and CD_3OD solvents. Chemical shift values and coupling constants are given in δ and Hz respectively. Mass spectral analyses of compounds were carried out using MS (Waters Q-Tof Ultima, Manchester, UK) in the ESI positive mode. Thin-layer chromatographic (TLC) analysis was performed on silica gel 60 F_{254} (Merck, Germany) coated on alumina sheet with

3% methanol in chloroform as the developing solvent. Samples were analyzed on HPLC (Waters, LC 10 A, USA) using a reverse phase C-18 column with methanol : water (70:30) containing trifluroacetic acid (0.1%) as the mobile phase at flow rate of 1 ml/min and monochromatic detection at 423 nm.

(b) Synthesis methyl 1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranuronate (Leu, et al., 1999; Liu, et al., 2005**)**

Glucuronic acid *γ*-lactone, [(*2R*)-2-[(*2S, 3R, 4S*)-3,4-Dihydroxy-5-oxo-tetrahydrofuran-2-yl]-2-hydroxy-acetaldehyde (25 g)], was added to MeOH (50 ml) that contained sodium (75 mg). The mixture was stirred at room temperature for 60 min after which the solvent was distilled under reduced pressure. The syrupy product was diluted with acetic anhydride (10 ml). This solution was added drop wise to a mixture of acetic anhydride (30 ml) and $HClO_4$ (0.15 ml), prepared below 4 °C. During this addition, the reaction temperature was maintained below 40 °C. The mixture was stirred for 24 h at room temperature and the solution stored overnight at 4 °C to yield 12 g of crystalline product. For further work up of the product, the mother liquor was poured onto crushed ice and neutralized with $NaHCO_3$ and the filtrate was extracted with $CHCl_3$. The $CHCl_3$ extract was dried over anhydrous Na_2SO_4 and concentrated to syrup. Upon storage at 4°C an additional 5 g of crude crystalline material was obtained. The product was re-crystallized from MeOH: 62%, mp 140 °C. 2.05 (s, 3H, $OCOCH_3$), 2.06 (s, 3H, $OCOCH_3$), 2.07 (s, 3H, $OCOCH_3$), 2.14 (s, 3H, $OCOCH_3$), 3.77 (s, 3H, -CH_3), 4.20 (1H, d, J = 9.5 Hz), 5.16 (1H, dd, J = 8 Hz), 5.26 (1H, t, J = 9 Hz), 5.33 (1H, t, J = 9 Hz) & 5.78 (1H, d, J = 8 Hz).

(c) Synthesis methyl 1-bromo-2, 3, 4-tri-*O*-acetyl-α-D-glucopyranuronate

A solution of compound methyl 1, 2, 3, 4-Tetra-*O*-acetyl-α-D-glucopyranuronate (10 g, 2.66 mmol) and $TiBr_4$ (11 g, 3.19 mmol) in CH_2Cl_2 (25 ml) was stirred at room temperature for 24 h. The mixture was washed with ice water and $NaHCO_3$ solution, dried over Na_2SO_4, and evaporated to dryness to give 4 g of white solid. m. p. 151-153 ^{o}C, ESI – MS (M+ Na)$^+$ 410, ^{1}H NMR (500 MHz, $CDCl_3$); 2.07 (s, 3H, $OCOCH_3$), 2.08 (s, 3H, $OCOCH_3$), 2.12 (s, 3H, $OCOCH_3$), 3.78 (s, 3H, -$COOCH_3$), 4.60 (d, 1H, J = 10.5 Hz), 4.87 (dd, 1H, J = 10 Hz), 5.26 (t, 1H, J = 10 Hz), 5.63 (t, 1H, J= 10 Hz), 6.66 (d, 1H, J = 4Hz).

(d) Synthesis of methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-gluco-pyranuronate (14´)

To a solution of curcumin (**1**, Fig. 1, 1 g, 2.7 mmol) in chloroform (40 ml), aqueous KOH (0.6 g, 10.8 mmol in 20 ml water) was added and stirred for 5 min. A solution of methyl 1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (2.2 g, 5.35 mmol) in chloroform (40 ml) was added. To the reaction mixture an aqueous solution of benzyltributylammonium chloride (0.5 g, 1.6 mmol in 20 ml water) was added. The mixture was sonicated until the completion of the reaction (4 - 6 h), which was indicated by the formation of clear aqueous layer. TLC analyzed the reaction product for the disappearance of curcumin. Then the chloroform layer was washed with water, dried over anhydrous sodium sulfate and distilled under reduced pressure. The crude sample was loaded on to silica gel (200-400 mesh) column and eluted out with $CHCl_3$. Pure fractions were pooled and evaporated to afford curcumin mono β-glucuronide triacetate derivative in 50 % yield (**14´**).

^{1}H NMR (CDCl$_3$): 2.07 (s, 3H, -OCOCH$_3$), 2.08 (s, 3H, -OCOCH$_3$), 2.12 (s, 3H, OCOCH$_3$), 3.79 (s, 3H, -COOCH$_3$), 3.99 (s, 6H, –OCH$_3$), 4.50 (1H, d, J = 10.5 Hz), 4.87 (dd, 1H, J = 4 Hz), 5.26 (t, 1H, J = 10 Hz), 5.62 (t, 1H, J = 10 Hz), 5.84 (t, 1H, J = 8 Hz), 5.91 (s, 1H), 6.80 (d, 2H, J = 15.5 Hz), 7.15 (d, 2H, J = 8 Hz), 7.28 (d, 2H, J = 3.5 Hz), 7.42 (s, 2H), 7.60 (d, 2H, J = 15.5 Hz).

(e) Methyl 1-curcuminyl-β-D-glucopyranuronate (14)

To methyl 1-curcuminyl-2,3,4-tri-O-acetyl-β-D-gluco-pyranuronate **(14´,** 1 g) in dry methanol (10 ml), a solution of LiOH (2.2 mmol) in methanol (8 ml) was added and the mixture was stirred. The deacetylation reaction was monitored on TLC. At the end of the reaction (0.5 h), the solution was neutralized by the addition of freshly regenerated Dowex (IR-120) H$^+$ resin. The resin was filtered and washed with methanol. The solvent distilled under reduced pressure to afford pure methyl 1-curcuminyl-β-D-glucopyranuronate in 95% yield (**14**). m.p. 65 ^{o}C; ^{1}H NMR (CD$_3$OD): 3.17 (s, 3H, -COOCH$_3$), 3.85 (s, 6H, –OCH$_3$), 4.10 (1H, d, J = 7.5 Hz), 5.11 (m, 2H, J = 5 Hz), 5.76 (1H, s), 6.04 (s, 1H), 6.10 (s, 1H), 6.78 (d, 2H, J = 15.5 Hz), 7.13 (d, 2H, J = 8 Hz), 7.26 (d, 2H, J = 3.5 Hz), 7.39 (s, 2H), 7.57 (d, 2H, J = 15.5 Hz).

4.1.3 RESULTS AND DISCUSSION

Reaction of curcumin with methyl 1-bromo-2, 3, 4-tri-*O*-acetyl-α-D-glucopyranuronate with potassium salt of curcumin under biphasic conditions using phase transfer catalysts was studied under the influence of ultrasound for the synthesis of glucuronide derivative of curcumin (Fig. 4.1). Curcumin is prone to rapid degradation in the presence of alkali and high temperature. Reaction of a solution of curcumin in chloroform with aqueous KOH led to facile formation of potassium salt of curcumin indicated by the decolorization of the organic layer with concomitant transfer of the pigment to the aqueous phase. A solution of methyl 1-bromo-2, 3, 4-tri-*O*-acetyl- α -D-glucopyranuronate in chloroform was added, which constituted a biphasic system with the sugar halide in the organic phase and the potassium salt of the phenolic substrate, curcumin in the aqueous phase. Aqueous solution of benzyltributylammonium chloride was then added and the contents were stirred which resulted in the formation of a homogeneous mixture. The use of ultrasound as an alternative source for providing the activation energy for this reaction was envisaged. The reaction mixture was sonicated for 4 h until the aqueous and organic phases got separated and the aqueous layer became colorless. The organic layer after work up and chromatographic separation afforded methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate (>50 %).
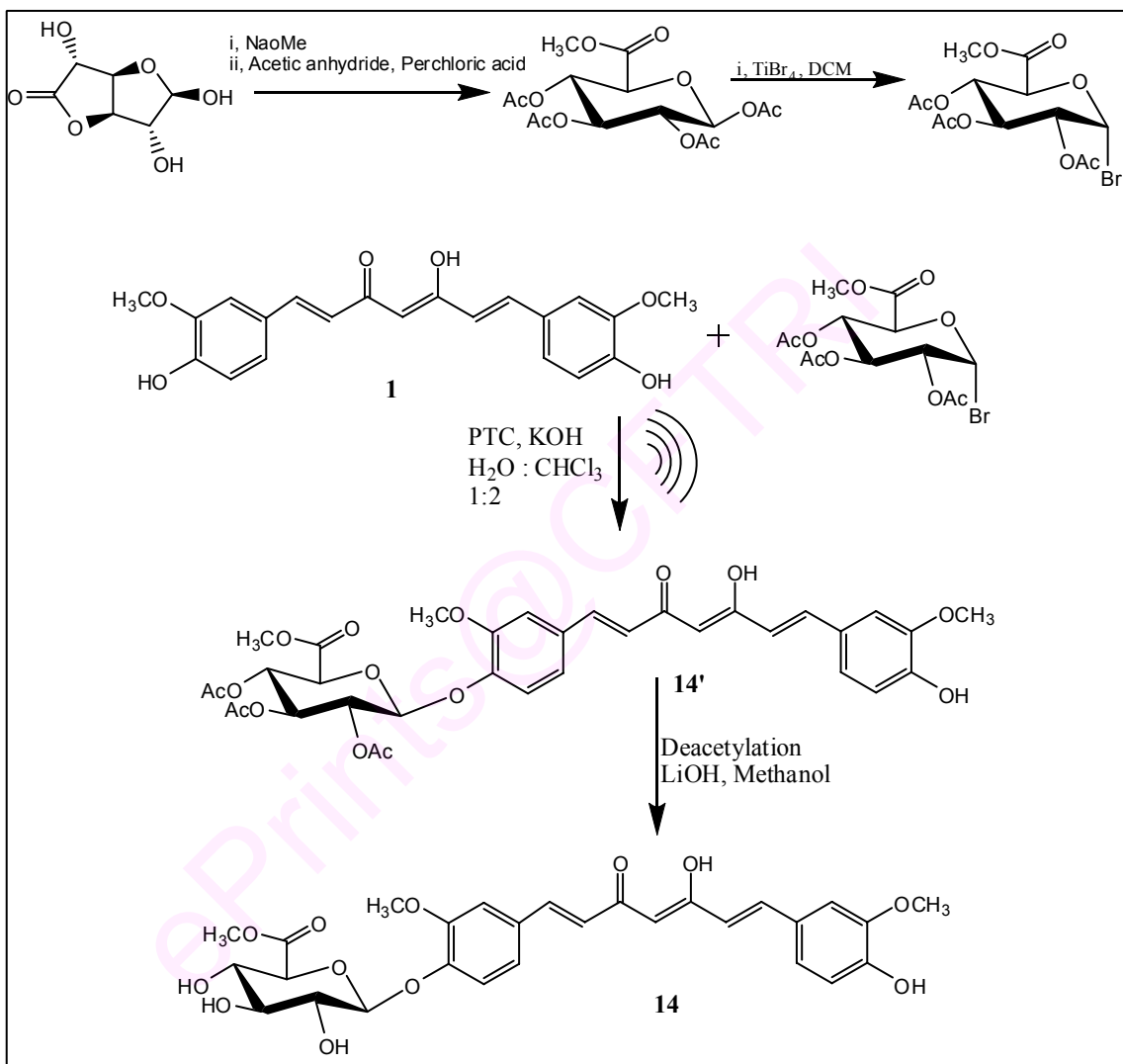
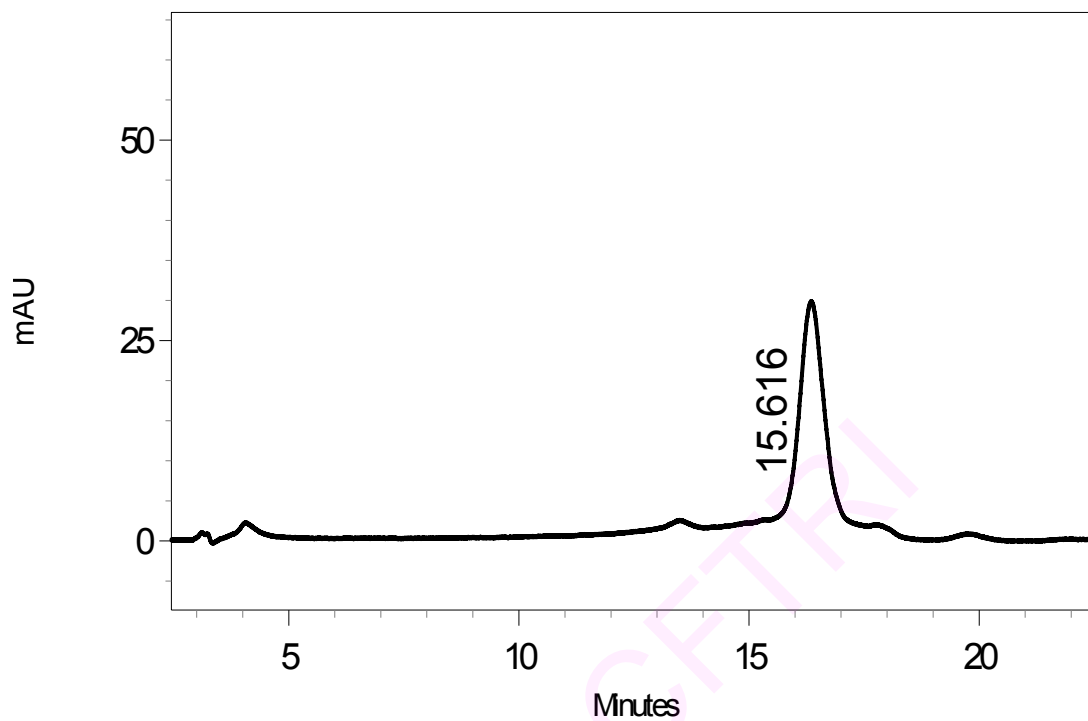**Fig 4.1 Synthesis of methyl 1-curcuminyl-β-D-glucopyranuronate**

Fig. 4.2 HPLC profile of methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-gluco-pyranuronate (14´)
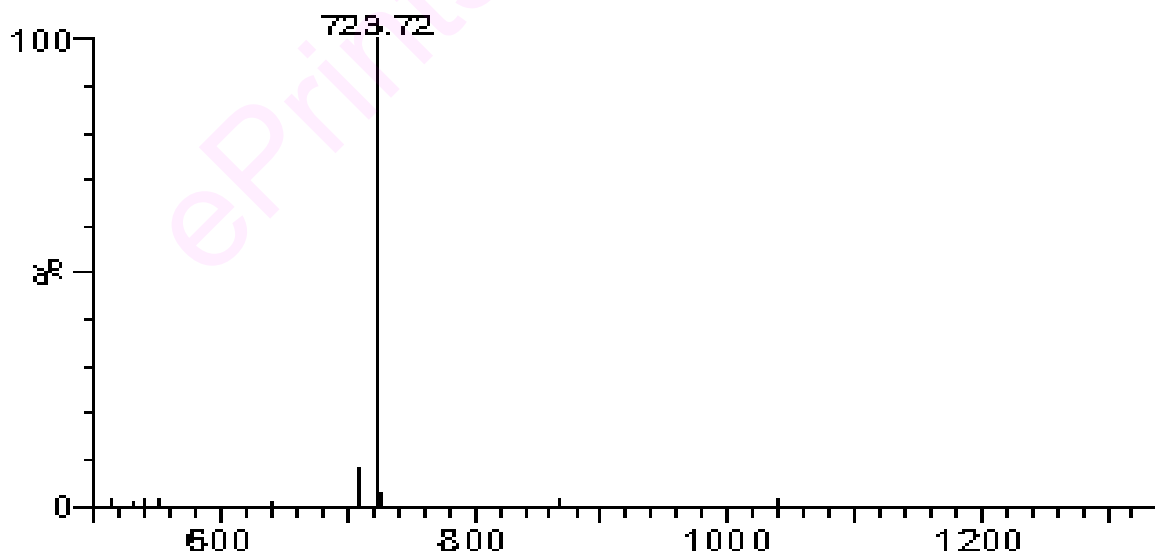

Fig. 4.3 ESI MS of methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-gluco-pyranuronate (14´)

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(a) Spectral characterization of the products

