SYNOPSIS

The thesis entitled "**CHEMO-ENZYMATIC SYNTHESIS OF NOVEL PHENOLIC LIPIDS AND THEIR EVALUATION FOR ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES**" has been divided into 6 chapters.

Chapter 1: Introduction about Antioxidants

An antioxidant is a substance that when present at lower concentrations than those of an oxidizable substrate (lipids, proteins, DNA, carbohydrates) considerably delays or prevents oxidation of that substrate. Different classes of antioxidants, based on their mode of action have been outlined in this chapter. Chapter 1 also describes the two major lipid oxidation processes, like free radical-mediated autoxidation and photoxidation. A number of diseases triggered by free radical induced cellular damage have also been explained. Various reactive oxygen species (ROS) generated as byproduct during the normal oxygen metabolism and their subsequent prevention by the endogenous defense mechanism has been briefed. This chapter also describes the antioxidant activity of natural phenolics and their different classes. An important aspect of this chapter is outlining the mechanistic aspects of different *in vitro* assays. Biological activities, especially antioxidant activity of natural phenolic lipids or phenolipid and studies conducted so far in measuring the effectiveness of phenolipids using different assays conducted in different mediums has been covered briefly. The chapter ends with the proposed research plan.

Chapter 2: Chemo-Enzymatic Synthesis and Evaluation of Novel Structured Phenolic Lipids as Potential Lipophilic Antioxidant and Antimicrobial Agents*

Synthesis of structured phenolic lipids has been carried out so far by the incorporation of phenolic acids into vegetable oils resulting in a mixture containing compounds such as diacylglycerols, monoacylglycerols and free fatty acids along with phenolic structured lipids. The reported radical scavenging activity is due to the mixture of phenolic lipids and not for the individual structured phenolic lipids. There were no reports on the synthesis of pure structured triacylglycerol containing phenolic acids. The present chapter deals with the synthesis of novel lipophilic phenolic compounds containing ferulic acid. The synthesized phenolipids were evaluated for antioxidant activity using three different *in vitro* antioxidant assays to find applications in oil-based food products. These assays are 2, 2-diphenyl-1-picrylhydrazyl free radical ($DPPH^{\cdot}$) radical scavenging assay, rancimat assay and the rate of inhibition of autoxidation of linoleic acid in Tween 20 micellar medium. Four structured phenolic lipids of varying fatty acid chain lengths and unsaturation (**Figure 1**)

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*Chemo-enzymatic synthesis and evaluation of novel structured phenolic lipids as potential lipophilic antioxidants. **Kunduru Konda Reddy**, Kaki Shiva Shanker, Thumu Ravinder, R.B.N. Prasad, and Sanjit Kanjilal. **Eur. J. Lipid Sci. Technol.** 2010, *112*, 600-608.

were prepared. All the compounds were characterized by NMR, Mass, and IR spectroscopy.

