# IMPROVING BIOAVAILABILITY AND BIOEFFICACY OF CARNOSIC ACID USING LECITHIN-BASED NANOEMULSION SYSTEM

by

HUIJUAN ZHENG

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Professor Qingrong Huang

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

OCTOBER, 2018

ABSTRACT OF THE DISSERTATION

IMPROVING BIOAVAILABILITY AND BIOEFFICACY OF CARNOSIC ACID USING LECITHIN-BASED NANOEMULSION SYSTEM

By HUIJUAN ZHENG

Dissertation Director:

Professor Qingrong Huang

Carnosic acid (CA) is a phenolic terpenoid mainly existed in rosemary, sage and other Labiate herbs. It possesses powerful antioxidant, anti-inflammatory and other health-promoting activities for treating degenerative and chronic diseases. The low solubility and dissolution in aqueous environment of carnosic acid posed a challenge for its application in functional food and accessibility for intestine absorption. The target of my research is to enhance the bioavailability and evaluate bioactivities of carnosic acid by encapsulated in a lecithin based nanoemulsion system.

The first part of the research is mainly about the investigation of the properties of carnosic acid and the development of the carnosic acid nanoemulsion (CA-NE). The CA-NE was formulated by medium chain triglycerides as oil phase, lecithin as the emulsifier and prepared by a two-step high speed-high pressure homogenization method. The produced nanoemulsion possess good stability under various pH and low ionic strength

conditions. Storage under different temperatures showed good stability of formulated carnosic acid nanoemulsion (CA-NE).

The second part of current research is focusing on the bioaccessibility and bioavailability evaluation by in vitro/in vivo models. The pH-stat lipolysis results revealed the nano-emulsified CA was much more bioaccessible than the unformulated CA with 2.8-fold improvement by nanoemulsion system compared with the MCT oil suspension and more than 12-fold compared with the water suspension based on the in vitro lipolysis study. The human gastrointestinal tract (TIM-1) model which simulated the physiological conditions of human upper GI tract revealed that the nanoemulsion greatly improved the oral bioaccessibility of CA by 5.8-fold and indicated a better bioavailability. The pharmacokinetics (PK) study using rats as model animal further showed that the oral bioavailability of carnosic acid enhanced almost 2.2-fold when encapsulated in the nanoemulsion compared with the unformulated CA suspension. Being the first thorough study on nanoemulsion formulation for carnosic acid, by applying two complementary simulation models and animal study, the present research elucidated the reason why nanoemulsion encapsulation would influence the bioavailability of carnosic acid and nanoemulsion proved to be a useful method for improving the oral bioavailability of CA.

The third part of my research evaluated the bioactivities of carnosic acid after encapsulation by nanoemulsion system. The antioxidant activity of CA after encapsulation by nanoemulsion decreased from the result obtained by the cellular antioxidant assay (CAA) using HepG2 cells, which mainly attributed to the sustained release and longer endocytosis process as proved by the cellular uptake by confocal laser scanning microscopy. The efficacy on the inhibition on inflammation was conducted using lipopolysaccharide-

stimulated RAW 264.7 macrophage cells. Results showed significantly enhanced anti-inflammatory ability of CA-NE with inhibiting the pro-inflammatory cytokines NO and TNF-α production ($p< 0.05$). The antiproliferative efficacy of carnosic acid nanoemulsion (CA-NE) on various carcinoma cells originated from different tissues or organs was evaluated and discussed by MTT assay. Results showed the inhibition was significantly improved by the use of the emulsion delivery system. We also evaluated how the nanoemulsion affected the anti-bacterial activities of four common food pathogens of carnosic acid, which showed the encapsulation would not affect the activity of carnosic acid as anti-bacterial agent. This result indicated a potential application of the nanoemulsion in food and beverages acting as food preservatives.

The findings in the current research suggested encapsulation in lecithin based nanoemulsion delivery system opens new possibilities for the successful application of carnosic acid in functional food formulations with increased bioavailability and bioefficacies. This study also indicates the successful design and potential application of the lecithin-based nanoemulsion formulation to other hydrophobic nutraceuticals or drugs.

## ACKNOWLEDGEMENT

The Ph.D. training is a journey really bringing me so much to memorize. It is just like riding a roller coaster, with so many exciting moments, laughing with tears, up and downs. Now, upon almost finishing this part of adventure, looking back to the past six years, my gratitude gives towards all those who have accompanied and supported me during this wonderful adventure. I now would like to take this chance specially acknowledge a number of them who helped me so much.

Firstly, I want to give my sincere thanks to my advisor, Dr. Qingrong Huang, for his guidance and great support to my research and life in Rutgers University. Dr. Huang is a person with great patience and excellent academic thoughts. I have learned tremendously from him from his knowledge in chemistry, nanotechnology and other areas. Every time when I had obstacles in the research, he was always there to discuss with me with great patience and offers me experimental support and ideas to get rid of these difficulties. It's Dr. Huang's positive attitude towards life and research inspired me so much to explore the wonderful of doing research and enjoy the fulfillment that academic study brought. I was truly so lucky and honored to have Dr. Huang being my most important guide during this six-year PhD adventure.

Then, I want to say thanks to my committee members, Dr. Chi-Tang Ho, Dr. Qingli Wu and Dr. Shiming Li for their precious and valuable advices and help on my research. I am so grateful and lucky that Dr. Ho has been my course teacher on Food Chemistry and Flavor chemistry and provided me with several recommendations letters. It is his expertise, open minding and strict requirement on research has broaden my vision on being a scientist. I have learned and enlightened so much with Dr. Wu's great knowledge on plant extracts

and analytical chemistry. Dr. Li has very strong background on chemistry and the analysis of nutraceuticals; he is always so nice to talk with me about the details of my research and helped a lot being a teacher and friend. I also want to give my acknowledgement to Dr. Chikindas for offering me the resource and advice on the antimicrobial study. I would like specially thank Dr. Yong Cao from South China Agriculture University, for offering all the conditions on the pharmacokinetic study.

Then, I really would like to say thank you to all my lab mates and friends. It is great pleasure to work with you people and it is you people makes the life in lab so interesting even with so many difficulties. I will always remember the joys and interesting moments with you. Thanks were given to Konglong Feng for the in vivo pharmacokinetic study, Ammar Algburi for the antimicrobial study and Hongwei Zhang, Qianru Liu, Ting Zheng on the TIM-1 study. Thanks Peiyi Shen, Liang Wen, Jie Xiao, Li Zhang, Muwen Lu, Jun Li, Yunjiao Chen, Chunxin Xia and many others for the joys and help you bring to my life.

I would like to express my deepest thanks to my family. I always feel how lucky to have my parents for their unconditional love. I want to thank my elder sister and brother for accompany and trust on me. I would like to particularly, thank my boyfriend Haixiang Han, for the love, support, encouragement, time he has given to me. He is always by my side no matter joys and difficulties. Together with him, I always feel unfearful no matter what difficulties we may encounter in life. Words is not enough to express my thanks to my families.

Lastly, I sincerely give my thanks for the financial support from Chinese Scholarship Council making my study and research possible here.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF ILLUSTRATIONS

# CHAPTER 1. BACKGROUND INTRODUCTION

## 1.1. Functional food and nutraceutical

### 1.1.1. Functional food

During the past two decades, consumer began to view food in a new perspective, besides providing energy and nutrition, the health promoting and disease-preventing, especially chronic disease-preventing properties from food have attracted much attention. Such that, the idea of this concept of nutraceutical and functional food has evolved from consumer demand and studies on the relations between nutrition and health. [1]

Even though, there is no universal definition, the most basic principle of functional food can be regarded as the foods that possess health benefits additional to their nutritional functions.[2] This makes functional food bridged the link of pharma and nutrition. Not only health-aware consumers, but also food industries and research scientists are increasingly seeking the new and valuable functional foods.

The idea of obtaining health-promoting function from food is not new at all, however, it is the scientific research nowadays provide the support and promote the understanding of how food can be beneficial beyond their nutrition needs.[3] The earliest functional foods mainly focused on fortifying food with different vitamins or some food insufficient mineral molecules and so on.[4] Then, the scope was getting wider and the focuses have changed to fortified with all kinds of micronutrients such as DHA, EPA, phytosterol, and soluble fibers with health promoting or diseases preventing activities. Now, the consumers and industries are gradually focusing on functional food that providing multiple health benefits within one single product, which should naturally derived, contains different bioactives, with

minimum processing and can be instantly accessed with various health benefits such as anticancer, anti-inflammation and so on.[5] In addition to the importance of the health effects, sensory properties such as taste, texture, and flavor remain crucial factors for consumers.

1.1.2. Functional drinks

Functional foods can be found virtually in all food categories, such as functional meat, cereals, prebiotics, probiotics and functional drinks. Functional drink is one of the most important categories within functional food, it usually uses for non-alcoholic beverages fortified with various nutraceuticals, such as vitamin E, C, A, calcium, lutein and other bioactive nutrients such as beta-carotene.[6-7] Beverages is one of the most active category because the convenience for daily consuming, easily to be distributed and bright potential to encapsulate different nutraceuticals.[8]

Functional beverages can be further divided into sports and energy beverages, vegetable and fruit beverages and nutraceutical drinks. As we know, milk has long been regarded as the only drinks containing all the essential nutrients for human. However, more and more researches also started focus on other nutrients based on different plants source such as vegetables, fruits and soybeans have been launched with some containing probiotic actives. This is also because some problems with dairy products, such as lactose intolerance and insulin-like growth factor (IGF-I)-contamination problems. Even though, dairy beverages, such as milk, yogurt, fermented milk are still very good choices for probiotic carrier beverages.[9-10]

As a useful method, many researches used nanoemulsion systems as the carrier for many health-promoting bioactives such as beta-carotene, curcumin, lycopene, with aiming to apply in functional beverages.[11-14] In my current work, carnosic acid was firstly

encapsulated as the nutraceutical in nanoemulsion systems and hopefully in future it can be incorporated in the designed functional beverage emulsion systems.

### 1.1.3. Nutraceuticals

Dr. Stephen DeFelice introduced the term “Nutraceutical” in 1979 from Nutrition and Pharmaceutical, both very important aspects to physiological health. Similar to functional food, until now, there is no regulatory definition for nutraceuticals, even though it is extensively applied in academic field as well as in food industry. DeFelice proposed the definition of nutraceutical could be, “a food (or part of a food) that offers medical or health benefits to prevent and treat of a disease”.[15] This kind of compounds are expected to enhance human well-being and prevent chronic diseases.

Nutraceuticals are those compounds aiming to prevent or treat of illness on a long-term physiological effect modes rather than immediate relief of symptoms compared with modern medicine. The list of nutraceuticals is very long, such as dietary fibers, various probiotics and prebiotics, fatty acids, bioactive peptides, minerals and secondary plant metabolites, such as polyphenols, carotenoids, , terpenes and alkaloids, etc., the list is still expanding because more new bioactives are found and extracted from different origins.[16] Even though structures and biological mechanisms within different nutraceuticals are different and the mechanisms of acting their bio-functions are varying, numerous researches had proved that many nutraceuticals can be functioned in the cell signaling and gene expression or regulation and then perform chronic disease-preventing activity like anti-obesity, anti-diabetes, anti-cancer and inflammation inhibition functions, prevent cardiovascular and cognitive disease.

## 1.2. Bioavailability of Nutraceuticals

Food Drug and Administration (FDA) defines bioavailability as "the rate and amount of an administrated compound that can get to the system becomes accessed to the location that being function". The overall bioavailability is affected consecutively by three factors, including bioaccessibility of the compounds for intestinal absorption, transportation through the gut wall for absorption, and finally the fraction that reaches the system circulation without metabolized or degraded.[17-18]

In many newly launched functional foods, the bioavailability study of the bioactives within these supplements are mainly focused on the hydrophilic bioactives because more complex for the situation of lipid compounds behaved in the small intestine. However, studying the bioavailability of these hydrophobic compounds is very important and meaningful.[19]

### 1.2.1. Solubilization

In order to become accessible for absorption by the enterocyte of gastrointestinal (GI) tract, the compound must be firstly solubilized or molecular dispersed within the aqueous intestinal fluids. For hydrophobic compounds, which cannot be solubilized in the intestinal fluid, the absorption can be greatly hindered by its dissolution rate. So one effective method to improve the solubility would be using lipid-based formulations, in this case, the solubility will be the portion of the bioactives that is within the colloidal particles in digestion tract liquid formed both by the exogenous lipids and stimulated endogenous compounds such as bile salts and phospholipids.[20] Such that, to what extent these colloidal systems can incorporate the nutraceutical become critical for its bioaccessibility. Lipids in the food can also contribute to the form of these colloidal carriers after hydrolysis by lipase.

Many researches have shown lipid-based formulations are capable of significantly enhance the absorption of low water-soluble molecules following oral delivery.[21]

1.2.2. Absorption

When talking bioavailability, the most significant part we should understand is the process of digestion, especially for the nutraceuticals which are supposed to be taken orally, as oral ingestion is the a easy and favorable way for nutrients taken in daily life or for nutraceuticals taken for chronic disease.

Digestion is a process that the digestion system breaks down ingested stuff into smaller molecules that is easier to be uptake by the gastrointestinal tract and either finally get into the bloodstream and systematic circulation or excreted out from the body after a food was taken orally. The digestive systems are composed mainly of small intestine and with other parts including mouth, pharynx, esophagus and large intestine.[22] As the biological membrane is lipophilic, such that lipophilic compounds could easily pass through it, while it is hard for hydrophilic compounds. In practical, if an interesting compound possess a lipophilic nature with low water solubility, the easier they can be interacted with the phosphatidylcholine membrane. In the pharmaceutical area, a higher logP of one compound usually means higher enterocyte transport ability.[23] Absorption is also affected by the transport route of the compound, such as paracellular, transcellular, the tight junction, active transporter and efflux transporter will also affect the absorption as well.

1.2.3. Transformation

Once again, many nutraceuticals may transform into inactive forms within the gastrointestinal tract by either chemical degradation or chemical and enzymatic

metabolism leaded functionality alteration. The chemical degradation usually including hydrolysis, reduction, and oxidation and so on.[24] Along the route of nutraceuticals from digestion to systemic circulation, metabolism of nutraceuticals happens mainly in two sites: the intestine in the digestive tract and after delivered by the portal vein to the hepatic metabolism in the liver. Two stages of metabolism are related to this process. In many situations, metabolism is one of the most crucial reasons result in the low bioavailability of a lot nutraceuticals. One example is curcumin may degraded and metabolized in the GI tract environment and finally produced all kinds of different compounds with various characteristics.[25]

For nutraceuticals that is not absorbed or uptake by our upper GI tract may be further utilized through metabolism and biotransformation by the gut microbiota in large intestine before absorption.[26] The final metabolites maybe get into the systemic transfer and circulation to perform the health benefits. However, those are not considered as the bioavailable of the original compounds.

In summary, due to various influence factors, nutraceuticals may have a poor bioavailability that cause the low concentrations to fulfill therapeutic functionalities.

1.2.4. Approaches to enhance the bioavailability

Within the analysis of the factors influence the final bioavailability, three ways can be applied to increase, including increase the solubility, improve the absorption and reduce the transformation including degradation and metabolism.

1.3.1.1. Increase aqueous solubility.

As discussed above, the solubility of hydrophobic compounds are low in gastrointestinal lumen, In order to be effectively absorbed by the intestinal enterocytes, the hydrophobic nutraceuticals need to be dissolved in micelle or mixed micelle. With the aim to enhance the solubility of these compounds, as food scientists, researchers apply delivery formulations, which are capable to improve solubility or dispersion of such nutraceuticals with safe ingredients. The delivery systems are expected to increase the amounts that could getting into the target action site within the biological systems. In the field of food science, some food ingredient based formulation are used to increase dissolution rate. (1) lipid-based delivery systems, like emulsions, SLN, self-emulsifying drug delivery system and so on. (2) Liposomes, (3) Polysaccharide and protein based nanoparticles, (4) Suspensions and dispersions.

In emulsion system, lipophilic bioactives are in solubilized state within the inner lipid phase. Once digested, these bioactives can be incorporated at molecularly dissolved state in the core of micelles.

1.3.1.2. Improvement of absorption

Many reasons would have influence on the procedure of compounds entering the epithelium cells linings of intestine as previously discussed. The mucus layer is the first barrier, especially for large molecules and those can be interacted with the mucin fibers and other mucus molecules. Secondly, the ability of a nutraceutical can transport through the phospholipid bilayer is another major factor influence the absorption, this is mainly dependent on the structures determined polarity of the compounds. Another way to enter the cells is through the tight junction; some molecules are very small or can increase the dimensions of it and travel through.[27] Active transporter within the cell also affect the

uptake process however this mainly relies on the nutraceutical concentrations. Another mechanism affect is the efflux transport.

So if any absorption enhancer function to help travel across the mucus layer, increase the gaps between adjacent epitheliums, or inhibit the activities of efflux protein transporters will help to improve the absorption.

1.3.1.3. Avoidance or minimizing transformation and metabolism

The metabolism and transformation occurred in the gastrointestinal tract as well as after absorbed by the intestine will affect the bioavailability a lot. Formulations may act as a protective vehicle to decrease the interaction of the nutraceuticals with other ingredients in the GI tract, however, they may not be able to manipulate too much about metabolism after absorbed. Only a few methods are known to inhibit or avoid metabolism: for example, long-chain triglycerides may help the lipophilic compounds bypass the liver metabolism by entering lymphatic circulation.[28] Other methods mainly relies on adding of enzyme inhibitor together with the compound. However, this may cause unexpected toxic effects and need to be carefully evaluated.

1.2.5. In vitro models for bioaccessibility evaluation

To study the bioavailability of the ingested nutraceutical is important for the appropriate oral dosage design and adequate concentrations reaching the targeted site. In order to get the accurate and complete information of the bioavailability of the ingested nutraceuticals, in vivo animal or human studies is more precise to be applied. However, due to the more costly and safety and ethical consideration of the in vivo and clinical studies, various in vitro Bioaccessibility methods have been developed. These in vitro methods are not only cost-effective, but can also help researchers to understand the mechanism through step by

step examine of digestion, absorption and metabolism in GI tract and act perfectly as a screening procedure prior to the in vivo studies.[29]

Usually, the systemic concentration of intravenous applied nutraceuticals or drugs is defined as 100% bioavailability. As aforementioned, the bioavailability of nutraceuticals administrated through other routes such as oral delivery can be influenced by various factors in the environments, just like pH, ionic strength, enzymes of the GI tract and metabolism during and after absorbed and transferred to the liver. Based on these factors, including the bioaccessibility in GI tract (B*), the absorption and transport efficiency (T*) and finally the survival of systemic metabolism (M*). The design of the in vitro instruments or machines and protocols in order to be closely related with the in vivo bioavailability of oral ingested nutraceuticals are usually focused on one or more of these factors.[30] Such that, these in vitro methods can be generally divided into single-factor and multi-factor models. Single-factor method usually focus on one region of the digestion tract and most important conditions, while multi-factor models usually includes several regions of the GI tract and incorporates more factors which is more complicated and maybe more correlated to the real situation.[31]

For the single-step/factor method, the pH-stat model is one of the mostly applied methods for assessment the bioaccessibility of lipid-based formulations for lipophilic compounds.

1.2.5.1 pH-stat in vitro lipolysis

Studies has shown that lipid based formulations can greatly enhance the bioaccessibility of hydrophobic compounds by incorporation of these compounds in mixed micelles and vesicles after digestion.[32-33] Thus this in vitro pH-stat model is a simulation of the environment of small intestine situation, as this is the most important region of lipids type compounds to be digested and absorbed. This model is very useful as a method to assess the hydrophobic molecules taking together with lipid-based formulations by oral route. The pH, ionic strengths and the enzymes are all controlled in this method and the mechanism, analysis and information we could obtained from this method was shown in **Figure 1**. Usually the experiment of this model can be conducted under two different situations, one is the fast-state simulated small intestinal fluid (FaSSIF) situation and another one is the fed-state small intestinal fluid (FeSSIF) composition, which mimic the digestion difference between the formulation is given either before or after a meal. No matter the different conditions, the basic simulated intestinal fluid contains the same components with different amounts, including pancreatin with lipase and other enzymes, bile salts, phospholipids and other salts formed buffer solution.

It is worth note that this model is a simulation of the small intestine only, which does not include the gastric part or accurately simulate different parts of small intestine, never the less this model can be combined with other methods that mimic the stomach or mouth to give a complementary profile of the digestion. However, these methods often lack the simulation of the physical movement of GI tract such as the peristalsis and transit time change and hard to give the systematic information on the digestion and absorption at different sites of small intestine in a consecutive manner. It was said that the methods like pH-stat method usually overestimates the oral bio efficacy.[34] Even though, this single

step/factor model is still very useful and applied by many researchers for the initial selection of the delivery systems and screening of the dosing amount as a cost-effective, simple to setup and interpret, fast and high throughput method.[35]

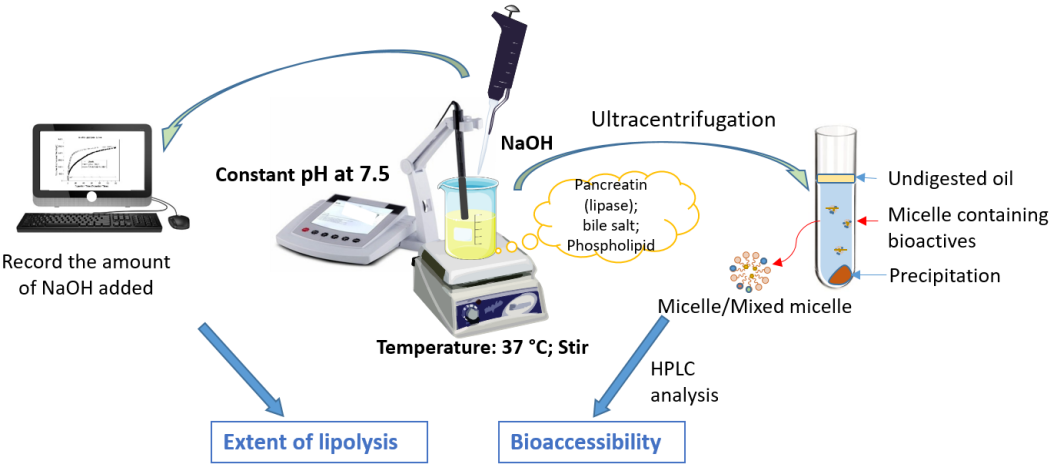



**Figure 1.** Schematic of pH-stat lipolysis for lipid digestion and bioaccessibility calculation of lipophilic nutraceuticals evaluation

1.2.5.2 TNO gastro-intestinal model (TIM)

TIM-1 model is by far the most precise and complicated in vitro model for simulation of the human digestion system and evaluation of the bioaccessibility of oral ingested food. TIM-1 (TNO, Zeist, Netherlands) is a computer-monitored, dynamic in vitro model for simulating the upper digestion systems of human. There are four compartments including stomach, duodenum, jejunum, and ileum in this model as shown in **Figure 2**. Moreover, two semipermeable fiber systems are attached at the end of jejunum and ileum, which are used to collect the bioaccessible portion of the ingested food at predetermined time. In the system, temperature, pH, enzyme, gastric emptying time, peristalsis, digestion fluid composition and secretion, transit time in each compartment are all precisely controlled by the computer program. The composition of digestive fluids in this model was described in

detail in previous published paper.[36] The secretion rate and amount of the digestive fluids and pH change in each section are simulated based on physiological data.[37] TIM-1 model stands for a consecutive multi-step in vitro digestion system that not only mimic the chemical, enzymatic degradation, metabolism but also the physiological movement. Another advantage of TIM-1 model is this design allows the systematic study on the digestion in different part of the small intestine and by collecting sample at different time period, the digestion profile can be established. What's more, the TIM-1 system also collect the samples of undigested part as in efflux, which stands for those part that could not be absorbed in small intestine and further entered into colon for degradation, biotransformation by gut microbiota and maybe reabsorbed by human. Such that, for food that has a higher efflux ratio are worth to further conducting the colon fermentation study. That is also why TIM-2 model, which mainly mimic the large intestine metabolism by gut microbes were also developed and worth to be applied. Besides this, the TIM-2 system is also useful for evaluating the functions of prebiotics on the microbes in colon and study the chronic disease preventing activity of those undigested and absorbed compounds such as resistant starch and so on.[38]

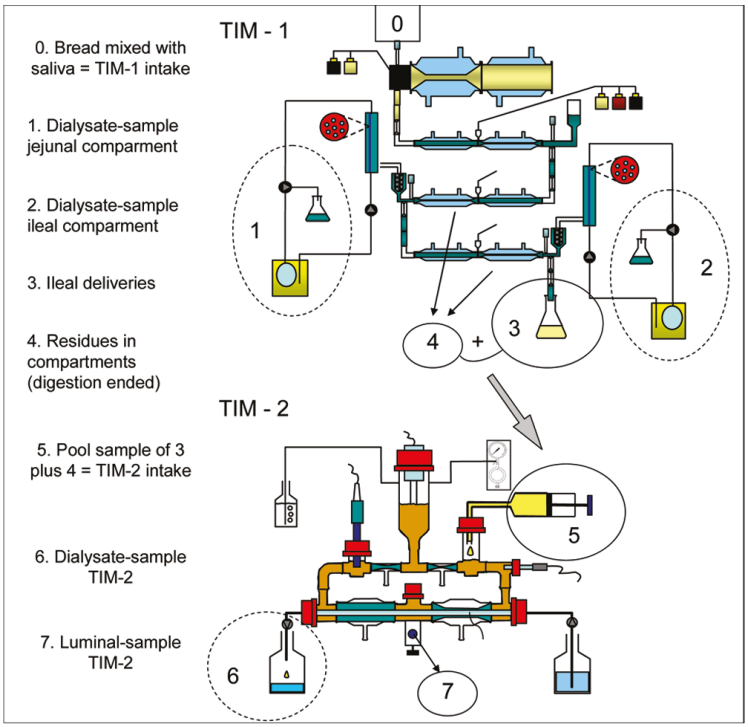



**Figure 2.** Schematic picture of the mechanism for upper GI lumen model (TIM-1) and colon model (TIM-2). (Reprinted from Ref. 36)

1.2.5.3. Transport or absorption study by Caco-2 cell models

As aforementioned, after solubilized by the colloidal systems composed of bile salts, phospholipid, digested monoglyceride and diglyceride, the nutraceuticals will then go across the mucous before getting to the small intestine epithelial cells, once reached the epithelia cells, there are generally two paths that the compounds will get through the cells, including paracellular and transcellular paths. Usually, the compounds with molecular weight less than 200 may pass through by the paracellular route and some compounds may help to increase the transport by opening the tight junction that between the enterocyte cells. Other compounds that are usually pass through the enterocyte by transcellular path, thus it might undergo metabolism in the cell and then exit from the cells. The transcellular

absorption including passive and active transport, depending on the hydrophobic/hydrophilic and molecular size properties with or without the help of transporter. Another way of the digested nutraceutical-containing micelle absorption is carrier-facilitated collisional mechanism or vesicular-mediated uptake.[20] Even after the uptake by the enterocyte, there is still a possibility that the compounds may be pumped out by the efflux transporters. Once the compound pass through the enterocytes, they will transfer to the liver for hepatic metabolism by portal vein or entering the lymphatic transportation to the systemic circulation. Finally get to the target site with adequate concentration to perform bio-function.

