SYNTHESIS AND ANTIOXIDANT, ANTICANCER, AND ANTIMICROBIAL ACTIVITIES
OF PALMITYL ESTER DERIVATIVE OF CARNOSIC ACID

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ABSTRACT

Carnosic acid (CA) along with carnosol (CAR) is the strongest phenolic diterpene antioxidants (PDAs) present in rosemary plant. However, CA has low antioxidant activity in emulsion-type food system due to its polar nature. The identification and characterization of the anticancer and antimicrobial properties of natural products and their semisynthetic derivative such as that of CA and CAR have received significant interest over the years.

The goals of this research were to synthesize lipophilic palmityl derivative (PE) of CA and study its antioxidant activity in bulk and emulsified corn oil. Anticancer properties against CCRF-CEM, K-562 and P388D1 leukemia cell lines and antimicrobial activity against Staphylococcus aureus (S. aureus), Bacillus cereus (B. cereus), Salmonella enterica, and Escherichia coli (E. coli) O157:H7 bacteria were also tested.

A four steps synthetic route was designed. In the first step CAR was converted into a benzyloxy protected benzyl ester of CA (yield 78%). Reduction of the benzyl ester to a primary alcohol (yield 63%) followed by esterification with palmitoyl chloride gave the palmityl ester derivative (yield 97%). Finally, double bond reduction followed by deprotection of benzyloxy group gave PE (yield 99%). Overall yield for the route was 47%.

The modification of CA affected functionality. PE had improved antioxidant activity in emulsified corn oil compared to bulk corn oil than the CA. However, CA was more effective in bulk oil. Compounds with hydroxyl groups were found to have cytotoxicity against three cell lines CCRF-CEM, P388D1 and K-562. Among compounds tested, CAR was found to be the most potent anticancer agent against all three cell lines. The study also indicated structure dependent activities for the compounds that had hydroxy group at the C-20 position.
CA and CAR had antimicrobial activity against *S. aeurus, B. cereus, Salmonella*, and *E. coli O157:H7*. *S. aeurus, B. cereus* were more sensitive to CA and CAR than *Salmonella*, and *E. coli O157:H7*. Other compounds, without hydroxyl groups, did not have antimicrobial activity. Study also indicated that antimicrobial activity varied depending on functional group present at C-20 position. Compound PE did improve antioxidant activity in emulsion but did not improve antimicrobial activity.
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LIST OF ABBREVIATIONS

TAG............................................. Triacylglycerides
TBHQ............................................ Tertiary Butyl Hydroxyquinone
BHA.............................................. Butylated Hydroxy Anisole
BHT.............................................. Butylated Hydroxy Toluene
SPME............................................ Solid phase micro extraction
CLSI............................................. Clinical and Laboratory Standards Institute
FDA............................................. US Food and Drug Administration
MIC............................................. Minimum inhibition concentration
CA.............................................. Carnosic acid
CAR............................................. Carnosol
$^1$H NMR...................................... Proton Nuclear Magnetic Resonance
$^{13}$C NMR..................................... Carbon Nuclear Magnetic Resonance
TMS............................................. Tetramethylsilane
CDCl$_3$........................................ Deuterated Chloroform
HRMS.......................................... High-resolution mass spectra
LiAlH$_4$........................................ Lithium aluminum hydride
Pd/C............................................ Palladium on Carbon
TLC............................................. Thin layer chromatography
MgSO$_4$....................................... Magnesium sulfate
O/W............................................. Oil in water emulsion
W/O............................................. Water in oil emulsion
HLB............................................ Hydrophilic-lipophilic balance
PE..............................................Palmityl ester
PDMS/DVB..................................Polydimethylsiloxane /divinylbenzene
ANOVA......................................Analysis of variance
ROS..........................................Reactive oxygen species
SOD..........................................Superoxide dismutase
CAT..........................................Catalase
GPx...........................................Peroxidase
GSH..........................................Glutathione
GST..........................................Gluthione-S-transferase
GR...........................................Glutathione reductase
DMBA....................................12-Dimethylbenz (a) anthracene
AFB₁.........................................Aflatoxin B₁
CYP1A2....................................Phase I cytochrome P450 enzyme 1A2
CYP3A4....................................Cytochrome P450 enzyme 3A4
1,25-D3...................................1, 25-dihydroxyvitamin D3
AML.........................................Acute myeloid leukemia
Ro25-4020..............................1,25-dihydroxy-16-ene-5,6-trans-cholecalciferol
ECM.........................................Excessive extra cellular matrix
MMPs......................................Matrix metalloproteinase
ALL..........................................Acute lymphoblastic leukemia
DTT.........................................1,3-propanedithiol, D, L-dithiothreitol
IMDM......................................Isocove’s Modified Dulbecco’s Medium
DMEM......................................Dulbecco’s Modified Eagle
DMSO........................................... Dimethyl Sulfoxide

CCCP.......................................... Carbonyl cyanide-m-chlorophenylhydrazone

TSB........................................... Tryptic Soya Broth

TSA........................................... Tryptic Soya Agar

ATP........................................... Adenosine triphosphate

CDC........................................... Centers of Disease Control

GC........................................... Gas Chromatography

MS........................................... Mass Spectrometry

HPLC........................................ High Performance Liquid Chromatography

THF........................................... Tetrahydrofuran

MgSO$_4$..................................... Magnesium Sulphate

Pd/C......................................... Palladium Sulphate

Pt............................................. Platinum

Ni............................................. Nickel

PGE2........................................ Prostaglandin E2 Synthesis
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CHAPTER 1. LITERATURE REVIEW

1.1. Lipid

1.1.1. Introduction

Lipid is defined as “a wide variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids, which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform or methanol” (Christie 1982). Lipids play an important role in the nutritional and sensory value of all kind of foods. They are key components of adipose tissue (Fennema 1996). When they combine with protein and carbohydrates, they form the principle structural components of all living cells (Fennema 1996). During the processing, storage, and handling of foods, lipids undergo complex chemical changes and react with other food constituents, producing numerous compounds both desirable and deleterious to food quality. Lipid oxidation is one of these processes that causes chemical changes and reduction in food quality. It is one of the major causes of food spoilage and is often referred to as rancidity. An oxidation reaction makes these foods less acceptable, decreases the nutritional quality of food, and certain oxidation products are potentially hazardous (Zdzislaw and Sikorski 2003).

Plant derived natural products have always been an important source of therapeutic agents. Approximately 25-30% of all drugs available as therapeutics at present are obtained from natural (plant, microbes, and animals) or natural product derivatives (Calixto 2005). Plant derived natural products are the main focus of many scientist to develop new medication for different diseases like cancer and microbial infection. This chapter will provide information on lipid oxidation, the anticancer and antimicrobial activities of plant derived natural products and
their derivatives, and some of common methods used to determine the anticancer and antimicrobial activities.

1.1.2. Lipid classification

Lipids can be classified as simple, derived or complex based on structure (Akoh and Min, 2008). Simple lipids include oils, fats and waxes (Figure 1.1). Oil and fats are ester of long chain fatty acids with glycerol. These esters are called triglycerides or triacylglycerols (TAG) and they are the major lipid form in our diet. The TAG that are solids or semisolids at room temperature are classified as fats and are the predominate form in animal lipid (Akoh and Min, 2008). They are generally the TAG containing saturated fatty acids. In contrast, TAG that are liquid at the room temperature are called oils and they originate primarily in plants. These are the esters of unsaturated fatty acids with glycerol (Akoh and Min, 2008). Waxes are esters of fatty acids and long–chain alcohol. Waxes are widely distributed in nature and they are usually inert due to the saturated nature of the hydrocarbon chain and serve to prevent water lose in animals, plants, and insects (Akoh and Min, 2008). For example, Carnauba wax obtained from carnauba palm of Brazil is an example of a tough and water resistant wax. It contains esterified fatty dialcohols (diols, about 20%), hydroxylated fatty acids (about 6%) and cinnamic acid (about 10%) (Akoh and Min, 2008).

Complex lipids are also esters of fatty acid, but in addition to these two components, it also contains additional groups such as phosphate or carbohydrate moieties (Figures 1.1 and 1.2). Complex lipids are further divided into different groups such as phospholipids, glycolipids, and other complex lipids. Phospholipids consist of phosphate group in addition to fatty acid and an alcohol. Phospholipids, which contain glycerol as the alcohol, are known as glycerophospholipids while those consisting of sphingosine as the alcohol moiety are called the sphinophospholipids.
Examples of glycerophospholipids in dairy products are phosphatidylethanol amine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine (Akoh and Min, 2008). Sphingophospholipids include sphingomyelin, glucosylceramide, and lactosylceramide, which are important dairy sphingolipids (Akoh and Min, 2008).

Figure 1.1. Examples of Simple, Complex and Derived Lipids.
Glycolipids comprise of a carbohydrate group, in addition to fatty acid and an alcohol. Glycolipids usually consist of galactose, but it can also contain glucose (Figure 1.1).

Cerebrosides are an example of a glycolipid and are common constituents of membrane of animals and plants. Other complex lipids (Figure 1.2) include sulpholipids, aminolipids and lipoproteins, which contain sulphur, amino, and protein molecules, respectively (Akoh and Min 2008).
Derived lipids consist of those lipids that do not belong to the simple and complex lipid categories (Figures 1.1 and 1.2). These are obtained from the complex lipids by hydrolysis and include carotenoids, steroids, and lipid soluble vitamin such as vitamin A, D, E and K (Akoh and Min 2008).

1.1.3. Lipid oxidation

The reaction of molecular oxygen with organic molecules has been a process of considerable interest. Although a wide variety of organic molecules are prone to chemical attack by oxygen, a great deal of attention has recently been focused on lipid because of the remarkable implications of their oxidative damage. The oxidation of lipid can occur in the biological system and affect nutrients level (Akoh and Min 2008). Lipid oxidation can damage the membranes, hormones and vitamins, which are vital components for the normal cell activity (Frankel 1991). Lipid oxidation of fatty constituents cause the loss of food wholesomeness by deterioration of flavor and aroma, as well as in decay of nutritional and food qualities (Frankel 1991). Lipid oxidation products can causes adverse effects such as carcinogenesis, premature aging and other diseases (Frankel 1991).

Lipid oxidation can occur in high fat contained food such as meat products, oil, and nuts and also in low lipid contained food such as milk and vegetable products. In practice, this process can affect all quality attributes of food (Frankel 1991). The food lipids contain fatty acid that can be saturated or unsaturated and can be part of the neutral TAG fraction or part of the phospholipids fraction (Frankel 1991). Free fatty acids are electron-deficient at the oxygen atom of the carbonyl group (C=O) (Wanasundara and Shahidi 2005); unsaturated fatty acids are also electron-deficient at the point of carbon-carbon unsaturation (C=C) (Frankel 1991). These
electron-deficient regions in fatty acids are susceptible to attack by a variety of oxidizing and high-energy agents, generating free radicals (Frankel 1991).

Autooxidation process consists of free chain radical mechanism. This process consists of three steps: initiation, propagation, and terminations (Wanasundara and Shahidi 2005). In the initiation process, alpha methylenic H atom is abstracted from the unsaturated lipid molecule and a lipid radical (L’) (Figure 1.3, Equation 1) is generated. Due to the high reactivity of lipid radical, it can react with atmospheric oxygen ($^3$O$_2$) and produce a peroxy radical (LOO’) (Figure 1, Equation 2) (Wanasundara and Shahidi 2005). In the propagation step, the peroxy radical reacts with another unsaturated lipid molecule and form a hydroperoxide and a new unstable lipid radical (Figure 1.3, Equation 3) (Wanasundara and Shahidi 2005). At each step, more and more oxygen is added to the system due to generation of free radicals (Wanasundara and Shahidi 2005).

<table>
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<td>LH + I → L’+ H’</td>
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<td>L’+ $^3$O$_2$ → LOO’</td>
<td>[2]</td>
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<tr>
<td>LOO’+ LH → LOOH + L’</td>
<td>[3]</td>
</tr>
<tr>
<td>LOOH → LO’+ ’OH</td>
<td>[4]</td>
</tr>
<tr>
<td>LO’+LH → LOH + L’</td>
<td>[5]</td>
</tr>
<tr>
<td>L’ + L’→ LL</td>
<td>[6]</td>
</tr>
<tr>
<td>L’+ LOO’ → LOOL</td>
<td>[7]</td>
</tr>
<tr>
<td>LOO’+ LOO’ → LOOR + O$_2$</td>
<td>[8]</td>
</tr>
</tbody>
</table>

Figure 1.3. Possible Reactions of the Autoxidation Process. “LH” is an Alkyl Group of an Unsaturated Lipid Molecule. “H” is an Alpha-Methylene Hydrogen Atom. “LO” is Lipid Radical, “LOO” is Peroxy Radical, and I is an Initiator. (Adapted from Wanasundara and Shahidi 2005).
Hydroperoxides that are formed in propagation step are unstable and decompose to radicals that speed up the propagation reactions. These are branching steps of lipid autooxidation process (Figure 1.3, Equations 4 and 5). This chain reaction proceeds, and termination occurs only when two free radicals combine to form a nonradical product (Figure 1.3, Equations 6-8) (Wanasundara and Shahidi 2005).

In autooxidation reactions, break down of substrate molecule and generation of new molecule occurs, this brings about the gross change in the chemical and physical properties of oxidizing substrate (Shahidi and others 1992; Frankel 1991). Decomposition of hydroperoxides can form new molecules such aldehyde, ketones, alkenes and alkanes that give undesirable odors and flavors to foods (Frankel 1991).

1.1.4. Lipid oxidation methods

1.1.4.1. Measurement of primary oxidation product. In lipid oxidation, there is continuous formation of hydroperoxides as primary oxidation products and there are several techniques available to measure the primary oxidation products. Out of these methods, iodometric titration and conjugated dienes methods are the most common (Shahidi and Zhong 2005). Iodometric titration assay is based on the oxidation of the iodide ion (I\(^{-}\)) by hydroperoxide (ROOH) and is the basis for standard method for determining peroxide value (Shahidi and Zhong 2005). In short, in this method, a saturated solution of potassium iodide is added to oil samples to react with hydroperoxides. The liberated iodine (I\(_2\)) is then titrated with a standardized solution of sodium thiosulfate and starch as an endpoint indicator. The peroxide value is obtained by calculation and reported as milliequivalent of oxygen per kilogram of sample (meq/kg) (Shahidi and Zhong 2005).
Chemical reactions involved are:

$$\text{ROOH} + 2 \text{H}^+ + 2 \text{KI} \rightarrow \text{I}_2 + \text{ROH} + \text{H}_2\text{O} + 2 \text{K}^+$$

$$\text{I}_2 + 2 \text{Na}_2\text{S}_2\text{O}_3 \rightarrow \text{Na}_2\text{S}_2\text{O}_6 + 2 \text{NaI}$$

1.1.4.2. Conjugated dienes and trienes. When hydroperoxides are formed, conjugated dienes are produced due to the rearrangement of the double bonds in unsaturated fatty acids. The resulting conjugated dienes exhibit an intense absorption of ultraviolet light at 233/234 nm (Shahidi and Zhong 2005). Theoretically, an increase in UV absorption reflects the formation of primary oxidation products in fats and oils and a number of studies have reported good correlations between conjugated dienes and peroxide value (Shahidi and others 1994; Wanasundara and others 1995). Conjugated dienes is based on ultraviolet light absorption and is therefore simple, fast, does require a derivatization of chemical reagents prior to testing and only small amounts of samples are needed. However, this method has less specificity and sensitivity than peroxide measurement (Shahidi and Zhong 2005).

1.1.4.3. Measurement of secondary oxidation product. Hexanal which is one of many decomposition products of lipid hydroperoxide is measured using headspace solid phase microextraction (SPME) method. In this method, samples are added into a headspace vial, sealed with a teflon faced silicone septa and heated for several minutes. The sample is then sonicated for a particular time period at 60-70 °C followed by desorption of the headspace gas onto a GC capillary column (Hall and others 2005).

1.2 Antioxidants

Antioxidants can be present as natural constituents of foods, and can be purposely added to products or produced during processing (Wanasundra and Shahidi 2005). Antioxidants have the capability to retard the oxidation of an organic substance and increase the shelf life of that
material (Wanasundra and Shahidi 2005). In fats and oils, antioxidants delay the onset of oxidation or slow the rate of oxidizing reactions (Wanasundra and Shahidi 2005). As mentioned earlier, when lipids undergo oxidation, different chemical compounds that have undesirable odors are produced. The main reason for adding an antioxidant is to maintain the quality and to extend the shelf life of food (Wanasundra and Shahidi 2005).

1.2.1. Classification of antioxidants

Antioxidants can be broadly classified according to their mechanisms of action: primary and secondary antioxidants (Wanasundra and Shahidi 2005). Some antioxidants can have more than one mechanism of activity and are considered multifunctional antioxidants (Wanasundra and Shahidi 2005).

12.1.1. Primary antioxidants. Primary antioxidants, due to their chemical nature, can act as free radicals acceptors/scavengers and delay or inhibit the initiation step or interrupt the propagation step of autoxidation (Wanasundra and Shahidi 2005). Bolland and tenHave (1947) conducted the first kinetic study of antioxidant activity and postulated Equations 11 and 12 (Figure 1.4) of the antioxidants mechanisms. The primary antioxidants (AH) react with lipid and peroxyl radicals \(\text{LOO}^•\) and convert them to more stable, nonradical products (Figure 1.4, Equations 11 and 12) (Wanasundra and Shahidi 2005). Primary antioxidants have the ability to donate a hydrogen atom to lipid radicals and produce lipid derivatives and antioxidant radicals \(\text{A}^•\), which are more stable and less readily available to participate in propagation reactions (Figure 1.4, Equation 12) (Wanasundra and Shahidi 2005). Primary antioxidants predominately react with peroxyl radicals due to the higher affinities for peroxyl radicals than lipid radicals (Wanasundra and Shahidi 2005). Primary antioxidants compete for peroxyl radicals with other compounds and they scavenge peroxyl and alkoxy free radicals formed during propagation
(Figure 1.3, Equation 3) and other reactions (Figure 1.3, Equations 4 and 5) in autooxidation
(Wanasundra and Shahidi 2005).

The antioxidant radicals, which is produced due to donation of a hydrogen atom, has low
affinity toward oxygen, and the unsaturated lipids, and they are relatively stable, so they do not
initiate a propagation reaction in autooxidation process unless present in very large quantities
(Wanasundra and Shahidi 2005). The antioxidant radical react with peroxy radicals (LOO•) to
stop chain propagation, and thus they inhibit the formation of peroxides (Equation 13). They
react with alkoxy radicals (RO•) and decrease the decomposition of hydroperoxides to harmful
degradation products (Equation 14) (Wanasundra and Shahidi 2005).

$$\text{LOO}^• + \text{AH} \rightarrow \text{LOOH} + \text{A}^• \quad [11]$$
$$\text{L}^• + \text{AH} \rightarrow \text{LH} + \text{A}^• \quad [12]$$
$$\text{LOO}^• + \text{A}^• \rightarrow \text{LOOA} \quad [13]$$
$$\text{LO}^• + \text{AH} \rightarrow \text{LOH} + \text{A}^• \quad [14]$$
$$\text{LO}^• + \text{A}^• \rightarrow \text{LOA} \quad [15]$$
$$\text{A}^• + \text{A}^• \rightarrow \text{AA} \quad [16]$$

Figure 1.4. Mechanisms of Primary Antioxidant Activity (AH is an Antioxidant Molecule).
(Adapted from Wanasundra and Shahidi 2005).

Antioxidant radicals participate in the termination reactions with peroxy (Equation 13),
alkoxy (Equations 14 and 15), or antioxidant (Equation 16) radical from the system
(Wanasundra and Shahidi 2005). Polyhydroxy phenolics and hindered phenolics are compounds,
which act as primary antioxidants. Several synthetic and natural antioxidants can act as primary
antioxidants (Wanasundra and Shahidi 2005).