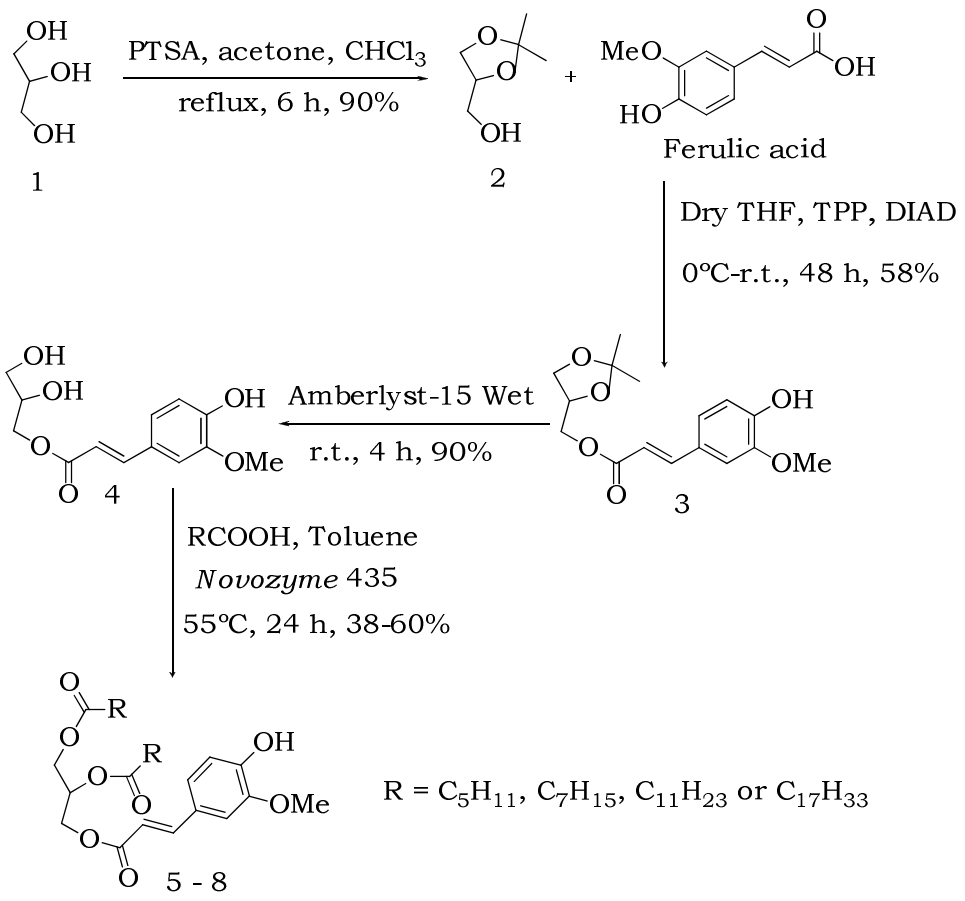

Figure 1: Chemo-enzymatic synthesis of structured triacylglycerol containing ferulic acid.

The DPPH· scavenging method was conducted in polar homogenous medium. It was observed that all the synthesized structured phenolic lipids exhibited good radical scavenging capacity but inferior to that of the reference compounds, ferulic acid and dodecyl gallate. Thus, the lipophilic modification decreased the radical scavenging capacity as

evident from FRSA of ferulic acid compared to phenolic structured lipids (compounds **5 - 8)**.

Antioxidant activity in lipid matrix was determined by measuring the oxidative stability of refined soybean oil spiked with reference antioxidants and the synthesized phenolic lipids using the Rancimat method. All synthesized compounds exhibited higher induction periods than the control soybean oil. In fact, activities of all the phenolic structured lipids are found to be at par with dodecylgallate and better than ferulic acid. Influences of the nature of fatty acids on their antioxidant activity are found to be negligible.

The rate of inhibition of autoxidation of linoleic acid in micellar medium was also studied in the presence of synthesized and reference antioxidants. After 5 days of incubation, the inhibition of conjugated diene formation by the structured phenolic lipids is found to be significantly higher than ferulic acid. However, activities of synthesized compounds are found to be similar irrespective of chain length. Antimicrobial activity of the synthesized phenolic lipids was also carried out in the present work and was found to display moderate to good activity against studied strains.

Chapter 3: Synthesis and Evaluation of Antioxidant and Antifungal Activities of Novel Ricinoleate-Based Lipoconjugates of Phenolic Acids[†]

Castor oil is also known as medicinal oil since ancient times, and was primarily used as purgative to counter constipation. The activity of castor oil is due to the presence of 88-90% of a unique fatty acid, 12-hydroxy-9-octadecenoic acid (or ricinoleic acid). In the present study, we make use of the ricinoleic acid of the castor oil, to prepare bio-based products from natural phenolic acids. The objective of this study is to find the influence of the presence of unsaturation in the alkyl chain and also in the side chain of phenolic acid on their antioxidant and antifungal activities (**Figure 2**).

The phenolics namely vanillic and ferulic acids were esterified with methyl ricinoleate as well as with its saturated analogue, methyl-12-hydroxystearate by the well-known Mitsunobu protocol. The secondary hydroxyl moiety of ricinoleate /12-hydroxystearate was being grafted to the phenolic moiety resulting in a unique class of phenolipid, having a terminal ester moiety and a pendant hydrophobic chain. To the best of our knowledge, there is no literature report on

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[†]Synthesis and evaluation of antioxidant and antifungal activities of novel ricinoleate-based lipoconjugates of phenolic acids. **Kunduru Konda Reddy**, Thumu Ravinder, and Sanjit Kanjilal. **Food Chem.** 2012, *134*, 2201-2207.

synthesis of ricinoleate/12-hydroxystearate based lipoconjugates of phenolic acids and their subsequent evaluation for the antioxidant and antimicrobial activities.

The DPPH$^{\bullet}$ scavenging assay conducted at three different concentrations of antioxidants (0.5, 1 and 2 mM) in a polar methanolic homogeneous medium. Results indicate decreased radical scavenging activity of lipophilic phenolic compounds compared to the corresponding free phenolic acid.