Given the complexity of the absorption process after digestion and dissolution, it is unlikely to apply in vitro static methods and materials to mimic the uptake process by the in vitro gastrointestinal chemical and physical models. In order to simulate and understand the transport and uptake of bioactives by this monolayer of epithelia cells lining in the intestinal walls, a cell-based models from differentiated Caco-2 cells was established as **Figure 3**. The Caco-2 cell was obtained from human colon carcinoma, after differentiation, it possesses the characteristic physiological functions of the human epithelial cells including the tight junction, transports and is effective to evaluate the transport and uptake by including the paracellular, transcellular, efflux activities. Many studies have been performed using this model to elucidate the absorption, metabolism rate and mechanism.[39-41] Even though, the Caco-2 cell model has all the necessary simulation of the chemical, enzymatic and biological activities occurred in the uptake, there are still some drawbacks of this model. Firstly, the successfully and consistency differentiation of the monolayer is not easy to monitored as location, passage of the cell, culture conditions will all contributed

to the differentiation process. Thus, the results is not easy to compare between labs and different batches. Secondly, as we have introduced, the mucus layer outside the epithelia cells is the first obstacle for the nutraceutical to get contact and absorbed into cells, however, Caco-2 cells is not mucus secreted.[42] Besides, there are other types of cell in the epithelia besides enterocytes. Given this situation, other cell lines, such as HT29, HT29-MTX intestinal epithelia cell model was also investigated by researchers. As an upgrade solution, co-culture of the Caco-2 and HT-29 was also developed.[43]

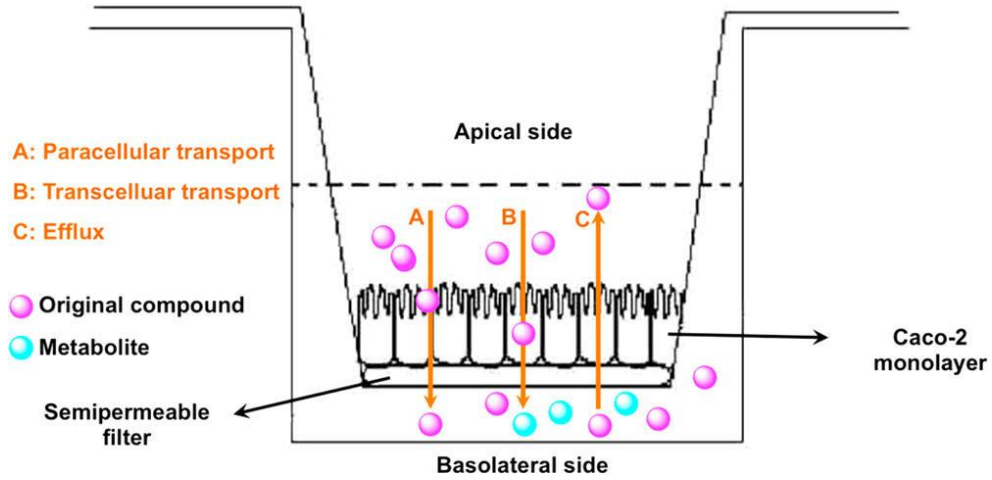



**Figure 3.** Transport mechanism study by Caco-2 monolayer. The Caco-2 monolayer mimic paracellular and transcellular routes into cells. (Reprinted from Ref. 29)

1.2.5.3 Correlations of in vitro and in vivo

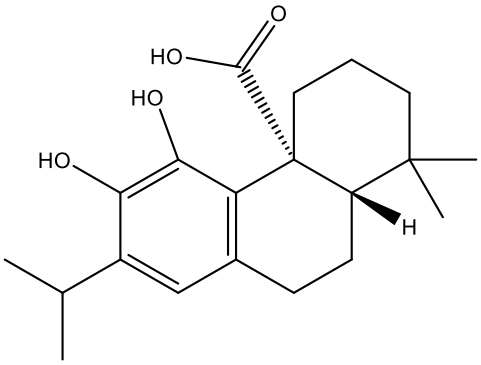
By the above introduced two simulation methods to test either the digestion and solubility by a machine based setup or the absorption models on a cell based model, there are still many steps hard to and impossible to be evaluated by in vitro simulations. These in vitro models can be used as a screening tool to compare and select formulations for the next step in vivo bioavailability study if indicating promising results from the in vitro

investigation. We talked about the metabolism after absorption, which accounts for the low bioavailability of a lot nutraceuticals. This can only be assessed by the in vivo methods.

Researchers have applied various models including both designed simulations models and animal or even human study to test the correlation of the in vitro and in vivo situations and using in vitro results for prediction of in vivo situations, we are very delightful to see a good correlation of many in vitro methods with the in vivo animal studies. However, as there are variations, strengths and limitations of these models exist, accurate correlation study between them is necessary. The most accurate bioavailability study is the clinical human trials; however, it is expensive and related to many ethical issues. Such that, the benefits of applying these in vitro methods to foresee the in vivo scenarios is vast and which makes it desirable to accurately study the in vitro and in vivo data correlations.

### 1.3. Carnosic acid and its challenges

Carnosic acid, a phenolic type diterpene that is typically obtained by extraction and purification of sage (Salvia officinalis L) and Rosemary (Rosmarinus officinalis L) leaves. Carnosic acid also existed in other herbs and plants within the close species of salvia. Carnosic acid has been proved possessing lots of benefits from food application to various disease inhibition, including antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory properties, such that it is used as an ingredient in food, nutritional supplements, and cosmetics.[44-46]




carnosic acid

**Figure 4.** Structure of carnosic acid

Sage and rosemary are common seen Labiatae herbs both used as spices and medicines world-widely especially in Mediterranean countries since old times and rosemary is the main form that people ingest carnosic acid in daily life. Among the plants proved to present antioxidant property, rosemary is among the most popular ones, besides using as flavor and spice in cooking, it is applied as nutritional supplements, antioxidant in foods, and cosmetics.[46-47] With the affirmation of the activity and under the request from industry, European Commission has classified rosemary extracts as food additives and assigned the number E392 to it. In the EU regulations, only deodorized rosemary extracts with CA and CAR as their major components are regarded as additives.

1.3.1. The molecular mechanism of bioactivity of CA

The antioxidant effect within carnosic acid was proposed related with the radical scavenging activity mechanism similar to other antioxidants, with the o-dihydroxylated phenolic ring and a carboxylic acid group, carnosic acid, also regarded as phenolic diterpene possess powerful antioxidant activity in food systems (catechol moiety).

Furthermore, the anticarcinogenic effects of carnosic acid has been suggested may be related to the capacity of modulating kinase activity, membrane proteins and/or receptors involved in signaling processes. In various cell models, the anticarcinogenic of carnosic acid might be related to its capacity to decrease membrane fluidity, as it has been reported that tumor cells usually contain more unsaturated phospholipids and less cholesterol compared to normal cells and therefore the membranes exhibit a higher fluidity. In addition, as we know, that the happening of cancer includes a series of sophisticated and complex procedures and the carnosic acid is believed to inhibit the secretion and express of several enzymes that are related to the COX-2 pathways, which is necessary for the cancer cell to migrate and grow.[48-49]

Carnosic acid exhibit high anti-inflammatory effect as proved in many studies, carnosic acid was found greatly inhibited the express of IL-1b and TNF-α and thoroughly hindered the expressing of COX-2. The suppression of iNOS and COX-2 expression because of the effect of inhibition in NF-κB signaling pathway was suggested involved in the carnosic acid inhibition on LPS-induced NO and TNF-α production.[50]

Mechanisms of the antimicrobial properties of phenolic diterpene like carnosic acid has not yet been well explained. Nevertheless, it was proposed that the hydrophobic nature of these bioactives helps them penetrate inside the bacterial phospholipid bilayer, in which hydrogen-bonding may occurred between the hydrogen-donor groups of carnosic acid with the membrane-phosphorylated groups of the cell membrane.[51] Another possible mechanism is that CA has the ability to react and enter into the hydrophobic part of the cell membrane to make changes to its charging situation, further influence the proteins related with the pump of expelling the internalized compounds out.[52] These studies indicated

carnosic acid could be applied as an antimicrobial agent in food area or as a therapeutic agent against drug-resistant bacteria.

1.3.2. Bioavailability of carnosic acid

Carnosic acid was reported as the most effective antioxidant in rosemary extracts. The antioxidant effect of carnosic acid in vitro is proved better than the commercial synthetic antioxidant BHT and BHA which is related to the power to react with the reactive oxygen species.[53]

Nevertheless, the scenarios in vivo is more complicated and the metabolism and final bioavailability in vivo of carnosic acid is so far barely know, there were very few studies on the bioavailability and less discussed the metabolism and relative amount deposited in different organs.[54-57]

Song et al [54] proposed and summarized the degradation and metabolism of carnosic acid. They found that the difference of the metabolism pathways and main products compared between in vitro and in vivo. Glucuronidation, oxidation and methylation are the pathways they found happened in vitro and in vivo with different priority. In vitro, the former two reactions occurred most, while in vivo, the first and third reaction takes the most important role. Overall, they found carnosic acid was not metabolically stable under all the tested situations. The following picture shows the position in the structure of carnosic acid that these metabolic happened.

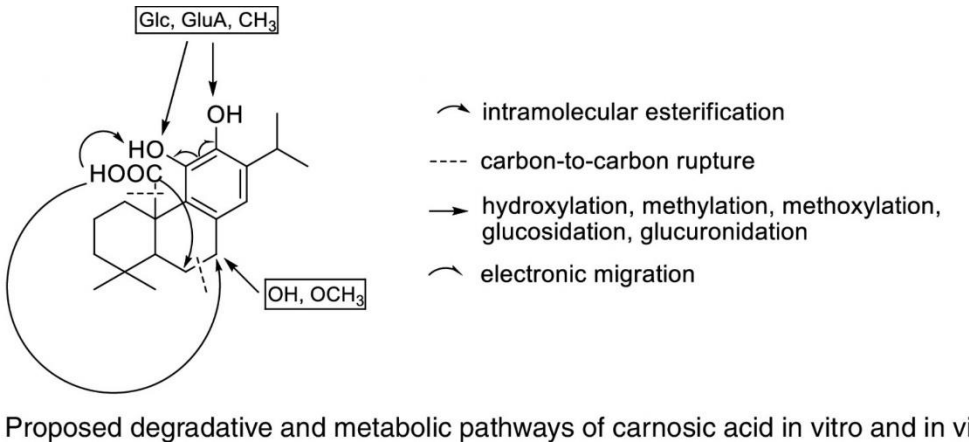



**Figure 5.** The proposed degradation and metabolism of carnosic acid in vitro and in vivo (Reprinted from Ref 54)

From the structure, it is obvious to know that carnosic acid is a highly hydrophobic compound thus having low water-solubility, poor bioaccessibility and proved unstable in alkaline environment.

Yan et al.[56] showed bioavailability around 65% of CA by using rats models. In this paper, information on the tissue distribution and excretion was not given and the degradation products was not detected.

Doolaege et al [55]applied two in vivo experiments, either by injection to the vein or by orally gavaged to the stomach (by dissolved CA in PEG400), carnosic acid was then studied in various tissues in experiment animals. Summarizing the data they obtained, they gave an overall in vivo results about the absorption, distribution, metabolism and elimination of carnosic acid. The intravenously absorbed accounts for one hundred percent absorption of carnosic acid. They can find carnosic acid existed in the blood after six hours and the relatively bioavailability calculated around 40 % and 15.6± 8.2% of the administrated carnosic acid was found in the feces. They also demonstrated that the uptake

of carnosic acid after oral ingestion is slow with the maximum time to reach highest concentration is 5.7 hours, indicated carnosic acid stays for quite a while in the circulation.

Based on the above analysis, we can see that firstly, there is difference between different studies, we are not sure whether the carnosic acid has a high bioavailability yet it is bioavailable. Secondly, in these oral studies, carnosic acid was gavage to rats with the help of other dispersed agent, such as PEG400, which may facility the solubility and protection during digestion, which resulted in a high bioavailability. Thirdly, it is still necessary to use a suitable delivery system to increase the solubility and dissolution in GI tract and be suitable applied in functional food systems and other areas by properly designed formulation. More importantly, it is worth to apply simulation methods to find out the mechanism of carnosic acid digestion before/after encapsulation.

1.3.3. Current formulations for carnosic acid

Currently, there is insufficient research about the incorporation solutions of CA in different carriers. Siva Ram Vaka et al[58] formulated a Chitosan-TPP nanoparticle formulation of carnosic acid and given to nasal mucus to regulate a nervous system molecule in the brain. The nano-formulation prepared by this method and materials strongly adhered and promptly anchored onto the mucus that has the opposite charge of chitosan. Their results showed that the nanoparticulate delivery system they formulated for intranasal given of CA might need fewer amounts of carnosic acid than pure carnosic acid to perform the necessary equivalent response, because the fabricated nanoparticle carrier could anchor onto the nasal mucus area and offer sustained release of CA for longer time in their formulation.

In another study, [59] the researchers used hydroxypropyl-beta-cyclodextrin for improving the solubilization in water phase of carnosic acid; however, HP-beta-CDdoes not have a GRAS status even though β-cyclodextrin is. Besides, it is mainly for pharmaceutical applications.

There is another study by Russo et al, [60] in which they made a CA microsphere to treat the basil microbial contamination problems. The microspheres consisted of 85% deacetylated chitosan, poloxamer P407 and carnosic acid, and was processed via a spray dry method. The yielded microspheres were 5-8μm in diameter and contained 17% carnosic acid. The antimicrobial activity of the CA microparticle was similar to the same concentration of CA in methanol exhibited. However, this formulation may not be best choice for functional beverage and nutraceutical aiming for the prevention of the chronic disease, either because the components or the size of the particles. It is acceptable that subcellular droplet size could increase the effectiveness for drug solubilization and increase their passive absorption by cells thus increasing the bioactivity.

In conclusion, there are not enough study on the delivery system development for carnosic acid, and most of the existed are for pharmaceutical use. So that a formulation with carnosic acid loading aimed to be applied as oral delivery of carnosic acid and may further be utilized in functional drinks are needed.

### 1.3.4. Approaches to improve the bioavailability of carnosic acid

Considering the potential health benefit of carnosic acid and its low bioavailability, design effective formulation may be one of the important approaches to achieve better bioavailability and thus health promoting benefit. Especially for its application in water-rich phase functional food.

Various carrier formulations for functional bioactives in food area that could be formulated totally by 'green' materials and components. The most common seen formulations are emulsion-based formulations, liposomes, nanoparticles, biopolymer gels, microspheres, and complex coacervates and conjugates.[61]

From a food scientist view, unlike in pharmaceutical area, any non-food-grade synthetic polymers and ingredients are better to be eliminated as carrier of nutraceuticals, with this in mind, there are not so many suitable materials for food applications. The following table list some components for nutraceutical encapsulation.[62]

**Table 1.** Materials used for bioactives incorporation in food industry (Ref 62)

| **Class** | **Examples** |
|---|---|
| Proteins | lk proteins (e.g., whey protein isolates, caseinates, individual caseins, micellar sein, bovine serum albumin, β-lactoglobulin, lactoferrin, α-lactalbumin)[a]<br>nt proteins (e.g. soy protein isolate, wheat protein, oat protein, zein)<br>Gelatins (e.g., fish gelatin, collagen) |
| Carbohydrates | Sugars (e.g. glucose, sucrose, lactose, trehalose, glucose syrups, honey, oligosaccharides)<br>Starch and starch derivatives (e.g., native starches, modified starches, resistant starches, maltodextrins)<br>Nonstarch polysaccharides (e.g., alginate, pectin, carrageenan, chitosan, plant materials)<br>Cyclodextrins (e.g., β-cyclodextrin, γ-cyclodextrin) |
| Lipids and waxes | Milk fat and milk fat fractions (e.g., olein and stearin)<br>Vegetable fats and oils (e.g., soy oil, canola oil, palm oil, sunflower oil, and fractions of these)<br>Waxes (e.g., carnauba wax, candelilla wax, beeswax)[a] |
| Surfactants | Synthetic surfactants (e.g., Tweens, Spans, polyglycerol polyricinoleate, sucrose esters)<br>Natural surfacatants (e.g., milk phospholipids, soy phospholipids, quilaja saponin)<br>Mono- and diglycerides (e.g., glycerol monosterate) |

A number of lipid-based formulations were fabricated that can enhance the bioaccessibility and bioavailability especially for hydrophobic nutraceuticals, which vary in their compositions and structures, such as simple oil solutions, self-emulsifying formulations, solid lipid nanoparticles (SLN), surfactant dispersions, microemulsions, nanoemulsions, conventional emulsions, micelles and Pickering emulsion as well. In food applications, emulsion-based carriers are relatively easy method for incorporating, protecting, and carrying bioactive compounds that are not easy to disperse in the aqueous phase.

## 1.4. Emulsion and Nanoemulsion for nutraceutical delivery

### 1.4.1. Emulsion introduction

Emulsion is a colloidal system composed of tiny droplet dispersed within another immiscible liquid by help of surface-active agent, which fabricated mainly with oil, water and surfactant. It is widely used in research and various industries. Usually we can classify emulsions into four main categories based on their particle size, thermodynamic stability and formation mechanism: regular emulsion, Pickering emulsion, nanoemulsion and microemulsion.

Regular emulsion usually has a size between 0.1-100μm, appears opaque, is thermodynamically unstable, and tend to aggregation, flocculation, coalescence and creaming during storage. Conventional emulsions are widely used in food industry as the low energy input for preparation.

Pickering emulsion is relatively rare used in food area even though it was discovered back to 1903 and later named after Dr. Pickering. The main difference of Pickering emulsion with conventional emulsion is it is not stabilized by a surfactant. Instead, it could be fabricated from well-designed small particle. Advantages of Pickering emulsion is firstly it's quite stable, besides it is surfactant-free, which can avoid the problems brought by surfactants and meet the need of clean and green label in food. Another attracting fact is it can be easily prepared by the standard emulsifying instrument. This would allow quick adaption into existing production. In food area, Pickering emulsion has been fabricated with different food grade materials such as proteins and polysaccharide to encapsulate different nutraceuticals.[63-65]

Microemulsion usually refers to the thermodynamically stable emulsions with size range smaller than 100nm with an optical transparent appearance under certain environmental conditions. Typically, it requires a higher surfactant-to-oil ratio to prepare microemulsion.[66]

Nanoemulsions is one kind of emulsion with narrow size distribution and low particle size, usually the size is below two hundred nanometers. The main difference of nanoemulsion with microemulsion is nanoemulsion has a higher free energy than the separated phase, while microemulsion has lower free energy, which allows microemulsion to be form spontaneously and thermodynamically stable. Submicron and nano-sized emulsions are becoming more and more important as a good choice of delivery vehicle for hydrophobic nutraceuticals and other active materials because quite a few advantages it possess.

First, nanoemulsion are kinetically stable as their size is uniform and usually below 200nm, during quite a long storage time usually no apparent coalescence and flocculation happen.[67] The small droplet size with large surface area could increase the effectiveness for drug solubilization, dissolution and increase the absorption by cells thus increasing the bioactivity.[68] Nanoemulsions are advantageous for drug targeting and controlled release, and the incorporation of different kinds of therapeutic agents and nutraceuticals.

Nanoemulsions could also increase the stability of encapsulated nutraceuticals, preventing the interaction of the bioactive with other components in the systems. In addition, nanoemulsion has been and will continue to have a promising application in food area, because it is easy to be prepared by using natural originated components. As such, nanoemulsions offer an alternative for the administration of nutraceuticals that have low water solubility.

Numerous studies have applied nanoemulsion systems in order to incorporate active food nutraceuticals and to increase their solubility and bioavailability and so on. Jo et al[69] prepared β-Carotene nanoemulsions with different emulsifiers Tween 20, Tween 80, WPI, SPI and, SC that had been prepared by microfluidization technique. They found that the nanoemulsions remained physically stable about five weeks at 25℃ and WPI formulated carrier compared with Tween 20 nanoemulsions apparently decreased β-carotene changed to other compounds and increased its chemical stability. Sari et al[70] prepared a curcumin nanoemulsion by sonication method with Tween 80 and WPI as surfactants, the resulting curcumin nanoemulsion had particles of mean particle size around 141.6 nm and slightly positive surface charge. They possessed good stability in the various environmental situations may occurred in food systems, like heating, different pH and ionic strength. The

nanoemulsion was also quite stable in digestion fluid and released slowly in the mimicked small intestine digestion buffer. Belhaj et al[71] fabricated a nanoemulsion for the delivery of CoQ10 without any organic solvent and with a good stability during one month at 25 °C. Their formulation increased the CoQ10 bioavailability as twice compared to oily commercial mixture.

1.4.2. Nanoemulsion preparation methods

In order to get optimized emulsion formulation, not only specific ingredients composition and ratios, but also a precisely designed processing method need to be manipulated. The preparation method is crucial to form the desirable formulations with good properties. In the following part, common methods applied for emulsion preparation, functional mechanism will be introduced.

As nanoemulsions are not thermodynamically stable systems, so usually it can not be formed spontaneously, thus either the energy within the molecules themselves or the outside energy input by machine can be used to fabricate the nanoemulsions. Base on this, the preparation of nanoemulsion can be divided into two categories, the methods that applies mechanical devices to offer mechanical energy to emulsify is so regarded as the high energy input methods. Another method that the formation of the nanoemulsion relies on the molecules itself is regarded as low-energy input method. In the low energy method, the emulsion was formed spontaneously with extremely small oil droplets can be dispersed into the continuous phase, thus this preparation route can also called spontaneous emulsification.[72] In low energy input method, nanoemulsion can be produced by phase transition method which is either performed by changing the composition with unchanged temperature [73-74] or perform the production oppositely by the other way.[75]

High energy emulsification method are usually realized by the help of mechanical equipment. The common-seen high energy method include high speed and high pressure homogenization, microfluidizition, ultrasonication and colloid milling by different machines like homogenizer, microfluidizer, sonicator and milling machine.

- High shear homogenizer (HSH)

High-shear homogenizer is usually used as a premixing tool to prepare a coarse emulsion system by direct mixing the oil and water by the existing of the emulsifier and other components. The rotating blade of HSH creates a turbulent velocity gradient that interrupts the interface of oil and water and an emulsion with one dispersed in another by the help of emulsifier was then formed.[76] Three parameters of HSH can be adjusted as needed; they are the blade geometry, rotational speed and the rotational time, with the rotational speed has the most important effect on the property of produced emulsion. The prepared emulsion particles of HSH are relatively large and the distribution is also wide. During the mixing, the operator also need to consider preventing the foam production by careful mixing. High-speed homogenizer can generally produce particle size within micrometer range.

- High pressure homogenizer (HPH)

HPH is the widely applied in industrial level for food material processing and preparation. The production of nanosized particles of HPH mainly relies on the valve design and efficiency; the high-pressure effect can be produced either by an electric motor or by gas/air. The pressure typically range from 50 to 500 bar and the valve of HPH will have influence on the flow scenario. During the processing, droplet could be disrupted because of the cavitation, while the flows passes through the narrow gap at a high speed,

the broken of the cavities with gas will cause the decrease of the size of the fluid mixture.[77] The valve geometry, rheology properties of the two phases, gaps size, the pressure and number of cycles will all have effect on the final particle geometry and polydispersity index. The HPH is a technique that is commonly used to produce particle to nanometric size. In general, the higher applied pressure and more processing cycles in the system, the finer and smaller the emulsion droplet can obtain. However, continue increasing the pressure or number of cycles may cause the aggregation of particle and thus not decrease the size. The particle size produced by HPH is usually within nanometric range depending on the constitution of the formulation and the production parameters.

- Colloid mill

Colloid mills can be widely used in a single or multiple cycles' process for food materials. The machine can be used to help insoluble dispersed phase particle evenly suspended into another carrier aqueous phase with its rotor-stator design. It can be also regarded as wet milling for the preparation of colloid systems. It can efficiently reduce the droplet size of intermediate to high viscosity liquid. The residence time in the channel and exerted forces could be altered by the gap size. The size of emulsion droplet formed by colloid mill is typically 1 – 5 μm in diameter.

- Microfluidization

Microfluidization seems similar to HPH, however, it's much more efficient with one smart design: the two channels within mixing chamber. In addition to cavitation resulted from pressure changes, the high velocity and very vigorous collision between the two flows of mixture caused by the strong shear force and ultra-high pressure and sharply pressure drop in a very short time range also resulted in a dramatically reduction of the particle

size.[78] Generally speaking, microfluidization could break the droplet to very small particles even below 100nm, and the distribution are smaller than HPH. One shortage of microfluidizer is higher possibility to be blocked. The following picture shows the core part of difference between HPH and microfluidizer.

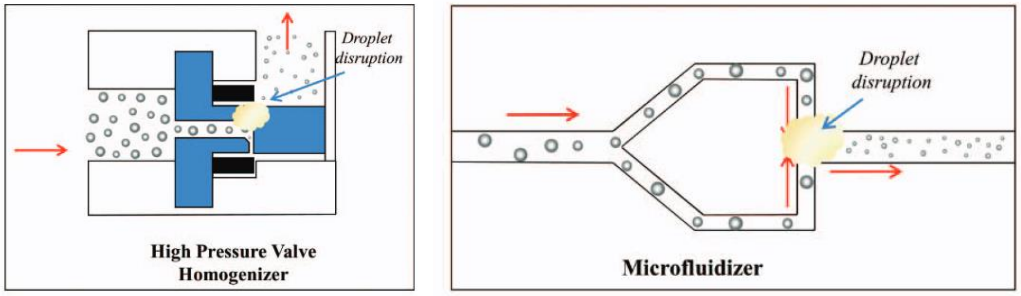



**Figure 6.** The difference between core parts of HPH and microfluidizer (Reprint from Ref 75)

- Membrane Homogenizer

This technique, pressure is used to get the dispersed phase or emulsion at surface of membrane into continuous phase. There are several advantages of the membrane homogenizer, such as the high-energy efficiency, narrow droplet distribution can be obtained and no heat created during the process.[79] The droplet size is typically 2-3 times of pore size. The disadvantage of this method is time consuming. The main operation factors can be adjusted are the property of the membrane used and the conditions such as transmembrane pressure, temperature, flowing speed of the dispersed phase and continuous phase. Certainly, the most affected factor is the properties of the dispersed phase and continuous fluid, such as the viscosity and so on.[80]

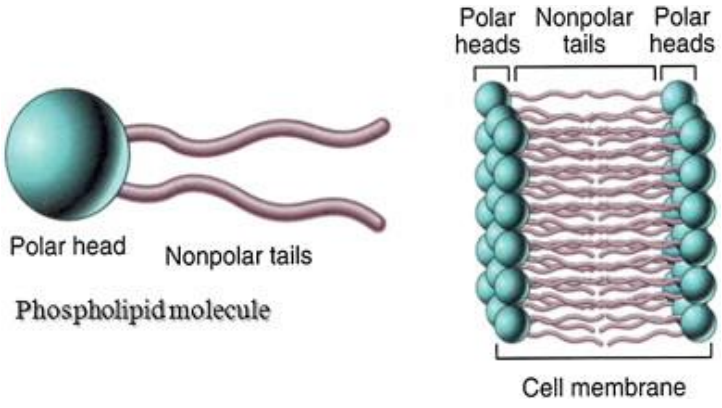
- Ultrasonic Homogenizer (Ultrasonicator)

This equipment applies sonotrode and sound waves with frequencies > 20 kHz to cause mechanical vibrations and formation of cavitation. As the local pressure is lower than the vapor pressure of the solution, cavitation force and great turbulent flow and high shear force also generated. Upon the collapse of these cavities powerful shocks waves will cause the deformation of droplets.[81] Ultrasonic homogenizer generates emulsion droplet of very high-energy efficiency than high-pressure homogenizer with small droplet diameter and size distribution. However, one serious shortage is excessive amount of heat generated at one spot during the process, which may cause unfavorable transformation of heat sensitive material, so an effective cooling system need to be applied together. The parameters for ultrasonicator mainly including frequency, sonication time, viscosity and temperature.

## 1.5. Lecithin

Lecithin also known as phosphatidylcholine (PC, the main constituent), is a natural mixture of the diglycerides of palmitic, stearic and oleic acids, which linked to the choline ester of phosphoric acid. It is the main structure constitute of the cell membrane of living plants and animals. In food industry, as a natural small molecule surfactant, lecithin is extensively applied as a surfactant in the food industry.[82] It is perhaps have been the natural surfactant in food area for the most use. Lecithin listed as one of the few emulsifiers that accepted as a natural ingredient by consumers. It has obtained generally recognized as safe (GRAS) by the US FDA under Title 21, part 184 for quite a long period, [83] which means that they can thus be safely employed in the development of lipid nanoemulsions for oral routes of applications.

Lecithin could be obtained from multiple different natural produces and food; examples are like milk, egg, soybean, rapeseed and sunflower seed and so on. Soybean oil has been the primary industrial source of vegetable lecithin for many years. The main component of lecithin is phosphatidylcholine, it contains non-polar tail which composed by two long fatty acids and a polar head with phosphate-choline. It is extensively applied in very broad areas for food such as emulsifier, wetting agent and so on. Besides used as emulsifier, lecithin also plays essential role during the digestion and absorption of lipophilic compounds, the intrinsic lecithin excreted to the intestine can help on the digestion of lipid components in food. In addition, even though there are other synthetic emulsifier and stabilizing agents, because the similarity of lecithin to biomembrane composition, they are still the emulsifying agents of choice in terms of biocompatibility. They are recognized as non-allergic, bio-friendly permeation enhancers and can be easily digested, metabolized and utilized by human body without exert toxic side effect. Its structure also makes it a good candidate to prepare delivery vehicles. The most common nano-formulations by lecithin are liposome, nanoemulsion and microemulsion (57, 44, 58).