The reactions were carried out with different phase transfer catalysts like cetyltributylammonium bromide, tetrabutylammonium bromide and benzyltributylammonium chloride. The reaction was found to occur efficiently in the presence of benzyltributylammonium chloride. TLC of the product showed a spot with RF value of 0.65. HPLC of the product showed retention time of 15.6 (Fig. 4.2), MS analysis of the product [ESI-MS] exhibited molecular mass ion at 723.72 [M + K]$^+$ corresponding **14´** (Fig. 4.3)**.** The ^{1}H NMR characterized the compound as the methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate of curcumin. While, the signals at 3.99, 5.91, 7.15, 7.28 and 7.42 correspond to the methoxyl, enolic and aromatic protons of the curcumin moiety, signals corresponding to the olefinic protons appear as doublets at 6.80 and 7.60 with J value of 15.5 Hz. The characteristic methyl group of –COOCH$_3$ was observed at 3.79. The three singlets at 2.07-2.12 corresponded to the acetyl groups. Signals corresponding to the sugar protons appeared in the range 4.5–5.84 accounting for five protons. The product was deacetylated with LiOH in methanol to yield methyl 1-curcuminyl-β-D-glucopyranuronate. HPLC showed retention at 4.76 min (Fig. 4.4). MS analysis of the product [ESI-MS] exhibited molecular mass ion at 581.71 [M + Na]$^+$ corresponding to curcumin conjugate of methyl mono-β-glucuronide **14** (Fig. 4.5, Table 4.1). The ^{1}H NMR characterized the compound as methyl 1-curcuminyl-β-D-glucopyranuronate. The signals at 3.85, 6.10, 7.39, 7.26 and 7.13 corresponded to the methoxyl, enolic and aromatic protons of the curcumin moiety. While signals corresponding to the olefinic protons appear as

doublets at 6.78 and 7.57 with J value of 15.5 Hz, the characteristic methyl group of –COOCH$_3$ was observed at 3.17. ^{1}H NMR showed the absence of acetyl groups, which confirmed the deacetylation. The sugar protons after deacetylation appeared in the range 4.1-6.04.
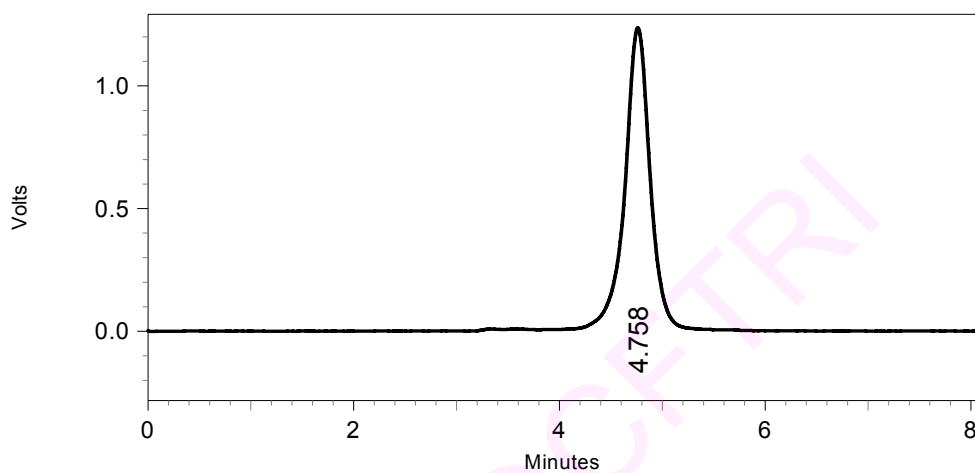

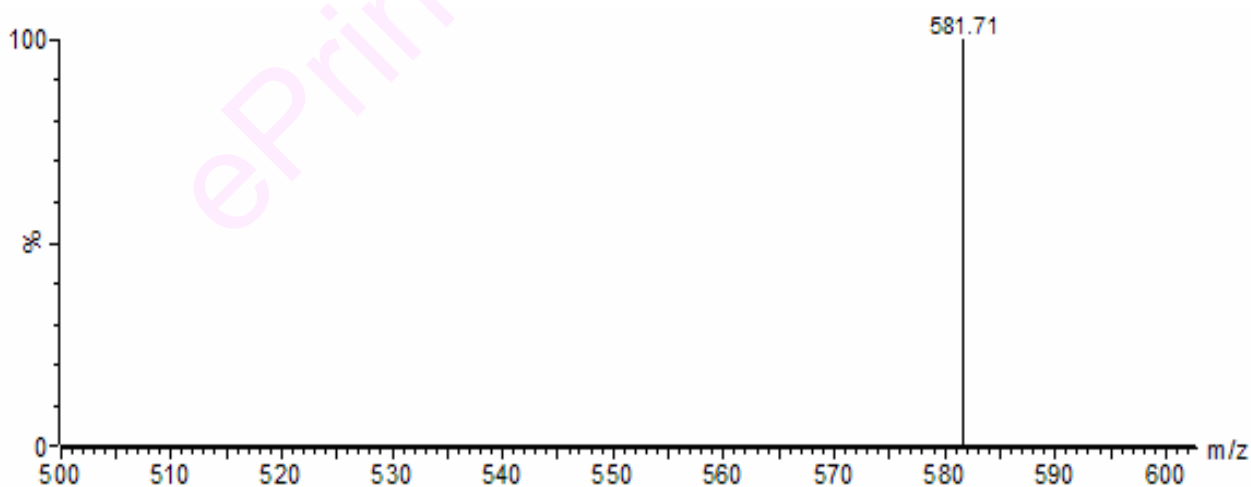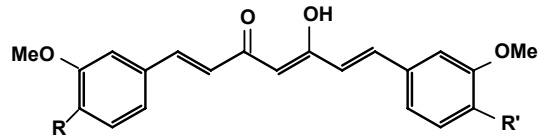**Fig. 4.4 HPLC profile of methyl 1-curcuminyl-β-D-glucopyranuronate (14)**

Fig. 4.5 ESI MS of methyl 1-curcuminyl-β-D-glucopyranuronate (14)

Table 4.1 Curcumin and its glucuronide derivative

Compound	Structure number	R	R'
Curcumin	1	OH	OH
Methyl 1-curcuminyl-2,3,4-tri-*O*-acetyl-β-D-gluco-pyranuronate	14´	OH	
Methyl 1-curcuminyl-β-D-glucopyranuronate	14	OH	

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4.2 GLUCAL DERIVATIVE OF CURCUMIN

4.2.1 INTRODUCTION

2,3-Unsaturated glycosides are versatile synthetic intermediates and also constitute the structural units of several natural products. Allylic rearrangement of 3,4,6-tri-*O*-acetyl-D-glucal, known as the Ferrier rearrangement, in the presence of a nucleophile leads to the formation of 2,3-unsaturated glycosides (Ferrier 1969; Ferrier 2001). This stereo-selective reaction is one of the most important methods for the preparation of glycosides utilized for synthesis of antibiotics. Its application to synthesis of natural products is well established (Marzabadi & Franck, 2000). Among glycosides, the 2, 3-unsaturated glycosides have a unique place and importance in carbohydrate chemistry since this double bond can be further functionalized. The reactions of glucal with phenolics are well established to yield bioactive natural products (Noshita, et al., 1994; Tosima & Tatsuta, 1993).

Several Lewis acids are employed for the Ferrier reaction. Reactions of glucal with various alcohols and thiols are reported in the presence of $BiCl_3$ (Swamy & Venkateswaralu, 2002). Different reagents such as, boron trifluoride etherate (Kunz & Sager, 1985), tin (IV) chloride, montmorillonite K-10, DDQ, $BiCl_3$ and N-iodosuccinimide have been employed for the Ferrier rearrangement. Glucosides are synthesized from glucose and fatty acid alcohols using Ferrier reaction catalyzed by $BF_3 \cdot Et_2O$ (Konstantinovic, et al., 2001). Ferrier rearrangement reaction is reported using acid zeolite as catalyst (Rauter, et al., 2007). A reagent trimethylsilylnitrate is employed for direct synthesis of 2-deoxy-

O-glycosides from glycols (Reddy & Vankar, 2004). Reports are available on microwave-induced, Montmorillonite K10-catalyzed Ferrier rearrangement of tri-*O*-acetyl-D-galactal (Shanmugasundaram, et al., 2002). Zinc chloride has been employed for the synthesis of 2,3-unsaturated 1-O-glucopyranosides of allylic, benzylic and tertiary alcohols (Bettadaiah & Srinivas, 2003).

Ferrier rearrangement for the synthesis of PEG-bound 2,3-unsaturated glycopyranosyl-amino acids (Bhat, et al., 2007), by a mild, efficient and selective method using potassium dodecatungstocobaltate trihydrate as catalyst, is reported (Rafiee, et al., 2004). Use of Indium chloride (Frost & Hartley, 2004), Ferric sulfate hydrate (Zhang, et al., 2008), Ceric ammonium nitrate (CAN), $Bi(OTf)_3$ and SiO_2-$Bi(OTf)_3$ (Babu, et al., 2005) as efficient catalysts is documented. Metal-catalyzed transformation of d-glucal to optically active furan diol (Hayashi, et al., 1999) is reported in literature. Other examples include, synthesis of 2,3-unsaturated C-glycosides by $HClO_4$–SiO_2 catalysis, (Tiwari, et al., 2005), Ferrier sulfonamidoglycosylation of D-glycals (Colinas, et al., 2007), Metal nitrates catalyzed *O*-glucosylation using acetyl glucal in organic solvents and ionic liquids (Naik, et al., 2005), Iodine catalyzed *C*-glycosidation of D-glucal with silylacetylene, (Saeeng, et al., 2003) and Niobium (V) chloride catalyzed microwave assisted synthesis of 2,3-unsaturated O-glycosides by the Ferrier reaction (Hotha & Tripathi, 2005).

In this present study, Ferrier reaction of D-glucal with curcumin was investigated with several catalysts with a view to synthesizing 2,3–dideoxy sugar derivative of curcumin.

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4.2.2 EXPERIMENTAL

(a) Synthesis of curcuminyl 4,6-di-*O*-acetyl-hex-2-enopyranoside (15´)

To the solution of anhydrous acetonitrile (20 ml) at 40 ^{o}C, curcumin (2.7 mmol, 1 g) was added. To the clear solution, 3, 4, 6-tri-*O*-acetyl-D-glucal (2 mmol x 3) in acetonitrile was added. To the mixture $Fe_2(SO_4)_3$ x H_2O (1 mol %) was added and stirred at 40 ^{o}C. TLC monitored the reaction for the disappearance of curcumin (6 h). The organic layer was washed with water, dried over anhydrous sodium sulfate and distilled under reduced pressure. The crude sample was loaded on to silica gel (200-400 mesh) column and eluted out with distilled $CHCl_3$. Pure fractions were pooled and evaporated to afford curcumin acetyl mono glucal derivative at 50 % yield.

^{1}H NMR (500 MHz, $CDCl_3$); 2.03 (s, 3H, -OCOCH$_3$) 2.07 (s, 3H, -OCOCH$_3$), 3.95 (s, 6H, -OCH$_3$), 4.28 (m, 1H), 4.41 (t, 2H, J = 6.5 Hz & J= 5.5 Hz), 4.85 (dd, 1H, J = 3 Hz), 5.19 (m, 1H), 5.99 (s, 2H), 6.15 (s, 1H), 6.64 (d, 2H, J = 15 Hz), 7.01 (d, 2H, J = 5 Hz), 7.11 (d, 2H, J = 7.5 Hz), 7.21(s, 2H), 7.58 (d, 2H, J = 15 Hz).

(b) Deacetlyation of curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside

To a solution of curcumin acetyl mono glucal derivative (**15´**, 1 g) in dry methanol (10 ml), sodium methoxide, prepared by dissolving sodium (45 mg) in dry methanol (6 ml), was added drop-wise and the mixture was stirred. The deacetylation reaction was monitored on TLC. At the end of the reaction (0.5 h), the solution was neutralized by the addition of freshly regenerated Dowex (IR-120) H^{+} resin. The resin was filtered and the solvent distilled under reduced pressure to afford curcuminyl hex-2-enopyranoside (**15**) in 90 % yield.

^{1}H NMR (500 MHz, CD$_3$OD); 3.70 (s, 6H, -OCH$_3$), 4.03 (m, 1H), 4.16 (t, 2H, J = 6.5 Hz & J= 5.5 Hz), 4.6 (dd, 1H, J = 3 Hz), 4.94 (m, 1H), 5.74 (s, 2H), 5.9 (s, 1H), 6.71 (d, 2H, J = 15 Hz), 7.04 (d, 2H, J = 5 Hz), 7.16 (d, 2H, J = 7.5 Hz), 7.30 (s, 2H), 7.50 (d, 2H, J = 15 Hz).
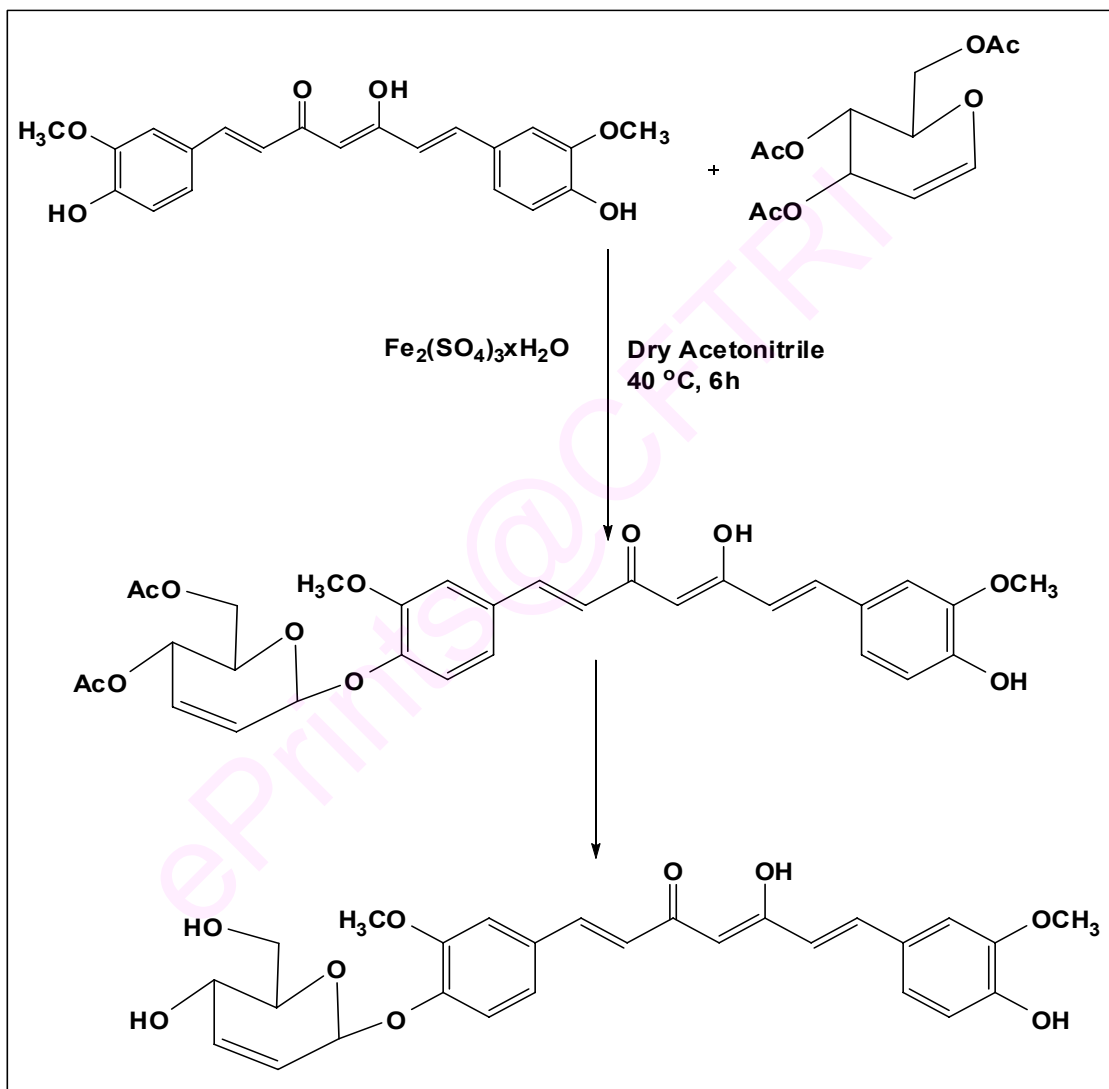

Fig. 4.6 Synthesis of Curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside

4.2.3 RESULTS AND DISCUSSION

Ferrier reaction is a well-known methodology for the synthesis of O-glycosides. Ferrier arrangement involves the rearrangement of the double bond from 1, 2 position with the removal of an acetyl group at the 3 position leading to the formation of 2,3-dideoxy derivatives. In this present study, reaction of a phenolic substrate, curcumin, with 3,4,6-tri-*O*-acetyl-D-glucal was studied. The reaction was explored in presence of several Lewis acid catalysts like $Bi(NO_3)_3$, $InCl_3$, $BF_3.EtO_2$, $ZnBr_2$, $ZnCl_2$ and Iodine. It was observed that the reaction was very slow in these cases. Curcumin mainly remained intact but the glucal was expended in the reactions. The latter is a competitive pathway reported in the Ferrier reaction, wherein the glucal gets dimerized leading to eventual formation of polymeric products. However, in presence of $Fe_2(SO_4)_3 \times H_2O$ as catalyst, the reaction progressed satisfactorily indicating the formation of a product. Reaction was investigated under different solvents viz., methanol, ethanol, dichloromethane, DMSO, chloroform, acetone and dioxane. The reaction in acetonitrile was found to provide optimum results. Curcumin showed moderate solubility in acetonitrile at RT and the reaction at ambient temperature was found to be very slow. However, curcumin showed good solubility at 40 ^{o}C and hence, the reaction could be conveniently carried out at this temperature. Accordingly, Ferrier reaction of 3,4,6-tri-*O*-acetyl-D-glucal with curcumin was carried out in presence of ferrous sulfate hydrate as catalyst under stirring at 40 ^{o}C to afford curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside in over 50 % yield (Fig. 4.6). The reaction carried out with different amounts of catalysts in acetonitrile solvent

showed varied yields (Table 4.2). 0.5 mol % of $Fe_2(SO_4)_3$ x H_2O showed little reaction. The results of 1 mol % of $Fe_2(SO_4)_3$ x H_2O showed 50 % yield. Further increase in the amount of catalyst amount did not result in improvement in the yield of the product.