Differential scanning calorimetry (DSC) was also taken up as second assay to measure the oxidative stability of linoleic acid measured as oxidative induction temperature (OIT) in the presence of synthesized phenolic lipids. The OIT of linoleic acid was found to be 112.1 °C. All the synthesized phenolic lipoconjugates exhibited higher OIT than the control linoleic acid, indicating their ability to penetrate inside the lipid matrix and providing protection to linoleic acid. In fact, activities of all the phenolic lipoconjugates are found to be at par or marginally better than reference antioxidants, namely BHT, dodecylgallate (DDG) and α-tocopherol. The order of reactivity of phenolic lipoconjugates are found to be **3** > **1** > **2** = **4**. The presence of unsaturation in the hydrophobic part of the ferulic-based lipoconjugate was found to decrease its activity (compound **1**) compared to its saturated analogue (Compound **3**). Moreover, ferulic

acid conjugated lipid showed better activity than the vanillic acid conjugated lipid (**1** vs **2** and **3** vs **4**).

The third assay model taken up in the present work is the rate of autoxidation of linoleic acid in Tween 20 micellar system, conducted in presence of reference antioxidant and the synthesized lipoconjugates of phenolic compounds. The order of inhibitions of autoxidation of linoleic acid by the lipoconjugates of phenolic acids (compounds **1**-**4**) is found to be the same as that observed in the DSC study and are at par with DDG, the well known food grade antioxidant.

All the prepared compounds were also evaluated for their antimicrobial activities and were found to exhibit moderate to good antifungal activities. None of the synthesized compounds showed any activity against studied bacterial strains. The prepared compounds were also evaluated for the drug-likeness profile using Lipinski's rule. All the lipoconjugates of phenolic acids are found to violate one of the Lipinski's parameters i.e., cLog>5, though they were found to be soluble in protic solvents. From the data of topological polar surface area (TPSA), it can be predicted that all four novel lipoconjugates of dietary phenolics have good penetrating power (TPSA<120) through cell membranes.