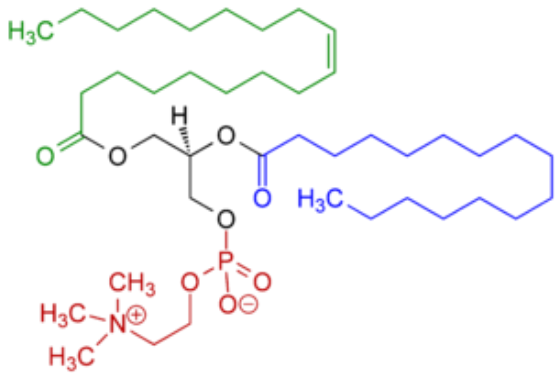


**Figure 7.** Structure illustration of phospholipid and chemical structure of phosphatidylcholine

Lecithin was also studied to possess many benefits, so that is a nutrient supplement that can be found in the market. Choline can also be obtained from lecithin for body requirement. In living organism, choline is critical for various biological functions, from lipid metabolism to the precursor for the synthesis of the neurotransmitter acetylcholine. Other benefits of lecithin include the liver protection from alcohol and fat liver and the maintenance of the function of brain by decreasing the cholesterol and increasing high-density lipoprotein.[84-85]

With a GRAS status, good emulsifying properties and physiological benefits, lecithin is an emerging material to be used as emulsifier or surfactant for oral formulations. The phospholipid-based carrier formulation has been proved effective to enhance the bioavailability for many lipophilic active ingredients.

# CHAPTER 2: HYPOTHESIS AND OBJECTIVES

## 2.1. Hypothesis

With the increasing conscious of healthy food for life, many potential bioactive ingredients from natural resources are extracted and studied; they are gradually applied as either a natural antioxidant or antimicrobial preservative for food and beverages, or for preventing metabolic disorder and chronic diseases as functional food supplements.

Carnosic acid is a nutraceutical that is mainly extracted from sage and rosemary leaves. It processes many health-promoting benefits, such as anticancer, anti-inflammatory, antioxidation and anti-bacterial properties. As most food systems are aqueous based and for daily intakes, oral route is preferred by people since it is the most convenient and non-invasive way. However, the health promoting benefits and food applications of carnosic acid maybe hindered by its hydrophobic nature and thus not easy to be dissolved in the water-rich aqueous phase, low gastrointestinal dissolution, not well studied absorption behavior. Based on this, Novel systems for delivering carnosic acid in solubilized forms need to be developed for improving its bioavailability and application.

As the solubilization and absorption of such water-insoluble compounds are greatly rely on the rate being incorporated into the micelle or mixed micelle, which are formed by the phospholipid and bile salts excreted endogenously or provided by the digested products of the formulations. The application of lipid-based formulations were well studied being an useful way to enhance the amount can be in the aqueous phase and lead to higher bioavailability of hydrophobic compounds. Among them, emulsions are the most extensively applied carrier formulations in academic and manufacturing because good food compatibility, relatively stability and improved absorption during digestion.

Based on the above analysis, I hypothesize that improving of carnosic acid's oral bioavailability and bioefficacy can be achieved by properly designed lecithin-based nanoemulsion delivery system, which is kinetically stable, able to maximize the solubilization and can be applied in aqueous food system. Lipids and lecithin in the formulation could offer extra components to form micelles after being digested in gastrointestinal tract.

## 2.2. Objectives

In order to fulfill the proposed hypothesis, following four specific aims were proposed to evaluate the effectiveness of emulsion-based carrier formulations to enhance the oral bioavailability and bio efficacy of carnosic acid. The eventual objective is to apply the formulation to food system served as a health promoting components.

### Aim 1. Formulate and optimize a lecithin-based nanoemulsion delivery system for carnosic acid

So far, there is no nanoemulsion system designed for carnosic acid applied in food area. In the current study, by a high energy input method, specifically, high speed and high-pressure homogenization combined method was utilized for the preparation of nanosized emulsion system. The optimized formulation are selected by designing ternary phase diagram with suitable oil with the aim to find the most stable yet high loading formulations for carnosic acid. The formulation were characterized regarding the stability, rheology and so on. Before the formulation fabrication, the basic properties regarding carnosic acid were studied.

### Aim 2. *In vitro* evaluation on bioaccessibility of carnosic acid nanoemulsion

The performance of emulsion-based carrier formulation on the bioaccessibility of carnosic acid was studied by *in vitro* lipid digestion model including pH-stat lipolysis and TIM-1 model, compared with the unformulated carnosic acid oil dispersion and water suspension.

**Aim 3. Investigate the nanoemulsion delivery system on the bioefficacy of carnosic acid.**

After evaluation of the bioaccessibility, we further did the bio-efficacy of carnosic acid nanoemulsion by in vitro cell studies. The in vitro anti-proliferative on cancer cells of carnosic acid nanoemulsion were evaluated using various carcinoma cells originated from patients with cancer such as human hepatic and colonic carcinoma cells. The cell-based antioxidant effect was also evaluated by HepG2 cell models. The in vitro inhibition on inflammation ability of carnosic acid nanoemulsion will be investigated using RAW 264.7 cell model. The antibacterial activity on Gram-positive bacteria and Gram-negative bacteria were performed. The mechanism of these effects were discussed.

**Aim4. Assess the oral bioavailability of CA in nanoemulsion by *in vivo* models (animal study).**

As our final target is to see whether the nanoemulsion formulation would improve the bioavailibity of carnosic acid, the in vivo animal study was necessary to perform. In *vivo* pharmacokinetic parameters (*C*max, *T*max, AUC) and the relative bioavailability of carnosic acid was determined and compared with the unformulated forms using animal models.

# CHAPTER 3. DESIGN AND CHARACTERIZATION OF THE NANOEMULSION BASED ON TERNARY PHASE DIAGRAM

## 3.1. Introduction

To prepare emulsions for food application, it is desirable that all the ingredients involved hold a GRAS status. Besides, carnosic acid has high melting temperature and low solubility, which brings most problems for processing procedure. The target of the current part is to formulate an optimized ratio of the three components to prepare a high loading and stable emulsion formulation for carnosic acid. Lecithin exhibits good emulsifying ability and claimed health promoting. Also, the main component in the lecithin in this study is phosphatidylcholine, which is the skeleton molecule of cell membrane, so the formulations is supposed to have better affinity to cells, which will bring a lot benefits for the encapsulated compound.

## 3.2. Materials and methods

### 3.2.1. Materials

Carnosic acid (95%) was bought from China, PC 75 was gifted from American Lecithin Company, US. MCT oil (Neobee 1053) was kindly provided by Stepan company. For every experiment, deionized water was used. All other chemicals were ACS grade without specifically specified.

### 3.2.2. Determination of carnosic acid by HPLC

CA was analyzed by a Dionex HPLC system with UV detector by our previous developed method.[86] The separation condition was conducted on a C18 TSKgel ODS-100Z column (5 μm, 100Å; 4.6 mm × 150 mm, Tosoh, Tokyo, Japan) with the mobile phase

composed of acetonitrile-0.1% phosphoric acid (60:40, v/v) at a flow rate of 0.6 mL/min with the detection wavelength was set at 230 nm. The injection volume was 20 μL.

3.2.3. Stability of carnosic acid in selected organic solvent

This step is to make a foundation for further detection or other characterizaiton that will need organic solvent, such as HPLC detection. Carnosic acid was dissolved in different solvent , namely, methonol, ethanol and acetone, then stored under four conditions separetely, 25℃-light, 25℃-dark, 4℃-dark and -20℃-dark. The amount of carnosic acid was detected at predetermined time.

3.2.4. Solubility and stability of carnosic acid in different oils

For the selection of oil phase, two factors were considered, one is giving highest solubility of carnosic acid, another one is the stability of carnosic acid. More than saturated amount of carnosic acid were added into different oils, namely, MCT, corn oil, canola oil and soybean oil. Heat up to make all the carnosic acid dissolved, then put the solution for 24 hours, then, the amount dissolved was dissolved and diluted by acetonitrile, the amount in each detected by HPLC.

3.2.5. Optimization of the formulation based on ternary phase diagram

The O/W nanoemulsions was developed using MCT oil, lecihtin and water. Based on previous study, twenty samples in the possible emulsion area of ternary phase diagram were prepared. We firstly prepared the dispersion phase (oily phase) by mixing 0.92 g of CA in 4.8 g in the carrier oil MCT, heated up to 90 °C, and maintained until the CA fully solubilized and in order to inhibit the recrystallization, the temperature was kept above seventy degree Celsius. The continuous phase (aqueous phase) was formulated by

dispersing the 0.9 g PC 75 lecithin into 24.3 g DI water and also heated up to the same temperature as oil phase. Once the two phases were ready, the aqueous phase was added into the oily phase, stir another two minutes and then the nanoemulsion was prepared by a two-step high-energy input method. Firstly, we can prepare the coarse emulsion with size usually around micrometers by using high-speed homogenizer at 12000rpm within 3 minutes based on a preliminary study. Then the crude emulsion was downsized by applying the high-pressure homogenizer instrument (EmulsiFlex-C6, AVESTIN Inc.) at 120MPa with four passes. Finally, the formulated nanoemulsion composed with 15.5 MCT, 2.9% lecithin, 3.0% CA and 78.6% DI water were collected from the equipment for each batch. After the preparation, the amount of carnosic acid in the nanoemulsion was determined by HPLC. MCT and water suspension was prepared by adding the same amount of carnosic acid in MCT or water as in emulsion formulation.

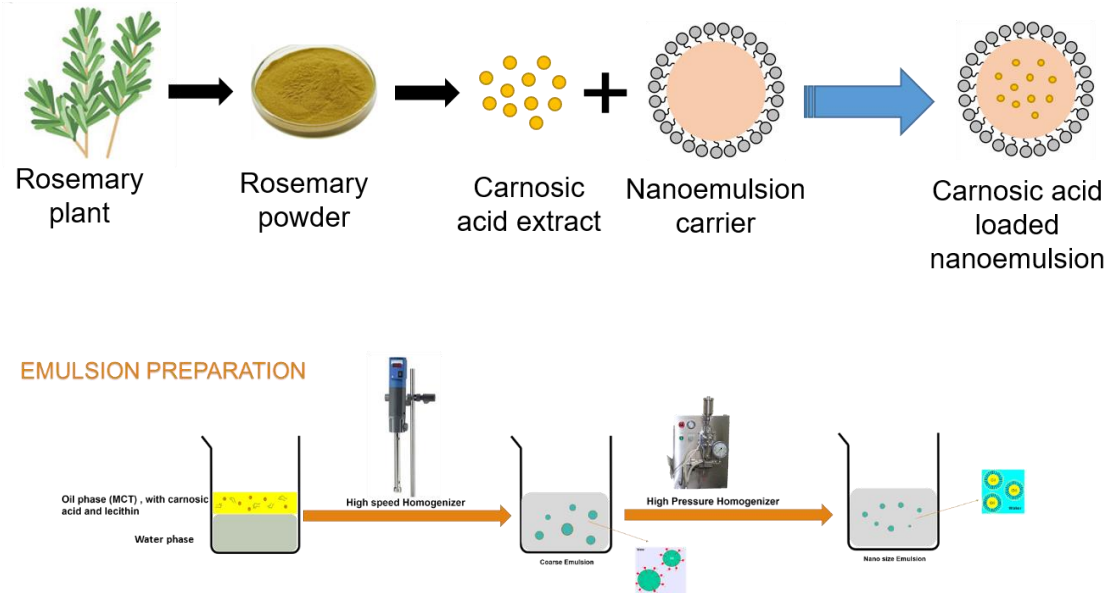



**Figure 8.** Preparaion emulsion with fine droplet using a combination of high-speed homogenizer and high-pressure homogenizer.

3.2.6. Morphology, size, size distribution and zeta potential measurement

The produced nanoemulsion was observed using freeze fracture scanning electron microscopy (Cryo-SEM). The nanoemulsion was firstly frozen by liquid nitrogen by placing in the stub used for the liquid sample preparation.Then the freezed nanoemulsion were put in the prepartion compartemnt for the fracture process and then the nanoemulsion was sublimited at −95 °C lasted four minutes to eliminate some water in order to observe the fractured surface, after coating with platinum, the nanoemulsion was obseved by the equipped SEM.[87]

Droplet size and polydispersity index (PDI) of the B-NE and CA-NEwas measured using a particle size analyzer (90 plus, Brookhaven Instruments Corporation, USA). The motion of the emulsion particles casused chaging of the intensity of laser light is measured by the DLS. All measurements were performed at laser light set at scattering angle of 90° and at ambient temperature in triplicate.

3.2.7. Stability of the nanoemulsion

10 grams of blank and carnosic acid nanoemulsions were weighted and kept in a 20 milliliter small container, filled with nitrogen gas on the top and then air-tightly screwed once prepared. All the small containers with CA-NE were prepared into two sets, stored at 4 °C and 25 °C, respectively and avoid of the light during the whole experiment procedure. The storage stability of particle size and size distribution were monitored for four weeks.

In antoher set of study, the physical stability and chemical stability of CA-NE samples was tested under the storage temperature at 25 °C, 37 °C and 4°C. The test was conducted at 1, 7, 14, 21, 28 days after preparation under dark conditions throughout the experiments.

The stability of carnosic acid nanoemulsion was also tested regarding its ability to keep unchanged physically under different pH and ionic strength. The pH selected was 4.0, 6.8, 7.4, 8.0 and 10.0. The ionic strength prepared by sodium choloride was in the range of 0-100mM. All the measurements were done at least three times.

3.2.8. Rheological properties

Rheological measurements were performed on pure MCT, blank nanoemulsion and CA-NE using ARES Rheometer (Rheometric Scientific, Piscataway, NJ) with a parallel plate design at ambient conditions. Flow curve of B-NE and CA-NE were performed first. Oscillation stress sweep with obtaining the linear viscoelastic range of stress value of the nanoemulsions. Dynamic strain sweep and frequency sweep was conducted.

3.2.9. Free radical scavenging effectiveness-DPPH assay

DPPH solution (DPPH in methanol or ethanol) was prepared at the concentration of 0.2mmol/L, CA and CA-NE sampels are prepared from 2000μg/mL to 15.625μg/mL in ethanol. Mix 190uL DPPH solution with 10uL sample of each concentration in a 96-well plate, then keep the mixture in dark at room temperature for 30 minutes. Each concentration do triplicate and the results are shown as the mean± standard deviation. Blank is the reaction mixture without your sample. After the 30 minutes reaction, the final mixture was measured at an UV wavelength of 517nm at a microplate reader.

Statistical analysis

All studies were conducted triplicate and were showed as the mean ± standard deviation. Where appropriate, The anlalysis was performed of variance (ANOVA) using the SPSS 16.0 software (IBM, NY, USA).

3.3. Results and discussion

3.3.1. Measurement of carnosic acid and stability in selected organic solvent

First, the carnosic acid dissolved in acetone or acetonitrile with different concentrations from 0.003mg/mL to 0.6mg/mL was prepared and the HPLC method described earlier was utilized for the determination. The HPLC profile and calibration curve was obtained as **Figure 9** showed below.

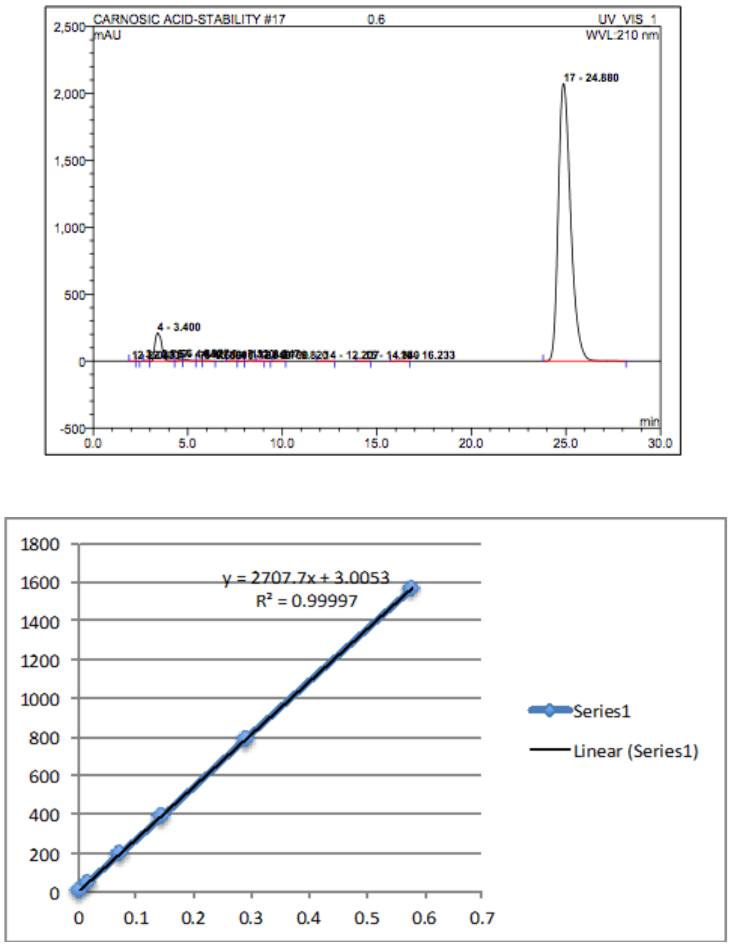
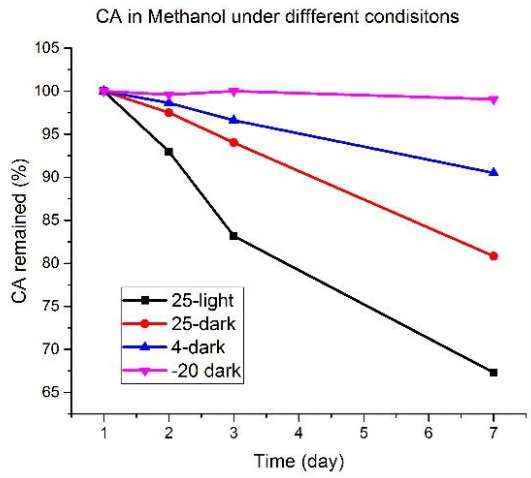
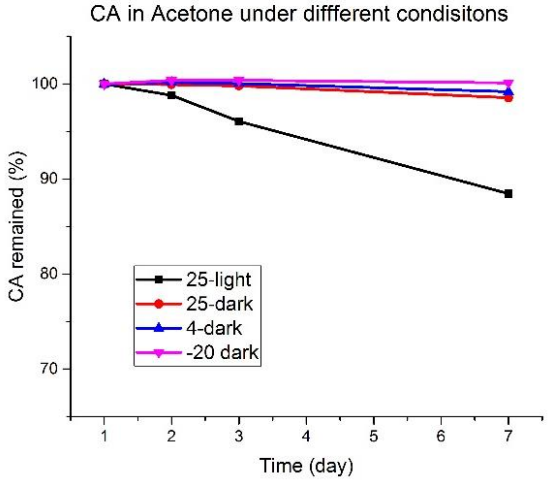
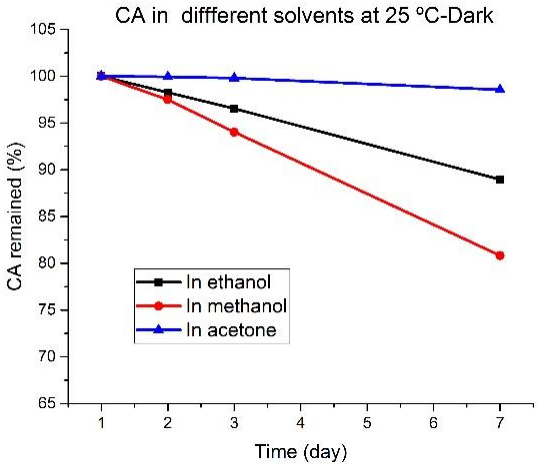


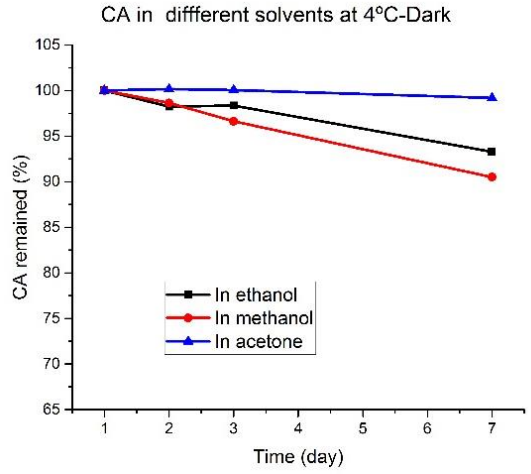

**Figure 9.** HPLC profile and standard curve of CA

The class of diterpenes inculding CA have been shown quite unstable in alkaline and polar solvent. They are sensitive to many factors and may undergo decomposition or transformation.[88-89] In order to use the apropriate solvent and avoid the use of those solvents cause unstable of carnosic acid in future. Here we tested the stability of carnosic acid in three different common solvents, namely, methanol, ethanol and acetone under three different temperatures which represent the ambient temperature with or without light exposure, refregerator temperature at 4 °C and the temperature in freezer. As we can see from **Figure 10**, the degradation of carnosic acid was faster at ambient condistions than the lower temperatures at 4°C or freezer for all the solvents tested during the 7-day experiment. This penomenon was also observed by other study of CA dissolved in THF.[90] We also found at all the tested temperatures, CA was most stable in acetone followed by ethanol and least stable in methanol, which is correlated to their polarity. Previous study reported that carnosic acid is more stable in DMSO than in methanol, the amount of carnosic acid even kept unchanged in DMSO for several days, while carnosol, the lactone form of carnosic acid, degraded significantly in a few hours in methanol, DMSO, DMSO–acetonitrile and ethyl acetate–acetonitrile.[89] They also found that carnosic acid degraded fast in methanol from 94% to around 40% in four days under room temperature with light, which is a little faster degradation compared to the results we find here. However, the stablity of these compounds is affected a lot by the solvent, the light and the temperature, which have also been reported by other studies.[91]


CA in Methanol under diffferent condisitons
CA remained (%)
Time (day)
25-light
25-dark
4-dark
-20 dark


CA in Acetone under diffferent condisitons
CA remained (%)
Time (day)
25-light
25-dark
4-dark
-20 dark


CA in diffferent solvents at 25 °C-Dark
CA remained (%)
Time (day)
In ethanol
In methanol
In acetone


CA in diffferent solvents at 4°C-Dark
CA remained (%)
Time (day)
In ethanol
In methanol
In acetone

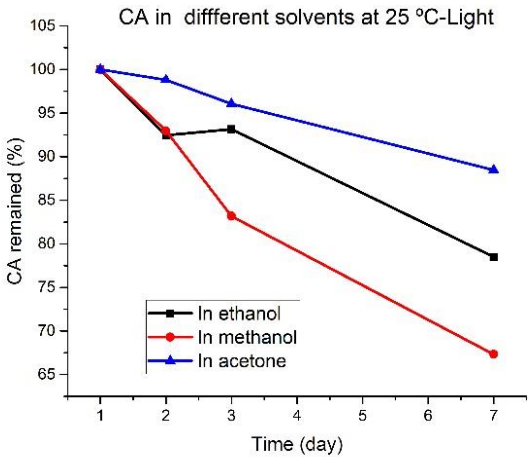



**Figure 10.** The amount change of carnosic acid in different solvents (methanol, ethanol and acetone) under various storage conditions (25 ℃-light, 25℃-dark, 4℃-dark, -20℃-dark

**Table 2.** Unchanged amount of carnosic acid

| | *Days/samples* | *25 light (%)* | *25 dark (%)* | *4 dark (%)* | *-20 **dark** (%)* |
|---|---|---|---|---|---|
| *Ethanol* | 1 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 2 | 92.45 | 98.252 | 98.241 | 99.85 |
| | 3 | 93.16 | 96.544 | 98.343 | 99.16 |
| | 7 | 78.49 | 88.960 | 93.281 | 98.78 |
| *Methanol* | 1 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 2 | 92.97 | 97.4902 | 98.6339 | 99.59 |
| | 3 | 83.17 | 94.0167 | 96.6215 | 100.01 |
| | 7 | 67.32 | 80.8218 | 90.5009 | 99.07 |
| *Acetone* | 1 | 100.00 | 100.00 | 100.00 | 100.00 |
| | 2 | 98.81 | 99.95 | 100.15 | 100.40 |
| | 3 | 96.07 | 99.80 | 100.06 | 100.40 |
| | 7 | 88.45 | 98.55 | 99.18 | 100.12 |

From the HPLC profile from day 1 to day 7 of CA in methanol as shown in **Figure 11**. It was clearly that there were mainly two new compounds formed during the storage of carnosic acid in methanol; however, these tow peaks are not observed in ethanol and acetone. The color change of carnosic acid in methanol also indicated that there were new compounds formed during storage, which further implied the stability of carnosic acid in methanol is worst in these three solvents, which can be explained by the polarity of methanol is the highest among these three solvents. The possible degradation products are carnosol according to the previous study and compared with the standard carnosol. Preliminary analysis revealed that CA is most likely to degrade in the methanol and sensitive to both temperature and light. Under 25℃, With Light or under dark affected a lot. The current study mainly focused on providing the knowledge on following handling of carnosic acid.

In **Table 2**, we summarized the percentage of the amount unchanged for carnosic acid as compared to the original amount in the samples when stored under different -20℃ in dark, 4℃ in dark, 25℃ in dark and 25℃ with light in methanol, ethanol and acetone, respectively.

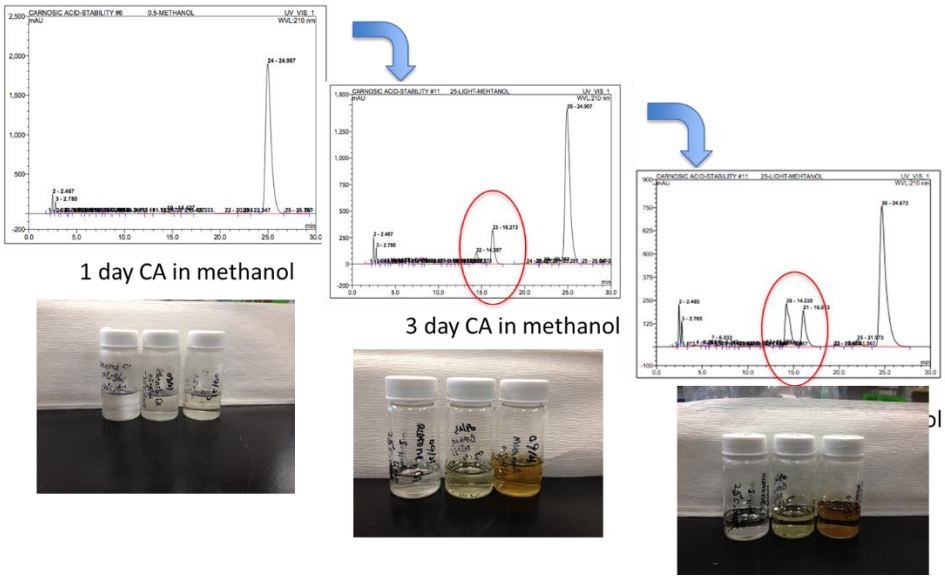



**Figure 11.** Degradation of CA in methanol at different storage time

3.3.2. Solubility in different oils and preparation of nanoemulsion

High solubility in the carrier oil is cruicial for us to design a nanoemulsion system, as the higher amount the bioactive can be solubilized, the higher possiblity that the high loading can be achieved. This is particularly important for oral formulation, as solubility of the nutraceutical in the inner oil phase will greatly infulence the ability of nanoemulsion to keep the nutraceuticals in solubilized condition. In the design of the nanoemulsion fromulaiton for carnosic acid, several important factors need to be considered. We need to minimize the crystalization or precipitation of the bioacitves while at the same time it is favorable to make a high loading formulaitons. These facotrs are crucial for fabrication of nano carrier with good stability.[92-93] We found that carnosic acid has the highest solubility in medium-chain triglyceride (MCT) among all the tested oils as shown in Table 3. As one of the important functions of the nanoemulsion for carnosic acid is to improve its loading

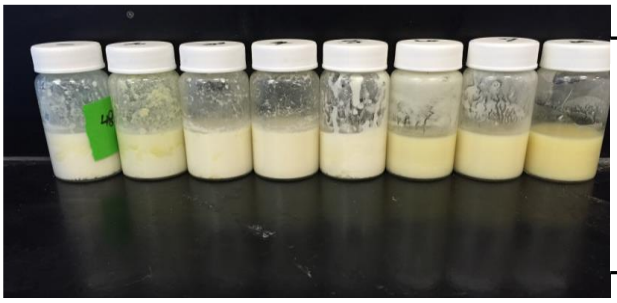
and then makes it possible for the further evaluation of the bioavailability and biofunctions. What's more, high loading means a less formulation dosage needed in a certain period of time, which is favorable for the functional food development. Based on this result, we choosed MCT for the preparation of formulation.