1.2.1.2. Secondary antioxidants. Secondary antioxidants have antioxidant activity
through various mechanisms to slow the rate of oxidation reactions. The secondary antioxidants
act as chelators of prooxidant or catalytic metal ions, provide H to primary antioxidants, decompose hydroperoxide to nonradicals ions, decompose hydroperoxide to nonradicals species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers (Wanasundra and Shahidi 2005). They generally enhance the antioxidant activity of primary antioxidants (Wanasundra and Shahidi 2005).

1.2.1.3. Synthetic antioxidants. Synthetic antioxidants are man made and are used to stabilize fats, oils, and lipid containing foods and are mostly phenolic-based (Wansundra and Shahidi 2005). Some examples of synthetic antioxidants include BHA, BHT, Gallates, and TBHQ (Figure 1.5).

![Butylated hydroxytoluene (BHA)](image1)

![Butylated hydroxytoluene (BHT)](image2)

![Ethoxyquin](image3)

![β-Carotene](image4)

![Lycopene](image5)

![Lutein](image6)

Figure 1.5. Examples of Primary and Secondary Antioxidants.
BHA is a monophenolic compound and present as a mixture of two isomers, 3-tertiary-4-hydroxyanisole or 3-BHA (90%) and 2-tertiary butyl-4-hydroxyanisole on 2-BHA (10%). BHA has better antioxidant activity in animal fats as compared to vegetable oils (Wanasundara and Shahidi 2005). When BHA is used in packaging material, it migrates to the contained food and delays lipid oxidation (Bolland and ten Have 1947; Buettner 1993; Decker and others 2002). BHT is also a monohydroxyphenol and is generally used in foods. BHT is effectively used to prevent oxidation of animal fats (Wanasundara and Shahidi 2005). Galletes are different esters of gallic acid (such as n-propyl, n-octyl, and n-dodecyl gallates) and are approved antioxidants for food. Propyl gallate is the most commonly used gallate (Wanasundara and Shahidi 2005). TBHQ is diphenolic antioxidants and is commonly used in a variety of fats and oils. TBHQ has excellent carry through properties and is a very effective antioxidant for use in frying oils (Wanasundara and Shahidi 2005).

1.2.1.4. Natural antioxidants. Plant parts such as bark, leaves, and seeds and their extracts have been used for centuries to preserve food (Wanasundara and Shahidi 2005). Even for the industrial materials, plant-based components were used as antidrying agents to prevent oxidation and polymerization of fatty acid-rich plant oil (Pratt and Hui 1995). Some examples of naturally occurring antioxidants are carotenoids, tocopherol and tocotrienols, and other phenolic antioxidant compounds from plants (Figure 1.5).

Carotenoids are lipid-soluble –colored compounds, which are mainly present in plants, fruits, and vegetables. Carotenes are polyene hydrocarbons and have varying degrees of unsaturation (Wanasundara and Shahidi 2005). Many fats and oils, which are obtained from plant sources, contain beta-carotene, and it contributes to the deep intense orange red color of many oils (Deshpande and others 1996; Reische and others 2002). Tocopherols and tocotrienols are
natural antioxidant compounds and found widely in different tissue. Tocopherols are present in vegetable oil-derived foods in abundance. Tocopherols and tocotrienols consist of the group of chromanol homologs, which shows vitamin E activity in the diet (Wanasundra and Shahidi 2005). Plants contain phenolic compounds in abundance in their secondary metabolite pool. Among these, a series of phenolic acids and polyphenolic derivatives are present, which have antioxidant properties. In foods, these compounds can act as radical scavengers or metal chelators (Wanasundra and Shahidi 2005).

1.2.2. Toxicity of natural and synthetic antioxidants

It is important that natural or synthetic antioxidants, which are added in foods, should have a low toxicity, should be effective in low concentration in a wide variety of fats, and should have approval by the Food and Drug Administration (FDA) (Shibamoto and Bjeldannes 2009). Overdose of vitamin C (106 g) induce perspiration, nervous tension and lowered pulse rate (Shibamoto and Bjeldannes 2009). World Health Organization (WHO) recommends daily intake of vitamin C be less than 0.15 mg/kg (Shibamoto and Bjeldannes 2009). Vitamin C toxicity has not been reported. But its oxidized form dehydroascorbic acid caused diabetogenic in rats after repeated intravenous injections of 80 mg of dose (Shibamoto and Bjeldannes 2009). Vitamin E appeared to be harmless when patients were given a dosage level of 300 mg/day orally and parenterally. However, 6 out of 13 patients complained of headache, nausea, fatigue, dizziness, and blurred vision when a given similar dose (Shibamoto and Bjeldannes 2009). Rats, fed a diet concentration 1.2 or 2.3 % of propyl gallate lost weight. The weight loss may have been due to reluctance of the rat which had the bitter taste of propyl gallates. When rats were given propyl gallates at the 2 to 3% level for 10 to 16 months, 40% of rats died within the first month and remaining rats showed severe growth inhibition. Autopsy results indicated kidney damage due
to ingestion of propyl gallates (Shibamoto and Bjeldannes 2009). BHT, it did not produce any toxicity when rats consumed feed mixed with 0.2, 0.5, and 0.8 and 1% of BHT for 24 months (Shibamoto and Bjeldannes 2009). Mild diarrhea occurred in dogs fed 1.4 to 4.7 g/kg of BHA for four weeks. “It also causes chronic allergic reaction, malformations, and damage to the metabolic system” (Shibamoto and Bjeldannes 2009).

1.3. Anticancer and Antimicrobial Activities of Plant Derived Natural Products

1.3.1. Anticancer activity of medicinal plants

Natural products that are obtained from plants have been used for the treatment of various diseases for thousands of years. In India, China, and Egypt, terrestrial plants have been used as medicines from ancient times and various modern drugs have been developed from them (Shoeb 2006). Documentation of the Ayrvedic system is recorded in the Sustruta and Charaka, which date from about 1000 BC (Fallarino 1994). The best-known Egyptian pharmaceutical record called “Ebers Papyrus” documented over 700 drugs, which indicates the history of Egyptian medicine dated from 1500 BC (Cragg and Newman 2005). The Chinese Materia Medica, which describes more than 600 medicinal plants, has been documented since about 1100 BC (Cragg and Newman 2005).

Cancer is a major public health burden in both developed and developing countries (Prema and others 2011). In 2002, it was estimated that, 10.9 millions new cases, 6.7 million deaths, and 24.6 million persons were living with cancer around the world (Parkin and others 2005). Cancer is second leading cause of death in the United States (Parkin and others 2005). It is the abnormal growth of cells in our body that leads to death (Cragg and Newman 2005). Cancer cells usually invade and destroy normal cells. The major cause of cancer is smoking, dietary
imbalances, hormones and chronic infections leading to chronic inflammation (Cragg and Newman 2005).

Recently, plant derived natural products such as flavonoids, terpenes, and alkaloids have received significant attentions, due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects. Plants have been used in treatment of cancer from a long time. Of the 92 anti-cancer drugs commercially available prior to 1983 in the USA and approved worldwide between 1983 and 1994, approximately 62% can be related to natural origin (Cragg and others 1997).

Vinblastine and vincristine alkaloids, which are obtained from the Madagascar periwinkle, *Catharanthus roseus* G. Don (Apocynaceae) introduced the new era of plant anticancer agents (Shoeb 2006). These were the first agents to advance into clinical use for treatment of cancer (Cragg and Newman 2005). Vinblastine and vincristine are used with other cancer chemotherapeutic drugs for the treatment of a variety of cancers such as leukemias, lymphomas, breast and lung cancers and advanced testicular cancer (Cragg and Newman 2005). Taxus species such as *Taxus baccata* was used in the Indian Ayurvedic medicine for the treatment of cancer (Shoeb 2006). The structure of paclitaxel was elucidated in 1971 and was clinically introduced to the US market in the early 1990s (Wani and others 1971; Rowinsky and others 1992). This compound has significant activity against ovarian cancer, advanced breast cancer and lung cancer (Rowinsky and others 1992). Topotecan and irinotecan, which are semisynthetic derivatives of camptothecin (camptothecin obtained from the Chinese ornamental tree called Nyssaceae) are used for treatment of ovarian and small cell lung cancers, and colorectal cancers, respectively (Creemers and others 1996; Bertino 1997). Etoposide and teniposide are semisynthetic derivative of epipophyllotoxin (epipodophyllotoxin obtained from
roots of *Podophyllum* species) are used in the treatment of lymphomas and bronchial and testicular cancers (Cragg and Newman 2005). Homoharringtonine obtained from the Chinese tree *Cephalotaxus harringtonia var* has been used successfully for treatment of acute myelogenous leukemia and chronic myelogenous leukemia in China (Kantarjian and others 1996; Cragg and Newman 2005).

Lingzhi is a mushroom rich in beta-D-glucose that has antitumor activities and it is currently used as an adjunctive in treatments because it reduces side effects of chemotherapeutic agents (Nagarani and others 2011). Amalkai contains vitamin C and superoxide dismutase (SOD) and other antioxidants, which prevents free radical oxidation (Nagarani and others 2011). Garlic contains dually sulfide compounds, which have anticancer property (Nagarani and others 2011). Ginger contains pungent ingredients in which gingerol and pardol have been shown to posses antitumor and antiprolifetaive effects (Nagarani and others 2011). *Aloe vera* and other species of aloe contain aloe emodin and acemannnan have anticancer activity (Nagarani and others 2011). Milk thistle seeds, as well as turmeric, had great liver protective effect against a number of toxins, including many chemotherapies (Luper 1998).

**1.3.2. Different methods for evaluation of cell proliferation**

There are different methods that are available to evaluate cell proliferation. These methods include the detection of proliferation associated antigens by immunohistochemistry, quantitation of DNA synthesis by measuring tritiated thymidine (3H-thymidine) or propidium iodide staining, quantitation of reduction of the intracellular environment by tetrazolium salt reduction (MTT assay) or qunatitation of intracellular ATP concentration (ViaLight Cytotoxicity/ Cell Prolifeartion Assay)(Anonymous 2009).
1.3.2.1. Detection of antigens associated with proliferation. This method basically detects the antigen, which are absent in non-proliferating cells but present in proliferating cells. The appearance of several antigens has been noted during cell proliferation. In case of humans, the frequently cited antigen used to monitor cell proliferation is recognized by the monoclonal antibody Ki-67. This monoclonal antibody is also suitable for examination of the cell cycle because this antigen is expressed during S, G2, and M phases. The use of monoclonal antibody Ki-67 requires that a cell, which is examined, be processed for immunohistochemical staining. This method is very useful to assess in-vivo tumor cell proliferation (Anonymous 2009).

1.3.2.2. $^{3}$H-Thymidine incorporation. $^{3}$H- thymidine incorporation method is used to measure cell proliferation. In this method, cancer cells are incubated with $^{3}$H-thymidine for 16-24 hours following the treatment with testing compounds. Incorporated $^{3}$H-thymidine is usually quantitated by scintillation counting of labeled cells collected by aspiration upon membrane filters. As only proliferating cells incorporate $^{3}$H-thymidine, this method is an accurate indicator of DNA synthesis (Wiepz and others 2006; Anonymous 2009).

1.3.2.3. Propidium iodide staining. Propidium iodide binds to DNA. Therefore, this method quanitatates the DNA content of cells as a measure of cell proliferation. Cells are lysed by a freeze-thaw cycle in the presence of 1% propidium iodide. A fluorescence plate reader measures fluorescence intensity and the fluorescence is directly proportional to the DNA content in the samples. DNA content values lower than control indicates cytotoxicity while higher DNA content value than control indicates proliferation (Patel and Bertics 2006; Anonymous 2009).

1.3.2.4. MTT assay method. MTT assay consists of qualified reagents that provide a rapid and convenient method to determine the viable cell number in proliferation, cytotoxicity, apoptosis assays etc. This assay is based on the cellular conversion of a tetrazolium salt into a
formazan product that is easily detected using a 96-well plate reader. In short, in this method the premixed optimized dye solution is added to the wells of a 96-well plate that usually contain various concentrations of growth factor or test substance. During a 4-hour incubation, living cells convert the tetrazolium component of dye solution into formazan product. After that solubilization solution / stop mix is then added to the culture wells to solubilize the formazan product, and absorbance is measured at 550 or 570 nm using 96-well plate reader. The 570 nm absorbance reading is directly proportional to the number of cells normally used in proliferation assays (Patel and Bertics 2006).

1.3.2.5. *ViaLight cytotoxicity/ cell proliferation assay*. *ViaLight* assay is used for mammalian cells grown either in suspension or on the surface as a monolayer. In this method, intracellular ATP is measured by luciferase enzyme that is catalyzed by bioluminescence. Since ATP is central to the metabolism of all cells the intracellular concentration is very precisely regulated. When a cell dies, the ATP levels drop instantly. Based on these two observations, the number of viable cells in a culture can be assessed by measuring the concentration of ATP in culture. Bioluminescent detection of ATP is very simple and sensitive. In this method, reagent containing luciferase and its substrate is simply added to the cell extract and the light emission is measured by microplate luminometer. The light emission is directly proportional to the ATP concentration in samples. ATP value lower than control indicates cytotoxicity and ATP value higher then control indicates proliferation (Anonymous 2009).

1.3.3. Toxicity of alkaloids and their derivatives

Natural products, especially alkaloids that are obtained from natural source and their synthesized derivatives have been highly effective conventional drugs for the treatment of many forms of cancer as discussed earlier. These drugs can have dose limiting or overdose toxicities
while treating different kind of cancer (Fattorusso and Taglialatela-Scafati 2007). Etoposide, which is semisynthetic derivative of epipophyllotoxin, can cause various side effects such as myelosuppression (i.e. generally dose dependent effect), including both neutropenia and thrombocytopenia in human patients (Kobayashi and Ratain 1994). The dose-dependent myelosuppression of etoposide is well documented (Joel and others 1994). A number of reports indicate that it causes hematological toxicity in human patients. The dose limiting effect vincristine is neurological and extensive peripheral neuropathy occurs at higher dose (Rosenthal and Kaufman 1974). Toxic effect of vincristine overdose was reported by a patient (53 year old) who had invasive cervical cancer. The toxic effects included parenthesis, bone marrow depression, severe oral mucositis, paralytic ilues, bladder atony, myalgia, muscle weakness, high fever, dearangements of various organs (liver, heart), hypertension, and insomnia (Lin and others 1998). The dose-limiting toxicity of vinblastine is myelosuppression in human patients (Fattorusso and Taglialatela-Scafati 2007). The syndrome of inappropriate secretion of antiduretic hormones (SIADH) has been also reported with the administration of vinblastine to human patients (Sorensen and others 1995). Treatment of lymphomas with vinblastine caused gastrointestinal toxicosis in cats (Sorensen and others 1995). Simon and others (2008) reported that 56% of the episodes of gastrointestinal toxicosis in feline lymphoma patients had received a multi-drug chemotherapy protocol, which included administration of vincristine. The dose limiting toxicity of topotecan is myelosuppression with the most common severe adverse event being grade 4 neutropenia in human patients (Creemers and others 1996). The chief dose-limiting toxicities of irinotecan therapy are myelosuppression and delayed-type diarrhea in human patients (Rothenberg and others 1993). Some of side effect of paclitaxel includes nausea,
numbness, and a reduction in the white blood cells in human patients (Srivastava and others 2005).

1.3.4. Antimicrobial activity of medicinal plants

The use of plants for treating diseases is as old as the human species. Medicinal plants are used on a larger scale due to their therapeutic properties. The different parts of medicinal plants include root, stem, leaves, flower, fruit, and twigs exudates (Asolini and others 2006). In addition to medicinal activity, medicinal plants also have antibacterial properties.

Millions of foodborne illnesses occur each year in United States. Center for Disease Control (CDC) estimates that 1 in 6 Americans get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases (Anonymous, 2011). Foodborne illnesses can be caused by eating or drinking a contaminated food or beverage due to contamination of bacteria, viruses, and parasites and toxins. Production of food from farm or fishery to the dining tables consists of several food production steps and contamination can occur at any point along the chain—during production, processing, distribution, or preparation. Therefore, controls are required to reduce or prevent foodborne illness (Anonymous, 2011).

The antimicrobial activity of “carqueja” decoction plant on gram-positive bacteria (e.g. *Staphylococcus aureus* (*S. aureus*) and *Streptococcus uberis* and gram–negative bacteria (e.g. *Salmonella gallinarum* and *Escherichia coli* (*E. coli*)) has been reported (Avancini and others 2000). The ethanol extract of chamomile plant has antimicrobial activity against *S. aureus* bacteria (Asolini and others 2006). The aqueous extract from the artichoke and the ethanol extracts from both artichoke and “macela” inhibited the growth of *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*), *Pseudomonas aeruginosa* and *S. aureus* (Ushimaru and others 2007). Extracts of *Caryophyllus aromaticus* (flower buds), *C. citratus* (leaves), *P. guajava*...
(leaves) and *M. glomerata* (leaves) had antimicrobial activity against *Enterococcus sp.*, *E. coli*, *S. auerus* and *Salmonella* (Ushimaru and others 2007). More and others (2008) studied the antimicrobial activity of eight South African plants against human oral cavity pathogens (i.e., *Actinobacillus actinomycetemcomitans, Actinomyces naeslundii, Actinomyces israelii, Candida albicans, Porphyromonas ginggivalis, Prevotella intermedia* and *Streptococcus mutans*). Out of eight, six plants, i.e. *Annona senegalensis, Englerophytum magalismontanum, Dicerocarym senecioiides, Euclea divinorum, Euclea natalensis, Solanum panduriforme* and *Parinari curatellifoli*, had antimicrobial activity against those microorganisms. In another study, the antimicrobial activities of hexane, chloroform, acetone, ethanol, methanol and aqueous extracts from roots and leaves of bushy lippia (*Lippia alba*) at the concentration of 2 mg/ disc were evaluated against *S. aureus, B. subtilis, Enterococcus faecalis* (*E. faecalis*), *Micrococcus luteus, E. coli, Pseudomonas aeruginosa, Seetia marecescens, Mycobacterium smegmatis, Monilia sitophila* and *C. albicans*. The chloroform, acetone and ethanol extracts from roots prevented the growth of *S. auerus, Micrococcus luteus* (*M. luteus*), *B. subtilis, Mycobacterium smegmatis* (*M. smegmatis*), *C. albicans* and *M. sitophilia*, while hexane, ethanol and methanol extracts from leaves inhibited *S. auerus, M. luteus, B. subtilis, M. smegmatis* and *M. sitophila* (Aguiar and others 2008). The hexane, dichloromethane, ethyl acetate and ethanol extracts of phloem of *Bowdichia virgilioides, Calophyllum brasiliense* (*C. brasiliense*), *Cariniana rubra, Lafoensia pacari* and *Stryphnodendron obovatum, Simaba ferruginea rhizomes, and C. urucurana latex* were tested against gram positive bacteria such as *S. auerus, S. epidermidis* and *S. agalactiae*. Ethyl acetate and hexane extracts from *C. brasiliense* phloem had marked antibacterial activity against gram-positive bacteria such as *S. auerus, S. epidermidis* and *S. agalactiae* (Silva and others 2009). Zampini and others (2009) studied the antibacterial activity of ethanol extracts.
from 11 Argentinean plant species. They observed growth inhibition in at least one of the following tested strains: S. auerus, E. faecalis, E. coli, Kneumoniae, Proteus mirabilis, Enterobacter cloaceae, Morganella morganii and P. aeruginosa.