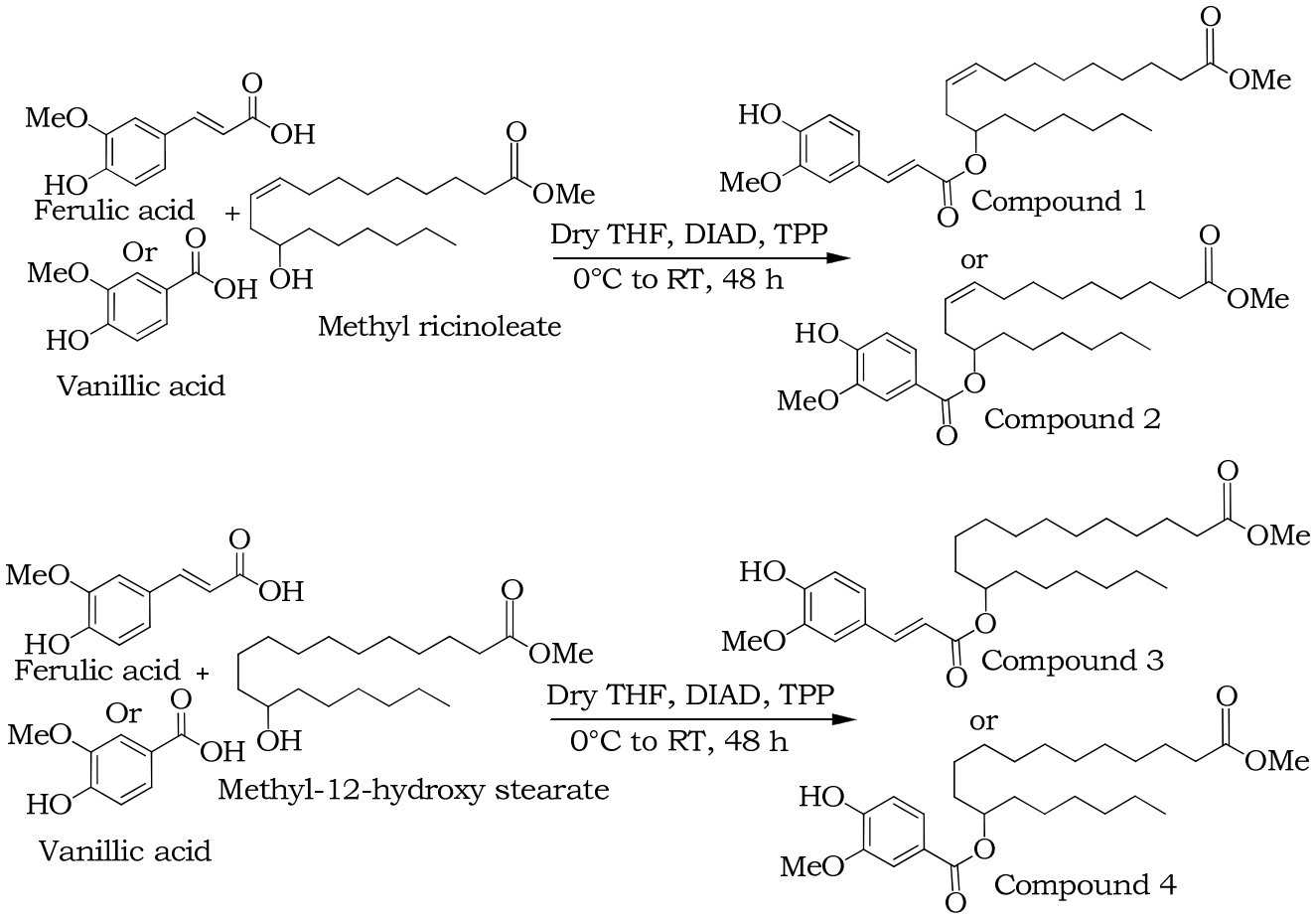

Figure 2: Synthesis of castor fatty acid-based lipoconjugates of phenolic acids.

Chapter 4: Chemo-enzymatic Synthesis of Capsiate Analogues and Their Evaluation for Antioxidant and Antimicrobial Activities[‡]

Capsaicinoids are naturally occurring lipophilic alkaloids derived from red peppers (*Capsicum annuum* L.). Capsaicinoids are responsible for the pungent spicy flavor of peppers. They are reported to possess many potent bioactivities, such as anti-inflammatory, antimicrobial, antimutagenic and antitumor properties. They are also used as topical analgesics for treating pain and for the enhancement of thermogenesis and fat consumption in mammals.

Capsinoids are nonpungent ester analogues of capsaicinoids and are known to share many potent biological activities of capsaicinoids. Because of their nonpungency, capsinoids are more palatable than capsaicinoids and may find application in the food and beverage industry as a useful ingredient. Capsinoids are structurally similar to capsaicinoids, only difference being an ester bond connecting the hydrophobic chain to the phenol moiety, instead of an amide bond as in capsaicinoids (**Figure 3**). The most natural abundant capsinoid is capsiate, 4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate. Other capsinoids such as dihydrocapsiate (4-hydroxy-3-methoxy benzyl-8-methyl-6-nonanoate) and nordihydro capsiate (4-hydroxy-3-

[‡] Evaluation of the antioxidant activity of capsiate analogues in polar, nonpolar, and micellar media. **Kunduru K. Reddy**, Thumu Ravinder, Rachapudi B. N. Prasad, and Sanjit Kanjilal. **J. Agric. Food Chem.** 2011, *59*, 564-569.

methoxy benzyl-7-methyloctanoate) have also been reported to be isolated from the fruit of sweet pepper cultivar, CH-19 sweet, of pepper *Capsicum annum* L.

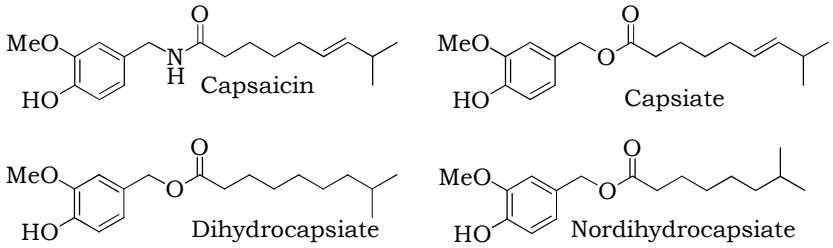

Figure 3: Chemical structures of capsaicin and capsinoids.

Limited availability of natural capsinoids and their complicated and costly chemical synthesis has restricted their applications in the food and cosmetic industry. This has necessitated the synthesis of their linear analogues. In the present work, synthesis of a series of capsiate analogues was conducted by lipase-mediated (Novozyme 435) esterification of vanillyl alcohol with different fatty acids (**Figure 4**).

The antioxidant activity of all the synthesized capsiate analogues was assayed in three different mediums, namely polar, nonpolar and micellar medium. The DPPH radical scavenging assay conducted in methanolic polar medium. All the synthesized capsiates exhibited excellent radical scavenging capacity; however reference antioxidants showed superiority.