(a) Spectral Characterization of products

Completion of the reaction in these experiments was confirmed by analysis of the product by TLC. The product was easily isolable from the reaction mixture by separation of the organic phase, its treatment with water, followed by drying of the organic layer and distillation. The crude product was purified by column chromatography on silica gel. TLC of the product showed a spot with RF value of 0.62. HPLC of the product showed presence of one compound with retention time of 11.7(Fig. 4.7). MS analysis of the product [ESI - MS] exhibited molecular mass ion at 603.95 $[M + Na]^+$ corresponding to diacetyl mono-glucal derivative of curcumin (Fig. 4.8). It was characterized as curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside by the ^1H NMR spectral data (**15´**). The signals at 3.95 correspond to the methoxyl and those at 6.85, 7.11 and 7.21 to aromatic protons of the curcumin moiety. While signals corresponding to the olefinic protons appear as doublets at 6.64 and 7.58 with *J* value of 15 Hz, the two singlets at 2.03 and 2.07 correspond to the acetyl groups of the glucal moiety. The anomeric proton of glucal shows a shift from 6.5 to 5.19 after getting attached to curcumin. The glucal sugar protons appereard in the range 4.28-5.99.

Table 4.2 Effect of amount of catalyst on the reaction

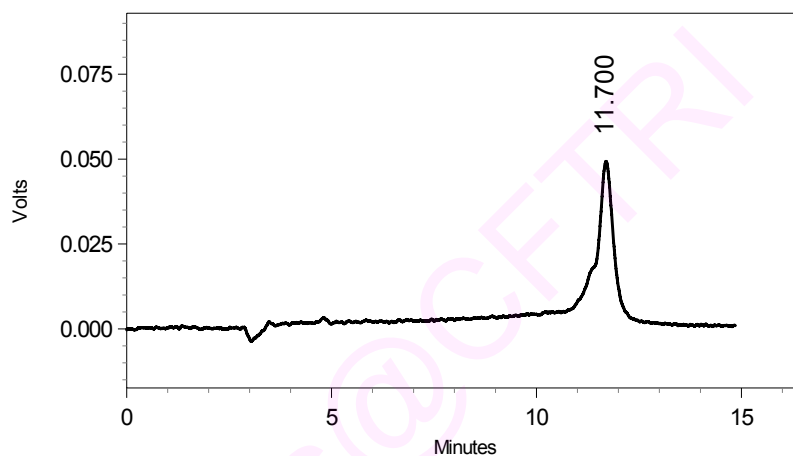| Entry	Catalyst (mol %)	Yield %
1	0.5	No reaction
2	1	50
3	2	35

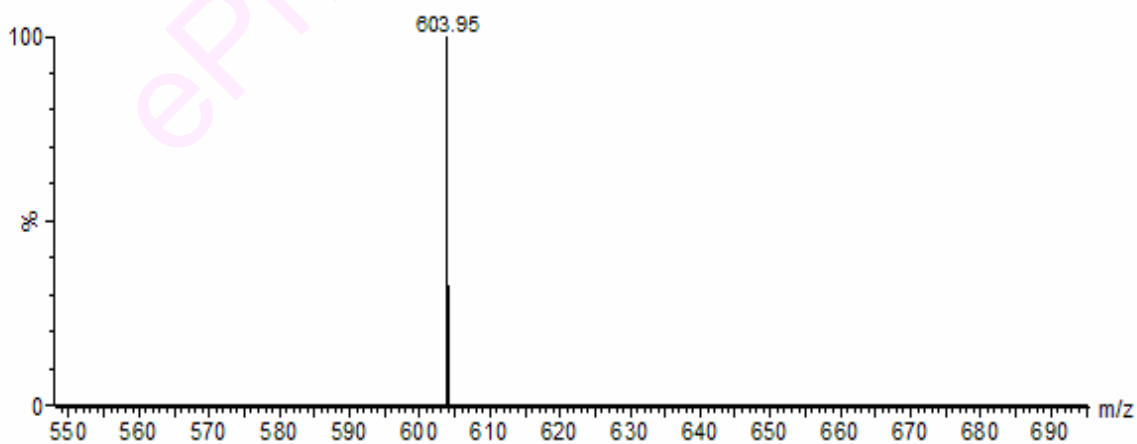**Fig. 4.7 HPLC profile of curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside (15´)**

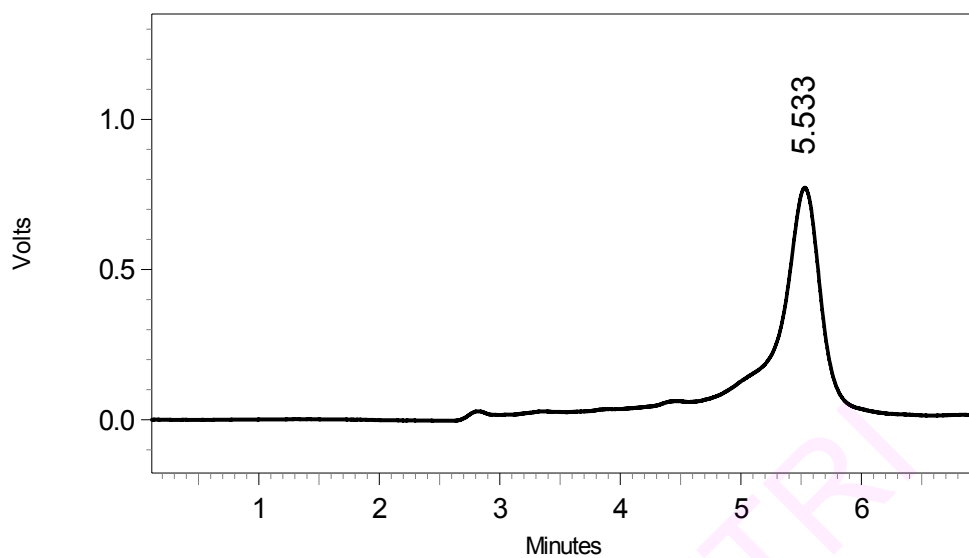**Fig. 4.8 ESI MS of curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside**

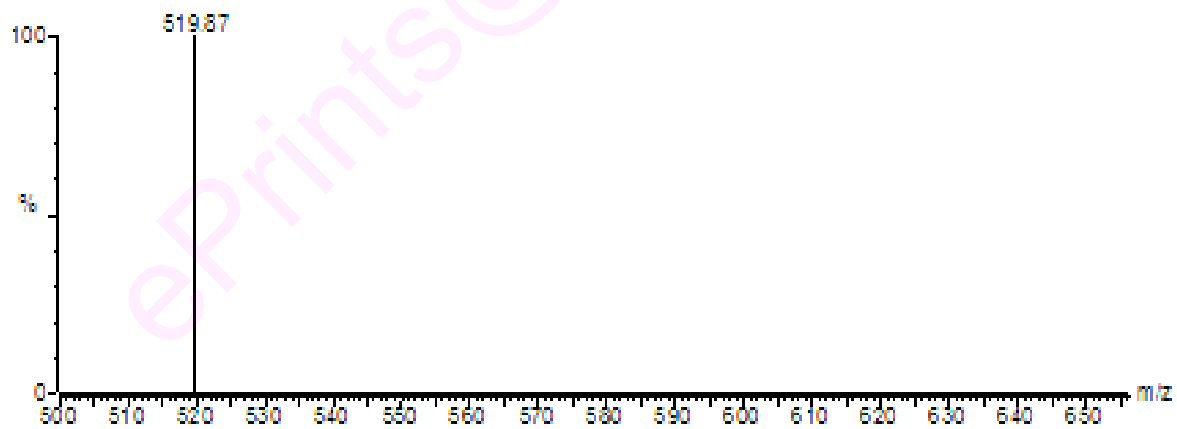**Fig. 4.9 ESI MS of curcuminyl hex-2-enopyranoside (15)**

519.87

Fig. 4.10 ESI MS of curcuminyl hex-2-enopyranoside

(b) Deacetylation of curcumin acetyl mono glucal derivative

Curcuminyl 4,6-di-O-acetyl-hex-2-enopyranoside could be conveniently deacetylated using sodium methoxide in dry methanol at room temperature. HPLC analysis showed one peak of retention time 5.53 (Fig. 4.9). The MS analysis of the products [ESI-MS] exhibited molecular mass ion (Fig. 4.10) at 519.87 [M + Na] [+] which were further characterized as curcuminyl hex-2-enopyranoside by NMR spectral data (**15**). The absence of acetyl groups in the region of 2.03-2.09 indicated complete deacetylation. The spectrum showed characteristic signals for methoxyl and enolic protons of curcumin at 3.70 and 5.90 respectively. The aromatic protons of curcumin apperared at 7.04, 7.16 and 7.30. The olefinic protons were seen at 6.71 and 7.50. The sugar protons were in the region of 4.41-5.99.

4.3 CONCLUSIONS

A synthetic methodology was developed for the synthesis of methyl 1-curcuminyl-β-D-glucopyranuronate starting from glucuronolactone. Ferrier reaction of 3,4,6-tri-O-acetyl-D-glucal with curcumin in presence of ferric sulfate catalyst at 40 ^{o}C in acetonitrile, followed by deacetylation afforded curcuminyl hex-2-enopyranoside in 45 % yield.

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CHAPTER 5

ANTIOXIDANT, ANTIMUTAGENIC AND ANTIBACTERIAL PROPERTIES OF CURCUMIN DERIVATIVES

5.1 INTRODUCTION

Curcumin is the nutraceutical component of turmeric. Its medicinal properties and its use in food are well studied. Antioxidant and wound-healing properties of curcumin, its' anti-cancer and anti-viral attributes, its' effect on lymphocytes, platelet aggregation, detoxification mechanisms, cell cycle and apoptosis and its' ameliorating role in diabetes and stress responses are well established (Joe, et al., 2004; Goel, et al., 2008). The antioxidant properties, antimutagenicity and antimicrobial studies of curcumin are well recognized (Araujo & Leon, 2001; Jayaprakasha, et al., 2006). Antioxidant activity of turmeric, turmerin and curcumin has been studied. Water-soluble extract turmerin, inhibits HIV infected T–cell proliferation and combination with 3' azido-3'-deoxythymidine decreases T-cell infection and increases cell viability (Cohly, et al., 2003). The effect of curcumin on lipid peroxidation has also been studied in various models by several authors. Curcumin is a good antioxidant and inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy, et al., 1994). The antioxidant capacities of curcumin extracts from fresh and dried rhizomes from different clones of turmeric grown *in vitro* are compared with their capacity to scavenge DPPH (Cousins, et al., 2007).

Curcumin, bisdemethoxycurcumin and demethoxycurcumin, are tested for their antioxidant activities by *in vitro* model systems, such as the phosphomolybdenum and linoleic acid peroxidation methods (Sun et al., 2004). Several theoretical calculations are also reported wherein DFT and TD-DFT calculations help to find out keto-enol equilibrium of curcumin in solution state

and low pK_a value for the dissociation of enol proton. This study shows the importance of proton-transfer/dissociation-associated radical-scavenging mechanisms of curcumin (Shen & Ji, 2007). Reports are available wherein, curcumin structure containing enol forms suppose to be more stable than diketo form. Attempts are made to understand the antioxidant action and mechanism of curcumin (Wright, 2002; Sun, et al., 2004). The antioxidant activities and antioxidant capacities of curcumin, dimethoxycurcumin and bisdemthoxycurcumin have been studied with *in vitro* model systems (Jayaprakasha, et al., 2006, Naidu et al., 2009). In order to understand the chemical basis of various biological properties of curcumin, the structural activity studies on curcumin, dimethoxycurcumin and bisdemthoxycurcumin are carried out. Curcumin is found to be more effective than its analogs in its cleavage reaction with DNA. The relative antioxidant activity has been examined by studying the effect of these curcuminoids on cleavage of plasmid DNA by Fe(II)-EDTA system (hydroxyl radicals) and the generation of singlet oxygen by riboflavin. The DNA cleavage activity is the consequence of binding of Cu(II) to various sites on the curcumin molecule and also anti-inflammatory and proapoptotic activities assigned to curcumin are mediated through its prooxidant/antioxidant mechanism. (Ahsan, et al., 1999; Sandur, et al., 2007).

Antioxidant properties of curcumin using EPR spectroscopic technique are investigated. Study reveals that curcumin is a potent singlet oxygen quencher at low physiological or pharmacological concentration. In aqueous solutions, this physiologically relevant property of this compound, can explain its effect in

protecting skin against UV light. Singlet molecular oxygen is an electronically excited species of oxygen that is produced in mammalian cells under normal and patho-physiological conditions (Das & Das, 2002). Antioxidant activities of curcumin extracted from turmeric by different techniques like hydro-distillation, low-pressure solvent extraction, soxhlet, and supercritical extraction using carbon dioxide and co-solvents have been compared. The solvents and co-solvents tested are ethanol, isopropyl alcohol, and their mixture in equal proportions. The soxhlet and low-pressure extracts exhibit the strongest antioxidant activities (Braga, et al., 2003). Curcumin protects islets against streptozotocin-induced oxidative stress by scavenging free radicals. Studies show curcumin prevents reduction in levels of cellular free radical scavenging enzymes (Meghana, et al., 2007). Antimutagenic activity and structure-activity relationship between curcumin and its naturally-occurring analogs, namely demethoxycurcumin and bisdemethoxycurcumin, and other structurally-related natural and synthetic analogs of curcumin, namely tetrahydrocurcumin, dibenzoylmethane, dibenzoylpropane, vanillin, ferulic acid, isoferulic acid and caffeic acid, by Ames test, against different classes of cooked food mutagens are studied. The results indicate that unsaturation in the side chain, a methoxy group on the benzene ring and keto-enol moiety in the curcumin molecule are the important structural requirements responsible for high antimutagenic potential of curcumin against mutagenicity of several food derived heterocyclic amines (Shishu & Kaur, 2008).

The antimicrobial activities of turmeric extracts are reported. Activites of ar-turmerone from the rhizome and curcumin show similar activity (Hoi-Seon-Lee,

2006). Antibacterial and antifungal properties of curcumin and its derivatives are reported wherein it is shown that synthetic derivatives of curcumin with piperine, glycine and alanine exhibit activities superior to curcumin (Mishra, et al., 2005). Curcuma oil when tested against cultures of *Staphylococcus albus*, *Staphylococcus aureus* and *Bacillus typhosus* show inhibition of the growth of *S. Albus* and S. Aureus at different concentrations (Chopra et al., 1941). The oil extracted from the spent turmeric oleoresin and its purified fractions are tested for antibacterial activity by pour plate method fraction eluted out with 5% ethyl acetate in hexane is the most active fraction (Negi, et al., 1999). GC and GC-MS analysis establish ar-turmerone, turmerone, and curlone are the major compounds present in these fractions along with other oxygenated compounds. The antifungal activity of turmeric oil, which is isolated from mother liquor after isolation of curcumin has also been studied. The turmeric oil is fractionated using fractional distillation under vacuum to get two fractions. These fractions tested for antifungal activity against different organisms by spore germination method (Jayaprakasha, et al., 2001).

Several derivatives of curcumin are synthesized with an aim to increasing the potency of biological activities of curcumin. Antioxidant properties of three curcumin derivatives, in which the 1,3-diketone is linked with nitrogen and sulfur moieties and their copper conjugates have been studied. Metal conjugation to curcumin imparts distinct advantage reflected in radical scavenging activities of curcumin compounds (Dutta, et al., 2001). Water-soluble cyclodextrin inclusion complex of curcumin shows enhanced radical scavenging activity compared to

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curcumin. Kinetics of binding is studied by stopped flow technique, from which the binding constants for inclusion complex are estimated. The influence of such inclusion complex on change in superoxide radical scavenging property of curcumin is examined using xanthine/ xanthine oxidase assay (Swaroop, et al., 2007). Manganese complexes of curcumin, diacetylcurcumin and ethylenediamine derivative are synthesized and evaluated *in-vitro* for antilipid peroxidation and superoxide dismutase activity. The results show enhanced radical scavenging activity (Vajragupta, et al., 2003).