Herbs and spice are important ingredients in cooking and food preparation and interestingly most have been found to have antimicrobial properties. Researchers in different parts of the world have studied the antimicrobial activities of indigenous herbs and spices. Recently, clove, cinnamon, black cumin, and garlic were found to have potent antimicrobial activities against different bacteria (Dauusch and Nixon, 1990). Italian, German, Australian and Polish investigators have also reported the antimicrobial activity of spices (Hammer and others 1999; Marino and others 1999; Burt and others 2003; Kalemba and Kunicka 2003; Fabio and others 2006). Bagamboula and others (2003) investigated the antimicrobial activity of 17 spices and herbs on Shigella sonnei (S. sonnei) and Shigella flexneri (S. flexneri) and out of which, clove, thyme, oregano, allspice, basil and marjoram had antimicrobial activity against both organisms. Garlic has been extensively used due to its antimicrobial properties (contributed by organo-sulfur compounds). Allicin, which is the principle bioactive compound of garlic, was found to exhibit antimicrobial activity against a wide range of gram-positive and gram–negative bacteria including multidrug-resistant enterotoxicogenic strains of E. coli (Hughes and Lawson 1991; Ankri and Mirelman 1999). Black cumin crude extracts have been tested for antimicrobial activity against different bacteria. The diethyl ether extract of black cumin seed showed concentration-dependent inhibition of the gram-positive S. auerus, and gram-negative P. aeruginosa and E. coli (Hanafy and Hatem 1991; Morsi 2000). Shelf and others (1984) have done studies to evaluate the antimicrobial activity of a combination of garlic and black cumin on the isolates of urinary tract infection. In vitro investigation indicated that a combination of garlic
and black cumin had more antibacterial activity on the isolates of the urinary tract infection, compared to the individual extract or drug such as cefalexin, cotrimoxazole and nalidixic acid. Block and others (1992) identified allicin, diallyldisulfide, 1,3-dithiin, and other related disulfide in water extract of garlic using gas chromatography (GC), mass spectrometry (MS) and high performance liquid chromatography (HPLC). In case of black cumin, they identified volatile oil such as thymoquinone, p-cymene, catechol, and pinene (Roy and others 1992). Basil herb, which contains active compounds such as linalool, methylchavicol, eugenol, methyl cinnamate had antibacterial activity against *Bacillus pumilus, Bacillus anthracae, S. aureus, S. sonnei* and *S. flexneri* bacteria (Cavallito 1946; Knobloch and others 1989; Suppakul and others 2003). Cinnamon spice, which contains cinnamaldehyde and eugenol, had antimicrobial activity against *S. enertica, Listeria monocytogens (L. monocytogens)* and *E. coli* (De and others 1999; Hammer and others 1999; Bagamboula and others 2003; Kalemba and Kunicka, 2003). Clove spice that contain eugenol had antimicrobial activity against *S. flexneri, S. Enterica, E. coli, and L. monocytogenes* (Hitokoko and others 1980; Azzouz and Bullerman 1982; Morsi 2000; Friedman and others 2002; Fabio and others 2003; Kalemba and Kunicka 2003). Oregano (herb), which consists of thymol and carvacrol, had antibacterial activity against *Salmonella spp. Vibrio parahaemolyticus, S. sonnei, and S. flexneri, S. enertica, L. monocytogenes* and *E. coli* (Cavallito, 1946; Beuchat, 1976; Hitokoko and others 1980; Azzouz and Bullerman 1982; Knobloch and others, 1989; Bagamboula and others 2003; Kalemba and Kunicka 2003). Thymol, carvacrol, caffeic acid and tannins are present in thyme herb, which have shown antibiotic activity against *Vibrio parahaemolyticus, S. auerus, B. subtiles, E. coli, S. sonnei, and S. flexneri, S. enertica and L. monocytogenes* (Beuchat 1976; Shelef and others 1980; Hao and others 1998; Ankri and Mirelman 1999; Hammer and others 1999; Kalemba and Kunicka 2003).
Herbs like sage contain thymol and eugenol as antibacterial agents against *B. cerus*, *S. aeurus*, and *Vibrio parahaemolyticus* (Fabio and others 2003, Shelef and others 1984).

*Rosmarinus officinalis* L. belongs to the Lamiaceae family of herbs (Suppakul and others, 2003). It is a household plant and grown in many parts of the world. This herb is known medicinally for its powerful antibacterial and antimutagenic properties. The components of rosemary extracts have anticancer activity and antimicrobial activities against different cancer cell lines and against many microorganisms, respectively (De and others 1999; Fabio and others, 2003; Kalemba and Kunicka, 2003). A detailed review on anticancer and antimicrobial activities of rosemary plant is presented in Chapters 4 and 5.

1.3.5. Different methods for evaluation of antimicrobial activity

1.3.5.1. Disk diffusion method. The disk diffusion method is well standardized and it is simple and practical method. In this method, bacterial load approximately 1-2 X 10^8 CFU/mL is applied to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Paper antibiotic disks (up to 12 commercially-prepared) with fixed concentration of test compound are placed on the inoculated agar surface. Plates are incubated for 16-24 h at 35 °C prior to determine the growth inhibition zones (mm) around each of the antibiotics disks are measured to the nearest millimeter. The diameter of growth zone indicates the sensitivity of the isolate and to the diffusion rate of the drug through agar medium. For interpretation of zone diameters of each drug, the criteria which is published by the Clinical and Laboratory Standards Institute (CLSI) or those included in the US Food and Drug Administration (FDA) approved product inserts for the disks is used. The disk diffusion test results are qualitative in that a category of susceptibility is derived from the test rather than an minimum inhibition concentration (MIC). However, some commercially available zone reader systems claim to calculate an approximate MIC with some
organisms and antibiotics by comparing zone size with standard curves of that species (Bauer and others 1966; Jorgensen and Turnidge 2007; CLSI 2009).

1.3.5.2. Agar dilution method. Agar dilution method is one of the most commonly used methods for determination of MIC of antimicrobial agents against bacteria. In this method, solutions that contain defined numbers of bacterial cells are spotted directly onto the nutrient agar plates that already have different antibiotic concentrations. After incubation, the presence of bacterial colonies on the plates indicates growth of the organism (CLSI 2009). One of the advantages of using this method is that the susceptibility of a number of bacteria can be tested in one plate. Another advantage is that the ability to test susceptibility of fastidious organisms since the agar with supplement is able to adequately support the bacteria growth. The disadvantage of this method is that it is time consuming and labor intensive (CLSI 2009).

1.3.5.3. Broth macrodilution method. In broth macrodilution method, a standardized bacterial suspension is added to test tubes that contained two-fold diluted antibiotics in a liquid growth medium. After overnight incubation at a suitable temperature, the tubes are examined for the visible bacterial growth as evidence by turbidity (Ericsson and Sherris 1971). The lowest concentrations of antibiotic that prevent growth represent the MIC. The advantage of this technique is that, it generates quantitative results. The main disadvantage of the macrodilution method are that it is tedious, and requires large amounts of manual task for preparation of antibiotic solutions. This method also requires large amounts of reagents for each test (Jorgensen and others 1999).

1.3.5.4. Broth microdilution method. In broth microdilution method, a standardized numbers of bacteria are inoculated into wells of 96-well microwell plates that contain various concentrations of antimicrobial agents. After that, the plate is incubated for 24 h at 37 °C. The
sensitivity of antimicrobial agents is measured by the presence of viable bacterial cells in culture medium based on quantification of ATP present. One of the most common reagents, which is used for quantification of ATP, is BacTiter-Glo™ reagent. The BacTiter-Glo™ microbial cell viability assay is a homogenous method for determining the number of viable bacterial cells in culture based on the quantification of ATP present. ATP is an indicator of metabolically active cells. The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of cells in culture (Klančnik and others 2009, Klančnik and others 2010).

After 24 hours of incubation, BacTiter-Glo™ reagent equal to the volume of cell culture medium present in each well (e.g., 100 µl of reagent to 100 µl of medium containing cells for 96-well plate format) is added. After reagent addition, contents are mixed properly on an orbital shaker and incubated for five minutes in dark and luminescence is measured. The MIC value is observed as the lowest concentration of antimicrobial agent, where there is no presence of bioluminescence signal (no metabolic activity) (Klančnik and others 2009, Klančnik and others 2010). The advantage of this method is that simultaneous testing of multiple antimicrobials with ease can be done using microwell plates. Compared with agar-based method, broth micro dilution can decrease much labor and time (CLSI 2009).

1.4. References


CHAPTER 2. SYNTHESIS OF NOVEL DERIVATIVE OF CARNOSIC ACID

2.1. Introduction

In the ancient times it was known that addition of herbs or plant-based material would prolong the shelf life of food (Wood 1997). With the advent of modern science we now know that certain molecules in these herbs are primarily responsible for the preservative action of these plant-based materials.

In the case of lipid-based foods, there are several reactions that can cause food degradation. Of which, oxidation is one of the most important cause of food degradation (Frankel 1991). Oxidative degradation of lipid based food products usually proceeds through radical mechanisms and leads to the formation of aldehydes, alkanals, alkenes, and alkanes as byproducts (Bolland and tenHave 1947). These by-products have negative aroma and taste, and therefore result in decreased nutritional, due to food avoidance, and sensory quality of food products (Frankel 1991; Shahidi and others 1992). Preventing or delaying these oxidative process from occurring in lipid based food systems is of great economic importance for food industry. Antioxidants are substances that prevent or delay the oxidation of lipid based food products by acting as radical scavengers (Wanasundra and Shahidi 2005).

In chapter 1, details about lipids, lipid oxidation and different antioxidants typically used in food systems were presented. Antioxidants whether natural or synthetic can be identified by their hydrophilic/lipophilic (Figure 2.1) nature, which is particularly useful for identifying antioxidants that are suitable to a particular type of food system. Lipid based food systems can be categorized as bulk oil and emulsions (i.e. oil in water type or water in oil type) (Pokorny and Korczak 2001). Since lipid oxidation is an interfacial phenomenon an ideal antioxidant has to be
present in the greatest concentration at this interface (Porter 1980; Porter and others 1989; Porter 1993; Frankel and others 1996).

Figure 2.1. Examples of Hydrophilic and Lipophilic Antioxidants.

It has been observed that polar antioxidants (hydrophilic) perform the best in non-polar medium such as bulk oils whereas non-polar antioxidants (lipophilic) work well for emulsion type food systems (Frankel and others 1996). For example Porter and others (1980, 1989, and 1993) have found that polar antioxidants such as propyl gallates, TBHQ and Trolox™ were superior antioxidants than non-polar antioxidants such as BHA, BHT and alpha tocopherol in bulk food systems. Similarly, Frankel and others (1996) found that hydrophilic antioxidants such
as carnosic acid (CA), and rosmarinic acid performed better than lipophilic antioxidants such as CAR and alpha tocopherol in bulk corn oil. In contrast, in corn oil emulsion systems the same lipophilic antioxidants worked better that hydrophilic ones. This trend was not limited to just corn oil systems but could be extrapolated to other oil systems. Hopia and others (1996) found that CA was a better antioxidant that carnosol in bulk methyl linoleate, the reverse trend was observed in emulsions of methyl linoleate.

Frankel and others (1996) explained the change in antioxidant effectiveness in different systems was an interfacial phenomenon. According to this phenomena, hydrophilic antioxidants prevent oxidation in bulk oil system by locating themselves at the surface of the oil where reaction of fat with molecular oxygen is most prevalent, and in emulsion food system, which is a multiphase system where lipids have large surface to volume ratio allows lipophilic antioxidants to concentrate at the oil-water interface and inhibit lipid oxidation. Chen and others (2012) explained the effective of polar antioxidant in bulk oil on the basis of formation of physical structure such as association colloids.

The efficiency of an antioxidant in a particular food system is dependant on its hydrophilic-lipophilic nature. With the development in the field of synthetic organic chemistry it is possible to modulate the hydrophilic or lipophilic properties of antioxidants via structural modification. Huang and others (1996) synthesized the methyl ester of carnosic acid and compared its behavior as an antioxidant against carnosic acid in bulk corn oil and emulsion systems. As expected, the non-polar synthetic derivative was found to be a better antioxidant than its polar starting material CA in corn oil emulsion systems; however, in bulk corn oil the more polar CA performed better than its methyl ester. Furthermore, Frankel and others (1994) noted that ascorbyl palmitate, which is a non-polar synthetic derivative of ascorbic acid had
better antioxidant activity in corn oil emulsion systems whereas a reverse trend was observed in bulk corn oil. While the above two examples involved modification on a polar antioxidant to a non-polar variant the reverse is also possible. For example Trolox™, which is the polar analogue of alpha tocopherol, was best suited for bulk oil systems where the antioxidant activity of alpha tocopherol was limited (Huang and others 1996).

Most of the food systems are in the emulsion form (Pokorny and Korczak 2001). Therefore a greater need to develop newer non-polar antioxidants is needed. Lipophilic antioxidants such as ascorbyl palmitate, ascorbyl olate and ascorbyl linoleate synthesized via esterification of ascorbic acid have been previously reported and studied in detail (Shahidi and Zhong 2005).

Rosemary extract has been known to show significant antioxidant activity (Rac and Osterie 1955; Tavassoli and Djomeh 2011). Rosemary extracts contain three antioxidants of a phenolic diterpene nature. These antioxidants include CA and CAR, flavonoids and phenolic acid (Tavassoli and Djomeh 2011). CA along with carnosol is the chief antioxidants responsible for the activity of rosemary extracts (Tavassoli and Djomeh 2011). CA is very unstable and during extraction or storage is partially converted into carnosol or other diterpenes such as rosmanol (Wenkert and others 1965; Bracco and others 1981; Schwartz and Ternes 1992; Hall III and Cuppett 1997). CA is a hydrophilic antioxidant and has been shown to be a potent antioxidant in bulk oil systems compared with emulsion type food systems. In addition to antioxidant activity, rosemary extracts have antimicrobial activity against Shigella sonnrei, Salmonella typhimurium, and Listeria monoctyogenes (Jordan and others 2012). This antimicrobial activity was attributed to CA and rosmarinic acid. Similarly, CA was found to have anticancer activity against colorectal cancer (Barni and others 2012), and potent inhibitor of
Prostaglandin E$_2$ synthesis (PGE$_2$) (Bauer and others 2012). PGE$_2$ has been reported to play a role in tumorigenesis (Bauer and others 2012). Detail discussion on antimicrobial and anticancer aspect are covered in chapters 4 and 5.

Based on the significance of CA as a potent antioxidant, as well as its application as an antimicrobial and anticancer agent, suitable synthetic routes to generate derivatives which are more non polar or lipophilic have been reported. Three potential sites on CA for any synthetic manipulation were identified and include the two phenolic hydroxy groups and the carboxylic acid group. An addition of hydrophobic alkyl chains are possible on all these three sites. Literature evidence indicates that the phenolic hydroxy groups are essential for the antioxidant activity of this type of compound. Hence, the carboxylic acid group as the only site for modification was observed. CA was found to be unstable and has been shown to convert to carnosol (CAR) and other diterpenes (Wenkert and others 1965; Schwartz and Ternes 1992). The reason for this instability is thought to be due to the rapid oxidation of CA in the presence of oxygen. To negate some of the issues relating to the instability of CA, Huang and others (1996) prepared methyl ester of carnosic acid (Figure 2.2) by reacting it with diazomethane.

Luis and others (1996) reported that treatment of CAR 2.12 with methyl iodide and a potassium carbonate in acetone resulted in the formation of ester derivative of CA 2.16 in 70% yield (Figure 2.3). The postulated mechanism of this reaction involves initial deprotonation of
one phenolic hydroxyl group CAR leading to the formation of a quinone type structure 2.15. During this step the lactone ring of CAR is opened and the resulting carboxylate anion was trapped by a methyl group resulting in the formation of a methyl ester 2.16. It was also thought that protection of one phenolic hydroxy group occurs. Thus, two molecule of methyl iodide are consumed in the initial step. The intermediate quinone then reacts with another molecule of methyl iodide to give the final product. Overall three molecules of methyl iodide were consumed during the entire step.

Figure 2.3. Base Mediated Rearrangement of Carnosol.

2.2. Justification and Objectives

Obtaining pure samples of CA is difficult and very expensive. To address the issues pertaining to instability of CA and for economical reasons it was decided to design a route, which would avoid utilizing CA as starting material. Thus, the synthetic route was designed such that it started from a relatively stable starting material, was amenable for scale up and avoids use to reagent that would be difficult to handle. CAR, which is a stable molecule and was available in abundance, was an ideal candidate for our synthetic plan. Compound 2.16 was of great interest to us since hydrolysis of the methyl ester and saponification with a long chain alcohol followed by deprotection of the methoxy groups and a double bond reduction could result in the desired derivative. Alternately, transesterification with the long chain alcohol followed by the same deprotection and double bond reduction steps would also result in the same target. However,
hydrolysis of compound 2.16 gave a complex mixture of products and the desired carboxylic acid 2.19 was obtained in only 8% yield (Figure 2.4).

It was decided not to use compound 2.16 for the saponification or transesterification process. So alternate routes were developed for synthesis of palmtiyl ester derivative of carnosic acid (2.24) using CAR as starting material. The objective of this research was to synthesize palmityl ester derivative of CA (2.24) (Figure 2.5) from CAR.

2.3. Materials and Methods

Solvents generally used during the course of the study were anhydrous and distilled. Acetone was distilled over calcium sulfate and stored under an inert atmosphere of nitrogen or argon. Dichloromethane was distilled from calcium hydride under nitrogen atmosphere. Tetrahydrofuran (THF) was distilled from sodium under nitrogen. Toluene was purchased as anhydrous from Sigma Aldrich and used as such without further processing. Reagents used during the course of this work were generally purchased from Sigma Aldrich. CA and CAR were obtained from Chromodex Inc. Column chromatography was performed using silica gel (230-
$^1$H NMR was recorded on a Varian Mercury-300 (300 MHz), Varian Unity/Inova-400 NB (400 MHz), or Varian Unity/Inova-500NB (500 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from TMS, using residual CDCl$_3$ (7.27 ppm) as an internal standard. Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and coupling constants. $^{13}$C NMR was recorded on a Varain Unity/Inova-500 NB (125 MHz), Varian Unity/Inova-400 NB (100 MHz), and Varian Mercury-300 (75MHz) spectrometers, using broadband proton decoupling. Chemical shifts are reported in parts per million (ppm) downfield from TMS, using the middle resonance of CDCl$_3$ (77.23) as an internal standard. High-resolution mass spectra (HRMS) (ESI+) were obtained from the Mass Spectrometry Laboratory, North Dakota State University, Fargo, North Dakota.

All glassware utilized during the course of any organic reactions were cleaned with soap water and then rinsed thoroughly with water before rinsing them with acetone. The cleaned glassware were stored in a preheated oven maintained at 110 °C. Prior to setting up a reaction the required glassware were cooled to room temp and flushed with an inert atmosphere of nitrogen. Round bottom flasks were sealed with rubber septums and subjected to cycles of alternate vacuum and nitrogen flush. Nitrogen atmosphere was maintained throughout the course of reaction and was conveniently accomplished via a rubber balloon filled with suitable inert gas connected to the reaction assembly via a syringe. Solid reagents were typically introduced first into the reaction vessel followed by the reaction solvent and other reagents. This order was changed depending upon the reaction and in certain reactions the sequence of addition is critical. Progress of the reaction was monitored via thin layer chromatography of aliquots of reaction mixture drawn during specific intervals of time. On completion of the reaction, the reaction
mixture quenched with appropriate aqueous reagent and extracted with ethyl acetate. The organic phase was separated, dried over anhydrous magnesium sulfate and concentrated under reduced pressure to yield the crude product. Pure product was isolated from this crude mixture after column chromatography using silica gel as the stationary phase and mixtures of ethyl acetate and hexane as the mobile phase. Fractions containing the product were combined and the solvent stripped off under reduced pressure. Pure samples were subjected to high vacuum to remove residual amounts of volatile impurities. Structural analysis of pure samples was done via \(^1\)H and \(^{13}\)C NMR. \(^1\)H and \(^{13}\)C NMR of compounds 2.16, 2.21-2.25, 2.26a-d, and 2.27-2.29 can be found in the appendix (Figures A1-A22). Molecular weight of the sample was confirmed by high-resolution mass spectrometer.