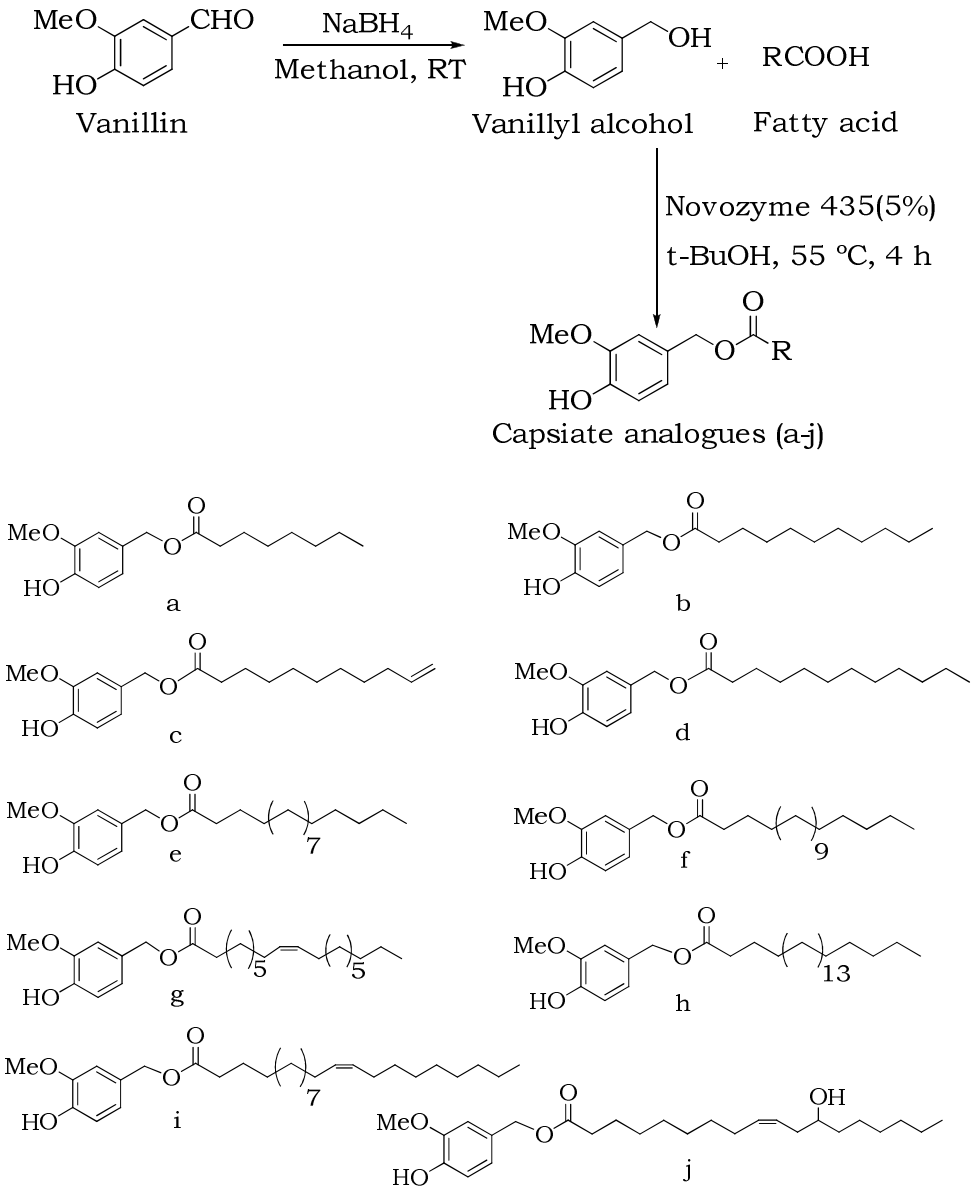

Figure 4: Synthesis of capsiate analogues

Rancimat assay was carried out in nonpolar medium. In this assay, the soybean oil spiked with antioxidant was subjected to accelerated oxidation at 110 °C under a purified air flow rate of 20 L/h. The volatile degradation products are trapped in distilled water influencing its conductivity. The induction time is defined as the time necessary to reach the inflection point of the conductivity curve. An

increase in the induction time indicates increased oxidative stability of soybean oil. All the synthesized capsiates inhibit the oxidation of soybean oil as is evident from their significantly higher induction period compared to soybean oil. In fact, activities of capsiate analogues are better than two references BHT and α-tocopherol.