In this present chapter, results of study of *in vitro* antioxidant, antibacterial and antimutagenic attributes of water-soluble derivatives of curcumin viz., curumin glucoside, amino acid conjugates of curcumin, curcumin glucuronide conjugate and curcumin glucal derivative, whose synthesis is reported in Chapters 2, 3 and 4, are presented and discussed.

5.2 EXPERIMENTAL

5.2.1 Materials and equipments

All the solvents and reagents used for the studies were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and β-carotene were procured from Sigma Chemical Co. (St. Louis, MO, USA). Spectrophotometric studies were done on a UV-visible spectrophotometer (GBC Cintra 10, Australia). All the chemicals and petri plates used for microbial studies were procured from Hi Media Ltd., Mumbai, India.

5.2.2 Radical-scavenging activity by DPPH method

Radical-scavenging activity of the derivatives was analyzed by DPPH method (Moon & Terao, 1998). DPPH is a free radical (**I**), stabilized by virtue of the delocalization of the spare electron over the whole molecule. The delocalization also gives rise to the deep violet color, characterized by an absorption band at about 515 -520 nm when measured in alcohol solution. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then it gives rise to the reduced form (**II**) with the loss of the violet color (Fig. 5.1). The reaction in Fig. 5.1 shows the formation of free radical. Radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorized) by one molecule of the reductant.

Individual samples were prepared by dissolving each compound in DMSO. The samples were assayed at different concentrations, making them up to 1 ml with 100 mM Tris-HCl buffer (pH 7.4), followed by addition of 4 ml of 2,2-

diphenyl-1-picrylhydrazyl (0.1 mM solution in methanol) and mixing of the contents by vigorous shaking. Control in these experiments was prepared by same protocol wherein the compound was not included. The tubes were incubated in dark at room temperature for 20 min. The absorbance was then recorded at 517 nm using DMSO for baseline correction. The experiments were carried out in triplicates.

The % radical-scavenging activity was calculated using the following formula,

Radical-scavenging activity (%) = [(Control OD – Sample OD)/ Control OD] X 100

5.2.3 Antioxidant activity by *β*-carotene bleaching method

In this assay, antioxidant activity was determined by measuring the inhibition of the formation of lipophilic organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Adegako, et al., 1998).

The mechanism of bleaching of *β*-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. *β*-carotene undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated *β*-carotene molecules. As *β*-carotene loses its double bonds by oxidation, its characteristic orange color is lost. The change is monitored spectrophotometrically.

A stock solution of *β*-carotene-linoleic acid mixture was prepared as follows: 0.2 mg *β*-carotene* was dissolved in 0. 2 ml of chloroform (HPLC grade), to which 20 mg linoleic acid and 200 mg Tween 40 were added. Chloroform was

completely evaporated using a vacuum evaporator. Then, 40 ml of distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking. 4 ml of this stock was dispensed into test tubes and 0.2 ml portions of the samples were added and the emulsion system was incubated for 2 h at 50 ^{o}C temperature. The same procedure was repeated for curcumin as positive control and a blank. After this incubation period, absorbance of the mixtures was measured at 470 nm at zero time (t = 0) and continued, at an interval of 15 min, till the color of β–carotene in the control tubes disappeared (180 min) and the antioxidant activity was determined with the formula,

Antioxidant property = 100 [1-(A_O - A_t)/ (A^o_O - A^o_t)] where A_o and A^o_O are the absorbance measured at zero time for the incubation of the test sample and control respectively and A_t and A^o_t are the absorbance measured in the test sample and control after incubation for 180 min.

5.2.4 Antimutagenicity by Ames test

The standard plate incorporation test was carried out according to Maron and Ames (1983). In the antimutagenicity test, the inhibition of mutagenic activity of the sodium azide/MMS by the test samples was determined. Two milliliters of top agar (0.6% agar & 0.25 ml of 0.5 mM Histidine–Biotin mixture) was distributed to 13 X 100 mm capped culture tubes held at 45 ^{o}C in a water bath and to it different concentrations of the test sample made in DMSO and 0.1 ml of 10 h old culture of either *Salmonella typhimurium* TA 1531 (MTCC 1254, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India) or *Salmonella typhimurium* TA 98 (MTCC 1251, Microbial Type Culture

Collection, Institute of Microbial Technology, Chandigarh, India) were added. It was mixed by vortexing at low speed and then poured on to the minimal glucose agar (1.5% agar with 40% glucose and VB salt) plate to form a uniform thin layer. Positive and negative controls were included in each assay. Sodium azide/MMS was used as a diagnostic mutagen (1.5 µg/plate) in positive control plates. Negative control plates were prepared with equivalent amounts of DMSO without sodium azide and the test sample, which was used as a check for the number of colonies that arise spontaneously. Only sample without mutagen was also taken as control. The number of histidine$^+$ (His$^+$) revertant colonies was counted after incubation of the plates at 37 ^{o}C for 48 h. Each sample was assayed using duplicate plates and the data presented as mean ± SD of the three independent assays. The mutagenicity of sodium azide/MMS in the absence of test samples was defined as 100% inhibition. The calculation of percent inhibition was done according to the formula given

$$\% \text{ Inhibition} = [1-T/M] \times 100,$$

Where T is number of revertants per plate in presence of mutagen (sodium azide/MMS) and test samples and M is number of revertants per plate in positive control (sodium azide/MMS) (Ong, et al., 1986). The number of spontaneous revertants was subtracted from numerator and denominator. The antimutagenic effect was considered moderate when the inhibitory effect was 25 – 40% and strong where, it was more than 40%. Inhibitory effects of less than 25% were considered as weak and were not recognized as a positive result (Ilken, et al., 1999).

5.2.5 Antibacterial activity

The antibacterial assay of curcumin and its derivatives was tested by pour plate method against *Bacillus cereus* (F 4810, Public Health Laboratory, London, UK)*, Staphylococcus aureus* (FRI 722, Public Health Laboratory, The Netherland)*, E.Coli* (MTCC 108, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India) and *Yersinia enterocolitica* (MTCC 859, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India) by the method of Negi et al., (1999). Samples were prepared by dissolving compounds in acetone. To flasks containing 20 ml melted agar, different concentrations of samples were added. Equivalent amounts of acetone were used as controls. One hundred μl (about 10^3 cfu/ml) of culture was inoculated into the flasks under aseptic conditions. The media was then poured into sterilized petri plates and incubated at 37 ^{o}C for 24 h for growth studies. Each experiment was done in duplicates and repeated three times.

The inhibitory effect was calculated using the following formula:

% Inhibition = (1 – T/C) x 100, Where, T is cfu/ml of test sample and C is cfu/ml of control. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium being tested.

5.2.6 Statistical analysis

All the experiments were repeated three times and the data were calculated as mean ± SD and analyzed by one–way ANOVA. The Duncan's multiple range test (DMRT), which involves the computation of numerical

boundaries that allow for the classification of the difference between any two treatments means as significant or non- significant. DMRT requires computation of series of values each corresponding to a specific set of pair comparisons (Gomez & Gomez, 1984). In all the plots and figures, the treatment means of the derivatives were calculated by Duncan's multiple range test (DMRT), the graph followed by different letters differs significantly (p<0.05).

5.3 RESULTS AND DISCUSSION

5.3.1 Curcumin glucoside

Curcumin di-β-glucoside tetracetate (Table 5.1, structure no. **3'**) was synthesized in high yields by Koeings-Knorr reaction of curcumin (**1**) with 2, 3, 4, 6-Tetra–*O*-acetyl-α–D-glucopyranosyl bromide in presence of benzyltributylammonium chloride as the phase transfer catalyst under biphasic conditions and under the influence of ultrasound. Deacetylation of **3** afforded the corresponding curcumin di-β-glucoside (**3**) (Discussed in detail in Chapter 2).

i) Solubility

The solubility of curcumin (**1)** in water was always a matter of concern because of its insolubility at neutral and acidic pH. The modification of **1** by attaching sugar moiety to its phenolic hydroxyl group rendered it water-soluble. The solubility of curcumin di-β-glucoside **(3)** was 10 mg/ml. In the present study, the antioxidant, antimutagenic and antibacterial activities of **3** were evaluated by *in vitro* methods and compared with those of **1**.

ii) Radical-scavenging activities (RSA) and antioxidant activities

Radical-scavenging assay was carried out with curcumin (**1)** as a reference for comparison with the water-soluble curcumin di-β-glucoside (**3**). Both **1** and **3** were dissolved in DMSO at 0.05-0.25 µM concentrations. The radical-scavenging activity (RSA) by DPPH method showed 40.65% and 49.5% activity at 0.05 µM of **1** and **3** respectively (Fig. 5.2). At 0.15 µM, RSA of **1** and **3** was 67% and 100% respectively. However, RSA of **1** and **3** at both these concentrations were significantly different from each other ($p < 0.05$). In the

antioxidant activity assay by *β*–carotene bleaching method (Fig. 5.3), 41% and 55% activity was observed at 0.05 µM for **1** and **3**, which were statistically ($p < 0.05$) different. At 0.15 µM concentration, **3** showed significantly ($p < 0.05$) higher activity (100%) than **1** (68%). These results clearly showed that **3** exhibited higher antioxidant activity than **1** at all concentrations.
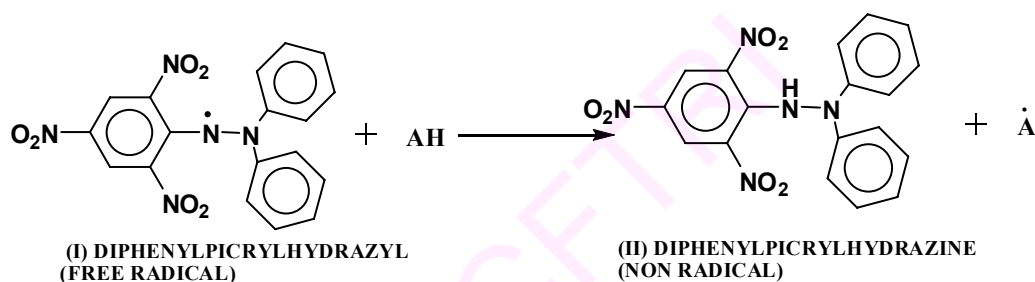
Fig. 5.1 Radical scavenging activity of DPPH

(I) DIPHENYLPICRYLHYDRAZYL
(FREE RADICAL)

(II) DIPHENYLPICRYLHYDRAZINE
(NON RADICAL)

Table 5.1 Curcumin and its glucoside derivatives

Compound	Structure number	RR'
Curcumin	**1**	OH
Curcumin di-β-glucoside tetra acetate	**3´**	
Curcumin di-β-glucoside	**3**	

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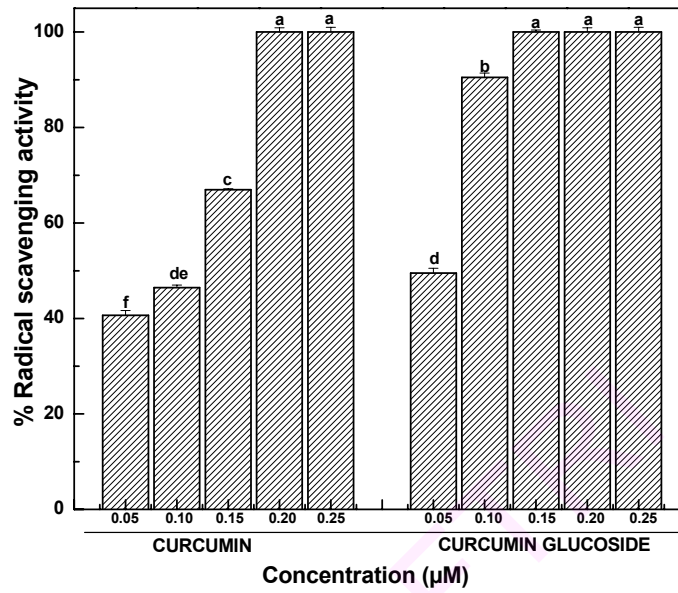**Fig 5.2 Radical-scavenging activity of 1 and 3 by DPPH method**

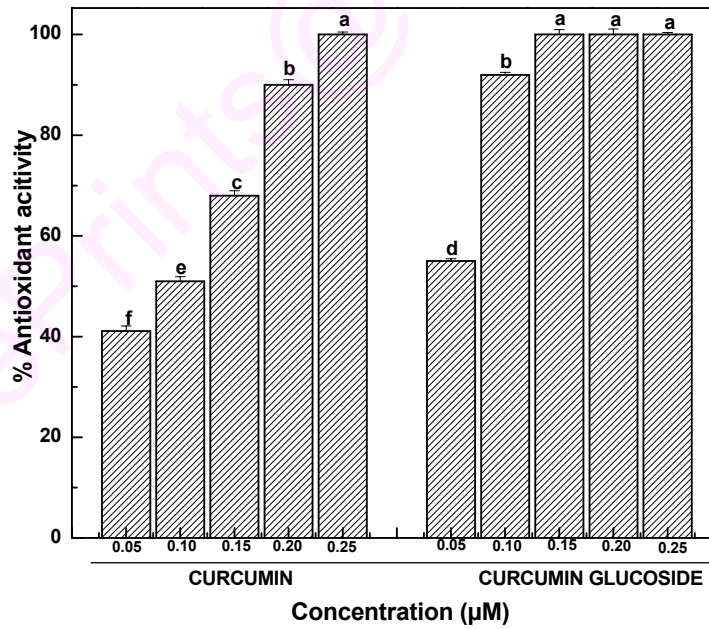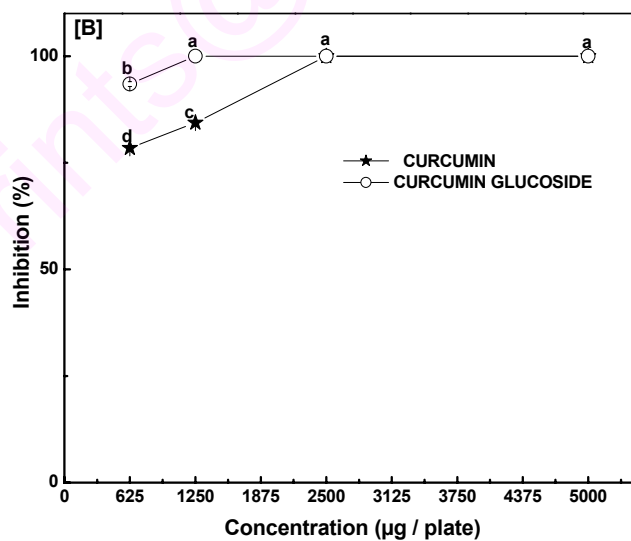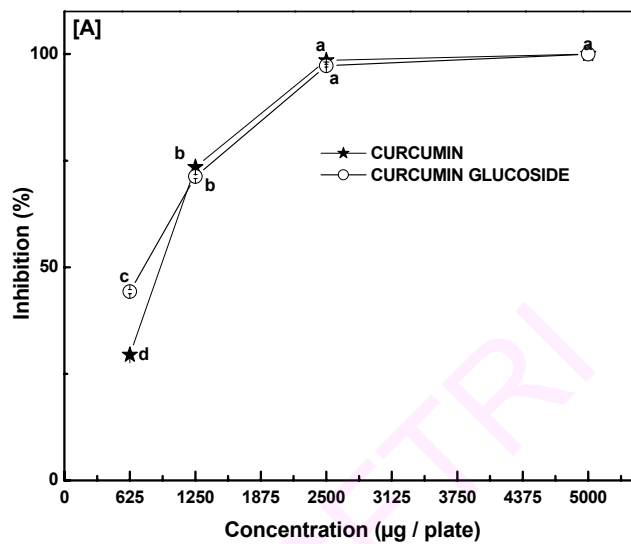**Fig. 5.3 Antioxidant activity of 1 and 3 by *β-carotene* bleaching method**

Fig. 5.4 Inhibitory effect of 1 and 3 against the mutagenicity of sodium azide to[A] S. *typhimurium* TA 1531 and [B] S. *typhimurium* TA 98

iii) Antimutagenicity of curcumin and curcumin glucoside by ames test

Antimutagenicity studies of curcumin (**1**) and curcumin di-β-glucoside (**3**) were carried out with *Salmonella typhimurium* TA 1531 as well as TA 98 by Ames test against sodium azide as a mutagen. The antimutagenic effect was considered moderate when the inhibitory effect was 25 – 40% and strong where it was more than 40%. Inhibitory effects of less than 25% were considered as weak and were not recognized as a positive result (Ilken, et al., 1999). **1** is known to be a good antimutagen (Goud, et al., 1993). The results of the present study are shown in Fig. 5.4. In case of TA 1531 (Fig.5.4 [A]), **1** had moderate activity at 625 μg/plate, whereas **3** showed strong and significantly higher (p < 0.05) antimutagenic activity at similar concentration. **1** had strong antimutagenic activity at higher concentrations, which were statistically similar to **3**. In case of TA 98 (Fig. 5.4[B]) **3** had significantly higher (p < 0.05) antimutagenic activity at 625 and 1250 μg/plate concentration than **1**, but at higher concentrations both showed similar activity. These results indicated higher antimutagenic potential of **3** compared to **1**. Free radicals are implicated in many physiological disorders such as inflammation, aging and carcinogenicity (Namiki, 1990), but oxygen scavenging can reduce these effects (Surh, 1999). Hochstein and Atallah (1988) suggested that compounds having antioxidant potential could also inhibit mutagenicity and cancer. Results in this study supported these observations, as reflected by antioxidant and antimutagenic activities of **1** and **3**.