2.4. Results and Discussion

2.4.1. Development of different routes for synthesis of palmityl ester derivative of carnosic acid

The synthetic route for the desired lipophilic derivative of CA 2.24 was based on the work of Luis and others (1996). This route utilized CAR 2.12 as the starting material. Ring opening of CAR using methyl iodide yielded compound 2.16, which has the carboxylic acid group protected as the methyl ester as well as the two phenolic hydroxyl groups masked as the methyl ether (Figure 2.6). Luis and others (1996) also reported that the double bond in the cyclohexyl ring of 2.16 was responsible for the rearrangement that occurred during base saponification (Figure 2.4). Therefore, it was decided to reduce this double bond by hydrogenation in order to avoid complication that may arise during subsequent steps. Compound 2.21 was then subjected to metal hydride mediated reduction, which converts the methyl ester to a primary alcohol in 2.22. It was presumed that this compound would then be stable to
esterification with suitable fatty acid to yield the lipophilic derivative 2.23. The methoxy groups could then be removed to reveal the free phenolic hydroxyls in the target molecule 2.24.

![Chemical structure](image)

Figure 2.6. First Route towards the Synthesis of the Lipophilic Derivative of Carnosic Acid.

The first step of the synthesis was the ring opening of CAR 2.12 using methyl iodide and potassium carbonate in anhydrous acetone (Figure 2.6). Care was taken to maintain an inert atmosphere of argon over the reaction mixture at all time. Luis and others (1996) obtained an isolated yield of 70% for compound 2.16. However, even after stirring the reaction mixture for 4 days, the highest yield for 2.16 obtained was 30%. Simply heating the reaction mixture to reflux for 2 days after previously stirring for 2 days resulted in doubling of the isolated yield from 30% to 60%. In order to make the process more user friendly, heating of the reaction mixture was commenced immediately after addition of all the reagents. This process was most beneficial and resulted in the isolation of the desired product 2.16 in 80% yield. This experiment was repeated several times with reproducible yields, and the reaction was easy to scale up (Table 2.1).
Table 2.1. Optimization of Conditions for Rearrangement of Carnosol.

After obtaining sufficient quantities of rearranged product 2.16, the next step in the synthesis plan was to reduce the double bond present in the cyclohexyl ring of the molecule (Figure 2.7). Hydrogenation of such double bond is typically accomplished with hydrogen gas using transition metal such as platinum (Pt), palladium (Pd) or nickel (Ni) as a catalyst (Gallezot 2003). These catalysts are commercially available, are required in extremely small amounts and can be easily removed from the product by simple filtration, thus minimizing the work up procedure. The general mechanism for this reaction involves the adsorption of hydrogen gas on the surface of the metal followed by coordination of the alkene substrate (Figure 2.8). Syn addition of hydrogen to the double bond results in the formation of the fully saturated product, which leaves the surface of the metal and the cycle, is repeated again for another molecule of the substrate. This reaction occurs under one atmospheric pressure of hydrogen. Typical reaction conditions are extremely mild and the aromatic double bonds, which require more rigorous conditions, are left untouched. Reduction of the double bond in 2.16 was thus accomplished by hydrogenation using 10% Palladium/Carbon (Pd/C) as the catalyst. The reaction was carried out in methanol and under 1 atm of hydrogen. A 91% yield of reduced product 2.21 after nine days.
of hydrogenation was achieved (Figure 2.7). Progress of the reaction was monitored by comparing $^1$H NMR of aliquots removed from the reaction mixture at different intervals of time and reaction was terminated when the protons corresponding to the double bond in the starting material were no longer observed.

![Figure 2.7. Hydrogenation of Compound 2.16 using Palladium/Carbon (Pd/C).](image)

Another reduction was completed to convert compound 2.21 into the corresponding alcohol using lithium aluminum hydride (LiAlH$_4$) (Figure 2.9). This reducing agent is typically employed for the reduction of esters to alcohol. The reaction proceeds by the nucleophilic addition of hydride to the ester leading to the in situ formation of an aldehyde (Figure 2.10). A nucleophilic addition of another hydride from a second molecule of LiAlH$_4$ then converts the aldehyde to the corresponding alkoxide, which yields the alcohol on aqueous work up. The reduction of the methyl ester in 2.21 was rather difficult and after stirring with 15 equiv. of LiAlH$_4$ for four days produced the corresponding alcohol in a disappointing 36% yield.
Repeating this experiment several times and increasing the reaction time did not improve the yield for the alcohol. Therefore, the reaction route was modified by reshuffling some of the steps. The major changes incorporated into the second route involved the reduction of methyl ester using LiAlH₄ followed by reduction of the alkene. The modified route is depicted in Figure 2.11.

Reduction of the ester with LiAlH₄ in THF in the presence of the double bond in 2.16 gave the corresponding alcohol 2.25 in 80% yield (Figure 2.12). The exact reason as to why the yields for this reaction is so drastically dependent on the presence of the double bond is unknown. This reaction was easily scaled up to 0.5 g.
Figure 2.11. Second Route towards the Synthesis of Carnosic Acid Derivative.

Figure 2.12. Reduction of Ester Group of Compound 2.16 with Lithium Aluminum Hydride (LiAlH₄) to the Corresponding Alcohol 2.25.

After obtaining sufficient quantities of the primary alcohol 2.25, attachment of the lipophilic side chain was the targeted. The palmitic acid substitution was the first derivative targeted. Esterification of carboxylic acids is traditionally accomplished by refluxing the carboxylic acid and the alcohol in the presence of an acid catalyst. This reaction is called the Fischer esterification and is aided by the removal of water, which is generated as a by-product (Fischer and Speier 1895). Attempts towards esterification of 2.25 by this route did not yield appreciable amount of the desired product. Another strategy commonly employed is to generate a reactive carboxylic acid equivalent in situ by coupling the carboxylic acid with a suitable coupling reagent. 2-Chloro-1-methylpyridiniumiodide is such a reagent (Mukaiyama 1979) and
reacting palmitic acid with 2.25 in the presence of this reagent resulted in formation the corresponding ester 2.26a in 32% yield (Table 2.2). The same transformation was also conducted using the acid chloride of the corresponding carboxylic acid in the presence of triethylamine as a base. This is a much milder procedure as it occurs at room temperature and thus minimizes the chances of side product formation. The yield for this process is very high and 92% of 2.26a was isolated (Table 2.2). The acid chloride of palmitic acid is commercially available but can also be prepared by the reaction of palmitic acid with oxalyl chloride.

Table 2.2. Optimization of Esterification Route.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Condition</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Palmitic acid, triethylamine, 2-Chloro-1-methylpyridiniumiodide, DCM, reflux, 1 d</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Palmitoyl chloride, triethylamine, DCM, r.t., 12 h</td>
<td>92</td>
</tr>
</tbody>
</table>

Additional ester derivatives 2.26b-d were synthesized after the optimization of the esterification step. The production of the esters was accomplished by reacting acid chlorides of varying lengths with the primary alcohol in 2.25. Commercially available acid chlorides were again chosen and reaction with octanoyl chloride (Table 2.3, entry 1), decanoyl chloride (Table 2.3, entry 2) and lauroyl chloride (Table 2.3, entry 3) gave the corresponding esters in 98%, 93% and 98% yield, respectively. Each of these derivatives were synthesized at the 100-150 mg scale.
Table 2.3. Preparation of Additional Lipophilic Derivatives.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>R</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.26b</td>
<td>CH$_3$(CH$_2$)$_6$-</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>2.26c</td>
<td>CH$_3$(CH$_2$)$_8$-</td>
<td>93</td>
</tr>
<tr>
<td>3</td>
<td>2.26d</td>
<td>CH$_3$(CH$<em>2$)$</em>{10}$-</td>
<td>98</td>
</tr>
</tbody>
</table>

Reduction of the double bond in 2.26a was achieved by hydrogenation with Pd/C at room temperature. These conditions were utilized in route 1 and required nine days for completion. In order to expedite the product formation, the reaction was done under methanol reflux. Small amounts of THF were utilized to aid dissolution of the starting material as it was observed that 2.26a did not have appreciable solubility in methanol. The reduced product 2.23 was isolated in 92% yield under reflux condition (Figure 2.13).

Figure 2.13. Palladium/Carbon (Pd/C) Catalyzed Hydrogenation of Compound 2.26a.

After making sufficient number of derivatives and obtaining sufficient amounts of desired compounds, deprotecting the methoxy substituents on the aromatic ring to reveal our target compound was investigated. The deprotection reaction was completed on palmityl derivative 2.23. Deprotection of methyl ethers of phenols with boron tribromide is a standard
approach (McOmie and others 1968). Under these conditions, coordination of the boron atom with the oxygen atom of the ether group, occurs and leads to the weakening, and finally cleavage of the carbon oxygen bond of the ether group, generating the corresponding borate and alkyl bromide as the product. The borate on hydrolysis under aqueous conditions yields the corresponding free alcohol. Literature evidence suggests that in case of aryl methyl ethers the carbon oxygen bond of the methyl substituent is cleaved selectively resulting in the formation of a phenol as a product and methyl bromide as a by-product. Palmityl ester 2.23, when subjected to boron tribromide, yielded a complex intractable mixture and the desired product 2.24 was not isolated (Table 2.4, entry 1). This experiment was repeated several times and the same observation was made. Curiously, during one such experiment a small quantity of rosmariquinone was obtained, which was confirmed by comparing proton NMR with authentic sample reported in literature. Deprotection of methyl ethers of catechol has also been shown to proceed with ceric ammonium nitrate and proceeds via a single electron transfer process (Kawasaki and others 1988). However, subjecting 2.23 to the same conditions again did not yield the product 2.24 and a complex mixture was obtained (Table 2.4, entry 2). Trimethylsilyl iodide as the deprotecting agent (Jung and Lyster 1977) was also tried, the mode of action of this reagent is very similar to that of boron tribromide, but this deprotecting agent also did not give the desired product (Table 2.4, entry 3).

The failure of the final step to deprotect the phenol led to a change in synthetic strategy that included a change in the type of protecting groups used during the first step. Furthermore, the functional group would need to survive strongly acidic reaction conditions through various step of the synthesis and yet could be cleaved off at the final step under less demanding conditions.
Table 2.4. Attempts towards Deprotection of Methoxy Groups.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions*</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BBr$_3$, DCM, 0 ºC–rt</td>
<td>Messy NMR, no methoxy protons observed in crude NMR but no product obtained</td>
</tr>
<tr>
<td>2</td>
<td>CAN, acetonitrile: H2O, reflux</td>
<td>No product obtained, crude NMR inconclusive</td>
</tr>
<tr>
<td>3</td>
<td>TMSI, DCM, -78 ºC</td>
<td>Messy reaction, no product obtained</td>
</tr>
</tbody>
</table>

*CAN = Ceric ammonium nitrate, TMSI = Trimethyl silyl iodide

The benzyl group was selected because it is stable to reaction conditions encountered during the course of the synthesis and could be cleaved off under either oxidative or reductive condition. The reductive conditions were selected because the compound (2.24) are relatively sensitive to oxygen.

The first step is one of the key steps of this strategy (Figure 2.14). Mechanistically the two reagents i.e. methyl iodide and benzyl bromide, should function similarly. Following the initial rearrangement of CAR (2.12), the reduction of the corresponding benzyl ester to the corresponding primary alcohol 2.28 with LiAlH$_4$ was expected. This primary alcohol can then be subjected to esterification using a long chain fatty acid followed by hydrogenation to remove the double bond and deprotection of the benzyl group to yield the final product. Another possibility included merging these two steps together, which would make the overall strategy more attractive.
The reaction of benzyl bromide with CAR under the optimized conditions yielded the corresponding tribenzylated derivative $2.27$ in 78% yield (Figure 2.15). Reduction with LiAlH$_4$ also proceeded smoothly and the desired primary alcohol $2.28$ was isolated in 63% yield. The esterification between the primary alcohol and palmitoyl chloride was completed in the presence of triethyl amine to yield the corresponding palmitic acid ester in 97% yield.

The final hydrogenation and deprotection was done in a single pot operation to give the target ester in $>95\%$ yield. The choice of toluene as a solvent was based on literature evidence that the rate of hydrogenolysis of benzyl ether in this solvent was considerable slower than that in any other solvents (Hawker and others 1990). While removal of the benzyl groups was the target, the reduction of the double bond in the ring was a concern, because considerable amount of side products could be generated if the reduction did not occur first or at least at a faster rate than the cleavage of the benzyl ethers (Figure 2.16).
2.4.2. Example of representative procedures

2.4.2.1. Synthesis of compound 2.27 from compound 2.12 using benzyl bromide and potassium carbonate (Figure 2.17).

Carnosol (1.0 equiv) and potassium carbonate (2.5 equiv.) were added to a three necked 100 ml round bottom flask fitted with a reflux condenser. Anhydrous acetone (0.04 M) was then introduced and the reaction was stirred using a magnetic stir bar. Benzyl bromide (5.5 equiv.)
was added to the reaction mixture and the reaction was heated to reflux using a preheat oil bath. Progress of the reaction was monitored using thin layer chromatography (TLC) and was deemed complete when no further change in the TLC of the reaction was observed. After the completion, the reaction mixture was cooled to room temperature and filtered through a celite plug to remove the unreacted potassium carbonate. The celite plug was washed with acetone (2 x 50 ml). Acetone was removed under reduced pressure to yield the crude product, which was subsequently purified by column chromatography using silica gel as the stationary phase and hexane: ethylacetate (100:0 to 95:5 v/v) as the mobile phase. Removal of solvent afforded viscous oil with the following characteristics (Appendix Figures A15-A16):

$^1$H NMR (500 MHz, CdCl$_3$) $\delta$ 7.41 – 7.34 (m, 4H), 7.31 – 7.21 (m, 6H), 7.18 – 7.12 (m, 4H), 7.09 – 7.01 (m, 3H), 6.78 – 6.73 (s, 1H), 6.49 – 6.39 (dd, $J$ = 9.6, 3.2 Hz, 1H), 6.12 – 6.00 (dd, $J$ = 9.6, 2.7 Hz, 1H), 5.18 – 5.11 (d, $J$ = 12.9 Hz, 1H), 5.08 – 4.99 (d, $J$ = 11.1 Hz, 1H), 4.96 – 4.85 (d, $J$ = 12.9 Hz, 1H), 4.83 – 4.75 (d, $J$ = 10.4 Hz, 1H), 4.74 – 4.68 (d, $J$ = 11.1 Hz, 1H), 4.63 – 4.50 (d, $J$ = 10.4 Hz, 1H), 3.97 – 3.77 (m, 1H), 3.40 – 3.16 (hept, $J$ = 6.8 Hz, 1H), 2.68 – 2.53 (m, 1H), 1.95 – 1.76 (m, 1H), 1.72 – 1.54 (m, 2H), 1.53 – 1.35 (m, 2H), 1.35 – 1.18 (m, 5H), 1.12 (d, $J$ = 6.9 Hz, 6H), 1.02 (s, 3H), 0.86 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 20.6, 21.9, 23.6, 23.8, 26.7, 31.5, 32.3, 34.1, 34.7, 41.2, 50.1, 51.9, 65.9, 121.2, 127.7, 127.8, 128.1, 128.3, 128.4, 128.5, 128.6, 131.0, 132.9, 136.6, 137.8, 138.1, 142.7, 149.6, 151.9, 173.8; HRMS (ESI); Calculated for C$_{41}$H$_{44}$O$_4$Na$^+$ ([M+Na]$^+$), 623.3095; Observed 623.3132
2.4.2.2. Synthesis of primary alcohol 2.28 by using lithium aluminum hydride (LiAlH₄) reduction from compound 2.27 (Figure 2.18).

![Figure 2.18. Synthesis of Compound 2.28.](image)

Compound 2.27 (1.0 equiv.) was added to a 100 ml round bottom flask containing a magnetic stir bar. To this flask THF (0.05 M) was introduced under an atmosphere of nitrogen. Lithium aluminum hydride (2.5 equiv.) was added in portions to the reaction mixture. Rapid effervescences was observed during this step and when required the rate of lithium aluminum hydride addition was reduced. Occasionally, cooling the reaction flask with water was necessary when exothermic reaction occurred. On complete addition of LiAlH₄, the reaction flask was closed using a rubber septum and vented by a nitrogen balloon. The progress of the reaction was monitored by TLC and the reaction was quenched when no further consumption of starting material was seen.

The quenching procedure required special attention due to the reactivity of lithium aluminum hydride. The reaction mixture was cooled to 0 °C and water (corresponding to number of grams of LiAlH₄) was added carefully. This was followed by addition of 15% NaOH solution (corresponding to number of grams of LiAlH₄) and then equal amount of water (corresponding to number of grams of LiAlH₄). The mixture was filtered through celite and crude reaction mixture was obtained after removal of solvent. Pure product was obtained by column purification using ethyl acetate and hexane (10:90 v/v) as elution solvents. Removal of solvent afforded viscous oil with the following characteristics (Appendix Figures A17-A18):
**1**H NMR (400 MHz, CdCl$_3$): $\delta$ 7.44 – 7.20 (m, 10H), 6.82 – 6.73 (s, 1H), 6.55 – 6.45 (m, 1H), 5.96 – 5.88 (dd, $J$ = 9.6, 2.9 Hz, 1H), 5.40 – 5.27 (d, $J$ = 11.0 Hz, 1H), 5.07 – 4.98 (d, $J$ = 11.0 Hz, 1H), 4.97 – 4.85 (m, 2H), 3.93 – 3.83 (m, 1H), 3.83 – 3.73 (m, 1H), 3.40 – 3.26 (hept, $J$ = 6.7 Hz, 1H), 3.25 – 3.14 (m, 1H), 2.50 – 2.38 (m, 1H), 1.85 – 1.43 (m, 4H), 1.37 – 1.10 (m, 9H), 1.04 – 0.94 (s, 5H); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 20.5, 23.8, 27.3, 30.9, 32.2, 35.0, 39.1, 75.5, 76.0, 121.4, 125.1, 126.0, 127.4, 127.9, 128.0, 128.2, 128.3, 128.6, 131.0, 131.5, 138.0, 138.1, 142.0, 143.0, 149.1, 149.2; HRMS (ESI): Calculated for C$_{34}$H$_{44}$O$_3$Na$^+$ ([M+Na]$^+$), 519.2841; Observed 519.2870

### 2.4.2.3. Synthesis of compound 2.29 from 2.28 (Figure 2.19).

![2.28](image1) → ![2.29](image2)

**Figure 2.19. Synthesis of Compound 2.29 from 2.28 and Palmityl Chloride.**

The alcohol 2.28 (1.0 equiv.) was added into a round bottom flask containing a magnetic stir bar. Anhydrous dichloromethane (0.1 M) was then introduced and the reaction mixture was maintained under an atmosphere of nitrogen. Triethylamine (1.2 equiv.) and palmitoyl chloride (1.1 equiv.) was added sequentially and dropwise to the reaction flask under vigorous stirring. The reaction mixture was stirred for 12 h and then quenched with saturated aqueous solution of ammonium chloride. The mixture was then transferred to a separatory funnel and the organic layer was removed. The aqueous layer was extracted twice with dichloromethane (25 ml). The organic layers were combined and dried over MgSO$_4$. Crude product was obtained upon filtration and concentration of the organic extracts. Pure product was obtained upon column
chromatography using ethyl acetate: hexane (5:95 v/v) as the eluent with the following characteristic (Appendix Figures A19-20):

\[ \textsuperscript{1}H NMR (400 MHz, CDCl}_3 \delta 7.44 - 7.13 (m, 11H), 6.73 (s, 1H), 6.51 (dd, \textit{J} = 9.3, 2.8 Hz, 1H), 5.91 (dd, \textit{J} = 9.5, 2.6 Hz, 1H), 5.31 (d, \textit{J} = 11.1 Hz, 1H), 4.95 (d, \textit{J} = 11.0 Hz, 2H), 4.78 (d, \textit{J} = 10.8 Hz, 1H), 4.57 (d, \textit{J} = 10.9 Hz, 1H), 4.15 (d, \textit{J} = 10.8 Hz, 1H), 3.29 (hept, \textit{J} = 6.8 Hz, 1H),

\[ \textsuperscript{13}C NMR (100 MHz, CDCl}_3 \]: \( \delta = 14.1, 18.8, 22.6, 23.0, 23.3, 23.8, 24.7, 26.5, 29.2, 29.3, 29.4, 29.5, 29.6, 30.0, 31.8, 32.3, 32.7, 33.1, 34.5, 40.5, 44.4, 51.2, 63.0, 74.7, 74.9, 120.6, 127.4, 127.4, 127.8, 127.9, 128.1, 128.2, 128.3, 128.9, 131.2, 132.0, 137.5, 138.2, 141.5, 149.3, 151.7, 173.9; \]

HRMS (ESI): Calculated for \( \text{C}_{50}\text{H}_{70}\text{O}_4\text{Na}^+ ([M+Na]^+) \), 757.5174; Observed 757.5166

### 2.4.2.4. Synthesis of compound 2.24 from compound 2.29 (Figure 2.20).

![Figure 2.20. Synthesis of Compound 2.24 (Target Ester).](image)

The synthetic route involve the addition of 10% Pd/C (0.7 equiv.) to a two necked round bottom flask and was completely wetted with anhydrous toluene (0.05 M). The palmityl ester 2.29 (1.0 equiv.) was dissolved in toluene (0.1 M) in a separate flask (small amount facilitated complete dissolution) and then added to the reaction flask. A hydrogen balloon was fitted to the reaction assemble and the reaction mixture was heated to 55 °C. Progress of the reaction was monitored periodically by TLC. On completion of the reaction mixture was cooled to room temperature and filtered through a celite plug. Pure product was obtained on removal of toluene
was removed under high vacuum. After the reaction, the collection flask was wrapped with aluminum foil to minimize the final product exposure to heat and light. Pure product was stored in the refrigerator under argon at all times to ensure product stability and increased shelf life.