The third *in vitro* assay conducted in the present work is the rate of inhibition of autoxidation of linoleic acid by antioxidant in Tween 20-based micellar medium. The autoxidation of linoleic acid was found to be markedly inhibited due to the addition of reference antioxidants as well as synthesized capsiates. When the formation of conjugated diene over 120 h, measured as absorbance at 234 nm is plotted against the chain length of fatty acids esterified to the vanillyl alcohol, a minimum is obtained near C18 chain length. This is observed both in the saturated as well as in the monounsaturated series. This indicates that in the studied Tween 20 micellar system, vanillyl ester attached with C18 alkyl chain (**f, g** and **j**), irrespective of functional moiety exhibited maximum inhibition of autoxidation of linoleic acid, when the study was conducted over a period of 5 days.

Antibacterial activities of the studied capsiate analogues revealed weak activities against the studied bacterial strains. However, with regard to antifungal activity, only capsiates prepared from shorter chain fatty acids have shown good activities towards *C.rugosa* and *S.cerevisiae*. Capsiates prepared from oleic acid and ricinoleic acid

showed good activities among the long chain compounds against *C.rugosa* and *S.cerevisiae.*

Chapter 5: Chemo-Enzymatic Synthesis of Capsiconiate Analogues: Effect of Lipophilization on their Antioxidant and Antimicrobial Activities[§]

Two non-pungent capsiate-like compounds are reported in the literature, isolated from the fruits of pepper, *Capsicum baccatum* L. var. *praetermissum*. These compounds were identified to be coniferyl esters, capsiconiate [coniferyl-(*E*)-8-methyl-6-nonenoate], and dihydro capsiconiate (coniferyl-8-methyl nonanoate) (**Figure 5**).

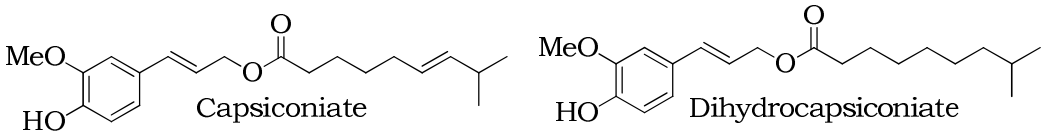

Figure 5: Chemical structures of capsiconiates.

The present chapter deals with chemo-enzymatic synthesis of capsiconiate linear analogues, which has not been reported earlier in the literature and their evaluation for antioxidant and antimicrobial activities (**Figure 6**).

[§]Synthesis and evaluation of antioxidant and antimicrobial activities of capsiconiate analogues. **Kunduru Konda Reddy** and Sanjit Kanjilal. Abstract presented at 67th OTAI Annual Convention & International Conference on "Latest developments in vegetable oil processing" and Lipids Expo-2012" on November 23-24, 2012, at ITC Maratha, Sahar Road, Mumbai, India.