The antioxidant activity and antimutagenicity studies showed, **3** was more potent as **1**. In the former, the phenolic hydroxyl of **1** was substituted with

glucose blocking the route of HAT (Hydrogen Atom Transfer) mechanism of antioxidant activity. **3** was found to have high antioxidant and antimutagenic properties after its modifications at phenolic hydroxylic positions. Here in this case, the mechanism involved may be sequential proton loss electron transfer (SPLET), which is supposed to be faster than other mechanisms, where it postulates that keto–enol moiety of **1** has a more easily dissociable proton, the enolic proton being more acidic than the other two hydroxyls of phenol rings in **1** (Litwinienko & Ingold, 2004). This hypothesis of sequential proton loss electron transfer (SPLET) in **1** was also supported by specific theoretical calculations like Density Functional Theory (DFT) and Time Dependent Density Functional Theory (TD-DFT) (Shen & Ji, 2007).

iv) Antibacterial activity

Further, curcumin (**1)** and curcumin di-β-glucoside **(3)** were tested for their antibacterial potential against *B. Cereus, S. Aureus, E. Coli* and *Y. Enterocolitica.* MIC values of the compounds are presented in Table 5.2. Fig. 5.5 (A -D) show the inhibition of bacteria at different concentrations of **1** and **3**.

1 and **3** showed 27% and 70% inhibition in the case of *E. Coli* at 0.14 μM and 0.15 μM concentrations respectively, which were statistically different ($p < 0.05$). **3** showed 100% inhibition at 0.47 μM but for **1**, 100% inhibition was obtained only at 0.68 μM. In case of *Y. Enterocolitica*, 0.68 μM of **1** showed 100% inhibition whereas similar inhibition with **3** was observed at 0.87 μM.

In case of *S. Aureus,* **1** showed 68 % inhibition at 0.07 μM (Fig 5.5, C), while **3** showed complete inhibition at 0.05 μM (Table 5.2). The growth inhibition
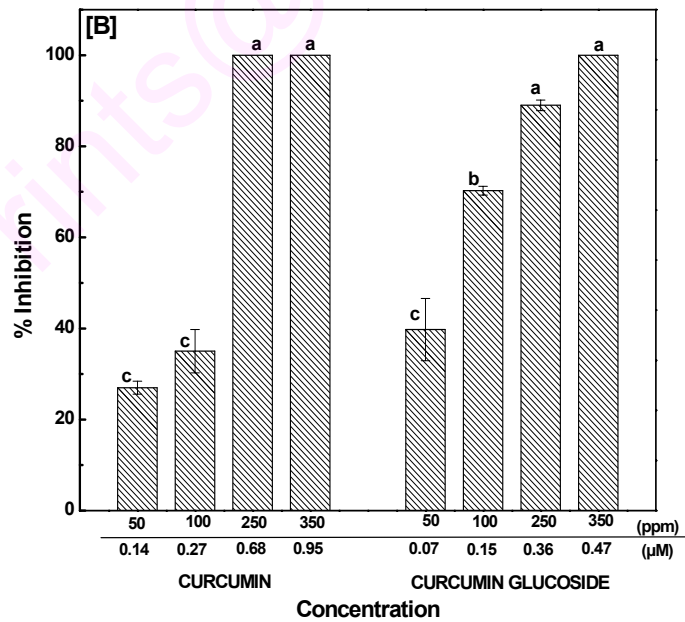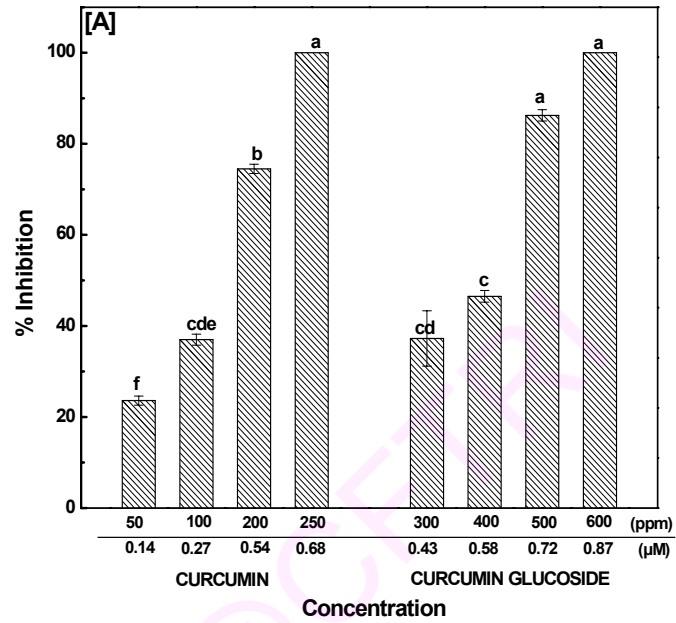
by these compounds against *B. Cereus* showed that **1** inhibited 30% growth where as **3** showed 67% inhibition at 0.07 µM, which were significantly different ($p < 0.05$). At 0.14 µM **1** showed 100 % inhibition while **3** showed only 78% inhibition at 0.15 µM, which was statistically lower ($p < 0.05$) than the inhibition showed by **1** at slightly lower concentrations.
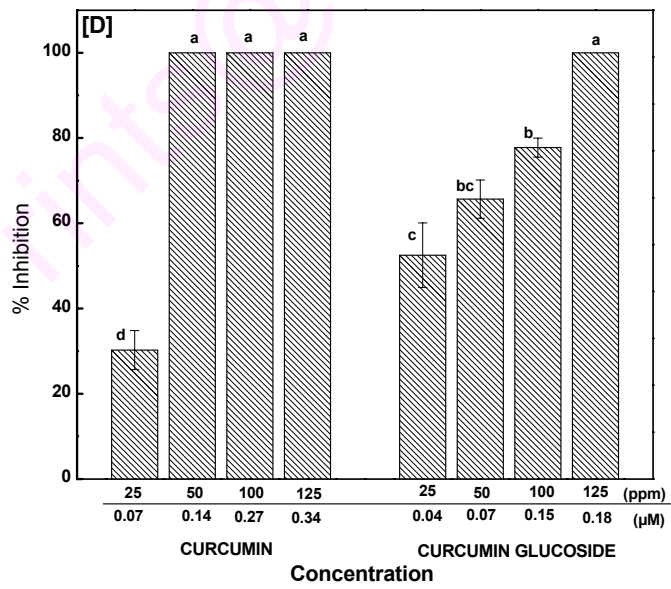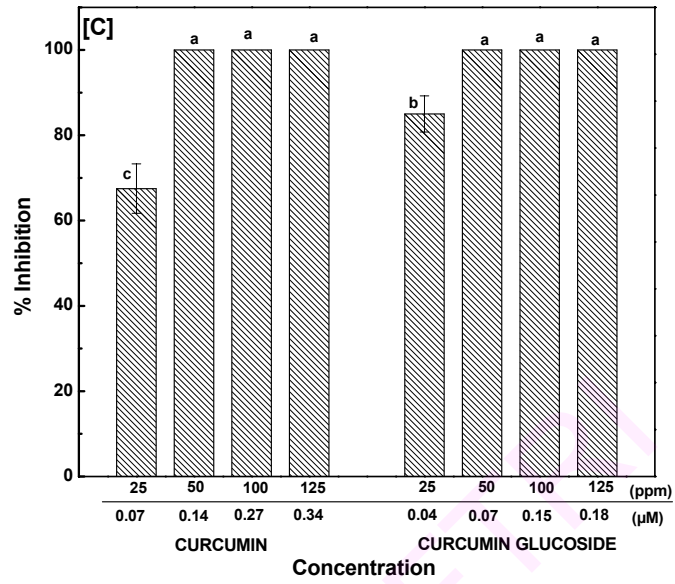
Table 5.2 Minimum inhibitory concentration (µM)* of 1 and 3

Bacteria	MIC of 1 (µM)	MIC of 3 (µM)
Escherichia coli	0.61	0.47
Yersinia enterocolitica	0.68	0.87
Bacillus cereus	0.14	0.18
Staphylococcus aureus	0.08	0.05

* The results of four experiments performed in triplicates

Fig. 5.5 Antibacterial activities of 1 and 3 against different bacteria
[A] *Y. enterocolitica* ; **[B]** *E. Coli*; **[C]** *S.Aureus* & **[D]** *B. Cereus*

[C]

% Inhibition

100
80
60
40
20
0

c   a   a   a   b   a   a   a

| 25 | 50 | 100 | 125 | | 25 | 50 | 100 | 125 | (ppm) |
| 0.07 | 0.14 | 0.27 | 0.34 | | 0.04 | 0.07 | 0.15 | 0.18 | (µM) |

CURCUMIN   CURCUMIN GLUCOSIDE

Concentration

[D]

% Inhibition

100
80
60
40
20
0

a   a   a   d   c   bc   b   a

| 25 | 50 | 100 | 125 | | 25 | 50 | 100 | 125 | (ppm) |
| 0.07 | 0.14 | 0.27 | 0.34 | | 0.04 | 0.07 | 0.15 | 0.18 | (µM) |

CURCUMIN   CURCUMIN GLUCOSIDE

Concentration

Certain curcumin bio-conjugates containing esters and peptides show enhancement in antifungal and antibacterial activities, which is attributed to better cellular uptake, increased cellular concentration and better receptor binding (Kapoor, et al., 2007), as observed in higher antibacterial activity of **3** against *S. Aureus* and *E. Coli.* However, in a certain study an enhancement in bacterial growth in the case of *Bacillus cereus* and *Y. Enterocolitica* was observed when the growth medium was supplemented with glucose (Rhee, et al., 1994). The high MIC value in the case of *B. Cereus* and *Y. Enterocolitica* may be possibly due to the fact that bacterial enzyme system may cleave the glycosidic linkage and the bacteria utilize the released glucose.

The higher antimicrobial activity observed for these curcumin bioconjugates may be due to,

- ❖ Enhanced metabolic stability due to masking of phenolic hydroxyl groups and delay in their glucuronide formation during metabolism.

- ❖ Better cellular uptake due to the transportation of the conjugate via the amino acid carrier protein, i.e. drug transport, and

- ❖ Superior aqueous solubility of the conjugates because of enhancement in polar character (hydrophilicity).

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 linked with **1** in order to facilitate the cellular uptake through receptor mediated endocytosis (Dubey, et al., 2008).

5.3.2 Curcumin Amino Acid Conjugates

A synthetic protocol was developed for the preparation of several curcumin-amino acid conjugates in high yields. Reaction of curcumin **(1)** with t-Boc–protected amino acids with dicylcohexylcarbodiimide in presence of dimethylaminopyridine and triethylamine as catalysts afforded t-Boc-amino acid conjugates of **1**. These were deprotected to afford the corresponding curcumin-amino acid conjugates (Table 5.3) in 45–76% yields (Discussed in detail in Chapter 3).

Table 5.3 Curcumin amino acid conjugates synthesized

(IV)

Structure No.	Compound	Structure IV, R =
4	1,7-Bis (4-*O*-L-leucinoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	H_3C–CH–CH_2–, H_3C
5	1,7-Bis (4-*O*-L-phenylalaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	CH_2 (benzyl)
6	1,7-Bis (4-*O*-L-alaninoyl-3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	—CH_3
7	1,7-Bis (4-*O*-L- isoleucinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	CH_3, H_3C–CH_2–CH—
8	1,7-Bis (4-*O*-L- valinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	H_3C, C, H_3C H
9	1,7-Bis (4-*O*-L- serinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	HO—CH_2—
10	1,7-Bis (4-*O*-L- cysteinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	HS—CH_2—
11	1,7-Bis (4-*O*-L- phenylglycinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	(phenyl)
12	1,7-Bis (4-*O*-L- glycinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	— H
13	1,7-Bis (4-*O*-L- prolinoyl -3-methoxyphenyl)-1,4,6-heptatriene-5-ol-3-one	—CH_2—CH_2—CH_2— (as part of a pyrolidine ring)

i) Solubility

All the amino acid-curcumin conjugates were tested for their solubility in aqueous systems. While all these derivatives were soluble in water at 1 mg / ml concentration pH 6, the conjugates of **1** with glycine (**12**) and proline (**13**) exhibited higher solubility in water (10 mg/ml).

ii) Radical-scavenging activity

These compounds were evaluated for their antioxidant behavior in comparison with **1** and the results are presented in Table 5.4. The antioxidant methods of DPPH clearly indicated that all the curcumin amino acid conjugates had lower IC_{50} values than **1**. The conjugates showed differences in their activities, which could be attributed to the various amino acids attached to **1**. Especially, alkyl substituted amino acids attached to **1** showed higher activity. Conjugates of curcumin with leucine (**4**, Table 5.4), alanine (**6**), isoleucine (**7**), valine (**8**), serine (**9**) and cysteine (**10**) exhibited 42%, 53%, 51%, 59%, 59% and 70% lesser IC_{50} values than curcumin (**1**). Among these, **10** showed highest activity, which could be attributed to the sulphur moiety present in it acting as a good radical-scavenging agent. Conjugates of curcumin with phenylalanine (**5**), phenylglycine (**11**) and proline (**13**) derivatives exhibited comparatively less radical scavenging activity than the other conjugates but still showed 31%, 22% and 17% lesser IC_{50} value than **1**.

Table 5.4 IC $_{50}$ value of curcumin-amino acid conjugates

Structure No.	IC $_{50}$, (μmol) by DPPH method	IC $_{50}$, (μmol), by β - carotene bleaching method
1	0.118[a]	0.109[a]
4	0.068[f]	0.064[f]
5	0.082[d]	0.077[d]
6	0.055[h]	0.047[h]
7	0.058[g]	0.06[g]
8	0.049[i]	0.037[j]
9	0.048[j]	0.043[i]
10	0.036[k]	0.03[k]
11	0.092[c]	0.086[c]
12	0.076[e]	0.066[e]
13	0.098[b]	0.091[b]

iii) Antioxidant activity

Antioxidant activity by β – carotene-bleaching assays also showed that all these derivatives had lower IC_{50} values than the parent molecule. The conjugates **6, 7, 8, 9** and **10** showed IC_{50} values 57%, 45%, 66%, 61% and 73% lower than **1**. The proline, phenylglycine and phenylalanine conjugates of curcumin (**13, 11** and **5**) also showed IC_{50} values 17%, 21% and 29% lower than **1**. Also in this assay the compound **10** showed highest activity substantiating the role of sulphur moiety in scavenging free radicals. Thus both the DPPH and β – carotene-bleaching assays established that curcumin - amino acid conjugates exhibited radical – scavenging and antioxidant properties superior than curcumin.

The enhancement in the antioxidant activities of curcumin amino acid conjugates could be attributed to two factors, firstly the radical scavenging activity of keto – enol group in **1**. The phenolic hydroxyl of **1** was substituted with amino acids blocking the route of HAT (Hydrogen Atom Transfer) mechanism of antioxidant activity. Here in this case, the mechanism involved may be sequential proton loss electron transfer (SPLET) where it postulates that keto – enol moiety of **1** has more easily dissociable proton, which is supposed to be faster than other mechanisms. The enolic proton being more acidic than the other two hydroxyls of phenol rings in **1** (Litwinienko, & Ingold, 2004). This hypothesis was also supported by some theoretical calculations like Density Functional Theory (DFT) and Time Dependent Density Functional Theory (TD - DFT), which also support sequential proton loss electron transfer (SPLET) in **1** (Shen & Ji, 2007).