**Table 3.** The highest dissolved amount of carnosic acid in selected oils

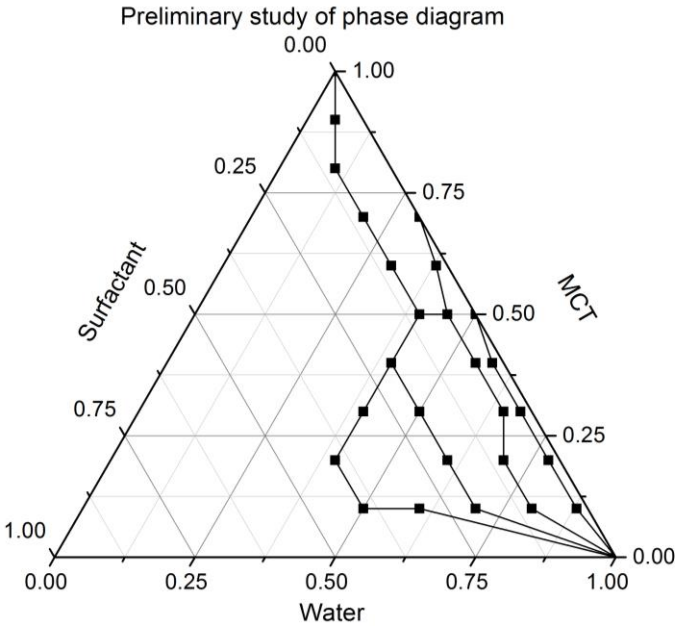
| Oil type | Area (m*Au) | percentage of solved | percentage | mg/ml |
|---|---|---|---|---|
| MCT | 78.97 | 0.2338 | 2.8055 | 28.0551 |
| corn oil | 35.55 | 0.1002 | 1.2019 | 12.0193 |
| canola oil | 26.585 | 0.0726 | 0.8708 | 8.7083 |
| soy oil | 15.805 | 0.0394 | 0.4727 | 4.7271 |

After determined the emulsifier and oil for the preparation of the emulsion, the next most important step is to optimize the formulation by turning the ratio between water phase, oil phase and lecithin. Such that, more than eighty different samples with various ratio of the MCT, lecithin and water was prepared by high-speed homogenizer. After 24 hours stay on the bench in the laboratory, we then divided those samples into different categories based on the observation of their stability and the measurement of their rheology properties, from the ternary phase diagram. Then in the category of stable emulsion, we further processed the formulation with high-pressure homogenizer of three best formulations showed in **Table 4**. The samples are stored at either 4 or 25 ºC for 30 days, and the formulation 81% water, 16% MCT oil and 3% lecithin with smallest size and size change

was selected for the next step.  and optimized the process regarding the cycles of high pressure and loading efficiency (LE%) of carnosic acid. As the experiment showed that when the loading of carnosic acid was above (including) 4%, even though the emulsion was formed, but the precipitation of carnosic acid happened. Thus The final formulation was chosen for 3% of carnosic acid.



| | Emulsion(o:w) |
|---|---|
| 1 | Pc75-1:1 |
| 2 | PC75-38:58 |
| 3 | PC75-28:68 |
| 4 | PC75-18:78 |
| 5 | PC75-58:38 |
| 6 | PC75-68:28 |
| 7 | PC75-78:18 |
| 8 | PC75-88:8 |



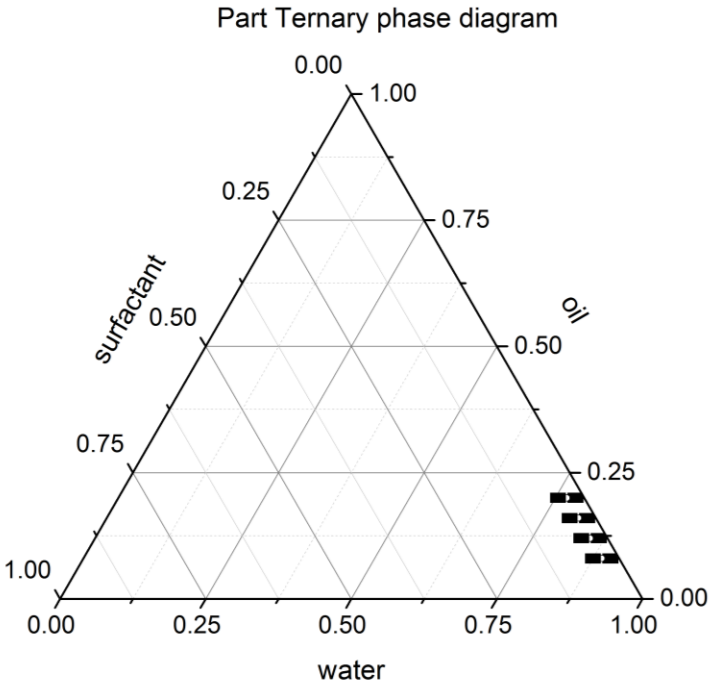



**Figure 12.** The optimization of lecithin based nanoemulsion formulations

**Table 4.** Stability and properties of the formulations

| day | Temperature(℃) | 16:81(O:W) | 12:85(O:W) | 8:89(O:W) |
|---|---|---|---|---|
| 1 | 4 | 137.5/0.266 | 164.9/0.207 | 161.9/0.221 |
| | 25 | 137.5/0.266 | 164.9/0.207 | 161.9/0.221 |
| 10 | 4 | 160.3/0.247 | 180.2/0.223 | 175.4/0.229 |
| | 25 | 156.1/0.241 | 182.3/0.247 | 177.8/0.256 |
| 20 | 4 | 142.6/0.219 | 189.9/0.183 | 189.3/0.179 |
| | 25 | 180.1/0.203 | 204.5/0.171 | 201.1/0.226 |
| 30 | 4 | 172.5/0.165 | 190.5/0.175 | 187.3/0.210 |
| | 25 | 158.7/0.198 | 180.0/0.235 | 197.9/0.227 |

| sample | cycles | size (nm) | PDI | zeta-potential(mV) |
|---|---|---|---|---|
| Blank | 3 | 200.9 | 0.203 | -59.2 |
| | 6 | 163.1 | 0.202 | -62.0 |
| | 10 | 144.9 | 0.250 | -60.4 |
| 1% | 3 | 260.7 | 0.203 | -61.1 |
| | 6 | 191.1 | 0.195 | -60.5 |
| | 10 | 162 | 0.204 | -59.9 |
| 2% | 3 | 218.3 | 0.239 | -61.8 |
| | 6 | 172.5 | 0.202 | -52.6 |
| | 10 | 156.3 | 0.196 | -59.6 |
| 3% | 3 | 197.2 | 0.229 | -62.6 |
| | 6 | 173.9 | 0.235 | -55.0 |
| | 10 | 197.2 | 0.229 | -57.9 |
| 4% | 3 | 222.5 | 0.243 | -61.9 |
| | 6 | 191.1 | 0.207 | -61.7 |
| | 10 | 144.8 | 0.205 | -50.2 |

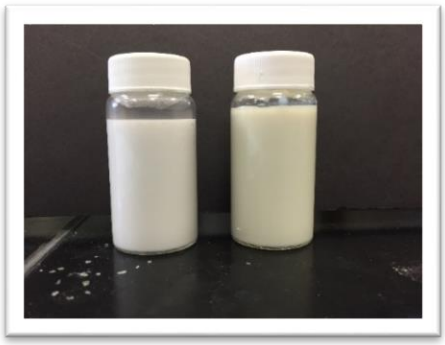


**Figure 13.** The picture of optimized nanoemulsion, left one is blank nanoemulsion, right one is carnosic acid

The final formulation we choose to prepare our carnosic acid nanoemulsion is 81% water, 16% oil and 3% PC 75 lecithin, the loading of carnosic acid was 3% percent of the

total weight. The final formulated nanoemulsion sample was then characterized within the following properties.

3.3.3. Morphology, size and size distribution, and zeta potential

In the following session, the most basic properties of CA nanoemulsion, including the morphology, the size, the uniformity and zeta potential were characterized.

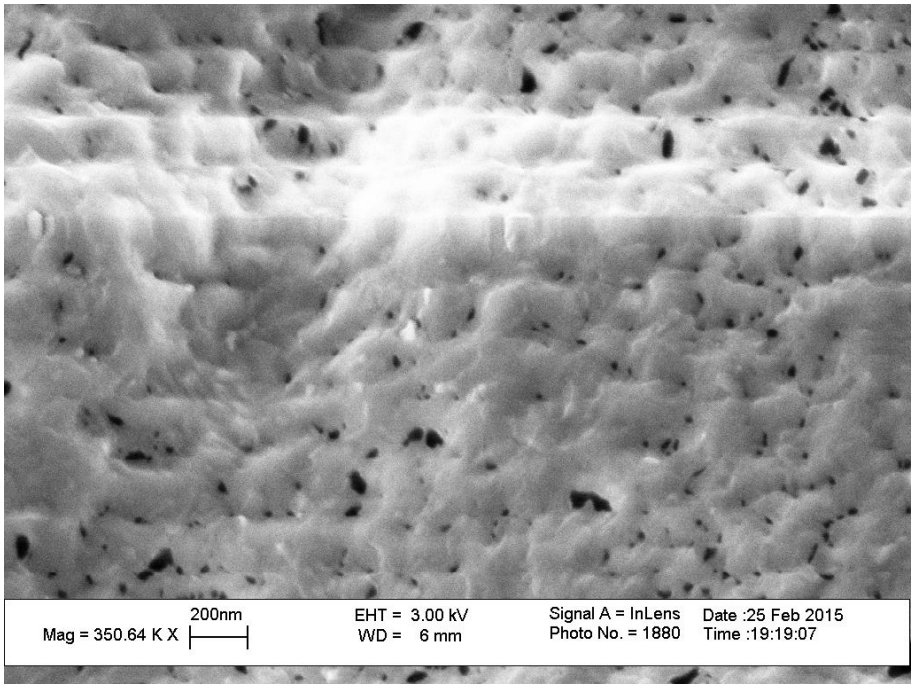



**Figure 14.** The Cyro-SEM picture of carnosic acid nanoemulsion

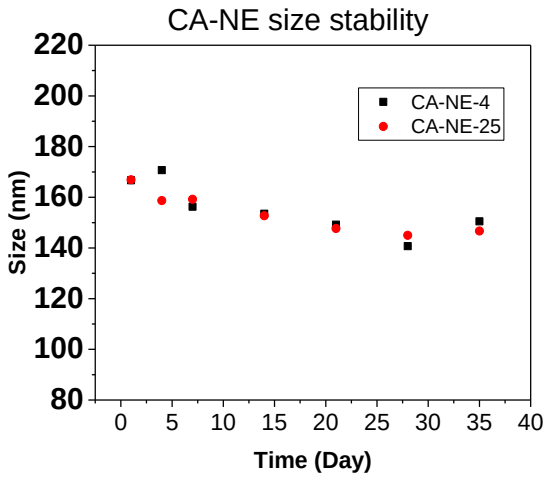
Freeze treatment of samples right before scanning environmental microscopy observation offer the possibility to observe a lot soft materials such as nanoparticle, nanoemulsion and liposomes in their original form. This technique could offer the information of both size and structure. The above picture is not in very good quality, the small droplets seemed very crowded, however, we can still see small bumps which is the

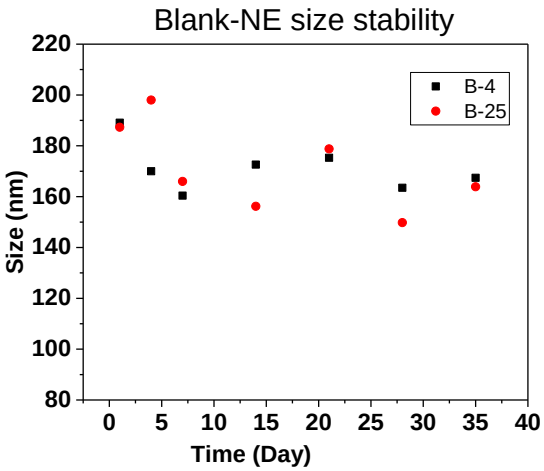
nanoemulsion droplets. They are sphere, and the size is below 200 am, around 150nm, which is similar to the data got from DLS as we will show below.

The size and size distribution is a fundamental indicator of the physical stability of nanoemulsion and need to be monitored. The size we got was 170.2±3.5nm with polydispersity index (PDI) was 0.231, which indicate the emulsion formed should be kinetically stable compared with the conventional ones, and relatively small PDI meaning the size distribution is narrow, also indicated slower happening of flocculation and coalescence. CA loading was 2.65%, zeta-potential was -57.2mV, and with a pH value of 4.55.

3.3.4. Stability of nanoemulsion

The size and size distribution change of CA-NE and B-NE stored at 25 ºC and 4 ºC were monitored of all the samples in predetermined time during four weeks. The average droplet size and polydispersity index (PDI) of both blank-nanoemulsion and carnosic acid nanoemulsion were obtained from the DLS instrument as **Figure 15** described. With the same preparation parameters and procedures, B-NE showed the droplet size around 190 nm, while carnosic acid nanoemulsion was around 167nm were obtained. Compared between the B-NE and CA-NE. Addition of carnosic acid showed no great influence on the droplet size, the changes of droplet size of both blank and carnosic acid nanoemulsion over four weeks storage were not significantly. In addition, no sedimentation or creaming was observed. It means that no coalescence of the droplets happened. The results indicate good physical stability of the formulation and carnosic acid loaded did not affect the physical stability.


CA-NE size stability
CA-NE-4
CA-NE-25
Size (nm)
Time (Day)
220
200
180
160
140
120
100
80
0
5
10
15
20
25
30
35
40


Blank-NE size stability
B-4
B-25
Size (nm)
Time (Day)
220
200
180
160
140
120
100
80
0
5
10
15
20
25
30
35
40

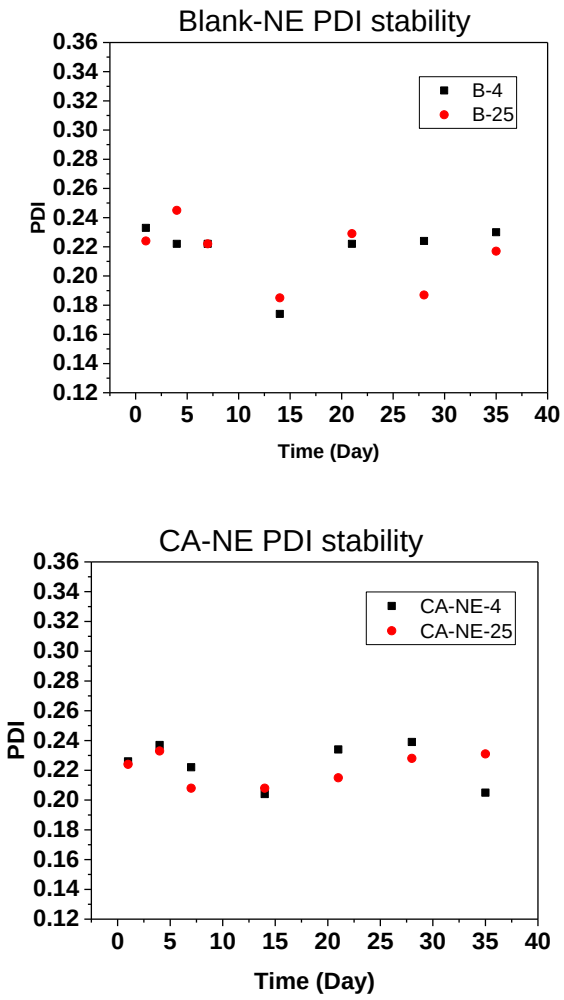



**Figure 15.** Size and size distribution change during different storage conditions of Blank nanoemulsion (B-NE) and carnosic acid nanoemulsion (CA-NE)

From the application viewpoint, it is crucial that the nanoemulsion formulation possess good physical stability during storage at different temperatures. Thus, we observed the changing of droplet size and distribution of the carnosic acid nanoemulsion over time when stored at different temperatures during four weeks were monitored as shown in **Figure 16 (A)**. In general, there is no obvious sedimentation or phase separation during the storage

when observed the appearance directly. When DLS was applied to measure the mean particle radius evolution, there was a slight fluctuation of the size during storage at every temperature, with the size change more obvious at higher temperature. However, it is still very clear from the figure that for all the samples, the size change through the entire period is not obvious, indicating the stability of the formulated carnosic acid nanoemulsion is relatively acceptable under all the temperatures.

The chemical stability of carnosic acid in the CA-NE was also tested at the same time during storage, as we can see from **Figure 16 (B)**, CA is more stable at lower temperature, and 90% percent of CA remain unchanged for a four-week storage. However, at elevated temperature of 37 °C, only 70% percent of carnosic acid left after a four-week storage. This result indicated two important facts; firstly, carnosic acid is not stable at high temperature even with the encapsulation of nanoemulsion, however, if compared with the aforementioned degradation speed of carnosic acid dissolved in the methanol, we can see a much slower speed it is in the nanoemulsion system. However, as the carnosic acid is barely dissolved in water, it is hard to compare with pure carnosic acid in water system without the help of organic solvent. Later we will study the stability of CA and CA-NE in buffer solution once the stability need to be examined. Nevertheless, the degradation of these bioactive compounds is hard to absolutely eliminate even with the encapsulation method. Yi.J et al [94] used beta-lactoglobulin (BLG) alone or BLG−catechin conjugates prepared emulsion to incorporate beta-carotene, They still found the amount of beta-carotene remained to 74.5% and 86.8% in the two emulsion systems they formulated.

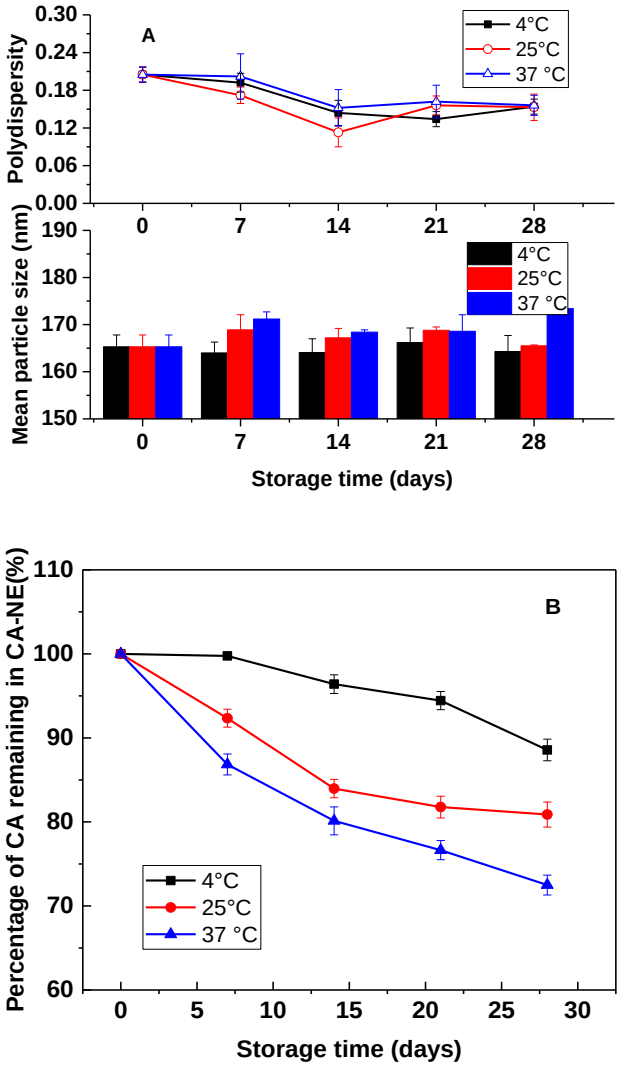



**Figure 16.** The stability of carnosic acid nanoemulsion (CA-NE) during 4 weeks storage at 4, 25 and 37 °C. (A) Size and size distribution (PDI) change (B) The amount of carnosic acid change during the storage.

We here evaluated how the different pH conditions will affect the physical property changes regarding the size and size distribution, as the pH is a key environmental factor need to be seriously considered in different food. The size and size distribution change under room temperature at dark (**Table 4**). Results showed the size and distribution of CA-

NE maintained stable under the pH from 4 to 10 during one week storage and we didn't find any visible phase separation indicated the CA-NE was stable to against particle flocculation at the tested pH conditions. Ionic strength is also a circumstance factor usually cannot be avoided in emulsion type food and beverage, thus we prepared different concentrations of sodium chloride to test the stability of CA-NE to the ionic strength. Results (**Table 5**) showed the high salt concentrations (> 100 mM) had considerably impact on the stability of the nanoemulsion. Strong ionic strength would disrupt the nanoemulsion droplets which can be the reason that the hindrance of the repulsion among the particles in the emulsion , at low level of ionic strength, the repulsion is strong enough to be greater than the forces leading to aggregation of the particles, thus the nanoemulsion could stay stable at low ionic strength environment.[95]

**Table 5**. The particle size and size distribution (PDI) of CA-NE under different pH

| Storage Time (Days) | Droplet size (nm) and PDI under different pH | | | | |
|---|---|---|---|---|---|
| | 4 | 6.2 | 7.4 | 8 | 10 |
| 1 | 173.8± 1.00$^{bc}$ | 177.5± 1.69$^{b}$ | 183.4± 1.06$^{a}$ | 178.8± 1.34$^{ab}$ | 181.2± 0.74$^{ab}$ |
| | 0.205± 0.012 | 0.211± 0.018 | 0.192± 0.018 | 0.205± 0.014 | 0.186± 0.009 |
| 2 | 172.0± 0.46$^{cd}$ | 167.2± 1.00$^{d}$ | 181.5± 1.15$^{ab}$ | 171.3± 0.92$^{cd}$ | 168.4± 0.95$^{cd}$ |
| | 0.205± 0.021 | 0.202± 0.020 | 0.197± 0.021 | 0.200± 0.023 | 0.188± 0.015 |
| 3 | 173.8± 1.50$^{bc}$ | 172.4± 1.47$^{c}$ | 179.4± 1.01$^{ab}$ | 175.4± 2.07$^{bc}$ | 177.5± 1.35$^{b}$ |
| | 0.215± 0.016 | 0.204± 0.014 | 0.175± 0.018 | 0.186± 0.019 | 0.196± 0.022 |
| 5 | 170.9± 1.27$^{cd}$ | 171.5± 0.90$^{cd}$ | 180.5± 0.71$^{ab}$ | 173.3± 1.02$^{bc}$ | 180.6± 0.86$^{ab}$ |
| | 0.234± 0.024 | 0.231± 0.015 | 0.181± 0.010 | 0.211± 0.031 | 0.201± 0.018 |
| 7 | 172.1± 1.84$^{c}$ | 172.2± 0.41$^{c}$ | 180.1± 2.05$^{ab}$ | 174.7± 1.59$^{bc}$ | 179.9± 0.77$^{ab}$ |
| | 0.221± 0.022 | 0.221± 0.016 | 0.179± 0.021 | 0.187± 0.009 | 0.200± 0.021 |

* Note: Different letters indicated significant differences ( $p <$ 0.05)

**Table 6.** The size and PDI of CA-NE under different ionic strength

| Storage Time (Days) | Droplet size (nm) and PDI under different ionic strength (mM) | | | |
|---|---|---|---|---|
| | 0 | 10 | 50 | 100 |
| 1 | 173.0±1.63$^{fg}$ | 165.6±1.34$^{g}$ | 165.5±1.26$^{g}$ | 233.2±12.02$^{e}$ |
| | 0.204±0.024 | 0.213±0.016 | 0.191±0.016 | 0.241±0.031 |
| 2 | 177.1±1.23$^{fg}$ | 174.5±1.60$^{fg}$ | 170.8±1.64$^{fg}$ | 284.6±2.74$^{d}$ |
| | 0.211±0.015 | 0.224±0.015 | 0.223±0.019 | 0.278±0.023 |
| 3 | 182.9±2.16$^{f}$ | 175.7±2.73$^{fg}$ | 174.9±2.30$^{fg}$ | 322.0±16.57$^{c}$ |
| | 0.200±0.025 | 0.204±0.020 | 0.207±0.018 | 0.282±0.055 |
| 5 | 176.9±1.74$^{fg}$ | 173.9±0.90$^{fg}$ | 176.6±1.31$^{fg}$ | 361.4±18.09$^{b}$ |
| | 0.186±0.014 | 0.211±0.021 | 0.191±0.013 | 0.311±0.041 |
| 7 | 174.7±2.56$^{fg}$ | 175.8±0.95$^{fg}$ | 172.6±1.23$^{fg}$ | 452.4±5.60$^{a}$ |
| | 0.211±0.017 | 0.212±0.025 | 0.209±0.019 | 0.287±0.029 |

* Note: Different letters indicated significant differences ( $p < 0.05$)

3.3.5. Dispersion and Rheology properties

As the nanoemulsion was fabricated aiming to increase the bioavailability of carnosic acid by increasing its solubility in liquid systems, it was fundamental requirement that the carrier formulations can be evenly suspended into the water rich phase. Here, a fast and straightforward experiment was performed to evaluate the type of the nanoemulsion is W/O or O/W. We used the oil that was for the preparation of the formulation or water being the test medium, respectively. One milliliter of the prepared blank nanoemulsion or CA-NE was dropped into 10 milliliter of each medium respectively as shown in Figure 16. After gently swirled by hand both samples dropped in DI water readily dispersed homogenously,

while the CA-NE in MCT oil beaded up into a big bead. From the dispersion behavior study, we can see that the CA-NE was an oil in water type nanoemulsion, which could be readily dispersed and used in the water-rich phase of food. More importantly, this test indicating that the emulsion formulation for carnosic acid could fulfill the goal of increasing the solubility or dispersity into water phase and may further increase its Bioaccessibility and bioavailability.

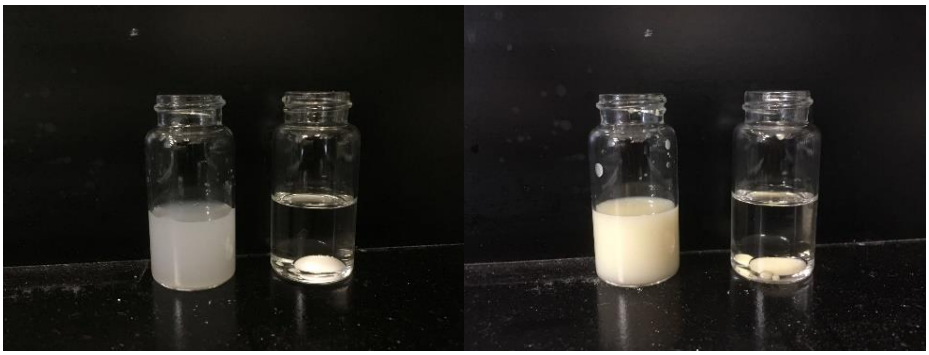


**Figure 17.** Dispersity of B-NE and CA-NE in water and MCT oil

As we can see from the **Figure 18** that both the blank naoemulsion and carnosic acid nanoemulsion are shear-thinning non-newtonia fulid. The carnosic acid added didn't affect much of the rheology behavior and the viscosity change, and has a relatively low vioscosity. And also when we dispersed the formulation in water or MCT oil, it's clear that the nanoemulsion can readily dissovled in water, but barely dissolved in MCT, which means the nanoemulsion is O/W type. The dynamic viscoelastic properties of samples made at various conditions were studied by a frequency sweep mode, the results are shown in Figure 18 (B), As we can see, CA-NE showed gel-like property (G'>G"). G' increased as frequency changed. Below the critical strain, for a structured or solid-like material, the frequency would not influence the storage modulus G'. The tested samples is regarded as

more closely to a fluid behavior if G' is changed as the changing of frequency. From this perspective, CA-NE contains a high ratio of water, so it is a little bit more fluid like rather than a solid one. Moreover, CA-NE exhibited rheological characteristics more like those of a weak gel or a viscoelastic suspension, as the storage modulus G' is always higher than G'' but with not much different, whtat's more , as the moduli having a stronger frequency dependence, especially G'' at the higher frequencies.[96] In some scenarios, the formed weak gel pattern in the system may siginficantly enhance the physical stability. In conculsion, the formed CA-NE is a viscoelastic fluid emulison system.