Pure product was in the form of viscous oil with following characteristics (Appendix Figures A21-A22):

\[
\begin{align*}
^1\text{H NMR} & \ (400 \text{ MHz, CDCl}_3) \ \delta \ 6.90 - 6.73 (s, 1\text{H}), 6.65 - 6.48 (s, 1\text{H}), 5.89 - 5.61 (s, 1\text{H}), 4.98 - 4.74 (d, J = 11.0 \text{ Hz}, 1\text{H}), 4.38 - 4.17 (d, J = 10.9 \text{ Hz}, 1\text{H}), 3.25 - 3.13 (d, J = 13.9, 6.9 \text{ Hz}, 1\text{H}), 3.11 - 3.01 (d, J = 13.8 \text{ Hz}, 1\text{H}), 2.91 - 2.81 (m, 2\text{H}), 2.38 - 2.23 (m, 2\text{H}), 1.91 - 1.77 (m, 1\text{H}), 1.6-1.4 (m, 6\text{H}), 1.35-1.18 (m, 1\text{H}), 1.03 - 0.95 (m, 32\text{H}), 0.94 (d, J = 7.0Hz, 6\text{H}), 0.91 - 0.84 (t, J = 6.9 \text{ Hz}, 3\text{H}); \\
^{13}\text{C NMR} & \ (100 \text{ MHz, CDCl}_3): \ \delta \ 14.3, 19.0, 19.1, 22.4, 22.7, 22.8, 22.9, 24.9, 27.3, 29.2, 29.4, 29.5, 29.6, 29.7, 29.8, 31.6, 32.1, 32.8, 33.7, 33.9, 34.5, 41.0, 42.1, 52.6, 69.5, 119.7, 127.9, 129.3, 132.9, 141.4, 142.1, 172.8; \ \text{HRMS} \ (\text{ESI}); \ \text{Calculated} \ \text{for} \ C_{36}H_{60}ONa_4 \ ([\text{M+Na}]^+), 579.4389; \ \text{Observed} \ 579.4212
\end{align*}
\]

2.5. Conclusion

A route towards the synthesis of lipophilic derivative of CA was achieved. This four step synthetic route utilizes chemical manipulations that are relatively easy to execute and high yielding. In addition, the initial ring opening/rearrangement of CAR could be accomplished with reagents other than methyl iodide, which would possibly open the door for a variety of other transformations. The highlight of this route was the high yielding one pot strategy of alkene hydrogenation accompanied by deprotection of the benzyl ethers to yield to final derivative. A considerable amount of knowledge was gained regarding chemical synthesis and functional group tolerance through route 1 and route 2 even though the method did not work, as proposed.
Yet a small number of derivatives were generated, which could be useful in the antimicrobial and anticancer studies.

2.6. References


CHAPTER 3. EVALUATION OF ANTIOXIDANT ACTIVITY OF NOVEL DERIVATIVE OF CARNOSIC ACID

3.1. Introduction

Herbs and spices contain many phytochemicals, which are sources of natural antioxidants, such as flavonoids, phenolic diterpenes, tannins and phenolic acids (Tavassoli and Djomeh 2011). Among these herbs and spices, rosemary is an important source of natural antioxidant. Rosemary is widely used as an antioxidant in Europe and the USA. The first use of rosemary leaves as an antioxidant was reported in 1955 (Rac and Osterie 1955). The antioxidant activity of rosemary extracts is primarily due to the phenolic compounds (Tavassoli and Djomeh 2011). Phenolic diterpenes, which have abietic acid structure, flavonoids and phenolic acids are the three categories of phenolic compounds in rosemary. Carnosic acid (CA) is the strongest antioxidant present in rosemary and it accounts for 90% of its antioxidant activity. In addition to CA, carnosol (abietane-type) and rosmarinic acid (hydroxycinnamic acid ester) are the other main antioxidant compounds present in rosemary (Tavassoli and Djomeh 2011). The other minor diterpenes such as rosmanol, epirosmanol, and isorosmanol, rosmariquinone, and rosmaridiphenol (Figure 3.1) also contribute to rosemary extract antioxidant activity (Nakatani and Inatani 1984a, b; Houlihan and others 1984; Houlihan and others 1985). During the storage and extraction of rosemary, CA is partially converted either into CAR or into other diterpenes such as rosmanol (Wenkert and others 1965; Bracco and others 1981; Schwartz and Ternes 1992; Hall and Cuppett 1997). Other herb such as sage also consists of CA and CAR (Rau and others 2006; Poeckel and others 2008). Rosemary and sage leaves consist of carnosic acid between 1.5 to 2.5% and carnosol about 0.3 to 0.4% (Vevey and Lausanne 1993).
CA has much more antioxidant activity as compared to the synthetic antioxidants butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) (Richheimer and others 1996). To understand the antioxidant activity, Masuda and others (2001) determined the antioxidant mechanisms of CA in the presence of lipid such as ethyl linoleate and the radical oxidation initiator 2,2’-azobis-(2,4-dimethylvaleronitrile). During this process, CA was oxidized to an \( \alpha \)-quinone and a hydroxy \( p \)-quinone (Figure 3.2). Masuda and others (2001) proposed that multiple structures accounted for the antioxidant activity of CA.

Thus, the conclusion was based on the quantitative time course analysis of the production of these quinones. CA consists of two reactive phenol groups in its aromatic region. According
to the proposed mechanisms (Masuda and others 2001), the 11-phenolic group of CA first undergoes hydrogen donation to radicals species such as lipid peroxyl radical (LOO•), to produce the carnosate radical (Figure 3.3 Intermediate 1). Radical termination of intermediate 1 occurs via a second lipid peroxyl radical by a radical-radical coupling reaction. This coupling reaction occurs at the 12-or-14-position because they are the ortho-and para positions, respectively, to the oxygen radical and is the radical stabilizing positions by the captodative effect. When the coupling proceeds at the 12-position (ortho-position), a peroxyl hemiacetal 5 is formed. This acetal is unstable and affords 6 by elimination of a hydroperoxide (LOOH). When the coupling proceeds at the 14-position (para-position), peroxide (intermediate 2) is produced. This peroxide is also unstable because a reactive enol at the 13-position in 2 attacks oxygen of the peroxide to cleave the peroxide bond, affording an epoxide intermediate 3. Finally, a two-step isomerization occur in the epoxyquinone 3, affording compound 4.

![Figure 3.3. Proposed Antioxidant Mechanism of Carnosic Acid. (Adapted from Masuda and Others 2001).](image)

The example mechanism for CA may account for why small amounts of antioxidants can be used to control lipid oxidation. The production of intermediate compounds with activity suggests that one molecule can control multiple oxidation processes.
General antioxidant activity depends very much on the lipid substrate used for the evaluation and the hydrophilic/lipophilic nature of the antioxidant compounds. Edible products are in the form of either bulk oil or emulsion (Figure 3.4). The majorities of edible products are emulsion of the oil-in-water (O/W) or water in-oil (W/O) emulsion types (Pokorny and others 2001). Bulk oils do not contain more than minute traces of dissolved water and thus basically a homogenous phase. In contrast, an emulsion consists of two immiscible phase (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other. The substance that makes up the droplets in an emulsion is referred to as dispersed or discontinuous phase and the substance that makes up the surrounding liquid is called the continuous phase (Pokorny and others 2001). A system that consists of the oil droplet dispersed in an aqueous phase is called an O/W emulsion, for example milk, cream, dressing, mayonnaise, soups, and sauces (McClements and Weiss 2005). A system that consists of water droplets dispersed in an oil phase is called a W/O emulsion, for example, margarine and butter (Pokorny and others 2001).

![Figure 3.4. Different Types of Oil Based Food Systems.](image)

As mentioned earlier, most foods cannot be related to the bulk oil system, therefore evaluation of antioxidants in multiphase systems is more relevant to their physical and chemical nature. Therefore, antioxidants exhibiting strong activity against oxidation of lipids in bulk
system are often insufficient in an emulsion system (Wanasundara and Shahidi, 2005). In general, hydrophilic antioxidants have better antioxidant activity than lipophilic antioxidants in bulk edible oils, whereas lipophilic antioxidants are more effective in emulsions (Figure 3.5) (Frankel and others 1996). The general rule is that in food systems of low surface-to-volume ratio (e.g., bulk vegetable oils), polar antioxidants, i.e. high hydrophilic-lipophilic balance (HLB), such as propyl gallate, TBHQ, and Trolox™ are more effective than lipophilic antioxidants, such BHA, BHT, and α-tocopherols (Porter 1980; Porter and others 1989; Porter 1993). In contrast, lipophilic antioxidants, i.e. low HLB, were strongly favored in foods of high surface-to-volume ratio (e.g. emulsified oils). Frankel and others (1996) explained the change in effectiveness of antioxidants in different system was an interfacial phenomenon (Figure 3.6).

According to this phenomena, hydrophilic antioxidants prevent oxidation in bulk oil system by locating themselves at the surface of the oil where reaction of fat with molecular oxygen is most prevalent. In emulsion food system, which is multiphase system (i.e. lipids have large surface to volume ratio), lipophilic antioxidants would concentrate in the oil- water interface and inhibit lipid oxidation (Frankel and others 1996).

![Propylgallate (PG) and t-Butylhydroquinone (TBHQ) and Trolox™ and alpha-tocopherol](image)

Figure 3.5. Examples of Hydrophilic and Lipophilic Antioxidants.
Chen and others (2012) explained the effectiveness of polar antioxidant in bulk oil was based on formation of physical structures such as association colloids in bulk oil system. Bulk oil consists of many minor components, for example it contains free fatty acid, sterols, antioxidants, phospholipids, monoacylglycerol, diacylglycerol, and water (Chen and others 2011a; Xenakis and others 2010). Some of these minor components are capable of forming association colloids. The association colloids act as nonreactors in bulk oils and increases lipid oxidation reactions by enhancing the prooxidant activity of antioxidants in the presence of transition metals (originating from the seed or oil manufacturing equipment) (Chen and others 2010; Chen and others 2011b). Association colloids have altered the effectiveness of antioxidants, such as alpha-tocopherol, Trolox, and chlorogenic acid ester (Chen and others 2010; Chen and others 2011a; Laguerre and others 2011). For example, Trolox (i.e. polar analogue of alpha-tocopherol) was able to partition into association colloids more than non polar alpha-tocopherol (Chen and others 2012), this
could also explain why Trolox or other polar antioxidants are more effective antioxidants than alpha-tocopherol or other non polar antioxidant in bulk oils system (Huang and others 1996a; Chen and others 2012).

The interfacial phenomena has gained significant importance in food and medicinal applications of antioxidants, especially in developing new antioxidant strategies, such as producing lipophilic derivatives of naturally occurring antioxidants for use in emulsions, liposome, and other biological media, because there are very few promising lipophilic antioxidants from natural sources. A number of phenolipids such as ester products of phenol with fatty acid or phenolic acids with fatty alcohol have been synthesized. They showed enhanced lipophilicity (Shahidi and Zhong 2011). For example, vitamin C (L-ascorbic acid) is a widely used natural antioxidants used in food, cosmetics and medicine. However, vitamin C is highly hydrophilic and its effectiveness in stabilizing oil based formulae and emulsion is based on the lipophilic nature of the test media. To alter the solubility or to increase its lipophilicity, many fatty acid esters such as L-ascorbyl palmitate, L-ascorbyl oleate, and L-ascorbyl linoleate have been synthesized (Figure 3.7) (Qing-Xun and Dong-Zhi 2002; Qing-Xun and others 2004). In fact, at present, L-ascorbyl palmitate is the only ascorbic acid analogue commercially produced and used to stabilize high fat containing foods and emulsions (Song and Wei 2002; Viklund and others 2003; Torres and others 2008).

The lipophilic antioxidants α-tocopherol and ascorbyl palmitate were more effective in oil-in-water emulsion system than in bulk oil, while the hydrophilic antioxidants Trolox™ and ascorbic acid were more effective in a water-in-oil emulsion system in bulk oil system (Frankel and others 1994). Furthermore, the antioxidant activity of commercial rosemary extract, CAR, CA, and rosmarinic acid and α-tocopherol produced similar results (Frankel and others 1996).
Commercial rosemary extract was analyzed by high performance liquid chromatography (HPLC), and found to contain 10.3 % CA, 4.4 % CAR, and trace amount of non-detectable amounts of rosmarinic acid (Frankel and others 1996). Antioxidant activity was evaluated at 60 °C in tocopherol stripped corn oil and its O/W emulsion. Hydrophilic antioxidants CA, rosmarinic acid and commercial rosemary extract were significantly more active in bulk corn oil than in the corresponding corn oil emulsion, while CAR and α-tocopherol performed better in the emulsion (Frankel and others 1996). In another study, α-tocopherol was found more effective than Trolox™ in methyl linoleate emulsion (Huang and others 1996a).

The antioxidant activities of CA and its nonpolar analogue methyl carnosate (Figure 3.8), in bulk corn oil lipid system and its O/W emulsion were also evaluated (Huang and others 1996b). Methyl carsonate and α-tocopherol were found to be more effective antioxidants than CA in emulsified corn oil, whereas CA was better in bulk oil than its emulsion (Huang and others 1996b). Hopia and others (1996) found that CA was a better antioxidant than CAR in bulk methyl linoleate, but the reverse trend was observed in bulk linoleic acid (Hopia and others 1996). Therefore, all these studies support the importance of antioxidant polarity and lipid-
containing systems in controlled lipid oxidation. However, none of these studies have assessed increasing lipophilicity of CA by addition of a palmityl substitution.

Figure 3.8. Methyl Carnosate (Methyl Ester Derivative of Carnosic Acid).

3.2. Objective

The increase in lipophilicity of CA will improve the antioxidant activity in an emulsion compared to CA. The objective of the research was to evaluate and compare the antioxidant activities of CA and its palmityl ester (PE) derivative (Figure 3.9), on the basis of inhibiting the formation and decomposition of hydroperoxide in bulk and emulsified corn oil.

Figure 3.9. Novel Palmityl Ester Derivative of Carnosic Acid (PE).

3.3. Materials and Methods

3.3.1. Materials

Stripped corn oil was obtained from ACROS Organics Inc., CA was purchased from Chromadex and TBHQ was obtained from the Eastman Chemical Co. Hexanal standard was purchased from Alfa Aesar and Tween 20 was obtained from the Fluke chemicals. Polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre (65mm) was obtained from Supelco.
3.3.2. Purification of oil

Stripped corn oil obtained from ACROS Organics had a peroxide value of more than 10 meq/kg. Therefore, purification of oil was carried out using column chromatography. For purification, 10 g of stripped corn oil was dissolved in 100 ml of hexane and then passed through a silica gel (particle size: 200-425 mesh) packed column under positive pressure. The oil was further eluted using hexane. All the eluents were collected and evaporated using rotatory evaporator at 40 °C under vacuum to recover purified oil (Figure 3.10). Purified oil was stored in sealed amber bottle at 7 °C until used in oxidation studies. Corn oil purification resulted in a peroxide value of less than 1 meq/Kg.

![Figure 3.10. Corn Oil Before (Left) and After (Right) Purification using Silica Gel.](image)

3.3.3. Bulk oil oxidation

Stripped corn oil sample (50 g) was transferred into separate 150 ml amber glass jars. CA was added at 0.02% by weight of oil to the corn oil. The antioxidant was dissolved in corn oil by warming the oil at 60 °C for 10 min. This procedure was repeated three times to give triplicate sample of oil (50 g ea) that were placed and oxidized in the dark at 60 °C in an oven according to AOCS Recommended Practice Cg 5-67 (AOCS 1997). The procedure was repeated for synthesized derivative PE and commercial TBHQ. TBHQ containing oil sample was considered as the positive control. The negative control oil sample was also prepared in triplicate without
the addition of an antioxidant. Oxidative stability of oil (0.5 g) was determined by measuring peroxide value and hexanal concentration at 0, 2, 4, 6, 8, and 10 days using methods described below (Sections 3.3.5 and 3.3.6), respectively.

3.3.4. Oil in water emulsion preparation

A 30 % O/W emulsion (30 % oil and 70 % distilled water) was prepared by homogenizing the oil. CA (0.02%) by weight of oil was added to the stripped corn oil (45 g) contained in the amber glass jars. Antioxidants were dissolved in corn oil by warming the oil for 10 min at 60 °C. For emulsion preparation, distilled water (105 g) and Tween 20 emulsifier were added into a blender and homogenized for 1 min at high speed. After that, the antioxidant containing oil was added gradually under continuous blending. After complete oil addition, samples were blended for an extra 3 min at high speed. Emulsion aliquots were transferred into 250 ml amber glass jars and loosely covered to prevent evaporation of water from emulsion. This process was repeated three times to give triplicate sample of O/W emulsion. Similar procedure was repeated for CA and TBHQ. A negative control sample was also prepared in triplicate without adding any antioxidant. All triplicate samples were randomly placed in dark in oven at 60 °C according to AOCS Recommended Practice Cg5-97 (AOCS 1997). Oxidative stability of the emulsion was monitored as described for bulk oil.

3.3.5. Peroxide value determination

Peroxide value (primary oxidation product) of bulk corn oil and O/W emulsion was determined by using AOCS official method Cd8-53 as reported by Crowe and White (2001) with slight modification. Instead of 5 g of oil, which was used in the standard method, 0.5 g of oil from bulk or emulsion was used. Oil from emulsions (8 g) was extracted using 20 ml of chloroform: methanol (2:1 v/v) in 50 ml flask. The solution was stirred for 20 min at room
temperature. Chloroform layer was separated using a separating funnel and this layer was removed under vacuum at 30 °C using a rotating evaporator. Peroxide values were evaluated in duplicate for each triplicate sample (i.e., n=6).