Another reason may be attributed to the radical-scavenging activity of amino group with the hydrazyl moiety to enhance the total antioxidant activity (Ionita, et al., 2000). Amine group absorbs a hydrogen ion from the solution to form NH_3^+ (Kim & Thomas, 2007). Free radicals can react with the hydrogen ion from the ammonium ions (NH_3^+) to form a stable molecule. Reports are available on the functionality of the amino acid affecting the activity. The activity of basic and neutral amino acids supposed to be higher compared to others because of their functionality (Triantis, et al., 2007). In the present study, it was observed that curcumin conjugates of alanine, valine, serine, isoleucine and cysteine (**6, 8, 9, 7** and **10**) showed enhanced activity while leucine and glycine conjugates of curcumin (**4** and **12**) exhibited slightly better activity than **1** and proline, phenylalanine and phenylglycine conjugates of curcumin (**13, 5** and **11**) showed almost similar activity compared to **1**. The lower activity of the other amino acids may be due to the steric hindrance during radical-scavenging action because of the presence of bulky groups.
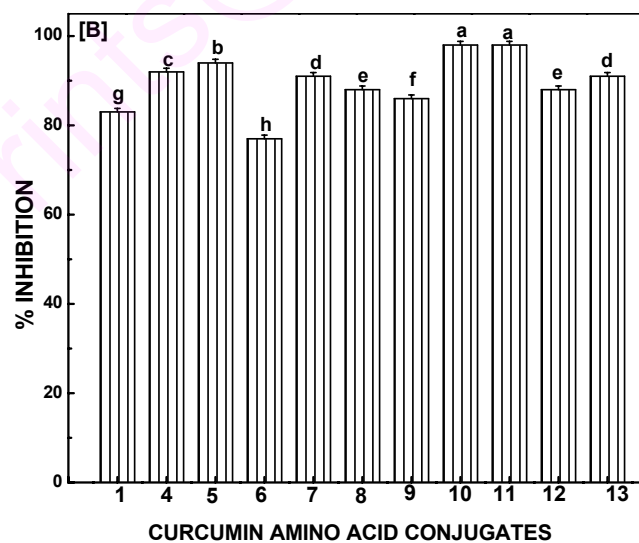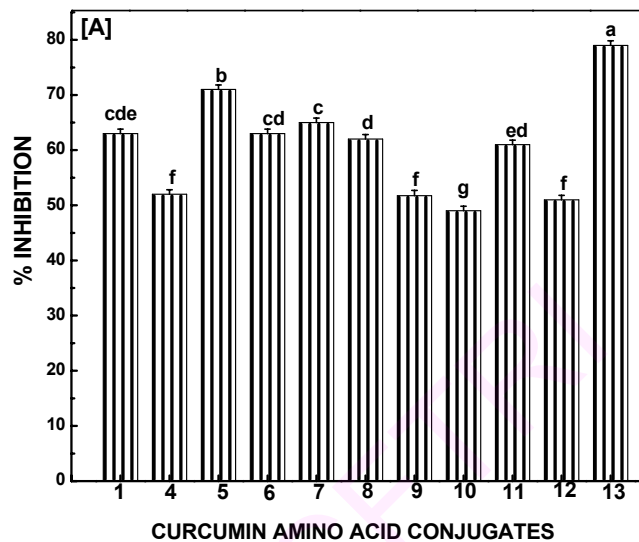
iv) Antimutagenic activity

Antimutagenicity studies of **1** and curcumin amino acid conjugates were carried out with *Salmonella typhimurium* TA 1531 as well as TA 98 by Ames test against sodium azide and MMS as a mutagen. The results indicated that curcumin amino acid conjugates had strong antimutagenic property similar to **1** against *Salmonella typhimurium* TA 98 when tested with sodium azide (Fig.5.6 [A & B]). Both **1** and curcumin amino acid conjugates showed strong antimutagenic activity against TA 98 with sodium azide at 100 and 250 µg / plate

concentrations. Alanine, isoleucine, valine and phenylglycine conjugates (**6, 7, 8** and **11**) showed the activities statistically similar (p<0.05) to **1** at 100 µg / plate concentration with sodium azide as mutagen. At 100 and 250 µg / plate concentration with sodium azide as mutagen **13,** showed strong antimutagenicity compared to **1**. Leucine, serine and glycine conjugated to curcumin (**4, 9** and **12**) showed statistically similar antimutagenic activity but it was significantly lower than **1**. Overall the conjugates as well **1** showed strong antimutagenicity at 100 and 250 µg / plate concentrations. In the case of MMS the conjugates showed strong antimutagenic activity against TA 98 at 100 µg / plate concentration for phenylalanine and cysteine conjugates of curcumin (**5** and **10**) and it was statistically similar to **1** (p < 0.05). **13** showed strong antimutagenic activity against TA 98 at 100 µg / plate concentration compared to **1** but showed almost similar activity at 250 µg / plate concentration. Alanine and glycine conjugates of curcumin (**6** and **12**) were statistically similar in their activity but had higher antimutagenic activity than **1**. Valine and phenylglycine conjugates of (**8** and **11**) also showed statistical similarity but significantly lower antimutagenic activity than **1**. At 250 µg / plate concentration all the conjugates showed strong antimutagenic activity which was statistically similar to **1** (Fig 5.6. [C & D]).

Fig. 5.6 Antimutagenicity of 1 and its amino acid conjugates against *Salmonella Typhimurium* **TA 98;**

[A] & [B] against sodium azide as mutagen at 100 & 250 μg/ plate concentrations

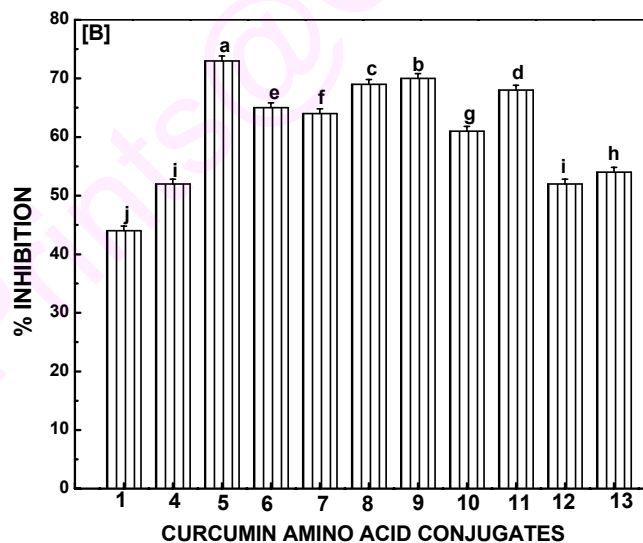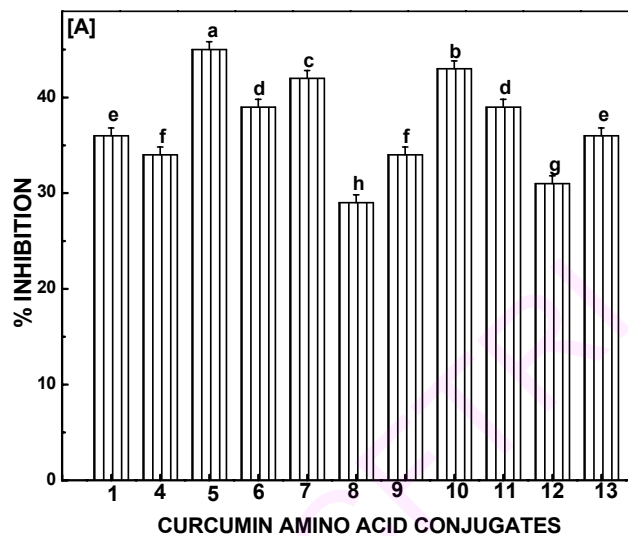**[C] & [D] against MMS as mutagen at 100 & 250 µg/ plate concentrations**
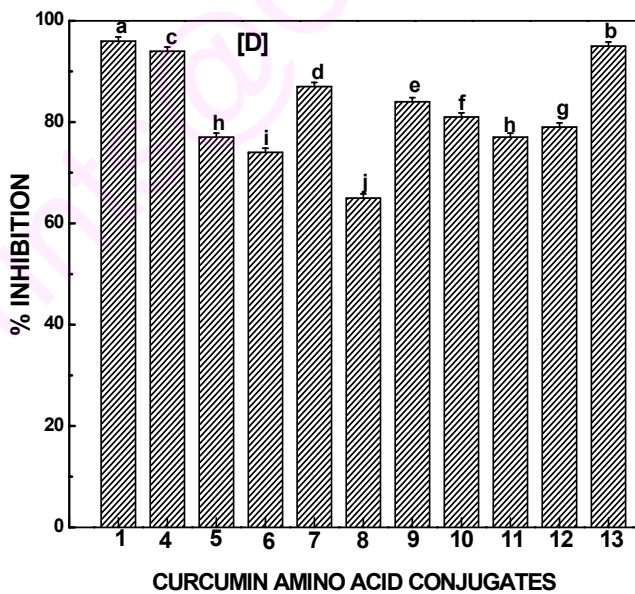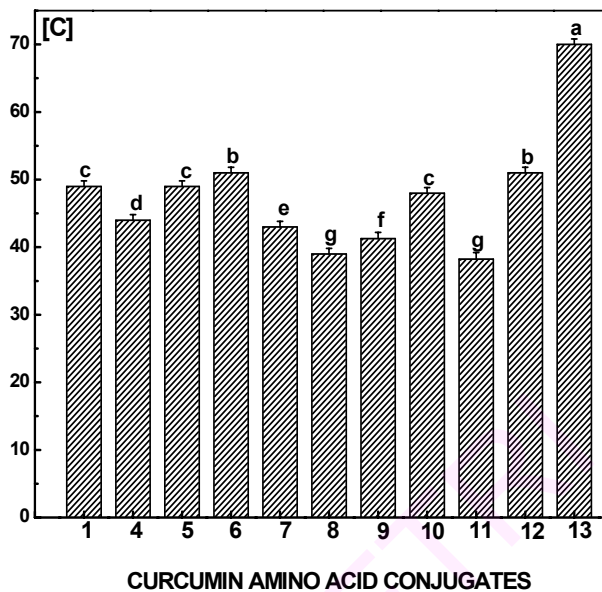
1 and curcumin amino acid conjugates were also tested for their antimutagenic activity against TA 1531 with sodium azide at 100 and 250 µg / plate concentrations (Fig. 5.7 [A & B]). Results indicated that at 100 µg / plate concentration against sodium azide as mutagen the activity of curcumin proline conjugate (**13**) was statistically similar to **1**. Leucine and serine conjugates of curcumin (**4** and **9**) showed similar activity but it was statistically lower than curcumin. **5** and **7** strong antimutagenicity compared to **1**. Other derivatives showed significantly higher activity than **1**. **8** and **12** showed moderate activity compared to curcumin. At 250 µg / plate concentration **1** showed lower activity than all the amino acid conjugates. In the case of MMS as mutagen cysteine, glycine and proline conjugates of curcumin (**10, 12** and **13**) showed lower activity than **1** while other derivatives showed significantly higher activity than **1** at 100 µg / plate concentration. At 250 µg / plate concentration curcumin-amino acid conjugates showed significantly higher activity than **1** except in the case of proline (**13**) derivative (Fig. 5.7 [C & D]).

1 is known to be a good anti-mutagen (Goud, et al, 1999). In the present study antimutagenicity studies of curcumin amino acid conjugates against *Salmonella typhimurium* TA 1531 as well as TA 98 by Ames test using sodium azide and MMS as mutagens were evaluated. The results indicated that curcumin amino acid conjugates exhibited strong antimutagenic property. **1** showed strong antimutagenic activity against sodium azide and MMS at both the concentrations tested, but the derivatives, in general, comparable and even superior antimutagenic activities at the concentrations tested. Strong

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antimutagenicity observed for curcumin amino acid conjugates can be attributed to their conversion to **1** in the bacterial cell. Earlier, it has been shown that even the traces of curcuminoids in **1,** curcumin-removed turmeric oil were found to impart strong antimutagenicity (Jayaprakasha, et al., 2002). Free radicals are implicated in many physiological disorders such as inflammation, aging and carcinogenicity (Namiki, 1990), but oxygen scavenging can reduce these effects (Surh, 1999). It was reported that compounds having antioxidant potential could also inhibit mutagenicity and cancer (Hochstein, & Atallah, 1988). Amino acids being the component of bacterial cellular environment, these conjugates may help to increase the intracellular delivery of **1** (Mishra, et al., 2005). Amino acids may cross the cell membranes gaining facile entry to the bacterial cells (Mishra, et al., 2005). The blocking of phenolic – OH group by amino acids in **1** helps in better endocytosis and their retention in the cell after the enzymatic cleavages (Kapoor, et al., 2007).

Fig. 5.7 Antimutagenicity of 1 and its amino acid conjugates against *Salmonella Typhimurium* TA 1531;

[A] & [B] against sodium azide as mutagen at 100 & 250 µg/ plate concentrations

[C] & [D] against MMS as mutagen at 100 & 250 µg/ plate concentrations
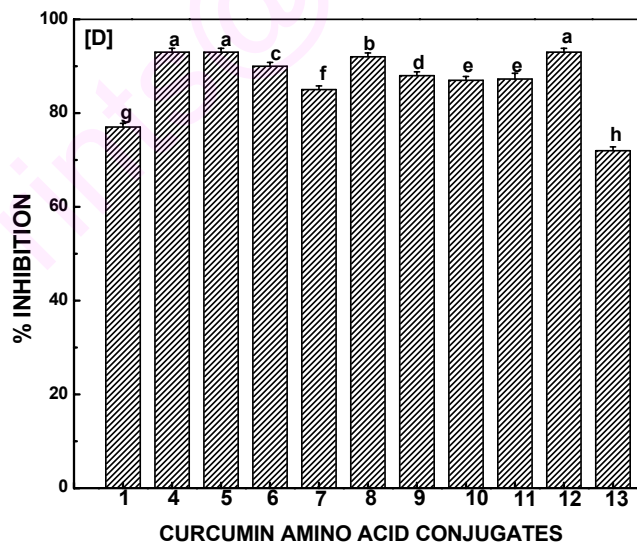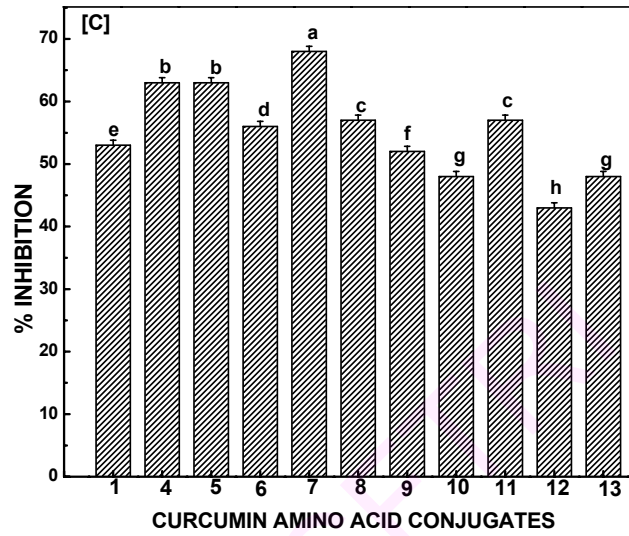
v) Antibacterial activity

Further, **1** and conjugates were tested for their antibacterial potential against *B. Cereus, Staphylococcus aureus*, *E.Coli* and *Y. Enterocolitica.* MIC values of the compounds are presented in Table. 5.5. Results showed the conjugates synthesized were more effective in inhibiting the growth of Gram –ve bacteri\a compared to the parent molecule. The MIC values of curcumin amino acid conjugates were lower compared to **1**. Against *E. Coli,* curcumin conjugates **4, 5, 6, 8, 10** and **11** (Table 5.5) showed 72%, 75%, 84%, 71%, 78% and 74% lower MIC values respectively compared to **1**. Conjugation of curcumin with isoleucine (**7**), glycine (**12**) and proline (**13**) showed moderate activity but curcumin conjugated to serine (**9**) showed lesser inhibition. Against *Y. Enterocolitica* lower MIC values were observed for all the curcumin amino acid conjugates compared to curcumin. Against *B. cereus* the conjugates **4**, **6**, **9**, **10** and **11** (Table 5.5) showed 63%, 42%, 45%, 70% and 53% lower MIC compared to **1**. Conjugates such as **5**, **7**, **8**, **12** and **13** showed higher MIC values than curcumin. Against *S. Aureus*, curcumin amino acid conjugates **7, 8, 9, 10, 11** and **13** exhibited lower MIC values compared to curcumin. Conjugation of **1** with phenylalanine (**5**), alanine (**6**) and glycine (**12**) showed higher MIC value compared to the parent molecule **1**.

The enhancement in antimicrobial activity of these curcumin amino acid conjugates may be due to, enhanced metabolic stability due to masking of phenolic hydroxyl groups and delay in their glucoronide formation during metabolism, better cellular uptake due to the transportation of the conjugate via

the amino acid carrier protein, i.e. drug smuggling, and more solubility of the conjugates because of enhancement in polar character (hydrophilicity). This makes these derivatives as potent prodrugs, which can get hydrolysed at the target sites and enhance the cellular uptake (Dubey, et al., 2008).

Table 5.5 MIC Values (µM*) of amino-acid conjugates of 1 against different bacteria

| Structure No.	E.Coli (µM)	Y. Enterocolitica (µM)	B. Cereus (µM)	S. Aureus (µM)
1	0.61	1.16	0.14	0.081
4	0.17	0.55	0.05	0.050
5	0.15	0.26	0.53	0.11
6	0.10	0.10	0.08	0.20
7	0.55	0.17	1.35	0.05
8	0.18	0.49	0.27	0.05
9	0.65	0.19	0.07	0.06
10	0.13	0.10	0.04	0.03
11	0.16	0.08	0.06	0.05
12	0.31	0.42	0.21	0.21
13	0.44	0.09	1.95	0.07

* The results of four experiments performed in duplicates

5.3.3 Glucuronide derivative of curcumin

Ultrasound-assisted reaction of **1** with methyl 1-bromo-2,3,4-tri-*O*-acetyl-α-D- glucopyranuronate in the presence of phase transfer catalyst under biphasic conditions followed by deacetylation to afforded methyl 1-curcuminyl-β-D-glucopyranuronate (Fig.5.8, Discussed in detail in Chapter 4)

i) Solubility: **14** was tested for its solubility in aqueous systems. It was soluble in water at 10 mg/ml concentration compared to the water-insoluble parent molecule.