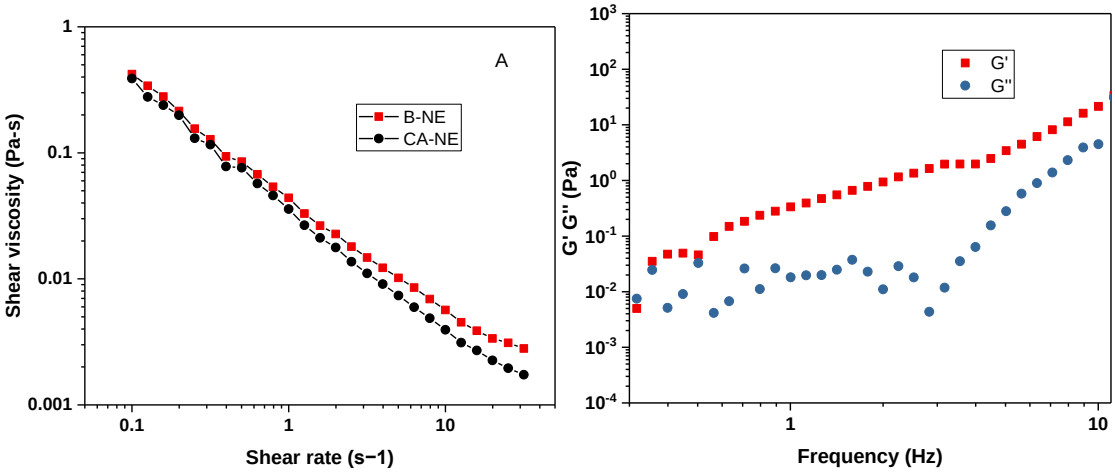



**Figure 18**. Rheology properties of carnosic acid nanoemulsion (CA-NE). (A) Steady shear viscosities of B-NE and CA-NE.

Previous studies have shown that carnosic acid is very effective as an antioxidant, however, there is no such report on how lecithin-based nanoemulsion encapsulation would affect its antioxidant property. In this research, the DPPH scavenging ability of CA and CA-NE were compared and the results are shown in **Figure 19**. As we can see from the figure, the capacity of carnosic acid to scavenge the DPPH radicals improved after encapsulation in

the nanoemulsion. As the blank nanoemulsion did not show DPPH scavenging activity, it is possibly because the protection effect of nanoemulsion on carnosic acid, makes the effective amount of carnosic acid in nanoemulsion to scavenge the free radicals is higher than that in the free carnosic acid solution.

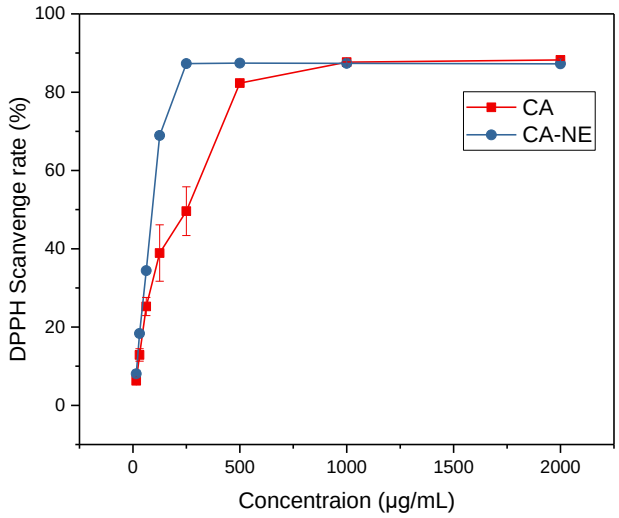



**Figure 19**. Antioxidant capacity of CA and CA-NE. DPPH radical scavenging activity; Results are the average of triplicates ± standard deviation.

## 3.4. Conclusion

In summary, we evaluated the basic properties of carnosic acid that related to the selection and preparation of carnosic acid nanoemulsion. Results showed medium chain triglycerids are the best choice as it has the highest solubility of carnosic acid. And the final formulation was selected based on a ternary phase diagram design from the one with relatively small size around 170 nm , small PDI (around 0.250) and high loading of carnosic acid (3.0%).Then the stability study under different storage temperaure and peroid was

conducted indicated lower teperautre and avioidance of light is better for the prolonged storage of the emulsion. The physical stability and chemical stability of CA-NE was also tested and results showed lower temperaure favorate the stability of carnosic acid degardation. When tested under different pH and inoic strength of the formulation, we found that the forumulaiton was stable from the pH 4-8, while it's not stable at high ionic strength, which is the underlying reason of the dramatically larger droplet size and even the separation to two phases of the whole CA-NE system. The viscosity data showed the nanoemulsion is a non-newtonian shear-thining fluid with low visocosity. Dispersion results confirmed the nanoemulsion is O/W type. The DPPH study showed encapsulation in nanoemulsion improved the scavenging capicity of carnosic acid. Overall, in this part, by optimizaiton of the preparation parameters and the characterization of the nanoemulsion system for carnosic acid, we successfully formulated the CA-NE nanoemulsion for future application and the method used to prepare the formulation can also be used for the oral delivery study of other similar compunds.

# CHAPTER 4. DETERMINING THE BIOACCESSIBILITY OF CARNOSIC ACID NANOEMULSION BY IN VITRO DIGESTION MODELS

## 4.1. Introduction

The objective of this work was to enhance the bioavailability of carnosic acid by the nanoemulsion systems. As we discussed, the bioavailability is affected by various factors during the digestion, absorption and metabolism after oral ingested. The Bioaccessibility is the first and a key factor for hydrophobic compounds like carnosic acid, as it is difficult for them to be solubilized and dissolution in the aqueous environment. In vitro digestion models could evaluate the bioaccessibility of carnosic acid nanoemulsion (CA-NE), which will be able to predict the bioavailability in vivo. Here, we applied two widely used methods, pH-stat lipolysis and TIM-1 models to kinetically and systematically determine the digestion process and bioaccessibility of carnosic acid nanoemulsion compared with unformulated oil suspension or water suspension.

## 4.2. Materials and Methods

### 4.2.1. Materials

Carnosic acid (95%) was obtained from China, PC 75 were a gift from American Lecithin Company, US. Medium-chain triacylglycerol (MCT) oil (Neobee 1053) was kindly provided by Stepan company. Fetal bovine serum (FBS) was purchased from Biowest (Riverside, MO).Sodium taurodeoxycholate was obtained from CalBiochem.Tris maleate, pancreatin, cell culture compatible dimethyl sulfoxide were obtained from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium, trypsin and the antibody penicillin-streptomycin were obtained from Life technologies (Grand Isaland, NY). Mobile phase

acetonitrile and water for HPLC was obtained with Pharmco- Aaper (Brookfield, CT). For all the experiments, chemicals were ACS grade without specifically specified.

4.2.2. Preparation for the testing formulation, MCT solutions and water suspension

Briefly, coarse emulsion was prepared by dissolve 3.0 wt% of carnosic acid in MCT oil (15.5%), lecithin (3%) were dissolved in water (78.6%). CA-NE was produced by high speed combined with high-pressure homogenization method. We firstly prepared the dispersion phase (oily phase) by mixing 0.92 g of CA in 4.8 g in the carrier oil MCT, heated up to 90 °C, and maintained until the CA fully solubilized and in order to inhibit the recrystallization, the temperature was kept above 70 °C. The continuous phase (aqueous phase) was formulated by dispersing the 0.9 g PC 75 lecithin into 24.3 g DI water and also heated up to the same temperature as oil phase. Once the two phases were ready, the aqueous phase was added into the oily phase, stir another two minutes and then the nanoemulsion was prepared by a two-step high-energy input method. Firstly, we prepared the coarse emulsion with size usually around micrometers by using high-speed homogenizer at 12000rpm within 3 minutes based on a preliminary study. Then the crude emulsion was downsized by applying the high-pressure homogenizer high pressure homogenizer instrument (EmulsiFlex-C6, AVESTIN Inc.) at 120MPa with four.

4.2.3. In vitro lipolysis of carnosic acid nanoemulsion and carnosic acid in MCT

In order to properly perform the simulation of our small intestine situation , the fed state *in vitro* lipolysis model was applied based on the previously published study with appropriate changes.[97] The required calcium and sodium salts, the other major components in bile salt with a certain ratio formulated the fed-state lipolysis buffer as in the published paper. Pancreatin solution containing the main digestion enzymes was prepared by

weighting more than the saturated amount dissolved by the lipolysis buffer freshly prepared right before the lipolysis study. The pancreatin in the supernatant from the oversaturated solution obtained by centrifugation at 2000 rpm for15 min and kept in the icy box until use. To begin lipolysis study, samples amount was adjusted to make sure the oil weight is 0.25g in the suspension or 1.60g of nanoemulsion sample. Samples were added into the 9mL lipolysis buffer. Then to initiate the lipolysis by pancreatin solution adding in and the experiment conducted under constant mixing. Within the two hours in vitro pH-stat simulation process, the temperature and pH was controlled around 37°C and 7.50, respectively. The pH was adjusted and controlled by intermittently adding with NaOH. The consumed amount of NaOH at every time point was keeping down by the computer and the entire mixture was used for further analyzing the amount of digested bioactive. Once the test is over, the obtained entire mixture was ultracentrifuged at 4 °C for 50 min at 50,000 rpm. The micellar carnosic acid which is the center phase was collected and either frozen in the freezer or immediately for HPLC analysis.

- Bioaccessibility of CA

After finishing the in vitro pH-stat lipolysis procedure, the center layer which contains our digested bioactive, here, carnosic acid was used to measure the amount of how much there is in the formed micelle or mixed micelle and other colloidal systems. As we introduced before, the micelle part of hydrophobic actives means the part that could be possibly absorbed by the gastrointestinal tract. Thus by measuring the concentration of carnosic acid in this phase by HPLC and times the volume of this micelle phase, we can get how much of the carnosic acid is bioaccessible. Then the bioaccessibility can be obtained by the following equation based on previous study.[97]

$$\%CA\ bioaccessibility = \frac{Solubilized\ \ CA\ of\ aqueous\ micelllar\ layer}{CA\ in\ the\ samples\ added} \times 100$$

- Extent of Lipolysis determination

We can convey the concept of the extent of lipolysis as how much and how easy of the lipid could be digested upon the finish of the in vitro lipolysis procedure, which can be reflected by the amount of NaOH added in. Based on the lipolysis curve, which is the NaOH consumed versus time, a digestion kinetic of bulk MCT and MCT in emulsion form can be compared.

The calculation for extent of lipolysis was based on stoichiometric ratio, which assumes that one triglyceride could release two fatty acids upon digested while consuming two molecules of NaOH, which was added to keep the pH constant. Since lecithin in the CA-NE also belongs to the lipid, the calculation of NaOH amount for CA-NE include both medium chain triglycerides (0.25 g) and lecithin (0.045 g). Extent of lipolysis was obtained by equation below referred to previous work.

$$\%Extent\ of\ lipolysis = \frac{Volume\ of\ NaOH \times Concentration\ of\ NaOH}{2 \times Mol\ triglycerides} \times 100$$

$$Mw\ of\ Triglyceride\ = \frac{3 \times 1000 \times Mw\ KOH}{SV}$$

4.2.4. Gastrointestinal Model

TNO gastrointestinal model-1(TNO, Zeist, Netherlands) is an in vitro simulation model mimicking the digestion process in human. It is maybe the most accurate model now for this purpose. In order to be better understand the situation in each step or parts of the upper

digestion system, the system is consisted with four parts, from up to down, gastric, duodenum, jejunum and ileum. In the system, temperature, pH, enzyme, gastric emptying, peristalsis, digestion fluid composition and secretion, transit time in each compartment are all precisely controlled by the program. Temperature was controlled at 37 ± 1 °C during the whole process. The composition of digestive fluids in this model was prepared according to previous published paper.[98]

Samples of either CA-Suspension or CA-NE were fed into the system the test was conducted for 6 h. The micellar fraction of CA, which represents the bioaccessible CA were obtained at 0.5, 1, 1.5, 2, 3, 4, 5 and 6 hours from both jejunum and ileum dialysate fluids, which were the amount that could passed through the 0.05 μm pore size hollow semipermeable fiber membrane (Spectrum Milikros modules M80S-300-01P). This system gives the possibility to analyze the effect of each part of the small intestine on the formulation digestion study. Meanwhile, the efflux part, which represented the amount will theoretically get into large intestine was also collected during 90 to 360 min. All the samples were immediately put into the freezer until analyzed. The experiment was conducted twice for each sample and analysis of each sample in duplicate.

### 4.2.5. Amount of carnosic acid determined by HPLC

CA was analyzed by a Dionex HPLC system with UV-Vis detector by our previous developed method.[86] The separation of the samples was performed on a C18 TSKgel ODS-100Z column (5 μm, 100Å; 4.6 mm × 150 mm, Tosoh, Tokyo, Japan) with the mobile phase composed of acetonitrile-0.1% phosphoric acid (60:40, v/v) at a flow rate of 0.6

mL/min with the detection wavelength was set at 230 nm. The injection volume was 20 µL.

Statistical Analysis

All data was depicted as means ± standard deviation. ANOVA and Tukey-test were conducted using SPSS 16.0 when necessary.

4.3. Results and discussion

4.3.1. pH-stat Lipolysis of carnosic acid nanoemulsion and carnosic acid in MCT

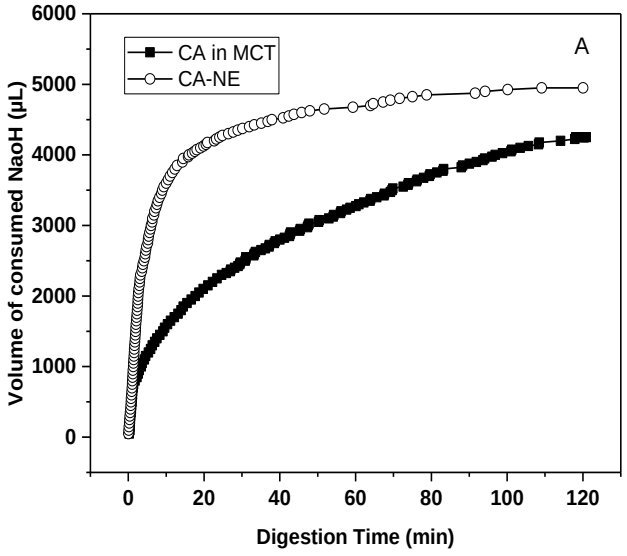
Because the low water solubility of lipophilic compounds, the beneficial bioactivities are difficult to reach. It was suggested that the bioavailability after taken orally of this kind of bioactives could be greatly enhanced if ingested together with lipids.[99] In this case, varieties of lipid delivery systems are developed to fulfill this target of increasing the water solubility and improving the bioactivity of the compounds. In vitro lipolysis has been applied to improve our understanding of ingested component solubilization and release, which is reliable, easy to perform and also have shown by many studies a good predicator of the digestion and final amount transfer to the circulation to be functioned situation of the body.[100] As we have shown that bioavailability can be affected by various reasons including the bioaccessibility, especially for the hydrophobic compounds. Usually, the higher amount of these compounds is bioaccessible for the absorption in small intestine, the higher chance they have increased bioavailability in the body. Improvement of oral bioavailability may achieve by increasing the solubility in gastrointestinal lumen.

4.3.1.1 Effect of emulsion on the digestion kinetic

To evaluated the digestion profile and Bioaccessibility of CA-NE. The in vitro lipolysis experiment was conducted between CA-NE and CA in MCT. The change of the digestion profile of CA-NE could be seen by the lipolysis curves in compared with CA in MCT by recording their volumes of NaOH consumed vs. digestion time (**Figure 20A**). As the ongoing of process, the pH in the mixture kept decrease as more free fatty acids were released by lipid lysis, NaOH is constantly added to keep the reaction situation at pH 7.5, the enzymes in pancreatin maintained their best function. From the titration profile, if we added the sodium hydroxide at higher rate meaning the higher rate of production of fatty acids from the lipase-digested lipids, which caused the decrease of the pH and indicated a fast digestion. What's more, upon the finish of the lipolysis process, the amount of triglycerides can be hydrolyzed by the lipase can be directly reflected by the total amount of NaOH used. As the more NaOH needed, the more fatty acids was released, meaning the higher ratio of triglycerides have been accessed for lipolysis.

After calculation, the digestion extent curve was obtained (**Figure 20B**). We can see more than 60% percent of the lipid digestion in CA-NE happened after 10 minutes of the in vitro simulated digestion process getting started, however, it took much longer time for the medium chain triglyceride containing carnosic acid sample exhibited this digesting extent, which appeared 50 min later. At 10 min, the extent of lipolysis of CA-NE was 68.4 ± 1.4%, while in MCT was 24.6 ± 1.4%, at 30 min, the digestion percent of CA-NE reached 85.8 ± 2.9%, while the percent in MCT was 44.6 ± 2.1%. Eventually, upon the 2 hours experiment finished, the extent of lipolysis of CA-NE reached 98.7± 2.6%, while in MCT it was 84.5± 3.2%. From the curve, we can see both CA-NE and CA in MCT oil showed a high degree of lipolysis within 2 hours digestion. The faster and more complete lipolysis

of nanoemulsion formulation than oil has also been observed by other studies.[34, 97] Previous studies have shown that the digestion rate is positively relative with the surface area of the formulation. As the emulsion is within nanosized, thus it has a relatively higher interface area for the enzymes to get access to, thus a faster digestion rate is expected. In our case, the CA-NE droplets has a larger contact area compared with the oil, CA-NE could be readily accessible by lipases which is solubilized in aqueous environment and thus facilitates lipids hydrolysis.



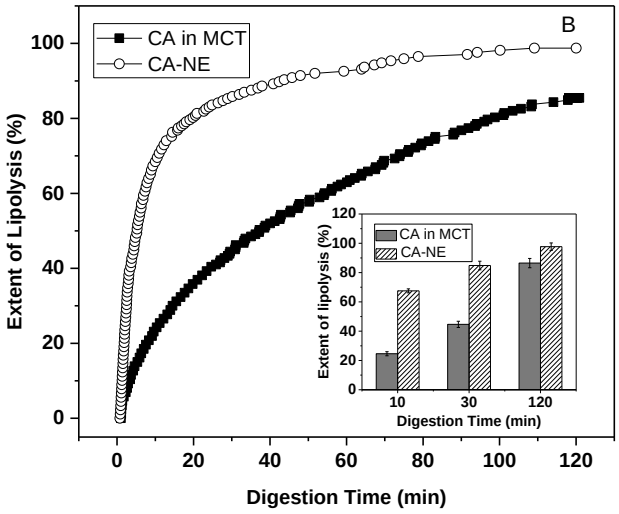



**Figure 20.** Comparison of pH-stat results. (A) Digestion profile of carnosic acid nanoemulsion, carnosic acid in MCT oil and carnosic acid in water as blank; (B) Extent of lipolysis of CA in MCT oil and nanoemulsion; (C) Extent of lipolysis at 10min, 30 min and 120min for carnosic acid nanoemulsion and carnosic acid in MCT oil.

4.3.1.2 Effect of emulsion on carnosic acid bioaccessibility

During lipolysis, as digested lipids are continuously formed into micelle and other colloidal structures with bile salts, phospholipids and lipids digestion products, any lipophilic compounds in the surrounding environment may also be encapsulated into the mixed micelle and thus the solubility of these hydrophobic compounds increased, which would be readily getting access to the small intestine absorption. As we know, the intestinal movement is always in a complicated and active mode, a lot of reactions and physiological are happening such as digestion, absorption and elimination. If the ingested hydrophobic bioactives can be efficiently, get involved into the constantly formed mixed micelle and other colloidal droplets and become soluble in the intestinal tract, a much higher possibility that they could stay longer in the intestine and, thus, a higher possibility to be absorbed.

Such that, the amount of carnosic acid in the clear micellar layer was measured to indicate the amount of carnosic acid that is bioaccessible. Results showed that the bioaccessibility of carnosic acid improved greatly by nanoemulsion when compared to MCT oil dissolved CA (**Figure 21**). In comparison to the MCT, dissolved CA, CA nanoemulsion enhanced the amount of carnosic acid in the micelle phase, which accounts for the bioaccessible part from 21.00% to 58.03%. The amount of CA in soluble form increased from 0.93±0.06 mg/mL to 2.65±0.06 mg/mL, which was a 2.8-fold improvement of the bioaccessibility of carnosic acid of formulated nanoemulsion compared with oil dispersion sample. The current finds were also proved by previous study that the fast digestion rate contributes to the higher amount to be bioaccessible for absorption of the hydrophobic bioactives.[101]

The percent of carnosic acid from the tested carnosic acid containing nanoemulsion sample and the control oil or water samples that account for the bioaccessibility calculation was detected by HPLC upon the finish of this pH-stat simulation of small intestine experiment. The bioaccessibility of carnosic acid improved greatly in nanoemulsion when compared to water suspension of CA (**Figure 21**). In comparison to CA suspension, CA nanoemulsion enhanced the amount of carnosic acid be possible for absorption from 4.6 % to 58.0%, which is almost 12.6-fold increase, and the results demonstrated the bioaccessibility of encapsulated actives could be greatly enhanced with the help of oil-based delivery systems. The possible reasons accounts for this significant bioaccessibility difference may be firstly, the presence of lipids during digestion in the CA-NE. Secondly, the small particle size of nanoemulsion could increase the water-solubility of CA, therefore a higher local concentration of CA within CA-NE than unformulated suspension, thus, a higher driving force to be solubilized to form the micelle.

There are several reasons that will affect the oral bioavailability of one compound. Such as the amount being bioaccessible to intestine lumen, the amount can be transported and absorbed by the intestine and finally the amount that could reach the system circulation. Such that, the target to have a have a higher bioavailability may be achieved by enhanced amount being bioaccessible.

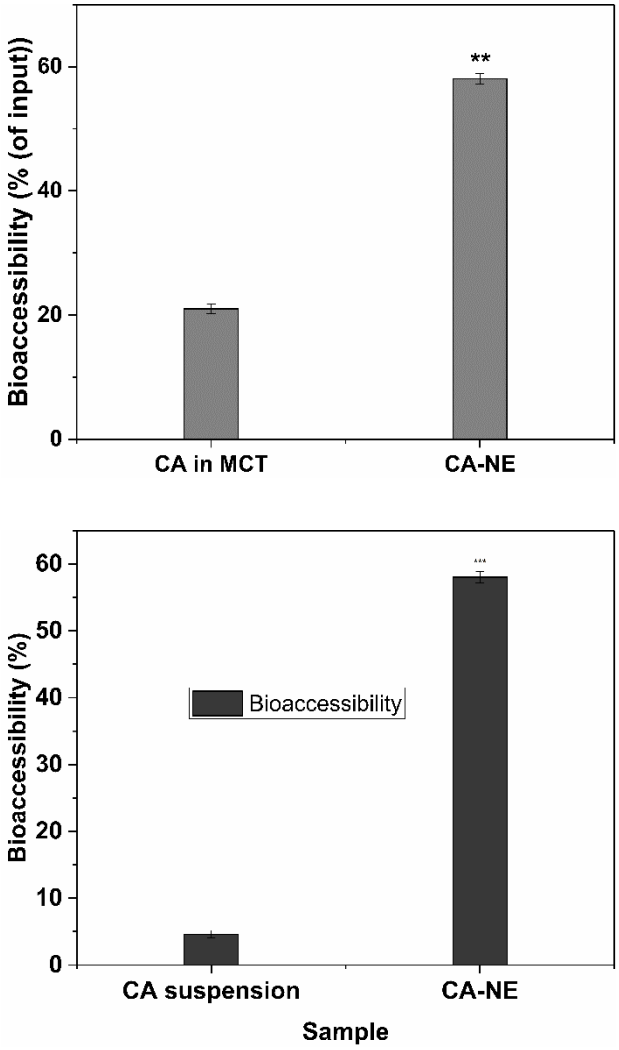



**Figure 21.** The carnosic acid bioaccessibility (% of input) after lipolysis in carnosic acid nanoemulsion, carnosic acid oil dispersion and water suspension samples. ($p<0.05$)

4.3.2. Comparison of Bioaccessibility of CA suspension and CA-NE by in vitro TIM-1 model

The digestion is a complex process that may be influenced by various parameters, even before absorption, such as the pH, temperature, ionic strength, peristalic movement, and GI digetion fluid secretions and so on. Such that, the precisely computer-controlled simulation instrument, TIM-1 model that simultes the different part of upper gastrointestinal lumen of human, was applied to investigate how the formulaiton altering the digesiton process in this work.[34] In the current study, we collected the digested samples from different parts that mimic the samll intestine inculding jejunum and ileum stands for the main absorption site for orally intake food at certain time intervals. Then the amount of CA in the jejunum and ileum filtrate can be obtained and detected after CA suspension or CA-NE were input into TIM-1 system. The concentraion of CA was measured by HPLC accordingly. The amounts of CA passed through the filters connected with Jejunum or Ileum are considered bioaccessible portion. The CA in the efflux of ileum is considered as those would enter human colon to be metabolized or interact with gut microbiota.

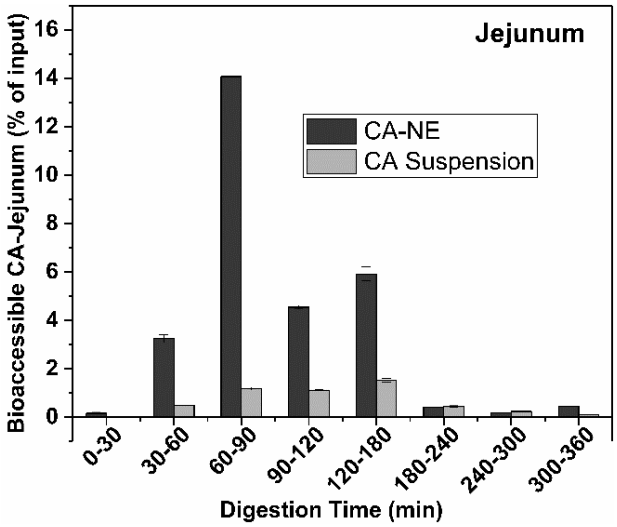
The bioaccessibility of CA was claculated as a percentage of the amount detected in different parts of small intestine to the amount of sample. The bioaccissiblity to the function of time as shown for duplicate experiments in **Figure 22**. In order to study the mechanism of CA absorption, the bioaccessibility was analyzed in different parts (Figure 21 A and B) separately.

When studied the digestion situation in Jejunum, for the CA Suspension sample, the digesion and absorption in jejunum of CA graduatlly increased with the first 2 hour after dosing, with the fastest increase observed during the second hour with 2.29%

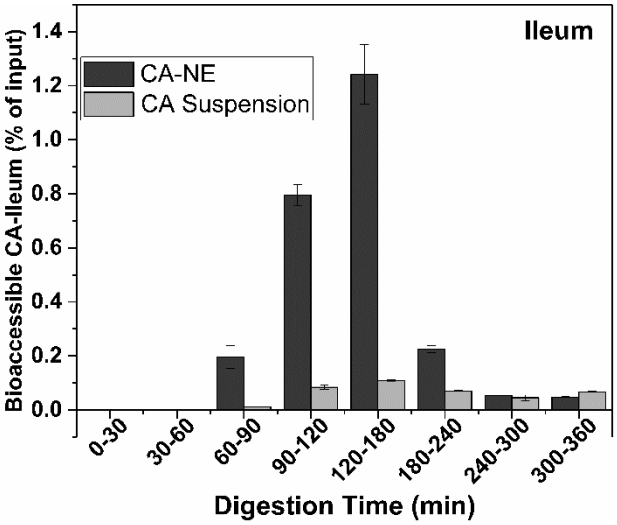
bioaccessibility increase, especially the highest increase was achieved between 60 and 90 min. The digestion rate then gradually decreased unitle the end of the digestion(**Figure 22A**). While for the CA-NE, the silimar digestion kinetic trends was observed as shown in Figure 3A, nevertheless, CA-NE showed a much hihger digestion rate at every time interval, the digestion rate of 18.60% was highest during 60-120min, especially 60-90min.

Compared with in jujunum, much lower amount of CA became bioaccessible in ileum for both formulations (**Figure 22B**) at every time interval, no CA was detected for the first hour, the digestion kept increasing, and the highest digestion rate occurred at 120-180 min with only 0.11% increase for CA suspension and 1.35% for CA-NE. From Figure 21C, we can easily conculde that the majority digestion and absorption of CA suspension and CA-NE occurred in jejunum and the fastest digesiton happened during the second hour with 19.6% increase, especially 60-90 min with a 14.26% increase of digesiton.

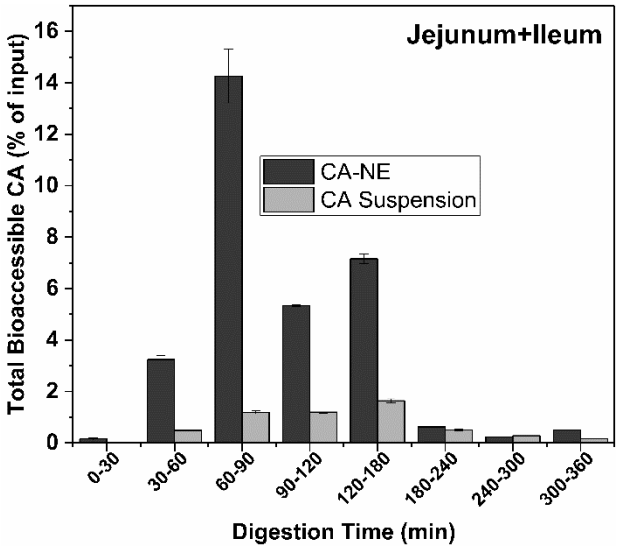
The mechanistic study suggested that the majority CA assessmebled into micelle and absorbed readily in the jejunum with the remaining was further formed in the ileum and the peak digestion and absorption of CA in ileum happened later than that in jejunum. The nanoemulsified CA exhibited higher dissolution rate in the intestine, which may be atrributed to the small droplet size and larger surface area, which would be benefical for a better oral bioavailability of CA.