### 3.3.6. Hexanal determination

Hexanal, which is one of many decomposition products of lipid hydroperoxide, was measured using a headspace solid phase micro extraction (SPME) method. Prior to headspace analysis by GC, oil or emulsion (0.5 g) was added to a headspace vial (2 ml), sealed with a Teflon faced silicone septa baked at 100 °C for 24 h before use, incubated at 95 °C for 10 min, and transferred to a sonicating water bath at 60 °C. In the sonicating water bath, the filament [polydimethylsiloxane/divinyl benzene (PDMS/ DVB) fiber (65 mm)] was inserted into the headspace of the vial, allowed to absorb headspace gases for 15 min, removed and immediately desorbed at 250 °C onto the GC column for 10 min. An HP 5890 Series II with FID detector GC was used to separate volatiles. The GC conditions were set as follows: helium head pressure (103.42 kPa), hydrogen head pressure (165.47 kPa), and air flow head pressure (303.37 kPa); Phenomenex zebronwax column (60 m x 0.25 mm (ID); and injection and detector temperatures were set at 250 °C. To separate volatiles, the GC oven initial temperature program was set at 40 °C for 10 min, then ramped at a rate of 1 °C/min to 55 °C followed by a second ramp set at a rate of 0.5 °C/min to 60 °C, held for 1 min and the third and final ramp was set at a rate of 20 °C/min to 250 °C, where it remained for 7 min. The hexanal concentration was determined using, a hexanal standard curve consisting of 0.1, 1, 2, 4, 6,8 10, 20, and 50 ppm hexanal were prepared in stripped corn oil in duplicate. These standard samples were injected on GC-headspace by the method described above. Hexanal values in oxidized oil and emulsions were determined in duplicate for each triplicate sample (i.e., n=6).
3.4. Statistical Analysis

Mean peroxide values of bulk corn oil, and emulsion and mean hexanal concentrations of bulk corn oil, and emulsions were considered independently in the statistical analysis. For all test systems, a significant difference between the mean peroxide value or hexanal levels for different antioxidants and days was determined using analysis of variance (ANOVA; SAS inc.). Tukey’s multiple comparisons were run to compare the antioxidants within each test system. Significance between treatments was determined at $p < 0.05$. ANOVA test was also run to compare the mean peroxide value or hexanal level for day 0 to day 2, day 2 to day 4, day 4 to day 6, day 6 to day 8 and day 8 to day 10 for each antioxidant and control in each test system. Significant difference was determined at $p < 0.05$.

3.5. Results and Discussion

3.5.1. Antioxidant activity of palmityl ester in bulk lipid system

Tukey’s multiple comparisons indicated that hydroperoxide formation was significantly different between all antioxidants in bulk oil system (Figure 3.11). It also indicated significant difference in hydroperoxide formation between PE and control; TBHQ and control; and CA and control. Statistical comparisons indicated that PE, CA, and TBHQ had lower hydroperoxide formation then control, overall. Furthermore, PE had higher hydroperoxide formation then CA and TBHQ overall.

ANOVA test for comparison by day indicated the control and PE had significantly different peroxide values between all comparisons (i.e., day 0 to 2 day, day 2 to day 4, day 4 to day 6, day 6 to day 8, day 8 to day 10). In case of CA, the first two comparisons (i.e., day 0 to day 2, day 2 to day 4) indicated that no significant difference in hydroperoxide formation
occurred. Furthermore, in case of TBHQ, peroxide values were not significantly different between all days except day 8 and 10.

Figure 3.11. Peroxide Value (PV) of Stripped Corn Oil without (Control) or Treated with Carnosic Acid (CA), Palmityl Ester (PE) or TBHQ over 10 Days.

In the case of hexanal formation in bulk lipid system (Figure 3.12), Tukey’s multiple comparison indicated significant differences between antioxidants. The control overall had higher hexanal formation then samples treated with PE, CA and TBHQ overall. Furthermore, oil with PE had higher hexanal levels then CA overall. TBHQ treated oil had significant less hexanal formation than oil with CA, PE and the control. In fact, hexanal in TBHQ treated oil was not detected through the entire 10 days.

Control oil had significantly different hexanal levels for day 2 to 4 and day 4 to 6. No hexanal formation was observed for CA and PE treatments up to day 2.
Figure 3.12. Hexanal Content of Stripped Corn Oil without (Control) or Treated with CA (Carnosic Acid), PE (Palmityl Ester) or TBHQ over 10 Days.

The antioxidant activities of hydrophilic CA and its lipophilic derivative PE in stripped bulk corn oil indicates that interfacial phenomena plays significant role on oxidative stability of lipid systems. The results are consistent with the observation of Frankel and coworkers (Frankel and others 1994; Frankel and others 1996), who showed that hydrophilic antioxidants such as ascorbic acid, Trolox™, CA, and rosmarinic acid were more effective in bulk than in O/W emulsion systems.

In case of bulk corn oil, TBHQ and CA were more effective antioxidants than PE. The greater effectiveness of both CA and TBHQ, as compare to PE, can be explained by their tendency to reside at the oil interface. Chen and others (2012) indicated better antioxidant activity of polar antioxidants on the basis formation of association colloids (which accelerate the
lipid oxidation process) in bulk oil system. They observed that polar antioxidant such as Trolox was able to partition into association colloids more than non polar antioxidants. This supports why polar antioxidants are better than non polar antioxidants in preventing lipid oxidation of bulk oil and may be the basis for the observed activity in this study.

TBHQ was more effective antioxidant than CA in bulk corn oil, primarily at preventing hexanal formation. Better antioxidant activity of TBHQ as compare to CA in soybean oil has been reported (Arouma and other 1992; Zhang and others 2010). TBHQ has superior antioxidant activity in various edible oils (Madhavi and others 1995). Jiang and Wang (2006) indicated that TBHQ has two para hydroxyl groups, which may makes the phenols donate hydrogen atoms more easily to active free radicals and interrupting the chain reaction of auto-oxidation.

3.5.2. Antioxidant activity of palmityl ester in oil-in-water (O/W) emulsion

In the case of O/W emulsion system (Figure 3.13), Tukey’s multiple comparison method indicated significant difference in hydroperoxide formation between TBHQ and PE, and PE and CA but not between TBHQ and CA. Furthermore, this comparison indicated PE prevented hydroperoxide formation better then control, TBHQ and CA overall. No significant difference in hydroperoxide formation between CA and control, TBHQ and control and TBHQ and CA were observed. This indicates that CA and TBHQ behaved like the control, as these compounds did not have antioxidant activity.

The between day comparison indicate that emulsion with CA, PE and control samples had significantly different hexanal levels for all days. Hexanal levels in the TBHQ sample was significantly different in all day comparisons except between day 4 to day 6, where it was non significant.
Tukey’s multiple comparison method indicated significant difference in hexanal level between control and TBHQ, CA and PE, TBHQ and PE emulsions (Figure 3.14). Furthermore, no significant difference in hexanal level between control and CA, control and PE, and CA and TBHQ emulsion was observed. CA, TBHQ and PE prevented hexanal formation better than the control overall. Statistical comparisons indicated that CA and TBHQ treated emulsions had higher hexanal formation than PE while CA had higher hexanal formation than TBHQ overall.

Comparison by day indicated significant difference in hexanal level when day 0 hexanal value was compared with the second day for CA and TBHQ treated emulsion. In case of PE and control, comparison by day indicated significant difference in hexanal formation between day 0 to day 2 and day 2 to day 4.
This result is in agreement with those of Frankel and others (1994) who observed that α-tocopherol and ascorbyl palmitate had improved antioxidant activity in emulsion systems as compared to bulk oil. In contrast, their polar derivative Trolox and ascorbic acid had better antioxidant activity in bulk oil systems than their emulsions (Frankel and others 1994). Methyl carnosate (non polar analogue of CA) was a more effective antioxidant than CA in emulsified corn oil (Huang and others 1996b). Furthermore, nonpolar antioxidants such as CAR and α-tocopherol were found to be more effective than polar antioxidants CA and rosmarinic acid in emulsion systems as compare to bulk oil (Frankel and others 1996). Finally, this study is also consistent with the observation, where CAR was found to be a better antioxidant than CA in bulk methyl linoleate as compare to linoleic acid (Huang and others 1996b).
3.6. Conclusion

The objective of this research was to study the antioxidant activity of CA and its lipophilic derivative PE in bulk corn oil and emulsion. The results showed PE-containing oil and emulsion had lower hydroperoxide and hexanal level compared to the control, which indicates that this derivative had antioxidant activity. Furthermore, the data supported our hypothesis that PE had improved antioxidant activity compared to CA in an emulsion system. This is the initial study of this derivative so it is important to further investigate antioxidant mechanisms of PE in order to understand and improve its antioxidant activity. Stability testing at different temperature should be conducted so that it can effectively applied in different type of food system accordingly. Furthermore, antioxidant activity of this novel derivative should be evaluated in other model systems.

3.7. References


CHAPTER 4. ANTICANCER ASSESSMENT OF SEMISYNTHETIC DERIVATIVE OF CARNOSIC ACID

4.1. Introduction

In chapter 3, information regarding the phytochemicals in rosemary and their potential antioxidant activity was presented. The different antioxidants that are present in rosemary extract are carnosic acid (CA), carnosol (CAR), rosmarinic acid, rosmanol, epirosmanol, isoromanaol, rosmariquinone and rosmaridiphenol (Figure 4.1) (Houlihan and others 1984; Nakatani and others 1984 a, b; Houlihan and others 1985; Tavassoli and Djomeh 2011).

The European Food Safety Authority (EFSA) in 2008 conducted a risk assessment study on CA and CAR. In this risk assessment study, the toxicological studies on rats were done using different solvent extracts of rosemary. These included rosemary extract prepared by extraction of dried rosemary leaves by means of supercritical carbon dioxide (D74), rosemary extract prepared from a partially deodorized ethanolic extract of rosemary (AR), extract prepared from a deodorised ethanolic extract of rosemary (ARD), rosemary extract produced from dried rosemary leaves by acetone extraction (F62), and deodorized rosemary extract obtained by a two-step extraction using hexane and ethanol (RES). These extracts consisted of CA and CAR as principle components (between 5 to 30%) (EFSA 2008). The result indicated low acute and subchronic toxicity in rats (EFSA 2008). The solvent extracts exhibited an effect only at higher doses of these rosemary extracts which was a minor increase in relative liver weight in rats (this effect was reversible) (EFSA 2008). Based on the results that were obtained, EFSA concluded that minor increase in weight of liver may be due to induction of Phase I and Phase II enzyme and it represents an adaptive response and are not of toxicological concerns (EFSA 2008).
CA and CAR are the major antioxidants present in rosemary extracts (Tavassoli and Djomeh 2011) and have been found in several studies to exhibit anticancer activities (Groopman and others 1992; Offord and others 1995; Dorrie and others 2001; Aoyagi and others 2006; Sharabanil and others 2006; James and others 2006; Costa and others 2007; Zunino and Storms 2009; Manoharan and others 2010; Yesil-Celiktas and others 2010).

![Figure 4.1. Different Phenolic Compounds Present in Rosemary Extracts.](image)

Living organisms have several antioxidants that counterbalance the potential deleterious effects of reactive oxygen species (ROS). The antioxidant defense system consists of enzymatic [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non-enzymatic antioxidants [vitamin C, vitamin E, and reduced glutathione (GSH)] (Halliwell and Gutteridge 2007). It is believed that the over production of ROS and/or reduction in the activities of antioxidants results in oxidative stress that leads to several disorders including cancer (Valko and others 2007). Gluthione-S-transferase (GST), glutathione reductase (GR) and reduced glutathione (GSH) act together to detoxify carcinogens either by destroying their reactive centers or facilitating their excretion by conjugation process (Renju and others 2007). Therefore, natural
or synthetic free radicals scavengers can be a potential chemo preventive strategy (Kleiner and others 2008; Renju and others 2007). Naturally occurring antioxidants such as CA and CAR have the capability to scavenge a number of reactive oxygen species including hydroxyl and lipid peroxy radicals and can be potentially used as an anticancer agent (Zhang and others 2010).

Manoharan and others (2010) investigated the chemopreventative potential of CA in 7, 12-Dimethylbenz (a) anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. DMBA is a carcinogen, which is used to induce oral cancer in hamsters (Manoharan and others 2010). The anticancer activity of CA was evaluated in hamsters randomly assigned into four groups (Manoharan and others 2010). Group I animals served as the control and were treated with lipid paraffin for 14 weeks on their left buccal pouches. Group II hamsters were treated with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group III hamsters also received the same treatment as group II in addition to oral administration of CA (10 mg/kg body weight/day), which was also done starting 1 week before exposure to the carcinogen and continued on alternate days to DMBA treatment until the animals were sacrificed. Group IV animals received oral administration of CA (10 mg/kg body weight/day) alone, as in group III, throughout the experimental period. Group II showed 100% tumor formation and these groups also showed significant alteration in the status of lipid peroxidation, antioxidants and phase I and phase II detoxification enzymes (Manoharan and others 2010). Group III, in which CA was administered to DMBA treated animals, were free of the tumor formation in the hamsters’ buccal pouches. CA also exerted potent antioxidative function and stimulated the detoxification cascade during DMBA -induced hamster buccal pouch carcinogenesis. This result suggested that the chemopreventive potential of CA is probably due
to its anti-lipid peroxidative potential and it can be used as a potent chemo preventive agent against oral carcinogenesis (Manoharan and others 2010).

Aflatoxin B₁ (AFB₁) mycotoxin is a potent hepatotoxic and hepatocarcinogenic agent (Groopman and others 1992). Oxidative stress (e.g. excessive production of ROS) plays an important role in the toxicity mechanism of several mycotoxins including AFB₁. Costa and others (2007) studied the cytotoxicity of CA on aflatoxin B₁ toxin exposed Hep G2 cells. For this study, they exposed HepG2 cells for 8 h and 24 h with 10 µM of AFB₁ toxin. The 8 h AFB₁ exposed cell was pretreated with CA for 24 h at 20 and 30 µM concentrations. The 20 and 30 µM CA pretreated cells had reduced ROS levels, by 146% and 173%, respectively (Costa and others 2007). The 24 h exposed cell with 10 µM of AFB₁ pretreated with 10, 20, and 30 µM of CA, led to a clear, dose-dependent protective effect on cell toxicity, reducing cell death induced by AFB₁ by 16%, 26% and 63%, respectively (Costa and others 2007). This result suggested that CA cytotoxicity was due to reduction in ROS (as CA scavenge free radicals), which were generated due to exposure of AFB₁. Fiander and Schneider (2000) found that CAR and CA induce GST and protect cell line IMR-32 against hydrogen peroxide promoted oxidative stress.

Slamenˇova` and others (2002) studied the protective effects of rosemary extracts against oxidative DNA damage, which was caused by H₂O₂ and visible light excited methylene blue in colon cancer cells CaCo-2 and hamster lung cells V79. Their finding showed that an ethanolic solution of rosemary extract reduced the genotoxicity of H₂O₂ and methylene blue after a 2 h (short term) and 24 h (long term) treatments at 0.3 µg/ml and 30 µg/ml concentrations, respectively. Sotelo-Felix and others (2002) found that CAR prevented the CCl₄-induced acute liver injury by blocking the free radicals that was generated during CCl₄ metabolism.
CA and CAR are potent inhibitors of DNA adduct formation induced by procarcinogens AFB₁. There are at least two mechanisms involved in the anticarcinogenic activity of CA and CAR: (i) inhibition of the metabolic activation of procarcinogens catalyzed by the phase I cytochrome P450 enzyme 1A2 (CYP1A2) and Cytochrome P450 enzyme 3A4 (CYP3A4); (ii) induction of the detoxification pathways catalyzed by the phase II enzymes such as GST (Offord and others 1997). Offord and others (1995) studied the effect of rosemary extract (mainly consisted of CA and CAR) on AFB₁-induced genotoxicity in human liver epithelial cells (T.142, T.3A4) and human bronchial (B.1A2, B.3 A4) cells. They tested the ability of rosemary extract to inhibit DNA-adduct formation in the above described cell lines. For the study, DNA adduct formation was analyzed by the incorporation of tritiated AFB₁ metabolites (3H-AFB₁) into DNA. The amount of DNA adducts, which was formed in CYP1A2-expressing cell lines B.142 and T.142 was 2.4 ± 1.5 and 10.7 ± 3.0, respectively (Offord and others 1995). For the CYP3A4-expressing cell lines B.3A4 and T. 3 A4, DNA adduct formation was 0.3 ± 0.1 and 1.8 ± 0.5 p mol/mg, respectively. These cell lines when treated with AFB₁ in the presence of rosemary extract (5 µg/ml), the amount of DNA adduct formation was greatly inhibited. Inhibition rate was 85% and 30% in B.142 and T.142, respectively; 67% and 48% in B.3A4 and T.3A 4, respectively after treatment (Offord and others 1995). This result suggested that rosemary extract strongly inhibit metabolic activation of AFB₁, leading to reduction of their genotoxic effects. Moreover, the induction of phase II enzymes such as GST provides an additional protective effect (Offord and others 1995).

Sancheti and Goyal (2006a) investigated the anticancer activity of rosemary extracts on two-stage skin carcinogenesis, which was induced by a single topical application of DMBA and promoted by treatment of croton oil for 15 weeks in Swiss albino mice. Rosemary extract, by
oral administration at a dose of 1000 mg/kg b. wt / day at pre, peri and post-initiaational phases reduced the tumor incidence (50 %, 41.7 %, and 58.3 %, respectively) in comparison to the control (100%). Rosemary treated animals also showed reduction in number of papillomas, tumor yield and tumor burden. Sancheti and others (2006b) also studied the antitumor activity of rosemary on DMBA initiated and croton oil promoted mouse skin tumorigensis. The anticancer activity was monitored on the basis of the average latency period, tumor incidence, tumor burden, tumor yield, tumor weight and diameter as well lipid peroxidation and gluthione level. Rosemary extracts treated mouse group showed prolongation in the latency period of tumor occurrences, decreased in tumor incidence, tumor burden and tumors yield. Lower tumor diameter was also observed in the rosemary treated mouse group. The level of lipid peroxidation was significantly reduced in the blood serum and liver and depletion in the gluthione were recorded in rosemary extract (500 mg / kg body wt) administered animal groups (Sancheti and others 2006b).

Differentiation therapy is an alternative or complementary approach to standard cytotoxic drug therapy of cancer, which targets inhibiting the growth of malignant cell by inducing normalization of cellular phenotype (Sharabanil and others 2006). 1A, 25-dihydroxyvitamin D3 (1,25-D3) is a powerful differentiation agent, and Acute myeloid leukemia (AML) cells have been found to be remarkably sensitive to 1,25-D3 and its analogs (Sharabanil and others 2006). They found in previous studies that CA markedly enhances 1,25-D3-induced differentiation and inhibits proliferation of HL60 and U937 myeloblastic cells (Kirstein and others 1996; Chandler and others 1997; Hidalgo and Eckhardt 2001; Yanf and others 2003). They have translated findings from previous study to the in vivo syngeneric murine myeloid leukemia model, using WEHI-3B D- murine myelomonocytic leukemia cell lines known to differentiate into
monocyte/macrophage lineage in response to 1,25-D₃. In this particular study, they have found out both CA and CA-rich ethanolic extract of rosemary greatly potentiated the in vivo differentiating and antiproliferative effects of 1,25-D₃ and its low calcemic analog, 1, 25-dihydroxy-16-ene-5, 6-trans-cholecalciferol (Ro25-4020) in WEHI-3B D- cells (Sharabanil and others 2006). Furthermore, they observed that rosemary extract alone had antitumorigenic activity, and in combination with a low dose of Ro25-4020 resulted in a strong cooperative inhibition of WEHI-3B D- tumor growth without toxicity. These results suggest that CA rich rosemary extract together with low calcemic vitamin D₃ analog can be used as effective and low-toxic differentiation therapy for acute myeloid leukemia (Sharabanil and others 2006).