Fig. 5.8 Methyl 1-curcuminyl-β-D-glucopyranuronate (14)
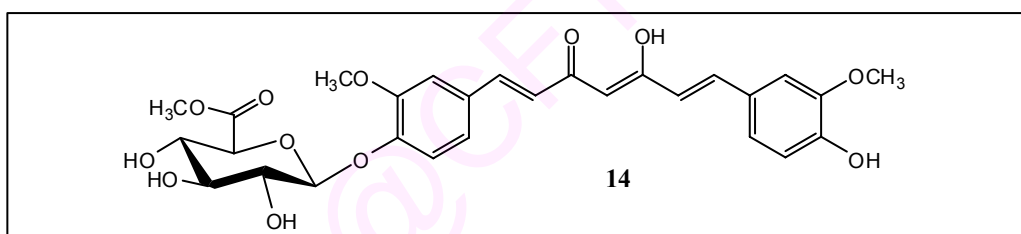
ii) Radical-scavenging activity

Radical-scavenging assay was carried out with **1** as a reference for comparison with the water-soluble **14**. **1** and **14** were dissolved in DMSO at 0.05–0.25 µM concentrations. The radical-scavenging activity (RSA) by DPPH method showed 41 and 46% activity at 0.05 µM for **1** and **14**, respectively (Fig. 5.9). At 0.1 µM, RSA of **1** and **14** was 47 and 56%, respectively However, RSA of **1** and **14** at both these concentrations were significantly different ($p < 0.05$). At 0.25 µM **1** showed 100% RSA but **14** showed the same result at 0.2 µM.

iii) Antioxidant activity

The antioxidant activity by β–carotene bleaching method (Fig. 5.10) was carried out wherein, **1** and **14** were dissolved in DMSO at 0.05–0.25μM concentrations. **1** showed 41% and **14** had 46% activity at 0.05 μM, which were statistically (p<0.05) different. At 0.1 μM concentration **14** showed significantly (p<0.05) higher activity (56%) than **1** (50%). Similarly at 0.2 μM concentrations **14** showed significantly higher antioxidant activity (100%) than **1** (90%). These results clearly showed that **14** exhibited higher antioxidant activity than **1** at all the concentrations.

iv) Antimutagenic activity

Antimutagenicity studies of **1** and **14** were carried out with *Salmonella typhimurium* TA 1531 as well as TA 98 by Ames test against sodium azide/MMS as a mutagen (Table 5.5). The results indicated that **14** possessed as strong antimutagenic property as **1**. Both **1** and **14** showed strong antimutagenic activity with TA 98 against sodium azide at all the concentrations tested. With MMS as diagnostic mutagen the derivative showed higher antimutagenicity compared to **1**, which were statistically different at the tested concentrations.

In case of TA 1531 **1** had 36% activity at 100 μg/plate, against sodium azide as mutagen, where as **14** showed strong 61% and strong antimutagenic activity at similar concentration. In case of TA 98, **14** had 74% and 76% activity for sodium azide and MMS respectively at 100 μg/plate concentration higher than **1**, which showed 63 % and 49% activity for sodium azide and MMS respectively. At 250 μg/plate concentration **1** showed strong antimutagenicity compared to **14**.

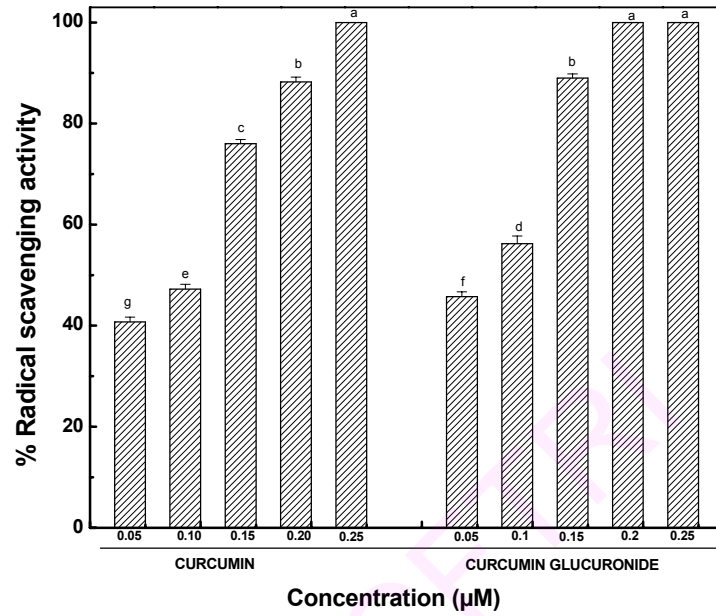**Fig 5.9 Radical-scavenging activity of 1 and 14 by DPPH method**

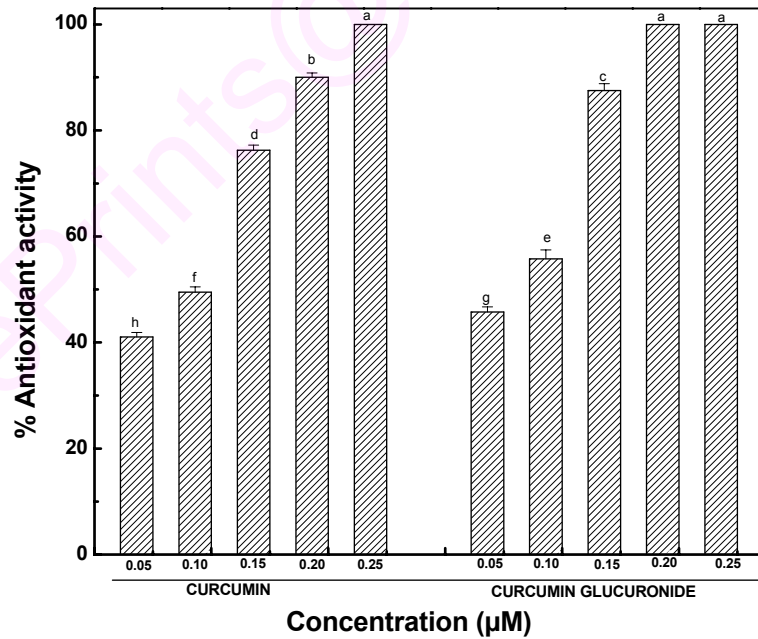**Fig. 5.10 Antioxidant activity of 1 and 14 by *β-carotene* bleaching method**

Table 5.5 Antimutagenic activity of 1 and 14 against sodium azide and MMS as diagnostic mutagens

Compounds	TA -1531		TA - 98	
	Sodium azide	MMS	Sodium azide	MMS
	100 µg/ plate			
1	36 ± 0.5	53 ± 1.8	63 ± 1	49 ± 1
14	61 ± 0.8	76 ± 0.5	74 ± 0.8	76 ± 0.5
	250 µg/ plate			
1	44 ± 1	77 ± 0.8	83 ± 0.6	96 ± 0.4
14	61 ± 0.6	74 ± 0.5	79 ± 0.6	88 ± 0.5

v) Antibacterial activity

1 and 14 were tested for their antibacterial potential against *B. Cereus, S. Aureus, E. Coli* and *Y. Enterocolitica.* MIC values of the compounds presented in Table 5.6 showed the inhibition of bacteria at different concentrations of 14. MIC values of 14 were higher than 1 for inhibition against *B. Cereus, S. Aureus* but lower than 1 in the case of *E. Coli* and *Y. Enterocolitica.*

The 1 and 14 showed 75% and 93% inhibition at 0.54 µM, which were statistically different (p < 0.05). 100 % inhibition was observed at 0.82 and 0.72 µM for 1 and 14 respectively against *Y. enterocolitica* (Fig. 5.11 [A]).

In the case of *E. coli,* 0.14 µM of 1 showed 25% inhibition, while 0.09 µM of 14 showed 36% inhibition, which was statistically different (p < 0.05). 100% growth inhibition for *E. coli* was observed at 0.68 µM for 1 whereas, 14 showed it

at 0.45 µM (Fig. 5.11 [B]). In the case of *S. Aureus* the 0.07 µM concentration of **1** showed 65 % while **14** showed 57 % inhibition at 0.05 µM level (Fig. 5.11[C]). Complete growth inhibition for *S. Aureus* observed at 0.13 and 0.14 µM concentrations for **1** and **14** respectively, which were statistically similar ($p < 0.05$). Against *B. cereus,* **1** showed 30% inhibition at 0.07 µM level, while **14** had significantly higher inhibition (52%) at 0.05 µM concentration (Fig. 5.11 [D]). Complete inhibition of *B. cereus* was observed at 0.14 µM level for both the compounds and were statistically similar ($p < 0.05$).

Table 5.6 Minimum inhibitory concentration (µM)* of 1 and 14

Bacteria	MIC of 1 (µM)	MIC of 14 (µM)
Escherichia coli	0.61	0.27
Yersinia enterocolitica	0.68	0.63
Staphylococcus aureus	0.08	0.13
Bacillus cereus	0.14	0.18

Fig. 5.11 Antibacterial activities of 1 and 14 against different bacteria
[A] *Y. enterocolitica* ; [B] *E. Coli*; [C] *S.Aureus* & [D] *B. Cereus*
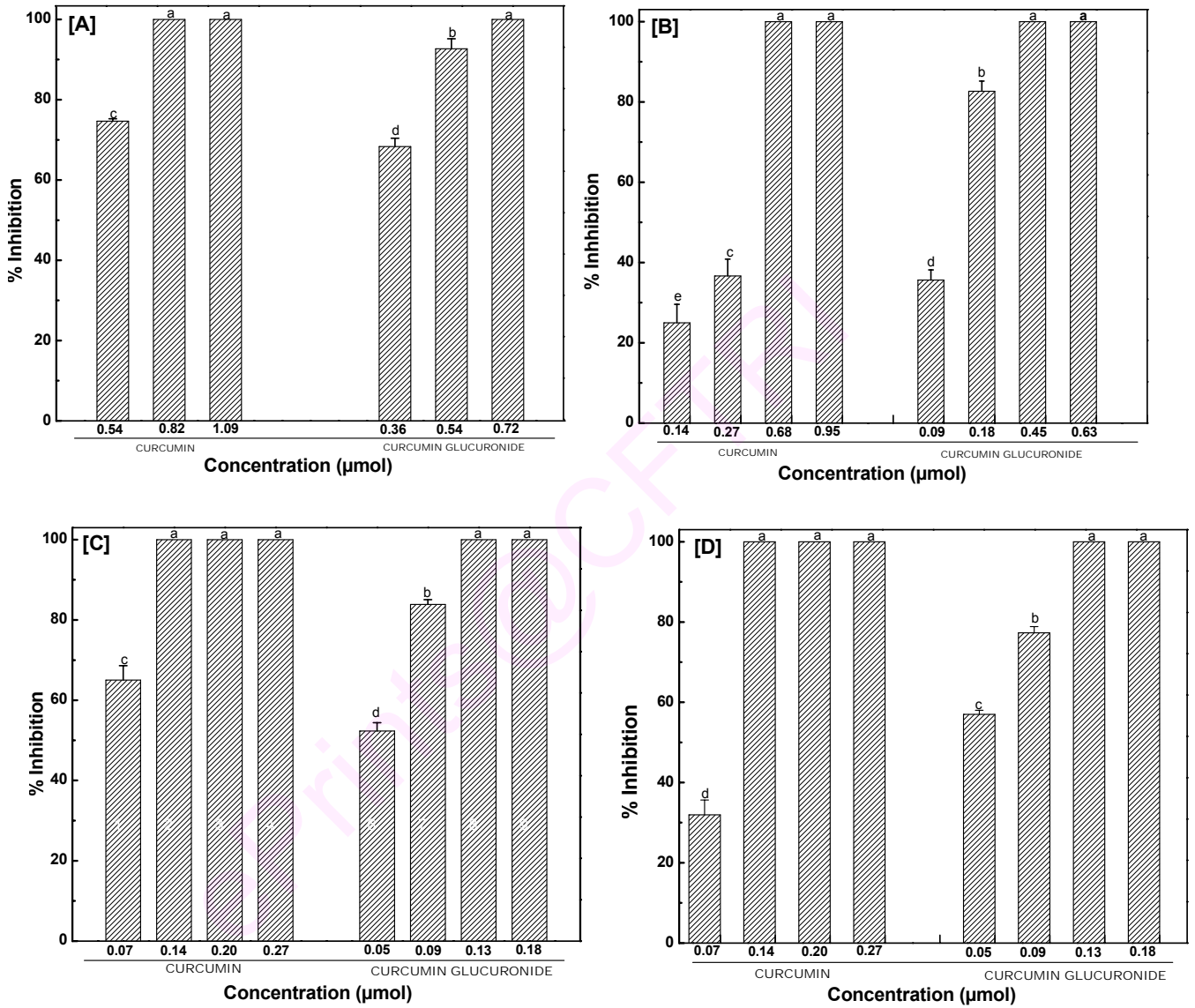
5.3.4 Curcumin Glucal Derivative

In an another approach to render **1** water-soluble Ferrier reaction of **1** was with tri-O-acetyl-D-glucal was carried out in the presence of $Fe_2(SO_4)_3$ x H_2O as catalyst under stirring at 40 ^oC in acetonitrile. The product formed was deacetylated to yield curcuminyl hex-2-enopyranoside (Fig. 5.12, Discussed in detail in Chapter 4).

i) Solubility

15 was tested for its solubility in aqueous systems. The derivative was soluble in water at 1 mg/ml concentration.

Fig. 5.12 Structure of curcuminyl hex-2-enopyranoside (15)
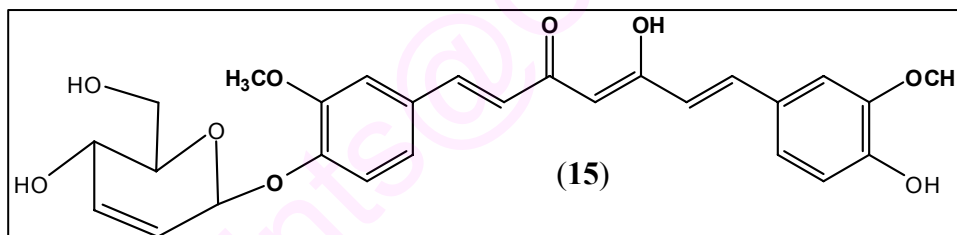
ii) Radical-scavenging activity

The radical-scavenging activity of **1** was compared to **15**. Both the samples were dissolved in DMSO at a concentration range of 0.05 -0.25 µM (Fig. 5.13**)**. At 0.05 µM concentration the compounds **1** and **15** showed 47% and 53% radical-scavenging activity respectively, which were statistically different ($p < 0.05$). Complete inhibition was observed at 0.25 and 0.20 µM concentrations for **1** and **15** respectively. An IC_{50} value 0.067 µM of **15** was very low compared to the parent molecule (0.118 µM).

iii) Antioxidant activity

Antioxidant activity by *β-carotene* bleaching method (Fig. 5.14) was carried out at 0.05-0.25 µM concentration in DMSO for **15** and compared to **1**. The antioxidant activity of 41 and 46 % was observed at 0.05 µM for **1** and **15** respectively, which were statistically different ($p < 0.05$). 100 % activity inhibition was observed for **1** at (0.20 µM) and **15** (0.25 µM) concentrations respectively.

iv) Antimutagenic activity

Antimutagenicity studies of **1** and **15** were carried out with *Salmonella typhimurium* TA 1531 as well as TA 98 by Ames test against sodium azide and MMS as mutagens. In the case of TA –1531, **1** had moderate and **15** had strong activity at 100 µg/ plate as well as 250 µg/plate concentrations against both sodium azide and MMS as mutagens (Table 5.7). In the case of TA 98, both **1** and **15** moderate antimutagenicity at 100 µg/plate but much superior activity at 250 µg/plate concentration.

Table 5.7 Antimutagenic activity of 1 and 15 against sodium azide and MMS as diagnostic mutagens.