Jejunum
CA-NE
CA Suspension
Bioaccessible CA-Jejunum (% of input)
0
2
4
6
8
10
12
14
16
0-30
30-60
60-90
90-120
120-180
180-240
240-300
300-360
Digestion Time (min)

(A)


Ileum
CA-NE
CA Suspension
Bioaccessible CA-Ileum (% of input)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0-30
30-60
60-90
90-120
120-180
180-240
240-300
300-360
Digestion Time (min)

(B)

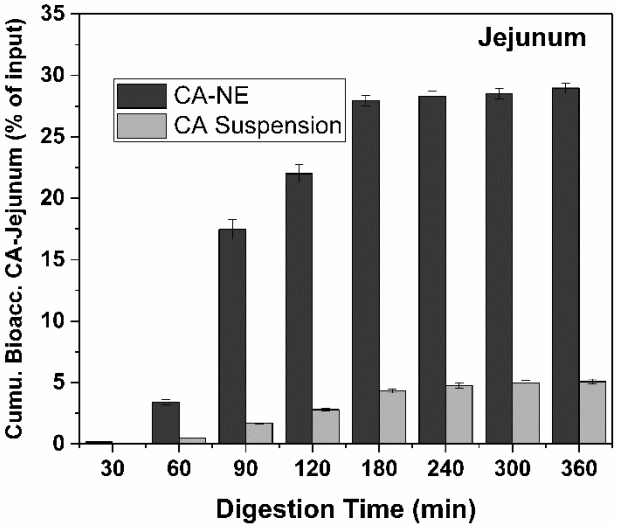



(C)

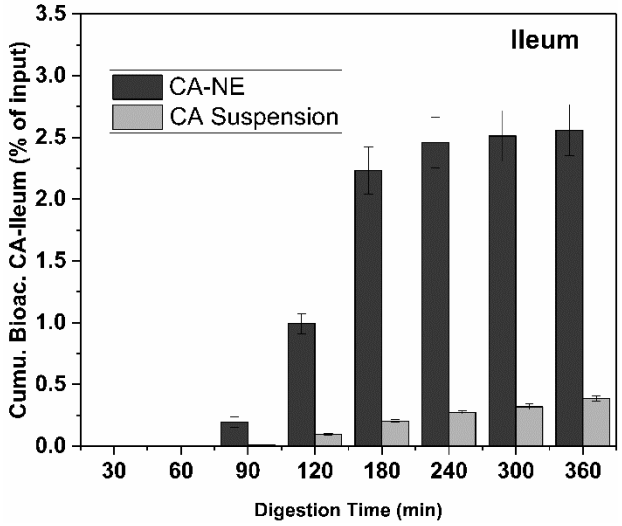
**Figure 22.** Bioaccessible CA fraction (% of intake) in every thirty minutes for first two hours and every one hour for following four hours' digestion period from jejunum and ileum of the TIM-1 model. (A) CA fraction in Jej.; (B) Bioaccessible CA fraction in ileum; (C) Entire bioaccessible CA fraction in jejunum adding on together with ileum filtrates as of the amount expressed as percent of input amount.

In a comparable overview of the digestion and absorption profile in the 6-hour digestion study, the cumulative bioaccessible CA in Jejunum, ileum and sum of jejunum and ileum were plotted in **Figure 23**. Upon finishing the digestion process of six hours, bioaccessibility in jejunum of the CA-NE was around 28.96%, while the overall bioaccessibility for CA Suspension was about 5.08%. This around 5.7-fold increase of bioaccessible. The same trend was found for the ileum samples, the amount digested from CA-NE (2.56%) was about 6.74-fold of the percent of original input digested from CA Suspension (0.39%). Combining the jejunum and ileum dialysates together, the cumulative

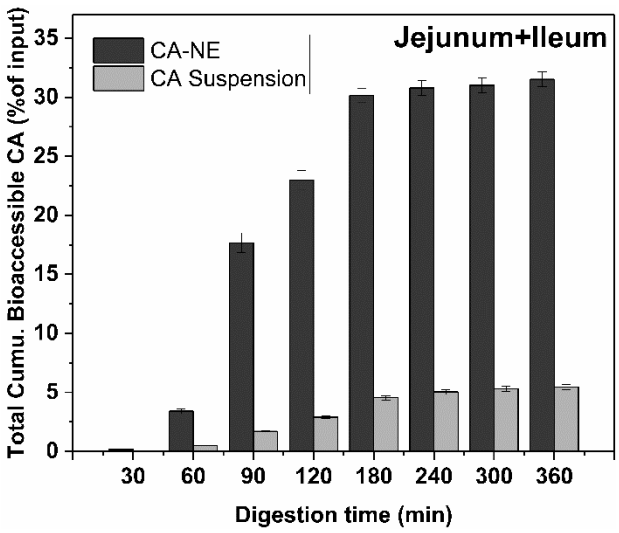
bioaccessibility of nanoemulsion formulation (31.52%) compared to the unformulated CA (5.46%), indicating a nearly 5.7-fold increase in the bioaccessibility obtained from this dynamic TIM-1 gastrointestinal model.




(A)




(B)




(C)

**Figure 23.** Cumulative CA bioaccessibility (% of input) recovered during the 6h of digestion in TIM-1 model for both CA-NE and CA Suspension. (A) Cumulative CA bioaccessibility in jejunum; (B) Cumulative CA bioaccessibility in ileum; (C) Overall cumulative CA bioaccessibility in both jejunum and ileum as of the amount expressed as percent of input amount.

**Table 7.** Bioaccessibility of CA as in CA suspension and CA-NE measured by TIM-1 Model

| Sample Name | CA intake (mg) | Bioaccessibility as of intake (%) | Efflux as of intake (%) |
|---|---|---|---|
| CA Suspension | 1500 | 31.52 ± 0.43 | 0.62 ± 0.02 |
| CA-NE | 1350 | 5.46± 0.22 | 0.18 ± 0.02 |

4.3.3. Discussion of the two in vitro Bioaccessibility models

In the current research, the nanoemulsion formulation was applied as a carrier for the hydrophobic compound carnosic acid. The surfactant used for the CA-NE formulation was soybean lecithin, which has a GRAS status, provided the CA-NE good stability and

solubility in aqueous systems. As the bioavailability of orally ingested compound are mainly affected by three parameters, including bioaccessibility, permeability and absorption, and metabolic stabilit.[102] For hydrophobic compounds such as carnosic acid, their low bioavailability is usually related with their low solubility and bioaccessibility in the gastrointestinal liquid, such that, improving the solubility of such compound is the main target to enhance the oral bioavailability. One convenient and efficient way to realize the target is to use a carrier formulation with lipid. pH-stat digestion simulation of small intestine study is a quick and reliable method to measure the bioaccessibility and thus could predict and screen the carrier formulations for hydrophobic compounds.[97] The in vitro lipolysis study in this paper demonstrated much higher bioaccessible CA after incorporated into the nanoemulsion system; oil is a necessary carrier for carnosic acid to be digested. Nanosized emulsion systems further offer the large surface area for the access of the lipase in the simulated intestinal fluids, thus faster and more micelle would be formed during the lipid breakdown, which resulted in a much higher (Bioaccessibility of CA compared with the CA suspension. However, this in vitro lipolysis model does not include a lot other physiological factors, which may overestimate the bioavailability in real situation.[103] Such that, more details controlled system, the TIM-1 upper gastrointestinal simulation model was further applied in our study to give a more comprehensive understanding and study of the dissolution and digestion process in the GI tract. TIM-1 model collected all the operation parameters from human data, and by precisely controlled physiological conditions simulating the upper digestion systems of human body. In our study using TIM-1 model, a 5.6-fold improvement of the bioaccessibility of nanoemulsion compared to suspension form was observed, which is lower than the 12.6-fold obtained from in vitro

lipolysis study. The results indicated that, besides the enzymes, other factors in the digestion tract will greatly influence the digestion and absorption and further on the bioavailability of carnosic acid. Such that, a more precise simulation should give a more accurate estimation of the how bioavailability would be in body and The TIM-1 model also gives the possibility to understand the details and mechanistically study the digestion activity in human upper GI tract.

## 4.4. Conclusion

With previously chapter, we formulated the carnosic acid nanoemulsion by lecithin, MCT and water. In this chapter, we tested the in vitro bioaccessibility of the formulated carnosic acid nanoemulsion by two in vitro models simulating the small intestine or upper gastrointestinal tract, namely, pH-stat in vitro lipolysis and TIM-1 models. Results from pH-stat showed an around 6-fold improvement of the bioaccessibility of carnosic acid in CA-NE compared with in oil suspension form, while this improvement was much higher compared with the water suspension. TIM-1 further showed a result of 5.6-fold improvement of CA bioaccessibility of nanoemulsion compared with the water suspension. What's more, the absolute bioaccessibility values obtained from the two methods are quite different, with the 58.0% and 31.5% of pH-stat and TIM-1, respectively. Considering the complex of GI tract, the TIM-1 model include more factors that mimic the digestion systems, thus the result of TIM-1 is more reliable. We did not test the oil suspension in TIM-1 study as the pH-stat method had firstly give us the idea that CA-NE had shown better bioaccessibility, considering the real situation consumers ingest nutrients, it's unlikely for people to drink an oil solution, so we just compared the water suspension with CA-NE.

We here proved that nanoemulsion could increase the bioaccessibility of carnosic acid compared with either the oil suspension or the water suspension.

# CHAPTER 5: IN VIVO EVALUATION OF THE BIOAVAILABILITY OF CARNOSIC ACID IN NANOEMULSION FORMULATION- PHARMACOKINETIC STUDY BY ANIMAL MODEL

## 5.1. Introduction

In order to perform its health-promoting activities, orally ingested carnosic acid must be able to reach into the systemic circulation and then bring to different targets tissues. As previously introduced, the bioavailability of carnosic acid is affected by many factors including the bioaccessibility we measured in last chapter. Other biological activities such as absorption, mechanism and transport to the circulation is hard to evaluate by the physical in vitro models. Thus, in order to get the bioavailability, which included all the factors, an animal study that study the pharmacokinetics of carnosic acid after oral administration of the nanoemulsion we designed here is necessary and meaningful.

Pharmacokinetics (PK) study is widely applied to evaluate the bioavailability of drugs, nutraceuticals *in vivo*.[104] We have introduced in the first chapter that right now there is few research on the bioavailability of carnosic acid and they gives quite different $C_{max}$, $T_{max}$ and even the bioavailability. Therefore, it is important to test the PK profile for carnosic acid in our nanoemulsion delivery system for better understanding of its relative bioavailability after oral consumption compared with the unformulated water suspension form.

## 5.2. Materials and Methods

### 5.2.1. Materials

Carnosic acid (CA, 95% purity) was obtained from Hunan, China. Gemfibrozil, NaCl, Lipase (150000 units/mg), Trypsin (7500 BAEE units/mg), fresh pig bile was obtained from Sigma (St. Louis, MO, USA), Alcolec PC75 was kindly provided by American Lecithin Company (ALC, Oxford, CT), Neobee 1053 MCT was gifted by Stepan. Ethyl Acetate were purchased from VWR (Radnor, PA). For all the experiments, Milli-Q water was applied. Mobile phase acetonitrile and water with HPLC grade was obtained with Pharmco- Aaper (Brookfield, CT).

### 5.2.2. Preparation of Carnosic Acid Nanoemulsion

CA-NE was produced by high speed combined with high pressure homogenization method. We firstly prepared the dispersion phase (oily phase) by mixing 0.92 g of CA in 4.8 g in the carrier oil MCT, heated up to 90 °C, and maintained until the CA fully solubilized and in order to inhibit the recrystallization, the temperature was kept above seventy degrees Celsius. The continuous phase (aqueous phase) was formulated by dispersing 0.9 g PC 75 lecithin into 24.3 g DI water and also heated up to the same temperature as oil phase. Once the two phases were ready, the aqueous phase was added into the oily phase, stir another two minutes and then the nanoemulsion was prepared by a two-step high-energy input method. Firstly, we can prepare the coarse emulsion with size usually around micrometers by using high-speed homogenizer at 12000rpm within 3 minutes based on a preliminary study. Then the crude emulsion was downsized by applying the high-pressure homogenizer high-pressure homogenizer instrument (EmulsiFlex-C6, AVESTIN Inc.) at 120MPa with four passes. Finally, the formulated nanoemulsion

composed with 15.5 MCT, 2.9% lecithin, 3.0% CA and 78.6% DI water) were collected from the equipment for each batch. Control group CA suspension was prepared by firstly prepared 0.5% CMC-Na solution, and a 30mg/mL CA suspended in the CMC-Na solution was prepared by bath sonication.

5.2.3. Experimental Animal

Male Sprague Dawley (SD) rats (weighted 265 ± 15g) were ordered from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). After one week accustomed to the environment, SD Rats were randomly housed into four groups (n=6), negative control, positive control and two experimental groups with three housed in one cage. All the rats were housed in a stable temperature (25 ± 1 °C) and humidity (50 ± 10%) with a standard 12 h light /12 h dark life cycles. The rats were fed with a standard diet and water libitum. The experimental protocol in this study has acquired approve by South China Agriculture University (No. 00185637) and the handling of animals obeyed to the International Guide for the Care and Use of Laboratory Animals principles.

5.2.4. Pharmacokinetics Study using SD Rats

The SD rats were divided to four groups with six rats for each, and the average weight of each group is around 300g. Group one is blank control which gavage only with NaCl solution; Group two is the Blank Nanoemulsion with no carnosic acid encapsulated; Group three is Control group with Carnosic acid Suspension, Group four is the Carnosic acid nanoemulsion.

The rats used in this pharmacokinetics study were fasted 12 h before administrations of 200mg/kg of CA in nanoemulsion or in CMC-Na suspension by oral gavage. At certain

time points (0.1, 0.5, 1, 2, 4, 8, 12 and 24 h after oral gavage); around 0.6 mL blood samples were collected by pre-heparinized 2mL vials. After every blood acquire, 0.6mL saline solution was administrated to compensate for the blood loss. The plasma samples were obtained by immediately centrifuged the whole blood at 4 °C at 4500 rpm for 15 minutes and then kept at -80 °C prior to HPLC measurement.

As for the sample preparation for analysis, 40 μL of gemfibrozil with concentration 64 μg/mL as internal standard was mixed with 200 μL of plasma. After thoroughly vortex, the plasma samples with IS was then subjected to a series of procedures, firstly extracted by 600 μL ethyl acetate repeated three times and then centrifugation was used to quickly separate the extraction phase with the blood at 4000 rpm for 3 min after each extraction. Ethyl acetate layer with extracted compounds from three times were combined. The solvent was then eliminated by nitrogen gas at ambient temperature, after this, the vial containing the extracted compounds were re-dissolved by 200 μL ACN, finally, centrifugation was performed again at 12000 rpm for 3 min at 4 °C to eliminate any insoluble compounds or impurities and then the samples are ready for detection.

From the figure of the concentration of CA in the plasma versus the time after oral gavage, two pharmacokinetic parameters can be obtained directly. One is $T_{max}$, meaning the time to reach the highest blood concentration of our target compound, and another Cmax, the highest blood concentration of the target compound during the entire experiment. The area-under-curve of plasma concentration-time curve ($AUC_{0\rightarrow t}$) were calculated by linear trapezoidal rule.

5.2.5. HPLC analytical conditions

CA concentration measured by HPLC system (Shimadzu, Japan) consisted with a quaternary solvent delivery system, a photodiode array detector equipped with automated injection. The column applied here is Agilent Zorbax Eclipse Plus C18, 4.6x250 mm, 5um (Santa Clara, CA). The detection of CA was conducted at 25℃ and a flow rate: 0.8mL/min. Mobile phase: Water with 0.1% phosphoric acid (Phase A) and ACN (Phase B). The chromatographic mobile phase gradient set as: 0-10min, 60%-70% B; 10-20 min, 70-70%B; 20-21min, 70-90% B; 21-25min,90%-90% B, 26-27min, 70%-60% B and the column re-equilibrated at 60% B for 9 min (total 36min), injection volume was 20 μL. Calibration curve was established by preparing of series of concentrations of CA (0.5, 1, 5, 10, 20, 50, 100 and 200 μg/m) in ACN with gemfibrozil as internal standard. The wavelength setting was 230nm.

Statistical Analysis

All data were depicted as mean ± standard deviation. ANOVA and Tukey-test were conducted by SPSS 16.0 when necessary. Significant difference was defined at $p < 0.05$.

5.3. Results and discussion

5.3.1. Oral bioavailability of CA-NE

In order to directly evaluate the influence of nanoemulsion encapsulation on the bioavailability of carnosic acid. Pharmacokinetics analysis was performed using rats model. In this single dose PO study, CA suspension, CA-NE, B-NE (positive control) or saline solutions (negative control) were oral gavaged to the rats to elucidate the digestion,

absorption and transfer to the systematic circulation of CA in either CA-NE or CA suspension (does: 200mg/kg body weight). The HPLC result obtained with blank nanoemulsion group (figure not shown) refer to a basic value of internal carnosic acid in rats, which showed no internal carnosic acid existed in the rats.

A plasma CA concentration versus time profile was calculated and determined for CA suspension and CA-NE groups was drawn as **Figure 24.** The basic and most important pharmacokinetic parameters are summarized in **Table 8**. The results showed a distinctive pharmacokinetics profiles between different carrier forms.

As shown in **Figure 24**, the nanoemulsion form of CA increased the plasma CA concentration at every time point compared to the CA suspension after oral intake. The profile of administration of CA suspension showed rapidly absorption peak with $T_{max}$=0.5 h while more interestingly, the dosing of CA-NE showed two absorption peaks at 1 hour ($T_{max}$=1 h) and 6 hour after oral dosing. The difference in $T_{max}$ may relied on the more viscous and sustained release of CA from CA-NE. The two peaks plasma peaks maybe because the redistribution and enterohepatic circulation existed, which exerted carnosic acid back to the gastrointestinal tract through bile for CA-NE administrated group. It was believed that this phenomenon could increase the absorption and effective concentration of bioactive compounds. Previous study on the rosemary extracts bioavailability also found this double-peak situation for carnosic acid even though the latter research found that this phenomenon is not very obvious.[56, 105] During 6 to 12 h, the plasma concentration of CA within CA-NE-fed group was significantly higher than that dosed with CA suspension at every time point, and at the last point of 24 hours, a very low concentration of CA was detected in CA-NE group while CA in the rats of CA suspension group was below the

detection. The delayed and sustained release and higher concentration of CA in plasma after second peak of CA-NE indicated that nanoemulsion is suitable as a controlled-release carrier for CA, which could maintain high and adequate bioactive levels in plasma and reducing the dosing frequency. From **Figure 24**, we also found that CA-NE gave a $C_{max}$ value of 53.79 μg/ mL, which was 13.3% higher than the unformulated CA.

There are three former studies showed various $T_{max}$ for oral administration of carnosic acid by different rats models, with 20min, 120 min, 137 min respectively,[55-56, 105] which maybe because different solubilizing agents and rats models were applied in these studies. Nevertheless, as the first formulated nanoemulsion delivery system for CA, our results here showed an apparent improvement of bioavailability by CA-NE compared with CA suspension. More importantly, CA-NE could be applied in various aqueous systems and is a promising delivery form to enhance the amount of CA that can be used by the body through enhancing the solubility, retention time and digestion kinetics in gastrointestinal tract.

The HPLC profile of the plasma samples after extraction and a series preparation procedure and finally the amount of CA was detected by HPLC. The **Figure 25 (A)** was a representative HPLC profile of one experimental rat from the CA-NE treated group after 10 minutes of oral intake of the carnosic acid nanoemulsion. As the figure showed, internal standard gemfibrozil (RT: 12.675min) can be successfully separated with our target compounds carnosic acid (RT: 16.227min). There are two unidentified peak between them, so by comparing with the blank control group rats, which take no carnosic acid or carnosic acid formulation, we found the same peaks in the HPLC profile as shown in **Figure 25 (B)**. It is clear that our HPLC method is capable and accurate to examine the amount of carnosic

acid in the plasma with standardized extraction procedure, appropriate choice of internal standard, optimized HPLC conditions and high sensitivity limitation.

**Table 8.** Relative bioavailability and pharmacokinetic parameters of CA when CA-NE and CA suspension were orally administered to male SD rats (n = 6)

| Formulation | $T_{max}$ (hour) | $C_{max}$ (μg/mL) | AUC $_{0-t}$ (μg /mL*hr) | AUC $_{0-\infty}$(μg /mL*hr) | Relative Bioavailability |
|---|---|---|---|---|---|
| CA Suspension | 0.5 | 47.46 ± 3.43 | 251.93 | 523.54 | |
| CA-NE | 1.0 | 53.79 ± 7.48 | 544.28 | 994.75 | 2.16 |

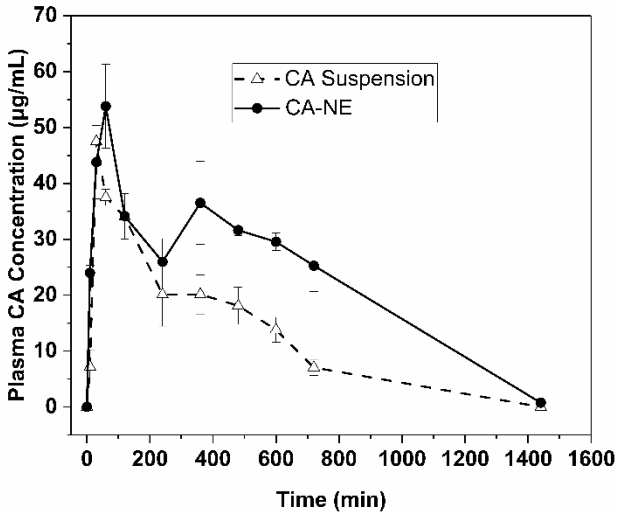
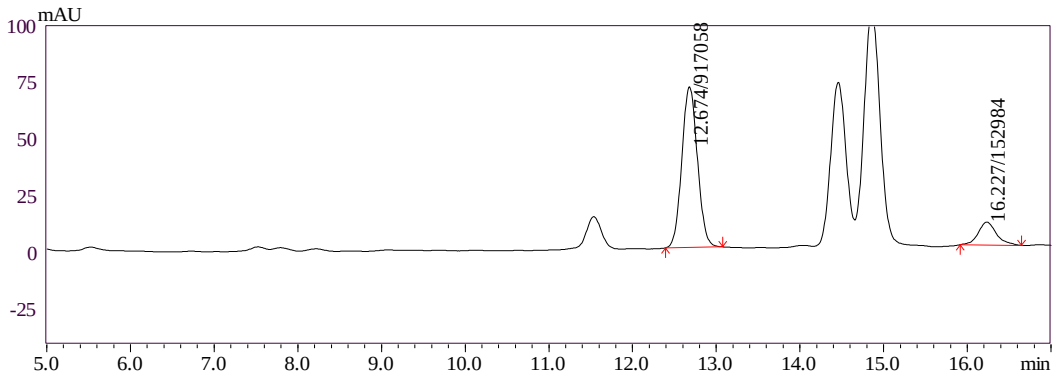
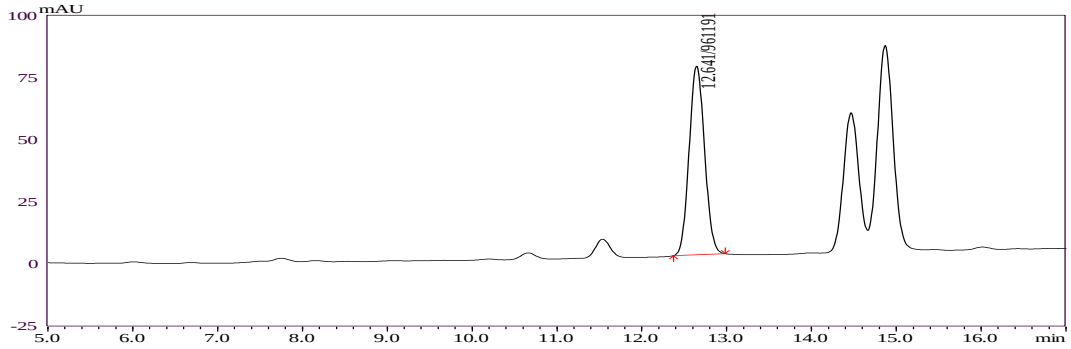



**Figure 24 .** Carnosic acid amount in the blood versus time dosing with CA-NE (solid line) or free CA (dashed line). Results are depicted as mean ± SD (n = 6).




(A)




(B)

**Figure 25.** HPLC picture of (A) Plasma of rats treated with CA-NE. Data showed were representative data at the 10 min collected sample, with internal standard: gemfibrozil (12.675min), carnosic acid (16.227min) (B) Blank plasma of rats spiked with internal standard gemfibrozil (12.675min) as a reference.

5.3.2. Discussion of the results

The gastrointestinal tract is the main digestion part for orally administrated nutraceuticals. The absorption of carnosic acid happened mainly in jejunum, which has been confirmed by TIM-1 results. The produced carnosic acid nanoemulsion (CA-NE) increased

significantly the concentration of carnosic acid in plasma compared to the CA suspension. The results can be seen from the calculated pharmacokinetic parameters (Table 7). In order to be uptake by the peripheral tissues, the plasma concentration of carnosic acid needs to be higher than the plasma basic values. The basic plasma AUC value can be obtained from the group of rats treated with physiology saline as control, as there was no CA detected, so the carnosic acid in plasma should be able to transfer to different tissues. In general, the carnosic acid peaked twice in the nanoemulsion dosage form at 1 hour and 6 hours, and came to baseline after 24 hours. The nanoemulsion increased the AUC value (544.28 μg /mL*hr) more than twice to the CA water suspension AUC value (251.93 μg /mL*hr). Thus, we can say the bioavailability of carnosic acid was improved by the nanoemulsion. In our study, after a series processing procedure for preparing the samples to be analyzed by HPLC, we didn't find any new peaks appeared in the HPLC profile of the plasma samples processed from all the tested rats, so the metabolites remain unknown, thus further analysis is needed.

## 5.4. Conclusion

As we know, it is necessary to conduct in vivo animal studies, if the bioavailability information is necessary, as in vitro methods are not able to offer this data. In our current study, by using the in vivo animal study, we found a significantly difference of the plasma concentration of carnosic acid between the nanoemulsion group, with a sustained $T_{max}$ and a higher $C_{max}$. The calculated AUC value proved a more than two-fold improvement of the bioavailability compared to the CA suspension group. The reason could be attributed to the enhanced solubility and more fast and more complete digestion of lipids, which promoted the mixed micelles formation to incorporate more carnosic acid in the digestion lumen.

Thus, in this chapter we further confirmed the nanoemulsion composed with soy lecithin, MCT and water could improve the bioavailability of carnosic acid. Thus, the nanoemulsified carnosic acid has a great potential to be used in functional food area. What's more, this optimized formulation may be further used to encapsulate other similar nutraceuticals aiming to expand their application or improved their oral bioavailability.