Metastasis is a characteristic of high malignant cancers (Liabakk and others 1996). Excessive extra cellular matrix (ECM) degradation is one of the distinctive features of tumor invasion and metastasis. Matrix metalloproteinase (MMPs) a ECM-degrading enzyme, are over expressed in a variety of malignant tumor types and their overexpression is associated with tumor aggressiveness and metastatic potential (Danilenko and others 2001). In the invasion of tumor cells, different types of MMPs are involved. MMP-1 has been observed in lung carcinomas and colorectal tumors. MMP-2 and MMP-9 found in malignant cancers, like melanoma and fibrosarcoma (Wang and others 2005; Steiner and others 2001) are abundantly expressed in various malignant tumors and are postulated to play a critical role in tumor invasion and angiogenesis (Danilenko and others 2003). CAR restricted the invasive ability of B 16/F10 mouse melanoma cells in vitro by reducing MMP-9 expression.

It has been found in studies that CAR induces apoptotic cell death in high-risk pre-B acute lymphoblastic leukemia (ALL) (Zunino and Storms 2009). Dorrie and others (2001) investigated the cytotoxicity of CAR against several pro-B and pre-B acute lymphoblast
leukemia lines. In all acute lymphoblastic leukemia cell lines tested, CAR induced apoptotic cell death distinguished by loss of nuclear DNA, externalization of cell membrane phosphatidylserine, and depolarization of mitochondrial membranes. James and others (2006) studied the antiproliferative activity of CAR and CA on Caco-2-cells. The effect of CAR and CA on growth of Caco-2 cells was measured by 3H- thymidine Incorporation method. Both CAR and CA inhibited 3H- thymidine incorporation in a concentration-dependent manner, with a 50% inhibitory concentration of 23 µM for both CAR and CA (James and others 2006). Yesil-Celiktas and others (2010) investigated the cytotoxicity of Rosmarinus officinalis leaves and active compounds such as CA against various human cancer lines such as NCI-H82 (human small cell lung, carcinoma), DU-145 (human prostate, carcinoma), Hep-3B (human liver, carcinoma, hepatocellular), K-562 (human chronic myeloid leukemia), MCF-7 (human breast, adenocarcinoma), PC-3 (human, prostate, adenocarcinoma) and MDA-MB-231 (human breast, adenocarcinoma) by MTT assay method. The extracts exhibited various cytotoxic effects against different cell lines, with IC<sub>50</sub> (i.e. concentration of drug which caused 50% inhibition of cell proliferation) values ranging between 12.5 and 47.55 µg/ml. CA also caused the lowest cell viability for above mentioned cell lines with values ranging from 13 to 30% at a concentration of 19 µM after 47 h of treatment (Yesil-Celiktas and others 2010). Crude ethanolic rosemary extract and its constituent’s CA and CAR have shown antiproliferative activity on human leukemia (HL-60 and K-562) and breast carcinoma (MCF7 and MDA-MB-468) cells (Cheung and Tal 2007). Aoyagi and others (2006) found the cytotoxic activities of CA and CAR against P388 murine leukemia cell lines. Therefore, modification to CA may provide additional benefit.

Mitomycin C (MMC) is an anticancer drug, which has been used for treatment of a variety of cancers in combination chemotherapy since 1974 (Paz 2008). This drug is considered
the prototype bioreductive drug: it is inert toward nucleophile in its original structure but during cascade of reactions initiated by reduction of the quinone ring, it is converted to an extremely reactive bis-electrophile (Figure 4.2 intermediate 3a that contains two electrophilic position—at C1 and C10) (Hoey and others 1988; Tomasz and Palom 1997; Palom and others 2002). This electrophile is able to alkylate biological nucleophiles, particularly DNA, to form four monoadducts and two cross-links. The formation of an interstrand DNA-DNA cross-link is consistently considered the cause of its cytotoxic effects (Tomaz and Palom 1997). Several reductases can attribute the cellular activation of MMC. In recent reports, it has been reported that GRP58 (a protein containing a dithiol active site in its thiodoxin (Trx)-like domains) caused the reductive activation of MMC in several cancer cells (Adikesavan and Jaiswal 2007; Celli and Jiasmail 2003; Su and others 2006).

Figure 4.2. Mechanism for the Reductive Activation of Mitomycin a. Mechanism for the Reductive Activation of Mitomycins a. aNu1H and Nu2H Represents two Nucelophilic Compounds. (Adapted from Paz 2010).
Paz (2010) studied that the reactions of MMC with simple dithiols [1,3-propanedithiol, D, L-dithiothreitol (DTT), dihydrolipoic acid] as a model for proteins, which contained a dithiol active site. They found that these dithiols could activate MMC by reduction (Paz and others 2009) and metabolites, which are formed by reductively activated-MMC, react further with additional dithiol to form cross-links by binding covalently the two sulfur atoms of dithiol (Figure 4.3) (Paz 2010). MMC also has been shown to alkylate other cellular nucelophiles in addition to DNA, such as glutathione (GSH) (Sharma and others 1994; Sharma and Tomaz 1994).

![Chemical structure](image)

**Figure 4.3.** Mechanism for the Reductive Activation of Mitomycin C (MMC) by Dithiols and Subsequent Formation of a Dithiol Cross Link. (Adapted from Paz 2010).

### 4.2. Objective

CA and CAR have anticancer activities against various cancer cell lines. Derivatives of CA (ME, MA, BE, BA, PEMeO and PE) were synthesized in which all compounds are novel except the PEMeO (Figure 4.4). The synthesis of these derivatives can be found in chapter 2. Thus, the objective of this research was to evaluate the cytotoxicity of these semisynthetic derivatives along with CA and CAR against leukemia cell lines and determine the relationship between cytotoxicity and structure difference.
Figure 4.4. Different SemiSynthetic Derivatives of Carnosic Acid.

4.3. Materials and Methods

4.3.1. Materials

K-562 Chronic myelogenous leukemia, and P388D1 murine leukemia cell lines were obtained from ATCC (American Type Culture Collection). CCRF-CEM acute lymphoblastic leukemia cell line was kindly provided by Core Biology Facility at North Dakota State University. Isocove’s Modified Dulbecco’s Medium (IMDM) for K-562 cell lines was also obtained from ATCC. Hyclone RPMI-1640 and Dulbecco’s Modified Eagle (DMEM) cell culture mediums for CCRF-CEM and P388D1 respectively were obtained from Thermo scientific. 5% penicillin/Streptomycin, 10% Fetal Bovine Serum, ultra pure DMSO, 96-microwell plate, (flat-bottomed, polystyrene treated) and Mitomycin C were obtained from Thermo scientific. CA and CAR were obtained from Chromadex. Other test compounds such as CA, CAR, ME, MA, BE, BA, PEMeO and PE were synthesized in our laboratory (Chapter 2). MTT Assay Kit were purchased from Promega.
4.3.2. Cell culture procedure

Frozen cells stored in cool cell controller prior to using them for cell cultivation. For cell
cultivation, 9 ml of IMDM, Hyclone RPMI-1640, and DMEM cell culture mediums were added
in K-562, CCRF-CEM, and P388D1 cell lines, respectively. These cells lines were then
centrifuged for 5 min at 1500 rpm at room temperature. The upper layer was then discarded and
each cell pellet then resuspended into 1 ml of their respective media. Each cell line was placed
into T-25 flask and 9 ml of cell culture media added. T-25 flasks were then kept in humidified
atmosphere of 95 % air and 5 % CO₂ at 37 °C for 2 days. Cell growth was then checked under
the microscope after 2 days. Cell lines containing culture media were then split into 1: 3 (3.3 ml
of cell containing media, and 6.7 ml of media) and 1:5 (2 ml of cells, and 8 ml of media) ratios
and kept under the above humidified atmosphere condition. Each cell line was maintained in
their respective culture medium by splitting them thrice in a week into the above mentioned ratio
and storing in humidified atmosphere.

4.3.3. Cytotoxic assay

Cell suspension (150 µl), which consisted of approximately 5000 cells, were added to
each well of a 96-microwell plate and incubated for 24 hrs in humidified atmosphere of 95% air
and 5% CO₂ at 37 °C prior to cell treatment. Mitomycin C was used as a positive control for all
three cell lines. Test compounds and mitomycin C were dissolved in dimethyl sulfoxide (DMSO)
in concentrations of 5, 10, 100, 250, 500, 1000, 2000, 4000, and 5000 µg/ml and added to each
well. The final concentration of DMSO in each well was 1% (v/v). A negative control was also
prepared in the same way as test compounds, but it did not contain test compound, it contained
1% DMSO (v/v) concentration in each well. For background absorbance, cell free well contained
150 µl of media, and test compound. The suspension was again incubated in same humidified
atmosphere condition for 48 hrs. After 48 hrs, 22.5 µl of MTT solution or dye solution were added to each well and again incubated at 37 °C for 4 hrs in a humidified atmosphere. After incubation, 150 µl of solublization solution /stop mix was added to each well and then incubated overnight under the described humidified conditions. The following day, the samples were mix for 10 min on a shaker. The plate was placed on a microplate reader (thermo scientific multiskan spectrophotometer) at 550 nm. For each cell line, all samples were prepared in sextet. IC$_{50}$ was calculated for each test compound for CCRF-CEM, P388D1 and K-562 cell lines. IC$_{50}$ value of compounds against each cancer line was defined as the concentration (µg/ml) that caused 50% inhibition of cell proliferation.

4.4. Statistical Analysis

The IC$_{50}$ data obtained in this study are expressed as mean ± SD. The data were analyzed by one way ANOVA test followed by Tukey’s multiple comparisons to determine the significant difference between test compound mean. A p-value < 0.0001 was considered significant between compound means.

4.5. Results and Discussion

CA and its semisynthetic derivative and CAR were assayed for their cytotoxic activities on P388D1 murine leukemia cells, K562 human myelogenous leukemia cells and CCRF-CEM human acute lymphocytic leukemia cells. The results are shown in Table 4.1.

CA, CAR, ME, MA, BA, and PE and mitomycin C showed cytotoxic activities on all three-cell lines. For all the compounds tested, CAR had better cytotoxicity as compare to CA for all three cell lines. The IC$_{50}$ or the concentration at which CA caused 50% inhibition of cell proliferation was 5.8, 5.7 and 2.5 µg/ml for CCRF-CEM, P388D1 and K562 cell lines, respectively (Table 4.1). Whereas, the IC$_{50}$ of CAR for CCRF-CEM, P388D1 and K562 cell lines
Table 4.1. Inhibition of Tumor Cell Proliferation by Carnosic Acid (CA), Carnosic Acid (CA) Derivatives, and Carnosol (CAR).

<table>
<thead>
<tr>
<th>Test Compound*</th>
<th>CCRF-CEM (IC₅₀ in µg/ml)</th>
<th>P388D1</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>5.8 ± 0.4</td>
<td>5.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>CAR</td>
<td>1.1 ± 0.4</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>ME</td>
<td>7.2 ± 0.7</td>
<td>2.6 ± 0.1</td>
<td>9.5 ± 0.8</td>
</tr>
<tr>
<td>MA</td>
<td>3.3 ± 0.3</td>
<td>2.5 ± 0.2</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>BE</td>
<td>22 ± 1.8</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>BA</td>
<td>2.4 ± 0.1</td>
<td>8.8 ± 0.1</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>PEMeO</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>PE</td>
<td>2.2 ± 0.1</td>
<td>46 ± 3.5</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.03 ± 0.0002</td>
<td>0.05 ± 0.0005</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>

* See figure 4.3 for compound identification; **NA = no activity

were 1.1, 2.2 and 2.0 µg/ml, respectively (Table 4.1). Compound ME, where both phenolic hydroxy groups were methylated and C-20 position were esterified as the methyl ester had lower cytotoxicity as compare to MA, where both hydroxy groups were methylated and C-20 position is in the form of an alcohol (Figure 4.4). Compound ME had IC₅₀ values of 7.2, 2.6 and 9.5 µg/ml for CCRF-CEM, P388D1 and K562 cell lines, respectively, whereas compound MA had IC₅₀ of 3.3, 2.6 and 2.5 µg/ml against CCRF-CEM, P388D1 and K562 cell lines, respectively. A similar trend was observed for compound BE, i.e. both hydroxy groups are benzylated and C-20 position is a benzyl ester where a reduced cytotoxicity as compare to BA, where both phenolic hydroxy groups are benzylated and C-20 position has an alcohol group was observed. Compound BE had an IC₅₀ value of 22 µg/ml against CCRF-CEM cell lines but did not have any activity against P388D1 and K562 cell lines (Table 4.1), whereas compound BA had IC₅₀ values of 2.4, 8.8 and 3.5 µg/ml against CCRF-CEM, P388D1 and K562 cell lines, respectively. Furthermore, compound PEMeO, i.e. phenolic hydroxy groups were benzylated and C-20 position had appended a long fatty acid group, did not have any activity against all three cell lines. Compound PE, which posses free phenolic hydroxy groups and C-20 position is tethered to a
long chain fatty acid group had cytotoxicity with IC$_{50}$ values of 2.2, 46, and 5.7 µg/ml against CCRF-CEM, P388D1 and K-562 cell lines, respectively. PE had better cytotoxicity (IC$_{50}$ of 2.2 µg/ml) as compare to CA (IC$_{50}$ of 5.8 µg/ml) against CCRF-CEM cell lines (compare CA and PE). However, compound PE had reduced cytotoxicity with IC$_{50}$ values of 46 and 5.7 µg/ml against P388D1 and K-562 cell lines, respectively as compare to CA, which had IC$_{50}$ values of 5.7 and 2.5 µg/ml against the same cell lines, respectively. Interestingly, ME, having both phenolic hydroxyl groups methylated and a methyl ester group at the C-20 position group had better cytotoxicity as compared to BE, where both phenolic hydroxy groups were benzylated and C-20 position had a benzyl ester group (compare ME and BE).

Cytotoxicity against all three lines was observed in compounds (i.e. CA, CAR, PE) with free phenolic groups. Whereas, compound PEMeO (with protected hydroxyl group) did not have cytotoxicity against any of the cell lines tested. It has been reported that organic molecules bearing a quinone structure exhibit various toxicities against living organisms due to the quinone’s characteristic reactivity (Masuda and others 2002). Bolton and others (2000) pointed out that the quinone acts as a Michael acceptor to alkylate important cellular protein and DNA and hence they are toxic to cells. In vitro studies using CAR have shown apoptotic cell death in leukemia cell lines (Zunino and Storms 2009; Dorrie and others 2001). Our observations are in line with the known literature regarding significance of phenolic/quinone moiety in the active molecule. In vitro studies have also shown that CAR can interfere with cell metatasis, chemotaxis and attachment (Zunino and Storms 2009). James and others (2006) found CA and CAR to have cytotoxicity activities against in vitro Caco-cell by inducing cell cycle arrest at G2/M phase. Steiner and others (2001) found CA had cytotoxic activity in HL-60 cells by transient arrest in G0/G1 phase.
The possible explanation of CAR showing better cytotoxicity than CA can be due to structural difference between CAR and CA. In case of CA, it has a carboxylic acid group moiety at C-20 position, whereas CAR bares lactone ring at C-20 position. Cytotoxicity of organic compound appeared in the cells after their intake from the environment (Sugawara and others 1994). Generally, a charged substance, especially a negatively charged does not permeate into cell via the lipophilic cell membrane (Sugawara and others 1994). CA has a carboxylic acid group at C-20 position that is negatively charged whereas the lactone ring at C-20 position in CAR is not. Masuda and others (2002) have reported better anticancer activity of CAR than CA in studies against K-562 and P388D1 cell lines, respectively.

Existing literature precedence discussed earlier stressed the importance of the phenolic/quinone core in the structure of CAR or CA as an important structural motif responsible of anticancer activity of these compounds. However, during the course of this study, compounds with masked phenolic hydroxy groups had activity comparable to CA (compare entries MA and BA with entry CA). In these compounds, the presence of a primary hydroxy group at the C-20 position was found to be an essential requirement as replacing this hydroxy group to an ester group resulted in drastic decrease in activity (comparing ME and BE with entries MA and BA).

4.6. Conclusion

Compounds with hydroxyl groups had cytotoxicity against all three lines CCRF-CEM, P388D1 and K-562. Among compounds tested, CAR was found to be the most potent anticancer agent in the above mentioned cell lines. Derivatives MA, BE, BE, BA, PEMeO and PE have been synthesized and their anticancer activity were tested against leukemia cell lines for first time. Observation indicates structure depended activities where hydroxy group at C-20 position was critical for activity of these compounds, this key finding has not been previously reported.
This study represents the first step towards under studying how these compounds act as potential anticancer agents. However, further studies to investigate the mode of action of these compounds should to be done.

4.7. References


European Food Safety Authority (EFSA). 2008. Scientific opinion of the panel on food additives, flavourings, processing, aids and materials in contact with food on a request from the commission on the use of rosemary extracts as a food additives. The EFSA Journal 721: 1-29.


5.1. Introduction

There is great interest in natural sources of antimicrobial agents. Some herbs, e.g. rosemary, have been known through traditional food uses to have antimicrobial activity against a wide spectrum of microorganisms. Various studies have documented the antimicrobial activity of rosemary. Rosemary extract consists of phenolic diterpenes such as carnosic acid (CA), carnosol (CAR), epirosmanol, and isorosmanol, and phenolic acids such as ferulic, caffeic, rosmarinic, and chlorogenic acids (Figure 5.1) (Chen and others 1996; Cuvelier and others 1996). Of these phenolic diterpenes, CA and CAR are present in highest concentrations in rosemary and are believed to be responsible for antimicrobial activity (Moreno and others 2006). The phenolic compounds generally have antimicrobial activity by weakening or destroying the permeability of cell membranes (Juven and others 1997; Vaara 1992; Taguri and others 2004).

Figure 5.1. Phenolic Diterpenes and Phenolic Acid in Rosemary Extracts.

Perez-Fons and others (2010) studied the mechanisms of action for antibacterial activity of CA using Staphylococcus aeurus (S. aeurus). They used membrane potential sensitive cyanine
dye DiS-C3(5) to evaluate the cell membrane permeabilization effect by CA. This dye, which is a fluorescent dye and is a caged cation, can distribute between cells and medium depending on the cytoplasmic membrane potential (Perez-Fons and others 2010). When this dye is inside the cell, it becomes concentrated and self quenches its own fluorescence. The fluorescence of this dye was monitored with an excitation wavelength of 610 nm and an emission wavelength of 670 nm. A blank sample was also prepared with only cells and the dye to subtract the effect of background absorbance. Two positive controls, i.e. carbonyl cyanide-m-chlorophenylhydrazone (CCCP), which functions via a proton motive force, and polymycin B, has the ability to decrease or increase the membrane potential, were used for this experiment. The time for fluorescence intensities to decrease was similar to that of the inhibitor CCCP (Figure 5.2) when 32 µg/ml CA was used in the assay while Polyimyxyn B produced an increase in the fluorescence. CCCP is a uncoupling agent that increases the proton permeability, and disrupts the electron transport chain preventing the formation of ATP, discharging the pH gradient, and destroying the membrane potential (Perez-Fons and others 2010).