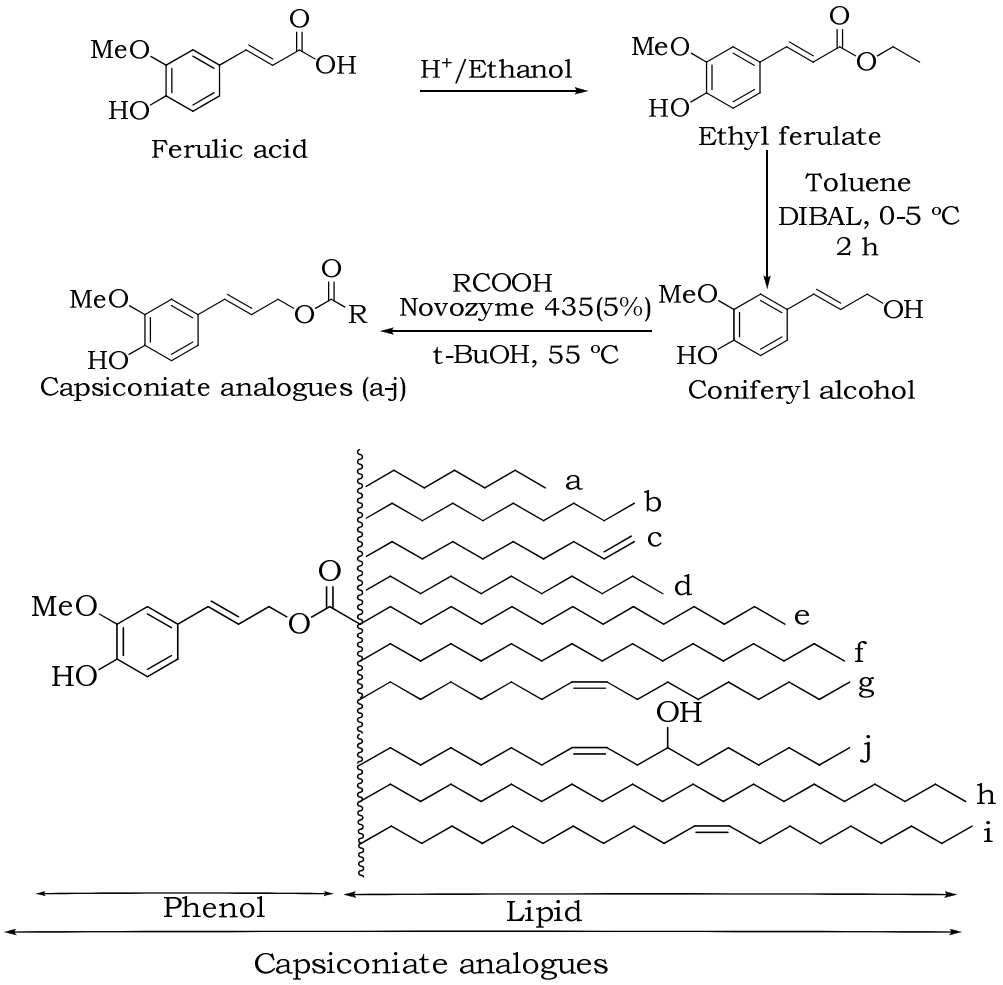

Figure 6: synthetic scheme of capsiconiate analogues (**a-j**).

All the prepared capsiconiates (**a-j**) exhibited excellent antioxidant activity in the studied *in vitro* antioxidant assays. In DPPH radical scavenging assay, all the compounds have shown better activity than α-tocopherol and at par with BHT. In the inhibition of oxidation of bulk oil, as the chain length increases, increased inhibition was observed up to C18 chain length. In the Tween 20 micellar system for the inhibition of autoxidation of linoleic acid, coniferyl esters attached to C11 and C12 alkyl chains (coniferyl undecanoate (**b**), undecenoate

(**c**), and laurate (**d**)) exhibited maximum inhibition of autoxidation of linoleic acid. The results in this assay are in support of the new cut-off effect reported in the literature.

All the compounds were also evaluated for their antimicrobial activities and found to be moderate in their antibacterial activity. However, capsiconiate analogues prepared with short chain fatty acids showed better activity than long chain compounds. All these synthesized capsiconiate (except **h** and **i**) showed very good antifungal activities. Activities are found to be very similar towards all the fungal strains irrespective of the chain length. Capsiconiates, prepared with behenic and erucic acids did not show any antifungal activity.

Chapter 6: Lipoconjugation of the Active Methylene Moiety of Curcumin and Its Effect on Antioxidant and Antimicrobial Activities

Curcumin can be structurally defined as diferuloyl methane, where two feruloyl moieties are connected by a methylene bridge (**Figure 7**). Curcumin contains two hydrophobic phenolic moieties that are connected by a flexible linker. Molecular docking studies confirmed that curcumin molecule can be adapted to many different confirmations to maximize the hydrophobic contacts with protein to which it is bound.

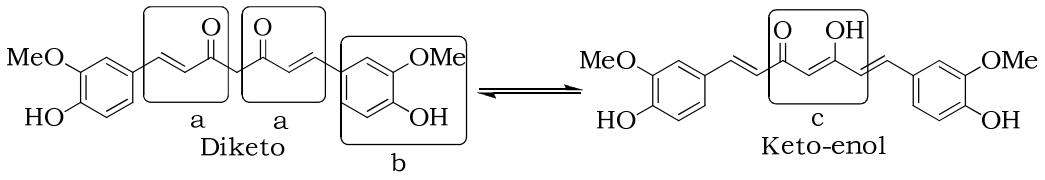

Figure 7: The structural features of curcumin: (a) α, β-unsaturated β-diketo linker; (b) O-methoxy phenolic group; (c) Keto-enol tautomerism.

Curcuminoids with a substituent at the central methylene moiety have been rarely studied for their antioxidant activity and most of the literature available is focused on anticancer, antiviral, or antibacterial activities. This lack of literature on the active methylene modification of curcumin has led us to study the antioxidant activity of curcuminoids where, the central methylene moiety was substituted with an alkyl group of different chain lengths ranging from butyl to hexadecyl. In the present work, synthesis of such lipoconjugated curcumin was carried out first by alkylating the active methylene moiety of acetyl acetone followed by its coupling to vanillin as shown in Figure **8**.