# CHAPTER 6 INVESTIGATION OF THE BIOACTIVITY OF CARNOSIC ACID NANOEMULSION BY IN VITRO CELL MODELS

## 6.1. Introduction

There will be oxidative stress when the produced reactive oxygen species (i.e. $O^2$–, $H^2O^2$) exceeded the antioxidant ability of cellular antioxidants in living organism. This excess ROS generated are highly reactive and they tend to initiate oxidation chain reactions and cause the chemical and functional changes of lipids, proteins and DNA,[106] which will in turn to cause the cell dysfunction and tissue injuries. In addition, the production of ROS which is mediated by NADPH oxidase (NOX) will cause the change of microenvironment in host tissue and oxidative stress on sites of chronic inflammation. This will activate the excessive release of many compounds that would promote the happening of inflammation, some of the most common seen compounds are like interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), nitric oxide (NO) and so on. It has been overwhelmingly documented that the uncontrolled production of ROS and inflammation stimuli will cause the progression of metabolic and degenerative diseases in human body, such as cancer, cardiovascular disease, neurodegenerative diseases and diabetes.[107-109] Usually there are two defense systems to combat the oxidative conditions, first systems include some antioxidant enzymes, like superoxide dismutase (SOD), glutathione peroxide, which are capable to react with either the formed free radicals or the molecules are very liable to produced reactive oxygen radicals. Physiological and dietary antioxidant are also performed as the secondary defense systems, such as glutathione, polyphenols and other natural antioxidants.[110]

Fruits, vegetables and herbs are excellent sources of bioactive compounds, consuming them may increase antioxidants and have desirable health benefits for prevention of chronic diseases as supported by former studies.[111-114]. Carnosic acid (CA) is one of those natural compounds that have attracted considerable attention. CA is a tricyclic diterpene that was firstly discovered in sage and later was found at much higher levels in rosemary leaves.[88] With the *o*-dihydroxylated phenolic ring and a carboxylic acid group, carnosic acid, also regarded as phenolic diterpene possess powerful antioxidant activity in food systems. More importantly, is has also been found with great chronic disease preventing functions such as anticancer, anti-inflammation, antiobesity, antidiabetic, anti-neurodegenerative disorders and so on.[44, 115-117] Oral delivery is considered as the most convenient and acceptable route for antioxidant, however, being a highly hydrophobic compounds, carnosic acid has a poor solubility in water and oil at room temperature. The low solubility and dissolution led to its poor bioaccessibility for the gastrointestinal absorption. Such that, development of a suitable carrier from natural materials that can increase the solubility in water phase and dissolution in gastrointestinal fluids is of great interest from functional food and pharmacological viewpoint, which will help expand the application of CA in food area and to enhance its oral efficacy, such that the bioactivities could be achieved. One of the edible delivery systems have been widely applied to achieve this target is nanoemulsion. The nanoemulsion formulation is proved capable of improve the bioavailability and anti-cancer activities of phytochemicals by many researches.[34, 118-119]

To our best knowledge, right now there was no study elucidating how the bioaccessibility would be changed of CA by nanoemulsion formulation. Therefore, the in vitro bioaccessibility of CA in nanoemulsion is necessary to be examined. As the

bioactivities and pharmacological properties of CA maybe primarily correlated with its ability to resist the oxidative stress by scavenge the free radicals and to inhibit the production of the inflammatory related molecules. The evaluation of the antioxidant and anti-inflammation capacity of CA after encapsulation in nanostructured carrier would be interesting and necessary. Right now, most of the studies applied chemical antioxidant assays to study the antioxidant property of nanostructured antioxidant, which doesn't take account into the pH, temperature, uptake and metabolism in the physiological situations. Physiological systems are very comprehensive and sophisticated and these chemical methods are hard to mimic or accurately predict the in vivo antioxidant assay. In this situation, in vitro cell culture models is a more reliable and cost effective approach to address the antioxidant capability and anti-inflammatory activity. Until now, there is insufficient study on how the encapsulation will affect the bioaccessibility of carnosic acid and also no study on the antioxidant and anti-inflammation properties of carnosic acid nanoemulsion using cell-based biological environment. Herein, the objective of the current study was to apply the cellular antioxidant assay (CAA) using HepG2 cells and anti-inflammatory property using RAW 264.7 cells to evaluate how nanoemulsion system will affect the antioxidant ant anti-inflammatory properties.

## 6.2. Materials and Methods

### 6.2.1. Materials

Carnosic acid (95%) was obtained from China, PC 75 were a gift from American Lecithin Company, US. Medium-chain triacylglycerol (MCT) oil (Neobee 1053) was kindly provided by Stepan company. Analytical standards for carnosic acid (>99%) and cell culture compatible dimethyl sulfoxide (DMSO) were obtained from Sigma Co..

DMEM, trypsin, penicillin-streptomycin, PBS were obtained from Life technologies and FBS was obtained from Biowest. For all the experiments, Deionized water obtained from a Milli-Q system was used. The mobile phase acetonitrile (ACN) and water for CA detection were purchase from Pharmco (Brookfield, CT). All other chemicals were purchased from Sigma-Aldrich and were ACS grade without specifically specified and applied without further treatment

6.2.2. Preparation of Carnosic acid nanoemulsion (CA-NE)

CA-NE was produced by high speed combined with high pressure homogenization method. We firstly prepared the dispersion phase (oily phase) by dissolving 0.92 g of CA in 4.8 g in the carrier oil MCT, heated up to 90 °C, and maintained until the CA fully solubilized and in order to inhibit the recrystallization, the temperature was kept above 70 °C. The continuous phase (aqueous phase) was formulated by dispersing the 0.9 g PC 75 lecithin into 24.3 g DI water and also preheated to 70 °C. Once the two phases were ready, the aqueous phase was added into the oily phase, the mixture was stirred for another two minutes and then the nanoemulsion was prepared by a two-step high-energy input method. Firstly, we can prepare the coarse emulsion with size usually around micrometers by using high-speed homogenizer at 12000rpm within 3 minutes based on a preliminary study. Then the crude emulsion was downsized by applying high pressure homogenizer instrument (EmulsiFlex-C6, AVESTIN Inc., Ottawa, Canada) at 120MPa with four passes Finally, the formulated nanoemulsion composed with 15.5% MCT, 2.9% lecithin, 3.0% CA and 78.6% DI water were collected from the equipment for each batch.

6.2.3. Droplet size and size distribution

In order to control the stability and consistence of the physical properties between each experiment, the droplet size and size distribution should be measured each batch for the following experiment. Droplet size and polydispersity index (PDI) of the nanoemulsion was measured by DLS technique based particle sizer (90 plus, Brookhaven Instruments Corporation, NY, USA). All the samples were measured at the same instrument setting with the laser light scattering angle of 90°. All the samples need to be diluted with the according continuous phase before measurement in order to get reliable data. All the measurements were conducted in triplicate.

6.2.4. Measurement of Carnosic acid by HPLC

CA was analyzed by a Dionex HPLC system with UV-Vis detector by our previous developed method.[86] The separation of the samples was performed on a C18 TSKgel ODS-100Z column (5 μm, 100Å; 4.6 mm × 150 mm) with the mobile phase composed of acetonitrile-0.1% phosphoric acid (60:40, v/v) and the flow rate was kept at 0.6 mL/min. 20 μL was injected for each sample and the detection was monitored under 230 nm.

6.2.5. Cell maintenance and cytotoxicity by MTT assay

HepG2 and RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2 cell was maintained in culture medium composed of DMEM added with 10% FBS and antibodies penicillin- streptomycin at constant temperature 37°C and humidified conditions with 5% $CO_2$. The medium for RAW 264.7 was DMEM supplemented with 10% deactivated FBS and antibodies and was incubated under the same conditions for HepG2.

The cell viability of CA, CA-NE and blank nanoemulsion (B-NE) were measured by a well-established MTT assay.[120] MTT assay has been applied to see the cytotoxicity of CA-NE as taking the unformulated carnosic acid with DMSO-dissolved one. On the first day of the experiment, cancer cells were started to culture in 96-well plate incubated in the culture media containing all the basic nutrients and balance salts they need for healthy growth. After 24h incubation, the media were discarded; the cells were then added with a series of concentrations (six replicates) of CA-NE or CA in DMSO diluted with the complete media at different ratios. Blank nanoemulsion and negative control (untreated cells) were cultured at the same batch in every independent experiment as reference value for the calculation and control of the culture conditions. After 24 h incubation with different samples, cells were incubated with 0.5 mg/ml MTT for 1.5 hours in dark. The final MTT dissolved in DMSO were tested by Biotek microplate reader at 570 nm, and 490nm as reference.

6.2.6. Cellular antioxidant activity (CAA) by HepG2 cell model

The method was based on a previous published paper with minor modification.[121] HepG2 cells were firstly inoculated at $6 \times 10^5$ /mL density on a black 96-well microplate first day and cultured overnight. On the second day, the old culture medium was aspirated and the cells were washed with PBS. Then different concentrations of samples plus 25 μM DCFH-DA added into the cells. After one-hour treatment at 37 °C, the medium was discarded and washed with PBS again. After that, ABAP solution was applied to all the wells except the blank control. The fluorescence was then read immediately by microplate reader preheated to 37 °C by setting the excitation and emission of 485/538nm. Totally thirteen points were collected and the data was collected kinetically for one hour. Cells

with the fluorescent indicator DCFH-DA and free radical generator ABAP are positive control, cells with DCFH-DA but no ABAP were blank control. The assay was conducted at least three replications, and the data from one representative experiment (n = 4 wells) were presented.

Calculation of CAA value. By subtraction the readings of blank control from the positive control or sample, the CAA value was calculated by doing integration of the area under the curve (AUC) of fluorescence to time.

$$CAA\ Value = \left(1 - \frac{AUC\ (treatment) - AUC\ (Blank\ control)}{AUC\ (Positive\ control) - AUC\ (Blank\ control)}\right) \times 100$$

Where AUC (treatment) is the integrated area of sample, AUC (Positive control) and AUC (Blank control) are the integrated area of the positive and blank curve, respectively.

6.2.7. Cellular uptake of CA-NE by HepG2

To study the cellular uptake qualitatively, cells were seeded on a 12-well dish covered with glass coverslips and cultivated in 1 mL complete medium. After overnight incubation, washed the cells, and equivalent volume of culture medium within the fluorescent compound-coumarin-6 stained CA-NE were treated to the cells and the cells were incubated with predetermined time periods in the incubator. When finished treatment, the solution was removed by aspiration, and cold PBS was applied three times to the cells for eliminating the nanoemulsion droplets that have not been got inside of the cells. Then the HepG2 cells were fixed with 4% paraformaldehyde for 15 minutes at ambient conditions. After another set PBS wash, the cells were further cultured with 10 μg/mL DAPI staining solution for 30 minutes. After washing again with the buffer, finally PBS was added to

maintain the HepG2. Now the uptake of the CA-NE by HepG2 cells are ready to be observed by confocal laser scanning microscope (CLSM, Zeiss LSM 710 Confocal Microscope) by appropriate settings. Fluorescence intensity was calculated and analyzed using Image J software and expressed as Relative fluorescence intensity as compared to the highest fluorescence obtained from the experiment.

6.2.8. RAW 264.7 Cellular release of nitric oxide (NO)

RAW 264.7 cells were cultured in 96-well plated at $4\times 10^6$cells/ml and incubated for 24h and then stimulated with LPS (100 ng/mL) with or without CA, CA-NE and controls at various concentrations for 24 h. Nitrite assays were performed using Griess Reagent System.[122] 50μL of treated culture media was mixed with 50μL of Griess reagents, the absorbance were measured at 570 nm after 10min reaction. Sodium nitrite was the standard preparing working solutions from 1-100 μM.

The RAW 264.7 cells with no treatment but only culture media are set as the blank control. Cells stimulated with LPS and samples were negative control, cells treated with LPS but not samples were positive control. The ability on the inhibiting of NO production can be obtained by the equation below:

$$(\text{Inhibition of NO})\ \% = (NO_{\text{Positive control}} - NO_{\text{sample}})/NO_{\text{positive control}} \times 100$$

6.2.9. RAW 264.7 Cellular release of TNF-α

RAW 264.7 were cultured for 24h, and the cells were then treated with three different media, namely, culture medium only, LPS alone or LPS together with samples for 24 h, then the cell culture supernatant was collected to measure the amounts of cytokines

followed the instructions within the ELIDA kit. Commercial ELISA kit (R&D Systems, Minneapolis, MN) was applied to detect the TNF-α produced in the supernatant.

Statistical Analysis

All measurements were done independently triplicate. Results are depicted as means± standard deviation (SD). The significance of the differences between groups was tested using ANOVA and Turkey test when necessary. All statistical analysis was done using SPSS 16.0.

## 6.3. Results and Discussion

### 6.3.1. Physicochemical properties of the CA-NE

The CA-NE formulation was fabricated as an oral intake carrier of CA by designing a ternary phase diagram from water, MCT and lecithin. The storage stability and high loading capacity makes it possible for further investigation of the in vitro bioaccessibility and bioactivities. In the present study, all the CA-NE were produced and characterized before conducting each experiment and the exact amount of CA each time is measured. The CA-NE used in this study possess a loading capacity 2.6-3.0%, size around 170nm, size distribution expressed by PDI around 0.240 and surface charge of the nanoemulsion droplets measured as zeta potential values around -57.2mV. The size of particle below 200 nm is usually regarded as nanosized with good stability and cellular uptake properties and PDI values smaller than 0.25 means a narrow size distribution which contributed to good stability during storage. Zeta potential has been used to characterize the surface charge of emulsions. In some literature it was suggested if the zeta potential measured is above 45mV, it’s very likely that the formulation possessing high stability. This mainly because high

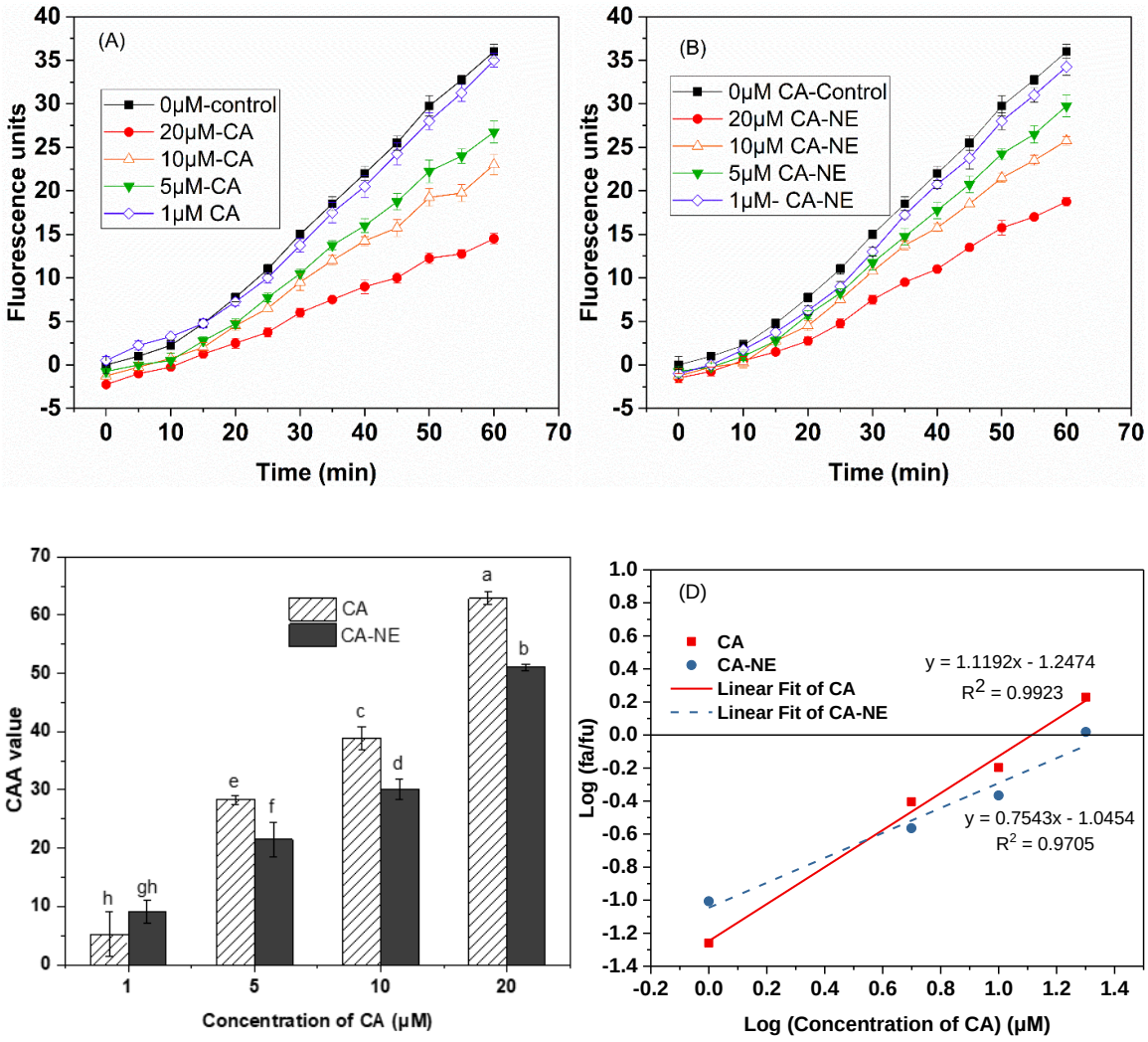
zeta potential in a colloidal system means strong repulsive force between the droplets to overcome the natural tendency to aggregates.[123]

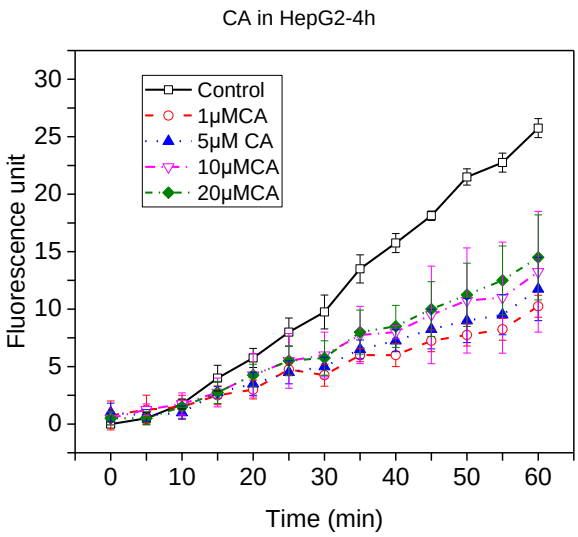
6.3.2. Cellular Antioxidant activity in HepG2 cells

Carnosic acid had been suggested and proved being a powerful antioxidant to be used for food area and health promoting activity by many studies.[124] Until now, there is no study about the antioxidant of CA by cell models, which would include the influence of cellular uptake, metabolism, enzymes, cell signaling and is expected to be more closely reflecting the antioxidant ability of in vivo circumstances.[125]

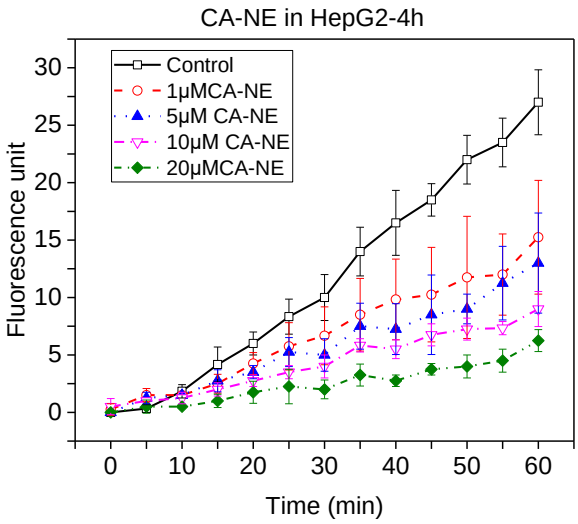
DCFH-DA is a non-fluorescent dye by itself, it firstly diffused into cells and become DCFH after elimination of acetate groups by intracellular esterases, free radicals oxidation of DCFH will produce fluorescent DCF. At the same time, the added antioxidant would scavenge the free radicals to decrease the fluorescence, such that, the lower of the fluorescence means the more powerful the antioxidant capability of the added compound exhibited. In our study, the cell viability experiment was firstly conducted for free CA, CA-NE and blank nanoemulsion by MTT assay to find the non-toxicity concentrations to perform the CAA assay (data not shown here). Results showed that when the concentration of CA below 50 μM, nearly one hundred percent of cell viability observed for all the samples. Such that the following treatment of HepG2 cells are below this concentration. When performing this cellular antioxidant assay, the cells were washed after treated with CA or CA-NE, which could eliminate the influence of the samples that neither absorbed into nor tightly adhered to the cells. In our CAA experiment, the fluorescence change during 1 hour of nanoemulsified CA, free CA and blank nanoemulsion were recorded and as the blank nanoemulsion showed no effect of the fluorescence, here we only showed the

kinetic fluorescence of DCF of free CA and CA-NE (**Figure 26A**, **Figure 26B**). As we can see, both free CA and CA-NE treatment inhibited the increase of fluorescence from DCF formation by free radical oxidation as low as 1 μM and this inhibition behavior is dose dependently, free carnosic acid showed lower fluorescence at each concentrations. The CAA value-dose was plotted by calculated CAA value by integration of the fluorescence-time curve for free CA and CA-NE at each concentration (Figure 25C).

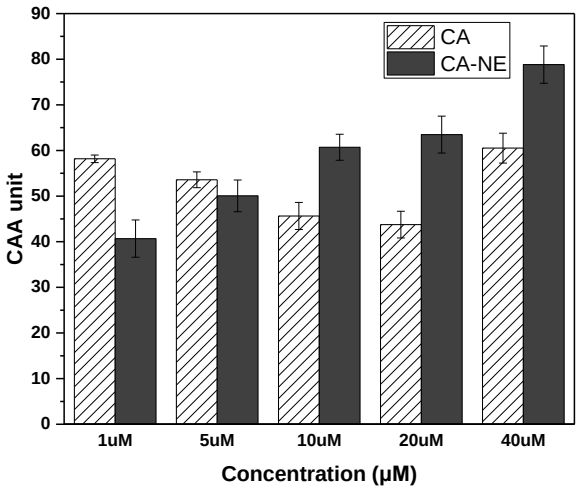





(E)




(F)




(G)

**Figure 26.** ABAP free radical-induced oxidation of DCFH to DCF and the inhibition of formation of free radicals indicated by DCF fluorescence by CA and CA-NE treated for 1 hour. Time–fluorescence profile for the inhibition of ABAP produced free radical on DCF

formation by free carnosic acid (A) and carnosic acid nanoemulsion (B); Dose-CAA value obtained by integration of each concentration of carnosic acid and carnosic acid nanoemulsion (C) (Mean ± SD, n = 4). $EC_{50}$ of free CA and CA-NE were calculated by median effect plot (D). Different letters indicated significantly differences ($p < 0.05$).

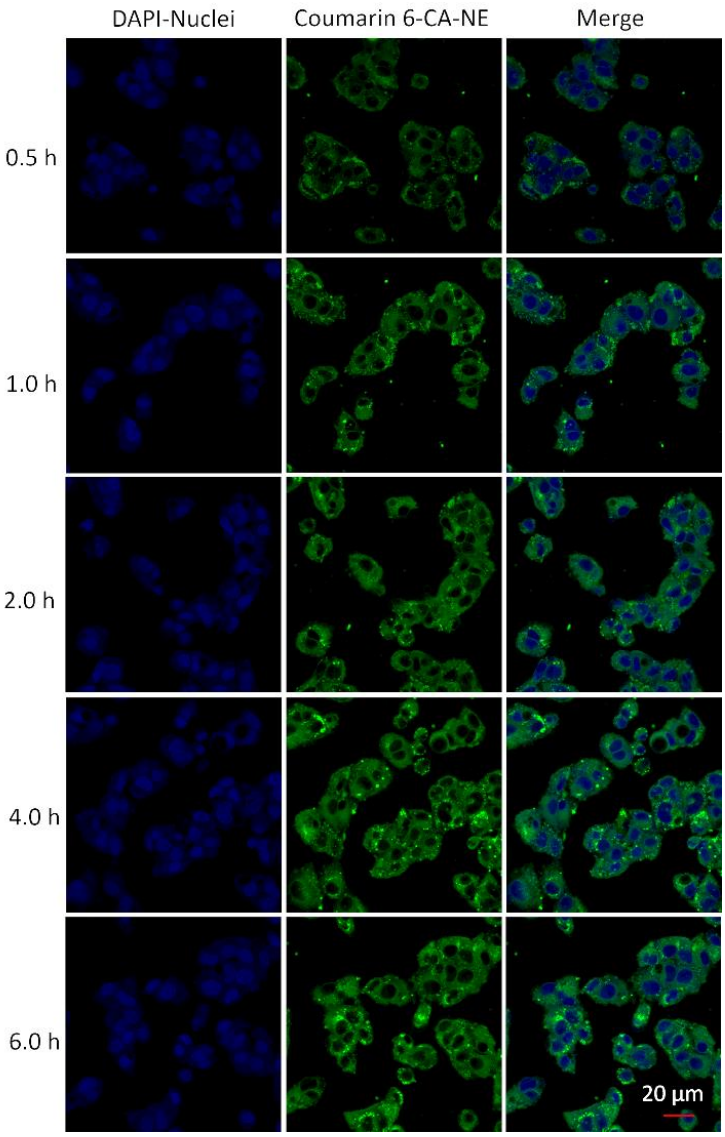
Results showed that CAA value of the free CA and CA-NE increased as the increasing concentration of CA. However, at each concentration, free CA in DMSO exhibited higher CAA value than the nanoencapsulated CA, indicating the nanoemulsion seemed decreased the antioxidant activity of CA during the 1-hour exposure of cells to CA-NE. In order to calculate the $EC_{50}$, the median plot was drawn (**Figure 26 D**), the $EC_{50}$ was 13.02±0.40 and 24.32± 1.27 μM for CA and CA-NE, respectively. We initially had several guess of the reasons of lower CAA value of encapsulated CA in nanoemulsion than the free DMSO dissolved carnosic acid. The first guess is the other components in CA-NE formulation might has pro-oxidant activity, which will compromise the antioxidant ability of CA, however, this was quickly eliminated because no pro-oxidant or antioxidant activity exhibited of the blank nanoemulsion by the study (data no shown here). Another more reasonable explanation is due to the sustained release character and slower cellular uptake profile resulted a low concentration of carnosic acid functioned in the cells which in turn a lower CAA value at one-hour treatment time of CA-NE compared with the DMSO delivered CA. There is previous study in our group using CAA assay to test the antioxidant activity of peptides/chitosan EGCG nanoparticles and found the oxidants resistance of EGCG could be improved after incorporation with the nanoparticulate system. While in another study, the researches applying liposome as carrier for curcumin found that the

encapsulation did not affect the antioxidant capability of curcumin.[126-127] However, our results indicated a lower antioxidant ability after nanoemulsified encapsulation. These different results may mainly rely on two reasons, one is the difference of the composition, size and surface characters of the nanodelivery systems, another reason is the encapsulated compound of EGCG, curcumin or CA has different solubility and cellular transport mechanism. As we previously mentioned, Cell based antioxidant assay included the effect of more factors than the chemical methods. In order to find out the reason of this decreased CAA value of CA-NE, the CAA assay was conducted for 4-hour treatment (based on the cellular uptake below) of cells with either CA or CA-NE at different concentrations, and then the fluorescence curve and CAA value was obtained. It was very interesting that after 4 hours treatment, the CAA value of CA-NE is greatly improved at all concentrations, and the $EC_{50}$ is almost the same as of 1-hour treatment by CA. However, the 4-hour CA treated cells didn't show concentration dependent and they are all possess a relatively high CAA value that is comparable or a little lower than 1-hour treatment. This result proved our speculation that the cellular uptake of nanoemulsion takes longer than the DMSO delivered compound, thus increase the treatment time up to 4 hours guaranteed all the CA was entered into the cells to perform the antioxidant activity. However, as for the DMSO delivered pure compound, during the 4-hour treatment, other reactions in the cells may already happened, caused the decrease of the antioxidant activity. Another experiment described below of cellular uptake of CA-NE was performed as another way to find out the possible reasons of lower CAA value of nanoemulsion than the DMSO one.

### 6.3.3. Cellular uptake of CA-NE by HepG2 Cells

Cellular uptake process of CA-NE was performed in order to give an explanation on how the nanoemulsion formulation incorporation affected the antioxidant ability in the HepG2 cells based on the Cellular antioxidant assay results. The uptake process of carnosic acid nanoemulsion by HepG2 at different treatment time from 0.5 hours to 6 hours is shown in **Figure 27**. As we know, it is impossible to clearly observe the CA-NE droplet by the CLSM because the nano-ranged small size. However, when the nanosized CA-NE accumulated on the cell membrane and in the intracellular area of the cells, the HepG2 cells became fluorescent and can be observed, which indicated the CA-NE was internalized into the cells. As we can see from **Figure 27** that the fluorescence intensity (green) in the cytoplasm that surrounded the nucleus (blue) was increasing as the time increased till 4 hours. This phenomenon indicated the cellular uptake of CA-NE is related to the time as more and more CA-NE droplets getting into the cells as the incubation time increases until 4 hours at 37 °C. The fluorescence intensity at 6-hour treatment is smaller than 4 hours suggested that the cellular absorption had reached saturation, and metabolism, elimination or exocytosis from cells may occurred.[128] There are possibly two ways that the compound formulated in nanoemulsion entering into the cells, both passive diffusion and endocytosis contributed to cellular uptake. As the size of CA-NE is bigger than 100 nm, the endocytosis is the most likely route for its internalization into cells. After a bioactive compound incorporated in the nanoemulsion, which possessing a slow sustained release behavior, the absorption or uptake of this compound is determined mainly by the endocytosis efficacy of this nanoemulsion droplets, the endocytosis is mainly mediated by clathrin and caveolae related pathways.[129] Usually as the endocytosis process needs more energy to enter into the

cells, so it's not as efficient as the passive diffusion, which is the route of small molecules enter into cells.[130] The free carnosic acid added in the cells this assay was initially completely dissolved by the DMSO before further introduced into the aqueous culture media. Carnosic acid, as hydrophobic drugs can diffuse readily by passive route. This cellular uptake experiment explains why the ability of scavenging free radicals in the cellular based antioxidant assay of carnosic acid dissolved in DMSO and formulated into nanoemulsion had a big difference and under the one-hour treatment of HepG2 cells, CA delivered by DMSO possessed lower $EC_{50}$.