Rosemary extract can be used for the topical treatment of skin disorder like acne vulgaris and seborrhoic eczema. Weckesser and others (2007) tested antimicrobial activity of a Rosmarinus officianlis CO2 extract and its CA against dermatological relevant aerobic and anaerobic bacteria using agar dilution method. Rosemary extract and CA had antimicrobial activity against a panel of aerobic and anaerobic bacteria (Tables 5.1 and 5.2).
Figure 5.2. Time Changes of the Fluorescence Intensities of Dis-C$_3$- (5) Upon Addition of Carnosic acid to *S. aureus* cells: 32 µg/ml (▲), 16 µg/ml (△), Cells Treated with CCCP (■), Cells Treated with Polyimyxyn B (□) and Untreated Cells (●). (Adapted from Moreno and Others 2012).
<table>
<thead>
<tr>
<th>Aerobic Bacteria</th>
<th>CO₂ Extract</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
</tr>
<tr>
<td><em>Streptococcus auerus</em> (ATCC 25923)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus auerus</em> Pen res. (E 12431/98)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus auerus</em>, MRSA (RV5/98)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus epidermidis</em> (DSM 1798)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus lugdunensis</em> (NG 3406/94)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> gr. A (E 12449/98)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em> gr. B (DSM 2134)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Enterococcus facealis</em> (ATCC 19212)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (ATCC 25922)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Morganella morganii</em> (E 10679/93)</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> (ATCC 27853)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> (E 83282/92)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> (ATCC 6633)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> (DSM 348)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

NI, no inhibition; NE not evaluated. *Adapted from Weckesser and others (2007).*

**Table 5.1. Antimicrobial Activity of *Rosmary officinalis* CO₂ Extract and Carnosic Acid (CA) against Aerobic Bacteria.**
Table 5.2. Antimicrobial Activity of *Rosmary officinalis* CO$_2$-Extract and Carnosic Acid (CA) against Anaerobic Bacteria.

<table>
<thead>
<tr>
<th>Anaerobic Bacteria</th>
<th>CO$_2$ Extract</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> (ATCC 13124)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> (FR 024/12-10)</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em> (ATCC 25586)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Bacteroides vulgatus</em> (ATCC 29327)</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td><em>Prevotella intermedia</em> (NCTC 9336)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em> (W 83)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Peptococcus magnus</em> (D 385-8/94)</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

*Adapted from Weckesser and others (2007).*

Bernardes and others (2010) studied the antimicrobial activity of rosemary extract, against microorganisms responsible for initiating dental caries. The rosemary extracts, i.e. crude EtOH/H$_2$O extracts from steam and leaves, CA and CAR had antimicrobial activity against *Streptococcus salivarius, Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus mitis*, *Streptococcus sanguinis*, and *Enterococcus faecalis* (*E. faecalis*) with MIC concentrations of 400, 30, and 35 µg/ml; 350, 50, and 35 µg/ml; 400, 15, and 35 µg/ml; 350, 30, and 75 µg/ml; 400, 40, and 50 µg/ml and 400, 70 and 100 µg/ml, respectively. Po-Jung and others (2007) studied the effect of rosemary extracts on *S. sobrinus* growth. The antibacterial activities of rosemary extracts were determined by microdilution broth method. The MIC of aqueous and methanolic rosemary extracts against *S. sobrinus* were 16 and 4 mg/ml, respectively.

Klančnik and others (2009) examined the antimicrobial activity of rosemary extract, and CA against gram positive (*Bacillus* and *Staphylococcus*) and gram negative (*Campylobacter* and...
Salmonella) bacteria, with different methods such as disk diffusion, agar dilution, and broth microdilution. All above test methods demonstrated the sensitivity of gram positive and gram negative bacteria toward rosemary extract formulations and CA. Rožmani and Jeršek (2009) studied the antimicrobial activity of rosemary extracts, containing 22.04% and 40.49% CA against different strains of Listeria monocytogenes (L. monocytogenes) species. Both the extracts had good antimicrobial activity against several strain of Listeria.

Rižnar and others (2006) studied the antimicrobial effects of commercially available rosemary VivOX 20 (CA, 20% w/w) and VivOX 4 (CAR, 4% w/w) extracts. Rosemary extracts were mixed into frankfurters prior to smoking and storage at 4 or 12°C. VivOX 20 and VivOX 4 formulated frankfurter sample had significantly lower aerobic plate counts (APC) then of control samples at both storage temperatures (Rižnar and others 2006).

Fernandez-Lopez and others (2005) also determined the antibacterial effect of rosemary extracts such as rosemary oil extract (OR), rosemary water extract (WR) and combination of rosemary oil and water extract (OWR) against the several food spoliation bacteria such as Listeria innocua 4202, L. monocytogenes 5105, Lactobacillus sake 550, Leuconostoc mesenteroides 824, Leuconostoc mesenteroides subsp. Dextranicum 882, Leuconostoc carnosum 558, Lactobacillus curvatus 860, Brochothrix thermosphacta CRA 7883, Brochothrix thermosphacta CRA 7884, Brochothrixthermospacta CRA 3235, and Lactococcus lactis FMRD 492 using agar diffusion method. OR, WR and OWR all had antimicrobial activity against each above bacteria tested. OR, WR and OWR had the zones of inhibition of 20.5, 17.4 and 14.2 mm; 25, 19.5, and 15.4 mm; 21, 15.4, and 14.2 mm; 26.2, 23, and 14.3 mm; 28.1, 19.5, 23.8 mm; 26.4, 24, and 25.1 mm; and 21.8, 18.2, and 13 mm, respectively for the above mentioned bacteria, respectively (Fernandez-Lopez and others 2005).
Uyttendaele and others (2003) measured the antimicrobial activity of ground rosemary along with other herbs and spices on *Shigella sonnei* (*S. sonnei*) CIP 82.49, *Shigella flexneri* (*S. flexneri*) CIP 82.48 and *E. coli* LMG along with two clinical isolates, *S. flexneri* serotype 6 (strain6) and *S. sonnei* (strain1), using the minimum inhibitory concentrations (MIC) assay. Rosemary extract along with other herbs had antimicrobial activity with MIC ranging from 0.5% to 1% (wt/ vol) depending on the *Shigella* strain used.

Commercial rosemary extract also called Oxy’less, which is used as an antioxidant in foods was dissolved in ethanol (100 mg/ml), and the solution was tested against gram-positive food borne microorganisms. Rosemary extract had antimicrobial activity with the MIC of 1% for *Leuconostoc mesenteroides*, 0.5% for *L. monocytogenes*, 0.55 for *S. auerus*, 0.13% for *streptococcus mutans*, and 0.06% for *Bacillus cereus* (*B. cereus*). CA and CAR phenolic diterpenes in rosemary extract were mainly responsible for antimicrobial activities (Campo and others 2000).

Weerakkody and others (2011a) studied the antimicrobial activity of two spice and herb extract combinations from galangal, rosemary, and lemon iron bark on inoculated *L. monocytogenes* and *S. auerus* and naturally present spoilage microflora on cooked ready-to-eat vacumm packaged shrimp stored for 16 days at 4 or 8°C. However, spice and herb extracts did not have inhibitory activity against *S. auerus* and *L. monocytogenes* in inoculated shrimps. A combination of galangal, rosemary, and lemon iron bark greatly reduced the levels of aerobic bacteria and lactic acid bacteria on day 12 at 4 °C by 1.6 and 1.59 log CFU/g, respectively. However, bacteria levels were equivalent to those of controls by day 16 (Weerakkody and others 2011b). Similarly, a combination of galangal and rosemary extracts significantly reduced levels of aerobic bacteria and lactic acid bacteria at 8 °C on day 8 by 2.82 and 2.61 log CFU/g,
respectively. Again, bacteria levels were equivalent to those of controls by days 12 and 16, respectively.

Both rosemary and CA inhibited *Listeria* at very low concentration of rosemary extract and CA, with MIC at 0.083 mg/ml and 0.166 mg/ml, respectively (Bubonja-Sonje and others 2011). Klančnik and others (2009) reported a strong antimicrobial activity of rosemary extracts, which contain CA as the main phenolic components, against gram positive bacteria *B. cerus* and *S. aeurus*. Synthetic CAR and CA had potent antibacterial activities against *Propionibacterium acnes* and *S. aureus* (Tada and others 2010).

Moreno and others (2006) studied the antimicrobial activity of methanolic extract of rosemary that contained 30% CA and 16% of CAR against gram positive bacteria such as *S. aureus* ATCC 25922, *Bacillus megaterium* PV447, *Bacillus subtilis* GSY1604, *E. faecalis* ATCC 29212 and gram negative bacteria such as *E. coli* XL1Blue, *Xanthomonas campestris pv campestris*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Methanol extracts had antimicrobial activity against gram positive bacteria with MIC ranging between 2 and 15 µg/ml and against gram negative bacteria with MIC ranging between 2 and 60 µg/ml.

Research indicates that rosemary and its predominant phenolic compounds, i.e. CA and CAR, have antimicrobial activity. It also indicates that gram positive bacteria are more susceptible than gram negative bacteria. Modification of CA structure may improve antimicrobial activity against gram negative microorganisms.

### 5.2. Objective

Semi-synthetic derivatives of CA such as ME, MA, BE, BA, and PE (chapter 2) were synthesized. The objective of this research was to evaluate the antimicrobial activities of these semisynthetic derivatives, along with CA and CAR, against gram positive and gram negative
bacteria. In addition, the effect of masking the free hydroxyl group of CA, with methoxy or benzyloxy groups and altering the C-20 position, with other groups such as ester or alcohol (i.e ME, MA, BE, BA, PE (Figure 5.3), on antimicrobial activity were investigated.

![Chemical structures](image)

Figure 5.3. Different Semisynthetic Derivatives of Carnosic Acid.

**5.3. Materials and Methods**

**5.3.1. Bacterial strains and growth conditions**

Four bacterial strains, namely *Salmonella enterica* 14028, *E.coli* O157:H7 ATCC 43894, *S. auerus* 29213, and *B. cereus* ATCC 10987 were used for antibacterial testing. *E. coli* O157:H7 ATCC 43894, *Salmonella enterica* 14028 and *S. auerus* 29213 strains were kindly provided by the Department of Veterinary and Microbiological Sciences at North Dakota State University. *B. cereus* ATCC10987 was purchased by ATCC (American Type Culture Collection (ATCC). The cultivation/ assay medium for all four strains was tryptic soy broth (TSB) or agar (TSA), which were obtained from BD. CA and CAR was purchased from Chromadex, (Santa Ana, CA). Other test compounds, i.e. semisynthetic derivatives of CA, were synthesized (Chapter 2). The antimicrobial activity of CA, CAR and other test compounds against above mentioned bacterial strains were done using broth microdilution method. Bactiter Glo reagent used, obtained
(Promega Madison, WI) was used for antimicrobial testings. Sterile 96-microwell plate and ultra pure DMSO were purchased from Thermoscientific (Waltham, MA).

Bacterial cultures for antimicrobial testing were prepared by isolating colonies from TSA slants and suspended in TSB medium (10 mL). Cultures were grown aerobically for 20-24 h under continuous shaking at 37 °C prior to use in the micro dilution assay.

5.3.2. Microdilution method

CA, CAR and other test compounds were prepared in ultra pure DMSO in different concentrations ranging from 200 µg/mL to 28, 000 µg/mL. For the broth micro dilution test, bacterial suspension containing 5x10⁴ CFU/100 μl was added to the each well of a sterile 96-microwell plate already containing test compound from above mentioned concentration range. Positive control wells were prepared by adding 5x10⁴ CFU/100 μl bacterial suspension to each well of a sterile 96-microwell plate already containing DMSO without test compound. Negative control (for background luminescence) consisted of 100 μl of growth media and test compound per well. The final concentration of DMSO was 4% v/v in each well of test compound, positive control and negative control samples. The content of each well were mixed on a microplate shaker (Cole Parmer, USA) for 1 min prior to incubation for 24 h at 37 °C. The MIC was the lowest concentration where no metabolic activity was observed after 24 h on the basis of the absence of a bioluminescence signal measured with a Microplate Reader after adding 100 μl per well of Bactiter Glo reagent and 5 min of incubation in the dark, as described in manufacturer’s instructions. All measurement of MIC value was completed in sextet.

5.4. Results and Discussion

Antimicrobial activities of CA, CAR, ME, MA, BE, BA, and PE was examined against the gram positive bacteria, *S. aeurus*, and *B. cereus* and gram negative bacteria, *Salmonella*
*enterica* and *E.coli* O157:H7. CA and CAR had antibacterial activities against the above mentioned bacteria (Table 5.3). Compounds ME, MA, BE, BA, and PE were inactive against tested bacteria (Table 5.3).

Table 5.3. MIC of Carnosic Acid (CA), Carnosic Acid (CA) Derivatives, and Carnosol (CAR) against *Salmonella enterica*, *E. coli* O157:H7, *S. auerus* and *B. cereus* in the Microdilution Method.

<table>
<thead>
<tr>
<th>Test Compound*</th>
<th><em>Salmonella enterica</em> 14028</th>
<th><em>E. coli</em> O157:H7 ATCC 43894</th>
<th>Staph. auerus 29213</th>
<th><em>B. cereus</em> ATCC 10987</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>360 ± 0</td>
<td>800 ± 0</td>
<td>40 ± 0</td>
<td>40 ± 0</td>
</tr>
<tr>
<td>CAR</td>
<td>280 ± 0</td>
<td>800 ± 0</td>
<td>100 ± 0</td>
<td>160 ± 0</td>
</tr>
<tr>
<td>ME</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>MA</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>BE</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>BA</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
<tr>
<td>PE</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
<td>NA**</td>
</tr>
</tbody>
</table>

*See figure 5.3 for compound structure; ** NA = No activity

CA and CAR both have phenolic groups in their structure. Fogg and Lodge (1945) indicated that phenolic compounds inactivate the cellular enzymes and hence produce antimicrobial action. In later studies, Judis (1963) suggested that phenolic compounds inhibit the microbial growth by weakening or destroying the permeability of cell membranes. Juven and others (1972) also demonstrated that phenolic compounds cause membrane permeability changes. According to Bemheim (1972), the changes in the permeability of cell membrane may be due to the interaction of phenols with phospholipids components of membrane. Compounds ME, MA, BE, and BA did not have phenolic groups, their hydroxyl position was occupied by another group (i.e., methoxy or benzyloxy group).

Furthermore, gram positive strains were more sensitive to CA and CAR than gram negative strains. For example, MIC of CA were 40 and 40 µg/mL for *S. auerus* and *B. cereus*.
respectively, while MIC of CAR were 100 and 160 µg/mL, respectively, for the same bacteria. Whereas, MIC of CA for gram negative strains _Salmonella enterica_ and _E. coli_ O157:H7 were 360 and 800 µg/ml, respectively, and MIC of CAR for the same bacteria were 280 and 800 µg/mL, respectively. Gram negative bacteria have outer membranes, that surrounds the cell wall, which restricts diffusion of antimicrobials through its lipopolysaccharides membrane (Wen and others 2003; Farbood and others 1976). Therefore, the restricted diffusion may be a reason for weaker antimicrobial activity of CA and CAR against gram negative bacteria. In contrast, gram positive bacteria have a single membrane, which allow for easy permeation of antimicrobials (Collins and Charles 1987). Several authors have reported that gram positive bacteria were more susceptible then gram negative bacteria to antimicrobial compounds including CA and CAR and rosemary extracts (Shelf and others 1980; Smith-Palmer and others 1998; Nychas and Tassou 2000; Rožmani and Jeržek 2009).

5.5. Conclusion

CA and CAR both had antimicrobial activity against both gram positive (_S. auerus_ and _B. cereus_) and gram negative bacteria (_Salmonella, E. coli_ O157:H7). Gram positive bacteria were more sensitive then gram negative bacteria. Compounds ME, MA, BE, and BA, in which both ortho hydroxy groups were replaced with other groups, did not have antimicrobial activity. Compounds in which the carboxylic acid group at C-20 position was replaced with other groups, including PE, were inactive against all tested bacterial strains. This indicates that antimicrobial activity may also vary depending on the functional group present at C-20 position. The antimicrobial assay method chosen for our research was a broth microdilution method where no bioluminescence signal indicated that there is no presence of live bacterial cell. It may be worthwhile to use other antimicrobial assay method to evaluate antimicrobial activity of CA,
CAR, ME, MA, BE, BA and PE. Literature sometime reports MIC as the minimum concentration required to reduce 50% of the bacterial population. Therefore, other methods might produce different results than the ones observed here.

5.6. References


CHAPTER 6. CONCLUSIONS

Three routes were developed to synthesize PE. The first two routes did not work, but a small number of derivatives were produced. The third route, which consisted of a four steps synthetic route, was found suitable for the synthesis of PE and executed successfully with high yield. The initial ring opening/rearrangement of CAR was achieved with reagent other than the methyl iodide and represented a significant finding in the synthesis. Another, highlight of the third route was that the alkene hydrogenation and deprotection of the benzyl ester was accomplished in one step with high yield. The PE was synthesized for the first time and it also opened the door to synthesize other derivatives than PE.

In the antioxidant activity study, the result indicated that oil and emulsion which contained PE had lower hydroperoxide and hexanal formation compared to the control, which indicated that this derivative had antioxidant activity. The PE had improved the antioxidant activity compared to CA in emulsified corn oil but was less active in bulk corn.

In the anticancer study, CA and its derivatives PE, MA, BA, and ME were found to have cytotoxicity against all three lines CCRF-CEM, P388D1 and K-562. Other derivatives of CA such as BE was found to have cytotoxicity only against CCEF-CEM cell lines. Among compounds tested, CAR was the most potent anticancer against the above mentioned cell lines. The study also indicated structure depended activities for the compounds with a hydroxy group at C-20 position. This was reported for the first time.

Antimicrobial study was achieved using microdilution method. CA and CAR was able to antimicrobial activity against S. aeurus, B. cereus, Salmonella enterica, and E. coli O157:H7. Other compounds, which had presence of hydroxyl groups, were not able to exhibit antimicrobial activity. PE contained hydroxyl groups, was not able to exhibit antimicrobial activity. This
indicates that antimicrobial activity could also vary on type of the functional group, which is present at C-20 position. Other methods can be used to study the antimicrobial activity of these derivatives.

Overall, increase in lipophilicity of CA improved antioxidant activity in emulsion as compare to CA. Cytotoxicity study indicated structure depended activities for the compounds with a hydroxy group at C-20 position. Antimicrobial activity can also depend on presence of functional group at C-20 position.
CHAPTER 7. FUTURE RESEARCH

Antioxidant activity of PE was evaluated for first time, so the antioxidant mechanisms of PE could be studied in the future to understand and improve its antioxidant activity. Further, PE can be used in other food system and its antioxidant study can be studied in the future. Furthermore, PE should be also tested for its stability at different temperature so that it can be effectively applied in different type of food system accordingly. The other fatty acid esters should be considered in future research as the length of the ester may produce different results. This is founded on the observation that methyl carnosate had improved activity in some test systems and that the palmitic may be too large to improve the activity (especially the anticancer and antimicrobial). Mode of action of derivatives, which exhibited anticancer activity, can be studied to better understand their cytotoxicity.
Figure A1. $^1$H Spectrum of Compound 2.16.
Figure A2. $^{13}$C Spectrum of Compound 2.16.

Figure A3. $^1$H Spectrum of Compound 2.21.
Figure A4. $^1$H Spectrum of Compound 2.22.

Figure A5. $^{13}$C Spectrum of Compound 2.22.
Figure A6. $^1$H Spectrum of Compound 2.23.

Figure A7. $^{13}$C Spectrum of Compound 2.23.
Figure A8. $^1$H Spectrum of Compound 2.25.

Figure A9. $^{13}$C Spectrum of Compound 2.25.
Figure A10. $^1$H Spectrum of Compound 2.26a.

Figure A11. $^{13}$C Spectrum of Compound 2.26a.
Figure A12. $^1$H Spectrum of Compound **2.26b**.

Figure A13. $^1$H Spectrum of Compound **2.26c**.
Figure A14. $^1$H Spectrum of Compound 2.26d.

Figure A15. $^1$H Spectrum of Compound 2.27.
Figure A16. $^{13}$C Spectrum of Compound 2.27.

Figure A17. $^1$H Spectrum of Compound 2.28.
Figure A18. $^{13}$C Spectrum of Compound 2.28.

Figure A19. $^1$H Spectrum of Compound 2.29.
Figure A20. $^{13}$C Spectrum of Compound 2.29.

Figure A21. $^1$H Spectrum of Compound 2.24.
Figure A22. $^{13}$C Spectrum of Compound 2.24.