	TA -1531		TA - 98	
	Sodium azide	MMS	Sodium azide	MMS
100 µg/ plate				
1	36 ± 0.5	53 ± 1.8	63 ± 1	49 ± 1
15	55 ± 0.7	59 ± 1.2	71 ± 0.7	55 ± 0.7
250 µg/ plate				
1	44 ± 1	77 ± 0.8	83 ± 0.6	96 ± 0.4
15	77 ± 0.8	82± 0.5	90 ± 0.4	99 ± 0.1

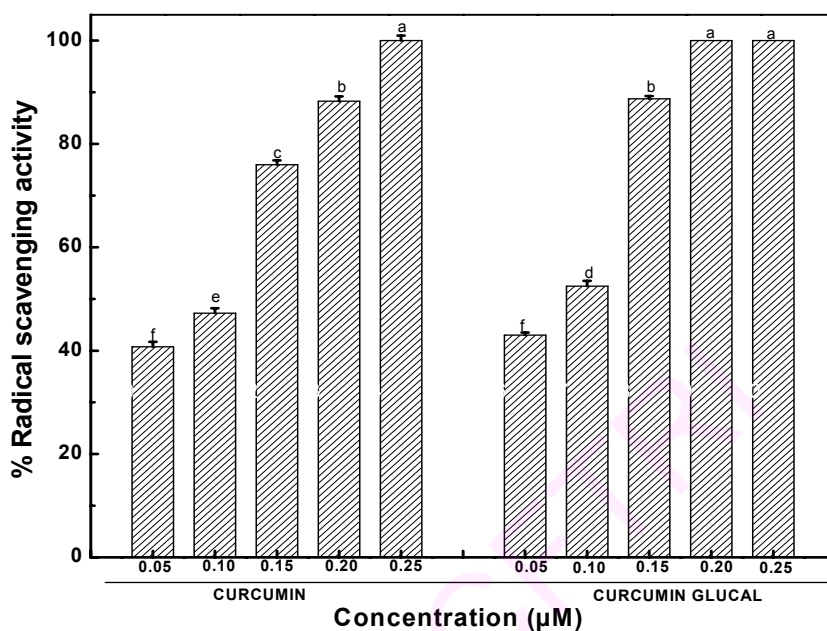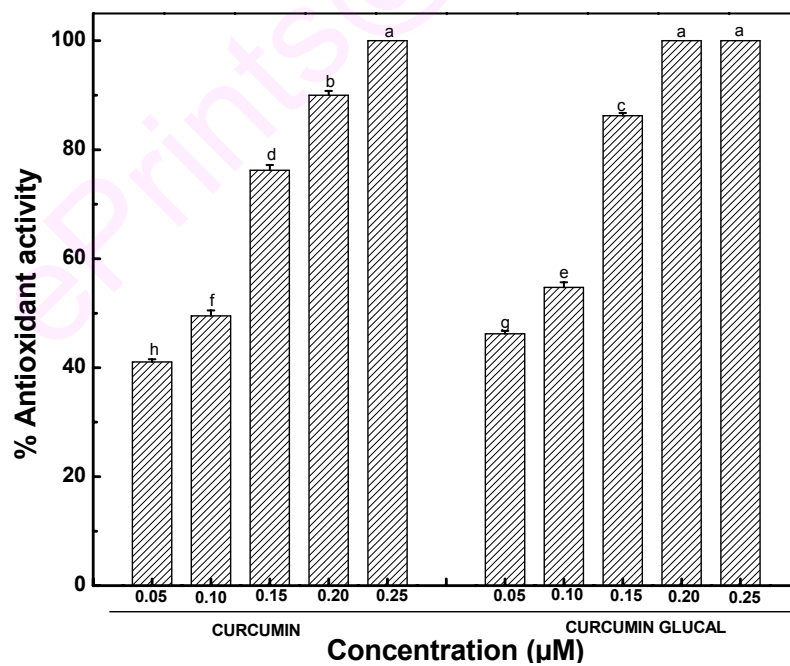**Fig. 5.13 Radical-scavenging activity of 1 and 15 by DPPH method**

Fig. 5.14 Antioxidant activity of 1 and 15 by *β-carotene* bleaching method

v) Antibacterial activity

Compounds **1** and **15** were tested for their antibacterial potential against *B. Cereus, S. Aureus, E. Coli* and *Y. Enterocolitica.* MIC of the compounds is presented in Table 5.8. Fig. 5.15 (a-d) shows the inhibition of these bacteria at different concentrations of both the compounds.

In the case of *Y. Enterocolitica*, **1** showed 75 % inhibition at 0.54 µM and **15** showed 66 % inhibition at 0.40 µM, which were statistically different ($p < 0.05$) [Fig 5.15 (A)]. Complete inhibition was observed at an MIC of 0.68 and 0.70 µM of **1** and **15** respectively (Table 5.8). Against *E. Coli* similar inhibition was observed at 0.27 and 0.10 µM respectively [Fig 5.15(B)] which were statistically similar ($p < 0.05$). The inhibition was 100% at a MIC of 0.61 and 0.40 µM concentrations of **1** and **15** respectively (Table 5.8).

The antibacterial studies on the **15** was carried out against *S. Aureus.* Inhibition was 65% and 41% at 0.07 and 0.05 µM for **1** and **15** respectively, which were statistically different ($p<0.05$). Inhibition was 100% at 0.08 and 0.20 µM (MIC) for **1** and **15** respectively (Fig. 5.15 [C]). Against *B. Cereus* **1** and **15,** showed 32 and 37 % at 0.07 and 0.05 µM concentrations, which were statistically different ($p<0.05$). Complete inhibition was observed at 0.14 and 0.20 µM (MIC) for **1** and **15** respectively (Fig. 5.15 [D]). Against gram +ve bacteria, MIC values of **15** were high compared to the parent molecule. Against *E. Coli,* the derivative **15** showed MIC value lesser than **1** but in the case of *Y. Enterocolitica* the derivative **15** showed MIC value comparable to the parent molecule.

Table 5.8 Minimum inhibitory concentration (μM)* of 1 and 15

Bacteria	MIC of 1 (μM)	MIC of 15 (μM)
Escherichia coli	0.61	0.40
Yersinia enterocolitica	0.68	0.71
Bacillus cereus	0.14	0.20
Staphylococcus aureus	0.08	0.20

Fig. 5.15 Antibacterial activity of 1 and 15 conjugate against, [A] *Y. enterocolitica* ; [B] *E. Coli*; [C] *S.Aureus* & [D] *B. Cereus*

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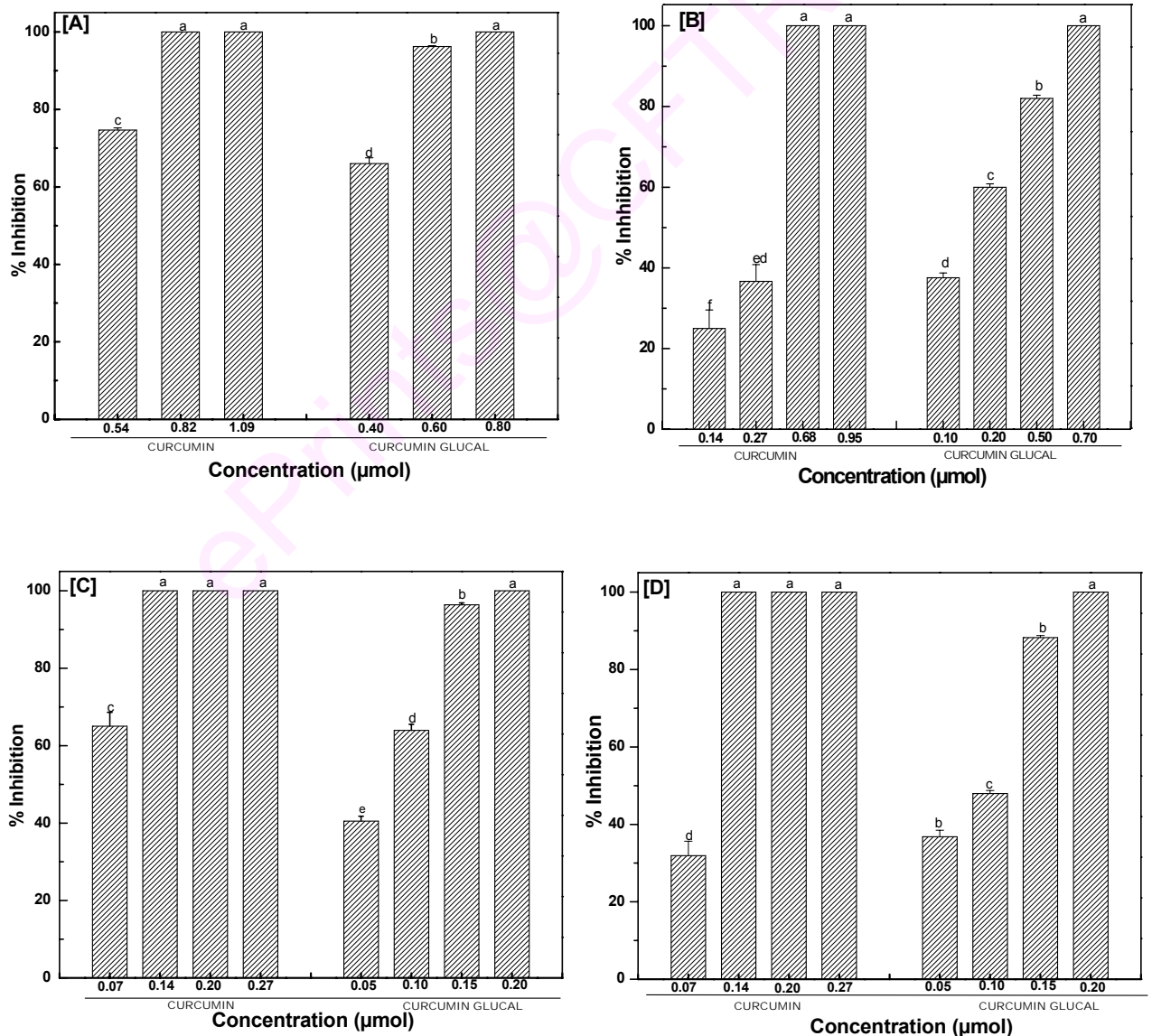
5.4 CONCLUSIONS

The antioxidant, antimutagenicity and antibacterial properties were evaluated for all the derivatives whose synthesis is discussed in Chapters 2, 3 and 4. Antioxidant activities of glucoside, glucuronide and glucal derivatives of curcumin, as determined by DPPH and β–carotene-bleaching assays, were higher than that of curcumin. Conjugates of curcumin with amino acids like alanine, valine, serine, cysteine and isoleucine exhibited even higher antioxidant potential as these were effective even at ~ 50 % of the concentration of curcumin.

Antimutagenicity studies were conducted for all the derivatives by Ame's test against *Salmonella Typhimurium* TA 98 and 1531 with Sodium azide and MMS as mutagens at 100 and 250 μg/plate concentrations. Results indicated higher antimutagenic potential for glucoside, glucuronide and glucal derivatives of curcumin compared to curcumin. Amino acid conjugates of curcumin showed strong antimutagenic activity against *S. Typhimurium* TA 98, with sodium azide and methyl methanesulphonate as mutagens at both the concentrations. With *S. Typhimurium* TA 1531 at 100 µg/plate concentration, against sodium azide as mutagen, conjugates of curcumin with proline, leucine, serine and valine showed activity similar to curcumin; other derivatives showed higher activity than curcumin. In the case of MMS as mutagen, cysteine, glycine and proline conjugates of curcumin showed lower activity than curcumin while other derivatives showed higher activity than curcumin. However, all the amino acid

conjugates showed higher activity than curcumin against TA 1531 at 250 µg/plate concentration.

Antibacterial potential of all the derivatives were evaluated against two gram +ve and two gram –ve bacteria by pour plate method. Results indicated curcumin glucoside possessed superior antibacterial activity against the tested bacteria. Curcumin amino acid conjugates, showed good inhibition against Gram –ve bacteria compared to Gram +ve bacteria. Inhibition potential was higher for these conjugates against *E. coli* and *Y. enterocolitica* compared to curcumin in the case of glucuronide derivative. Glucal derivative of curcumin also showed good inhibition against *E. coli* but inhibition was comparable to that of curcumin in the case of *Y. enterocolitica.*

The studies demonstrated that all the sugar and amino acid derivatives of curcumin, synthesized in the present study, were soluble in water and displayed *in vitro* antioxidant, antimutagenic and antibacterial properties superior than curcumin.

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SUMMARY

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CONCLUSIONS

Synthetic routes to several curcumin derivatives viz., glucosides, amino acid conjugates, glucuronide and 2,3-dideoxyglucose derivatives have been evolved and conditions standardized for obtaining these compounds in optimum yields. The water-soluble derivatives, thus prepared, exhibited more pronounced *in vitro* antioxidant, antimicrobial and antimutagenic properties compared to curcumin. Salient findings of the study are:

➢ A synthetic protocol was developed for the glucoside derivatives of curcumin in high yields (>65 %) and selectivity using ultrasonic waves.

➢ Ultrasound-assisted Koenigs – Knorr reaction of 2, 3, 4, 6-tetra-*O*-acetyl-α-D-1-bromoglucose with the potassium curcuminate under the biphasic reaction conditions in the presence of benzyltributylammonium chloride afforded curcumin di-β-glucoside selectively.

➢ The reaction under monophasic conditions in alcoholic solvents and DMSO led to the selective formation of curcumin mono-β-glucoside.

➢ This work establishes a simple synthetic methodology for the glucoside derivatives of curcumin in high yields and selectivity using ultrasonic conditions.

➢ Amino acid derivatives of curcumin were synthesized by reaction of the *N*-protected t-Boc amino acids with curcumin in presence of dicyclohexylcarbodiimide with dimethylaminopyridine and triethylamine as catalysts under inert atmosphere at ambient temperature.

- The reaction was demonstrated with several *t*-Boc-amino acids with different functionalities viz., i) an un-substituted one like glycine, ii) alkyl substituted ones like alanine, isoleucine and valine, iii) an amino acid that is an aryl substituted one like phenylalanine and phenylglycine, iv) an amino acid containing sulphur like cysteine, v) an amino acid present as an imino acid like in proline, and vi) an amino acid that contains a hydroxyl group like in serine.

- An improved accelerated protocol for the deprotection of *t*-BOC group with TFA (10%) under ultrasonic conditions has been developed.

- Several curcumin-amino acid conjugates were obtained in high yields (45 - 76 %).

- A synthetic methodology was developed for the synthesis of methyl 1-curcuminyl-β-D-glucopyranuronate starting from glucuronolactone.

- Curcuminyl hex-2-enopyranoside was obtained in 50 % yield by Ferrier reaction of 3,4,6-tri-*O*-acetyl-D-glucal with curcumin in presence of ferric sulfate catalyst at 40 ^{o}C in acetonitrile, followed by deacetylation.

- The glucoside, amino acid, glucuronide and 2,3-dideoxy derivatives of curcumin, thus prepared, exhibited good water solubility (1-10 mg/ml).

- The radical scavenging activity by DPPH method as well as antioxidant activity by *β*–carotene-bleaching assay showed 67-68% and 100 % activity at 0.15 μM concentrations for curcumin and curcumin di-β-glucoside respectively.

- Curcumin glucoside exhibited higher antimutagenic potential compared to curcumin in experiments with *Salmonella Typhimurium* TA 98 and TA 1531 against sodium azide as the mutagen.

- Curcumin glucoside showed antibacterial activity superior than curcumin against *E. coli, B. cereus* and *S. aureus*.

- IC_{50} values were found to be lower for curcumin amino acid conjugates compared to curcumin in both DPPH method and β–carotene-bleaching assay. Conjugates of curcumin with amino acids like alanine, valine, serine, cysteine and isoleucine were effective even at ~50 % of the concentration of curcumin.

- Amino acid conjugates of curcumin showed strong antimutagenic activity against *Salmonella Typhimurium* TA 98, with sodium azide and methyl methanesulphonate as mutagens at 100 as well as 250 µg/plate concentrations.

- With *Salmonella Typhimurium* TA 1531 at 100 µg/plate concentration, against sodium azide as mutagen, conjugates of curcumin with proline, leucine, serine and valine showed activity similar to curcumin; other derivatives showed higher activity than curcumin.

- In the case of MMS as mutagen, cysteine, glycine and proline conjugates of curcumin showed lower activity than curcumin while other derivatives showed higher activity than curcumin. However, all the amino acid conjugates showed higher activity than curcumin against TA 1531 at 250 µg/plate concentration.

➢ Curcumin amino acid conjugates showed lower MIC values compared to curcumin against the Gram - negative bacteria, E. c*oli* and Y. *enterocolitica*. Against *S.aureus,* all derivatives, except alanine and phenylalanine conjugates of curcumin, exhibited higher inhibition. Against *B. cereus,* isoleucine, phenylalanine, valine, proline and glycine conjugates of curcumin showed lower inhibition compared to curcumin.

➢ IC_{50} values of glucuronide and glucal derivatives of curcumin were lower than the parent molecule in antioxidant assays.

➢ Both the derivatives showed as strong antimutagenicity as the parent molecule against sodium azide and MMS at 100 and 250 µg/plate concentrations.

➢ Inhibition against *E. coli* and *Y. enterocolitica* was higher compared to curcumin in the case of glucuronide derivative. Glucal derivative of curcumin also showed good inhibition against *E. Coli* but comparable to curcumin in the case of *Y. enterocolitica.*

➢ The studies clearly demonstrated that the binding of the sugar and amino acid groups to curcumin imparted hydrophilic nature to the molecule and rendered it water-soluble.

➢ The derivatives possess high potential for use as food additives and for therapeutic applications in view of their superior biological attributes.