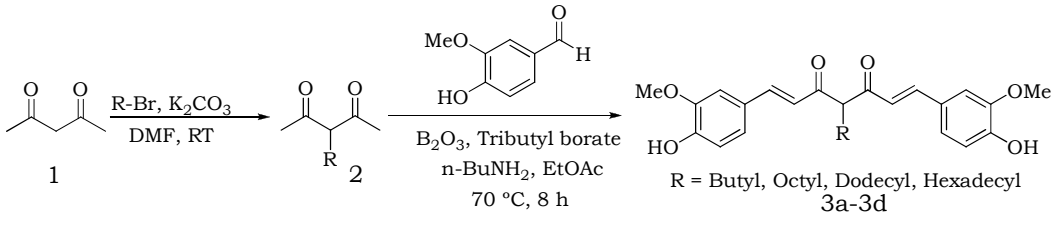

Figure 8: Synthesis of substituted acetylacetones.

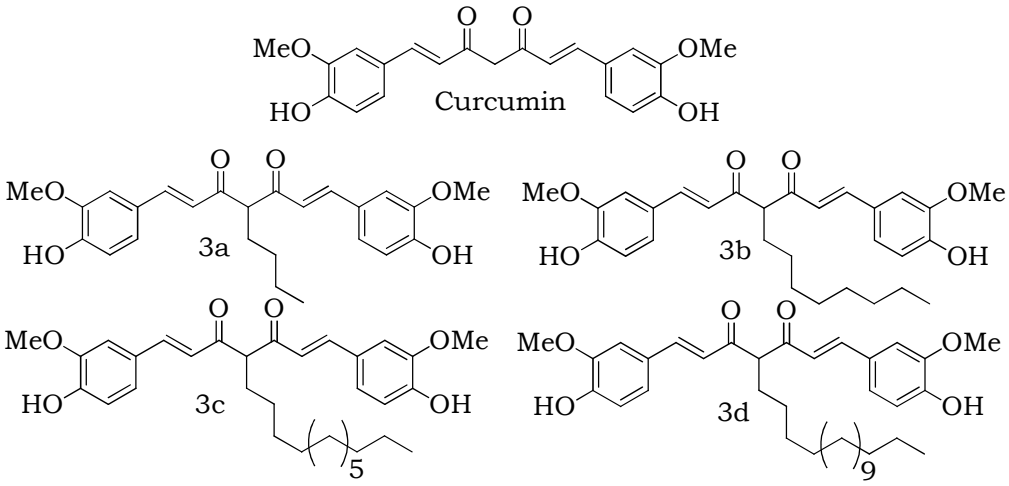

Figure 9: Structures of curcumin and alkylated derivatives (**3a-3d**).

All the synthesized compounds (**3a-3d**) have shown very good antioxidant activities in all the studied *in vitro* assays. In DPPH radical scavenging assay, substitution at central methylene moiety of curcumin by different alkyl chain lengths such as butyl, octyl, dodecyl, and hexadecyl (**3a-d**) did not improve the radical scavenging activity compared to curcumin. At 1mM concentration, their radical scavenging activity was found to be similar to that of curcumin. In the inhibition of oxidation of bulk fish oil, curcumin and its alkylated (**3a-d**) derivatives quite effectively inhibited the oxidation. Results obtained indicate that lipophililization of curcumin increased its activity towards the inhibition of the formation of lipid oxidation products significantly compared to curcumin. However, a decreasing trend was observed among the synthetic analogues. This is because increased hydrophobicity of a molecule renders the molecule to be soluble in fish oil. This may probably push the molecule at the core of the oil

medium, far away from the air-oil interface where lipid oxidation occurs. Thus, a mild increase in hydrophobicity of curcumin molecule through alkylation at the active methylene moiety increased its ability to counteract the lipid oxidation products. In the Tween 20 micellar system for the inhibition of autoxidation of linoleic acid, all the synthesized compounds inhibited the autoxidation of linoleic acid.

All these compounds were also evaluated for their antimicrobial activities and found that they were either similar or better than curcumin in their antibacterial activity towards the studied strains. In fact, all the compounds were more effective towards gram-positive bacteria compared to gram-negative bacteria. None of these compounds showed any antifungal activity at the studied concentrations.