DAPI-Nuclei
Coumarin 6-CA-NE
Merge
0.5 h
1.0 h
2.0 h
4.0 h
6.0 h
20 µm

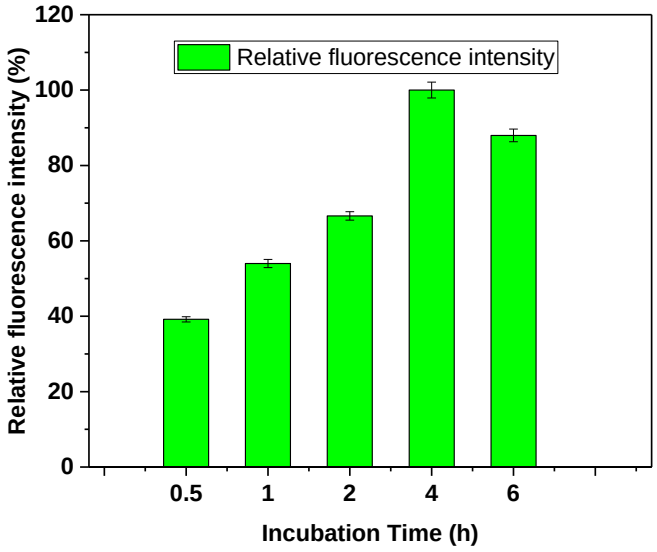



**Figure 27.** Upper panel: CLSM images of HepG2 cells incubated with coumarin 6 labeled nanoemulsion loaded with carnosic acid observed by CLSM at different incubation time. Lower panel: Relative fluorescence intensity was calculated based on fluorescence analysis using Image J. Result is depicted from the means ± SD from 3 different area calculations.

Based on the cellular antioxidant assay and cellular uptake study on HepG2 cells, a schematic figure showing the cellular uptake and cellular antioxidant assay of carnosic acid nanoemulsion (CA-NE) as in **Figure 28**.

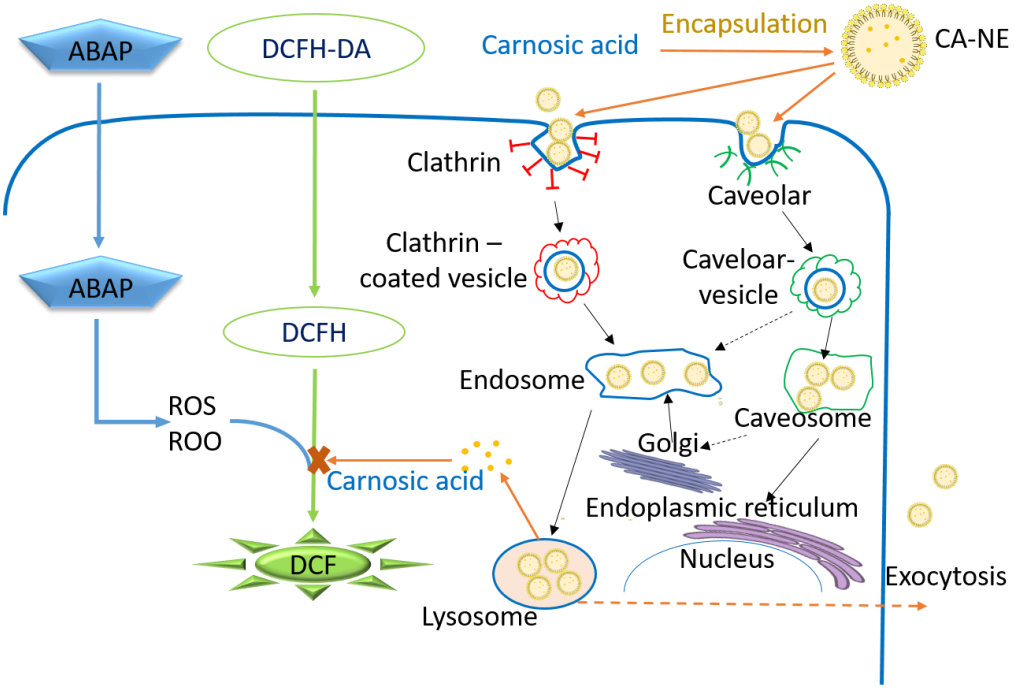



**Figure 28.** . Schematic figure of cellular uptake and cellular antioxidant assay of carnosic acid nanoemulsion (CA-NE).

6.3.4. Effect of nanoemulsion on release of Nitric Oxide (NO)

When stimulated using LPS, the macrophage cells produced and secreted excessive NO and inflammation condition in the cells was established. NO is involved in many inter- and intra-cellular signaling pathways of immune and other normal body functions, nevertheless, the uncontrolled and imbalance NO production indicated the abnormal and failure to control the inflammatory process and is closely related with carcinogenesis.[131] To determine whether nanoemulsion formulation would affect the NO regulation of CA, the NO production of LPS-activated RAW 264.7 macrophages treated with CA, CA-NE and B-NE was measured. The concentrations of each samples are within the no cytotoxicity range based on the MTT results (data not shown). The NO inhibition activity (**Figure 29**)

indicated that CA and CA-NE could dramatically inhibit the release of Nitric Oxide by a dose-dependent behavior for all tested concentrations. More interestingly, we found that the nanoemulsion showed prominent improvement of the anti-inflammatory activity regarding NO inhibition capability compared with free CA. From **Figure 29** we can see at the concentration of 6.25 μM, CA-NE exhibited about 20.0% inhibition of NO, which exceeded free carnosic acid inhibition rate around 7.2%, As the concentration increased, at 12.5 μM of CA, the NO inhibition of CA-NE reached 29.5 %, while CA only had 11.1% inhibition. The NO inhibition of CA-NE reached 62.3% at 25 μM equivalent CA concentration, which was dramatically efficient and powerful than the 37.8% inhibition rate of free CA according to the statistically analysis. More interestingly, we found the blank nanoemulsion without carnosic acid also possess slight NO inhibition activity with 25.6% decrease of NO production at highest diluted concentration (amount equivalent to the volume of CA-NE at 25 μM). The possible reason of the improved NO inhibition maybe related to the components of nanoemulsion formulation of CA-NE, it is apparently that the enhanced NO inhibition of CA-NE is contributed by both of CA and B-NE and also nanoemulsion has great possibility to be successfully applied as a carrier system for carnosic acid to be internalized into cells. Previous study has also reported the nanoemulsion exhibited slight NO inhibition activity and has a positive effect on the inflammation inhibition of nobiletin compared with unformulated one.[132]

Lipopolysaccharide (LPS) is a polysaccharide that can be obtained from the Gram-negative bacteria cell, which is related to various pathways and functions in the macrophage cells in human and animal body. The LPS could also promote the production of some compounds that secreted in the cell to mediate the inflammation procedure. The

various inflammatory mediators and cytokines such as such as Nitric oxide, TNF-α and IL- and enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The overproduction of NO by iNOS is a response to the stimulation of RAW 264.7 macrophages by LPS, which could be detected and quantified photometrically to reflect the anti-inflammatory ability of tested antioxidants and their formulations. Based on the current NO inhibition result on the stimulated-inflammation-RAW 264.7 cells, it is very likely that lecithin based nanoemulsion has the ability to inhibit the onset and developing of inflammation.

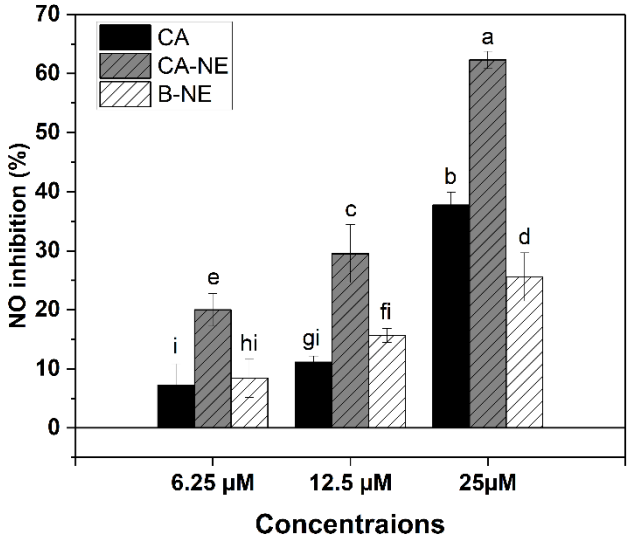



**Figure 29.** NO inhibition after 24 h-treatment of LPS-treated RAW 264.7 macrophage with CA, CA-NE, B-NE at concentrations of CA from 0 to 25µM, for B-NE is the corresponding dilutions of CA-NE. Data shown as mean ± SD from 3 experiments.

6.3.5. Influence on TNF-α production

Upon stimulation by LPS, macrophage cells would produce different inflammatory mediators such as NO, IL-1, TNF-α, IL-10. With uncontrolled secretion of these pro-inflammatory cytokines would possibly resulted in various chronic inflammation and diseases. Such that, the inhibition on the release of these cytokines is a useful way to treat the inflammation related disorders. In this study, we treated the RAW 264.7 macrophage with lipopolysaccharide and different concentrations by CA or CA-NE simultaneously to test the effect of nanoemulsion on the modulation of TNF-α by ELISA. LPS alone dramatically increased the release of TNF-α in the cell supernatant, the cells treated with either CA or CA nanoemulsion significantly decreased the pro-inflammatory cytokine TNF-α production amount followed a dose-dependent scenario if take the positive control LPS group for comparison ($p < 0.05$). The data showed (**Figure 30**) that at the concentration of equivalent 6.25 μM of CA, CA reduced the TNF-α level from 1060.90 pg/mL of LPS group to 938.23 pg/mL, while CA-NE reduced this number to 757.73 pg/mL. CA-NE at the equivalent concentration of 25 μM CA was found reduced the level of TNF-α to 492.56 pg/mL, which was around 53% inhibition rate compared with the LPS group, while CA also reduced this amount to 623.57 pg/mL with around 41% decrease of the amount of TNF-α. Blank nanoemulsion treated group also showed slight inhibition at each concentration with no significant difference and reduced the level of TNF-α release to 946.57 pg/mL from 1060.90 pg/mL of LPS group at the amount equivalent to the volume of CA-NE at 25 μM CA. The results here indicated that nanoemulsion could significantly improve the inhibition ability of pro-inflammatory cytokine TNF-α in this macrophage

cells after its stimulation in the inflammatory status, which may attributed to the effective delivery for carnosic acid to the cells. Macrophages play the central role in the inflammatory response and immune response. Once activated, they will secrete various cytokines and chemokines such as NO and TNF-α.[133] The secretion of these molecules are regulated by a series of pathways once the cells are activated by different stimuli and the following initiation of various transcriptional molecules such as NF-kappa B and MAPKs, which will cause the gene activation.[134] The mechanisms that makes the function of treating with inflammatory situation of CA-NE is related to its ability to inhibit the production of cytokines NO and TNF-α may through the hindrance of the activation of the NF-κB and MAPK activities.[132] Chronic inflammation is related with the occurrence of numerous different diseases such as inflammatory bowel, neurodegenerative problems and cancer.[135] Thus, by successfully delivery of carnosic acid and other phytochemicals to better perform anti-inflammatory activities may be a useful way to prevent and treat the related.[136-137]

In conclusion, a nanoemulsion formulation was developed in this study with loading efficiency 2.6-3.0%, size around 170nm, PDI around 0.240 and zeta potential values around -57.2mV. The formulation exhibited good physical stability at various pH and low ionic strength. Based on the in vitro pH lipolysis results, the nanoemulsion promoted faster lipid digestion and the bioaccessibility of carnosic acid was greatly improved by nanoemulsion systems by 2.8-fold compared to the oil suspension, indicating a possible enhancement of the bioavailability. The results indicating the importance of designing proper delivery systems for lipophilic nutraceuticals. In this study, we also evaluated bioactivity regarding the cellular antioxidant activity of carnosic acid nanoemulsion using HepG2 cell as model indicated the antioxidant activity is closely related to the cellular

uptake process as proved by the cellular uptake study of CA-NE by HepG2 cells using CLSM. The inhibition activity on the inflammation of nanoemulsified CA and unformulated CA was tested using the stimulation of RAW 264.7 cells showed the CA-NE could significantly decrease of the inflammatory mediator NO and cytokine TNF-α secretion compared to the free CA indicating a promising method to deliver CA to perform better bioactivity at relatively low concentrations.

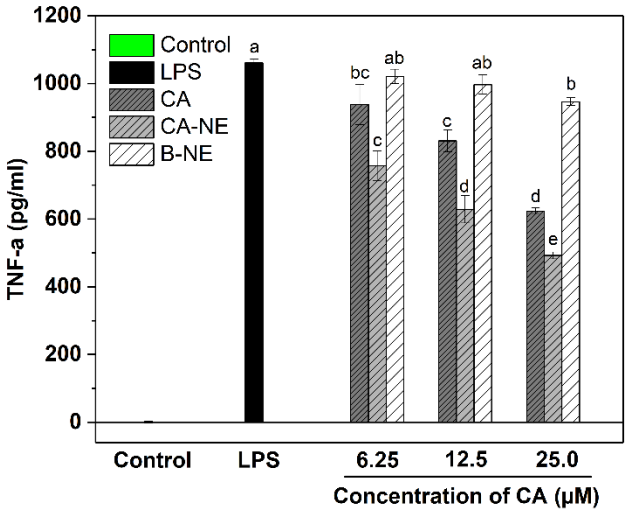



**Figure 30.** TNF-α release after 24 h-treatment of LPS-stimulated RAW 264.7 macrophage cells with CA, CA-NE and B-NE at concentrations of CA from 0 to 25μM, for B-NE is the corresponding dilutions of CA-NE. Reseult shown as the mean ± SD of 3experiments. Different letters depicted significant difference ($p < 0.05$).

## 6.4. Conclusion

In conclusion, a nanoemulsion formulation was developed in this study with loading efficiency 2.6-3.0%, size around 170nm, PDI around 0.240 and zeta potential values around -57.2mV. The formulation exhibited good physical stability at various pH and low ionic strength. Based on the in vitro pH lipolysis results, the nanoemulsion promoted faster lipid digestion and the bioaccessibility of carnosic acid was greatly improved by nanoemulsion systems by 2.8-fold compared to the oil suspension, indicating a possible enhancement of the bioavailability. The results indicating the importance of designing proper delivery systems for lipophilic nutraceuticals. In the current research, we also studied on the bioactivity regarding the cellular antioxidant ability of carnosic acid nanoemulsion using HepG2 cell as model indicated the antioxidant activity is closely related to the cellular uptake process as proved by the cellular uptake study of CA-NE by HepG2 cells using CLSM. The inhibition activity on the inflammation of nanoemulsified CA and unformulated CA was tested using the stimulated RAW 264.7 cells showed the CA-NE could significantly decrease of the inflammatory mediator NO and cytokine TNF-α secretion compared to the free CA indicating a promising method to deliver CA to perform better bioactivity at relatively low concentrations.

# CHAPTER 7. DETERMINING THE ANTI-PROLIFERATION ON CANCER CELLS AND ANTIBACTERIAL EFFECT OF CARNOSIC ACID NANOEMULSION

## 7.1. Introduction

Carnosic acid has been proved to possess a lot of chronic disease preventing activities including cancer. In this chapter, we would like to test how the nanoemulsion formulation would affect the anti-proliferation ability of carnosic acid. As we have introduced in the previous chapters, that carnosic acid is a molecule that highly hydrophobic and we tested its solubility in different oils and in water. It is hardly dissolved in the aqueous environment and difficult to dissolve at room temperature in oils. However, it is common sense that many pathogens and food-borne bacteria are inhabited in the aqueous or hydrophilic environment. Given this situation, the lipophilic nature of many bioactives that hard to be dispersed or dissolved in this hydrophilic circumstance have become a hurdle for their highest antibacterial activity to be exhibited. Furthermore, this limited their application as an antimicrobial agent in water-rich food prodcuts.[138] As we know, currently most synthetic preservatives have caused problems and concerns from consumers. The requirement of natural obtained anti-bacterial agents have promoted the finding and research on many new plant-derived molecules. Carnosic acid has long been used by Mediterranean people as preservatives for food and now research also showed its inhibition on various food-borne bacteria.[139] By applying nanoemulsion system, we have showed its ability to improve various biological functional properties and enhanced the bioavailability of carnosic acid using both systematically designed instrument and animal studies. Here,

we would like to further expand and test the possible application of this nanoemulsified carnosic acid formulation into our food antimicrobial area.

## 7.2. Materials and Methods

### 7.2.1. Materials

Carnosic acid (95%) was obtained from China, PC 75 were a gift from American Lecithin Company, US. Medium-chain triacylglycerol (MCT) oil (Neobee 1053) was kindly provided by Stepan company. Analytical standards for carnosic acid (>99%) and cell culture compatible dimethyl sulfoxide (DMSO) were obtained from Sigma Co.. DMEM, trypsin, penicillin-streptomycin, PBS were obtained from Life technologies and FBS was obtained from Biowest. For all the experiments, Deionized water was used. The mobile phase acetonitrile (ACN) and water for CA detection were purchase from Pharmco (Brookfield, CT). All other chemicals were purchased from Sigma-Aldrich and were ACS grade without specifically specified and applied without further treatment. The tested bacteria are Escherichia coli (ATCC 25922), Salmonella typhimurium (ATCC 13311) and Listeria monocytogenes (ATCC 7644) and Staphylococcus aureus (ATCC 63589).

### 7.2.2. Preparation for the testing formulation

CA-NE was produced by high speed combined with high speed-high pressure homogenization method. We firstly prepared the dispersion phase (oily phase) by dissolving 0.92 g of CA in 4.8 g in the carrier oil MCT, heated up to 90 °C, and maintained until the CA fully solubilized and in order to inhibit the recrystallization, the temperature was kept above seventy degrees Celsius. The continuous phase (aqueous phase) was

formulated by dispersing the 0.9 g PC 75 lecithin into 24.3 g DI water and also preheated to 70 °C. Once the two phases were ready, the aqueous phase was added into the oily phase, the mixture was stirred for another two minutes and then the nanoemulsion was prepared by a two-step high energy input method. Firstly, we can prepare the coarse emulsion with size usually around micrometers by using high speed homogenizer (IKA Works Inc., Wilmington, NC) at 12000rpm within 3 minutes based on a preliminary study. Then the crude emulsion was downsized by applying high pressure homogenizer instrument at 120MPa with four passes Finally, the formulated nanoemulsion composed with 15.5 MCT, 2.9% lecithin, 3.0% CA and 78.6% DI water were collected from the equipment for each batch. After the preparation, the amount of carnosic acid in the nanoemulsion was determined by HPLC. MCT and water suspension was prepared by adding the same amount of carnosic acid in MCT or water as in emulsion formulation.

7.2.3. Maintenance of Human Cancer Cell Lines

HepG2, Caco-2, HT-29, Hek, SK and Hela human cancer cells originated from different issues or organs were grown and cultured in DMEM added with 10% FBS and antibodies penicillin- streptomycin composed culture medium. non-essential amino acids (NAA) was also added for the Caco-2 culture. The incubator was set at 95% relative humidity, 5% $CO_2$ and a stable temperature of 37°C.

7.2.4. Anti-proliferation activity on cancer cells by MTT assay

The cell viability of CA, CA-NE and blank nanoemulsion (B-NE) on different carcinoma cells were measured by a well-established MTT assay.[120] MTT can be applied to understand the anti-proliferation ability of CA-NE as taking the unformulated carnosic acid with DMSO-dissolved one as control. On the first day of the experiment, cancer cells

were seeded to culture in 96-well plate with 1 x $10^4$ cells/well density as counted and appropriately diluted from the cell cultures and then incubated in the culture media containing all the basic nutrients and balance salts they need for healthy growth. After 24h incubation, the media were discarded; the cells were then added with a series concentration (six replicates) of CA-NE or CA in DMSO diluted with the complete media at different ratios. Blank nanoemulsion and negative control (untreated cells) were cultured at the same batch in every independent experiment as reference value for the calculation and control of the culture conditions. After 12, 24 or 48h h of incubation with different samples. Cells were incubated with 0.5 mg/ml MTT for 2 hours in dark. The final MTT dissolved in DMSO were tested by Biotek microplate reader at 570 nm with 490nm as reference.

The percentage of cell alive was obtained using the equation below:

$$\%Viability = \frac{OD\ of\ treated\ wells}{OD\ of\ control\ wells} \times 100$$

7.2.5. Preparation of inoculums

We have tested four most common seen bacteria in food products including Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, and Salmonella typhimurium in this part of experiment. The bacteria cells were transferred on the tryptic soy agar (TSB) plates and then we transferred one to two colonies into sterile TSB culture media and subculture twice at 37 °C to prepare inoculums. The different bacteria were cultured at 37°C overnight before the experiment.

7.2.6. Antibacterial activity evaluation by well diffusion method

We firstly applied a widely used and easy to observe the result method called well diffusion. We diluted the bacteria cultures into the concentration $1\times 10^6$ colony forming unit (cfu)/mL and was then swabbed the cultures onto entire TSB Agar surface. Then a sterile pipet tip was used to make holes on the agar surface, in order to mimic the interfere of each hole yet making the best use of the bacterial inoculum Agar surface, four holes were made on one agar plate. Once the bacteria were prepared, 100 μL of the tested either carnosic acid suspension or CA-NE at several concentrations by diluted with the culture medium are added separately in each well. Then the agar plates were incubated overnight at 37°C. We can then evaluate whether there is antibacterial activity by observing whether the added compound or formulation inhibit the growing of bacteria around the hole. If so, a inhibition circle around the hole will appear.[140] By this method, the antibacterial effect was tested on four bacteria mentioned above. For the preparation of the samples, CA was dissolved initially in 100 μL DMSO and then further dissolved to TSB. For the carnosic acid nanoemulsion, it was directly diluted by TSB. For both samples, making the highest concentration of carnosic acid 500 μg/mL (1500 μmol/L), totally eight concentrations were prepared by half-dilution, six replicates were made for each concentration. DMSO and blank nanoemulsion were diluted according to the concentrations as positive controls. Wells without samples were set as negative controls.

7.2.7. Inactivation kinetics and Minimum inhibition concentration by broth microdilution

Carnosic acid-loaded nanoemulsion was studied for the antibacterial activity and compared with pure carnosic acid. Growth curve determination was performed by microdilution method. Overnight grown (in nutrient broth medium) of S. typhi, Listeria monocytogenes,

Staphylococcus aureus and E. coli were cultured separately. Seven concentrations for each sample were prepared. The samples were carnosic acid, carnosic acid nanoemulsion, DMSO, blank nanoemulsion, because the turbidity of nanoemulsion, nanoemulsion alone without the bacteria were added as control. 96-well microplate was used for successive microdilution study. For each well, 100μL samples and 100 μL bacteria solution were mixed, except for the bacteria alone, broth alone and sample alone as control. For the first well of each series, 200 μL of carnosic acid solution or carnosic acid nanoemulsion (0.50 mg/mL carnosic acid) was added and successive transfer was done. All the wells except well number 8 were inoculated with 100 μL of inoculum ($10^5$ CFU/mL). The microplates were then cultured at 37°C for 24 hours and the OD was taken every one hour. The absorbance wavelength was set at 595nm. All measurements of MIC values were repeated in triplicate. MIC value means the lowest concentration of the CA or CA-NE or other samples that is necessary to fully inhibit the grow of bacteria. All the tests were conducted at least 3 times.

7.3. Results and Discussion

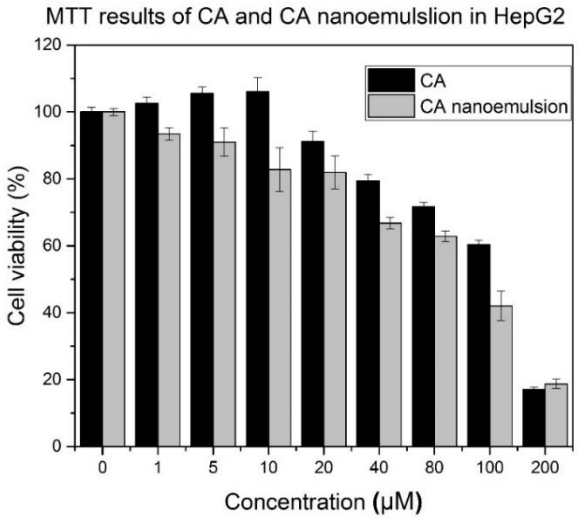
As previous published paper have demonstrated the anticancer activity of rosemary extract and its main component carnosic acid on various cancer cells by both cell studies and animal models.[141-142] However, right now, there is no published paper on the nanoemulsion encapsulated carnosic acid formulation on this area. Thus, it is interesting to test whether encapsulation in the nanoemulsion formulation will alter its effect compared with the organic solvent mediated unformulated carnosic acid.

In this part, we tested the anti-proliferation on various common seen and hard to cure carcinoma cells from different tissue or organs, such as Caco-2, which is from the colon

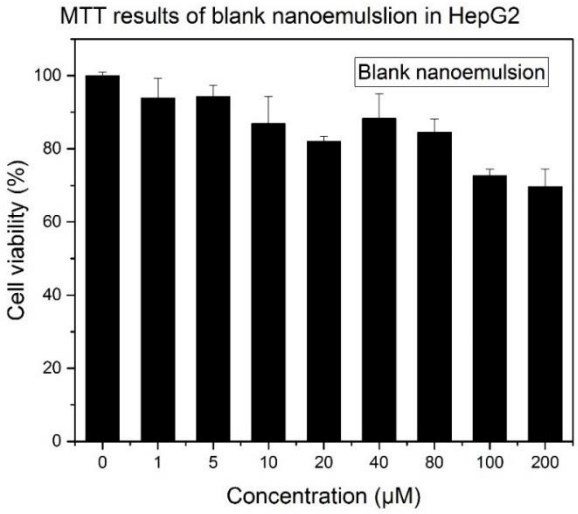
and HepG2, which is the hepatic carcinoma of human. We used MTT assay to test the viability after treated with different concentrations of unformulated and nanoemulsified carnosic acid formulations, and the anti-proliferation activity was calculated based on the cell viability results taking cells with no treatment but culture medium as control. The blank nanoemulsion and the diluted DMSO were also tested as a positive control.

7.3.1. Anti-proliferation effect on HepG2 cell line

Liver cancer, or called hepatocellular carcinoma (HCC) is one of the most common diagnosed cancers around the world. The occurrence of liver cancer is related to various liver diseases such as hepatitis B and C, alcohol liver and fatty liver and so on.[143] As HCC has a long latent period, most of the patients were first diagnosed are already in the intermediate or even advanced worst stage. As we know, chemotherapy is often the only option in this situation. Nutraceuticals are alternative therapeutic agents to prevent and control the cancer progression as a chemoprevention agents or chemotherapeutics.




(A)




(B)

**Figure 31.** Anti-proliferation on HepG2 cells of CA and CA-NE. HepG2 cells were cultured with 1 to 200 μM doses of carnosic acid. 0 denotes negative control (A) and blank nanoemulsion (B). The data were calculated from six wells for each assay, expressed as mean± SD (n=6).

As we can see from **Figure 31**, both carnosic acid dissolved in DMSO and carnosic acid nanoemulsion has the effect on the inhibition of HepG2 cell proliferation at various extent after 24 h treat in a dose dependent manner. At concentrations 200 μM, more than 80% cells were inhibited. With the nanoemulsion encapsulated carnosic acid showed slightly better inhibition activity than the pure carnosic acid. $EC_{50}$ of CA-NE is 107.71μM CA (CA in nanoemulsion), while 120 μM for pure CA. The results showed carnosic acid nanoemulsion was an effective anti-proliferative agent against liver cancer cell line HepG2. As compared the EC50 of carnosic acid in nanoemulsion with the unformulated one, we can see the CA-NE possess a little higher inhibition activity on the HepG2 cell grow. The

reason of this improved activity may be related to the protection effect of nanoemulsion on the encapsulated carnosic acid and thus higher amount of carnosic acid can be functioned on the cells. At all the concentrations, blank nanoemulsion showed slightly inhibition on the cells also on a concentration-dependent manner. Blank nanoemulsion was composed mainly by MCT and lecithin. Usually, it is not expected that the blank nanoemulsion could also possess anticancer activity, however, maybe the components for preparing the nanoemulsion also contributed a little bit on its anti-proliferation ability.

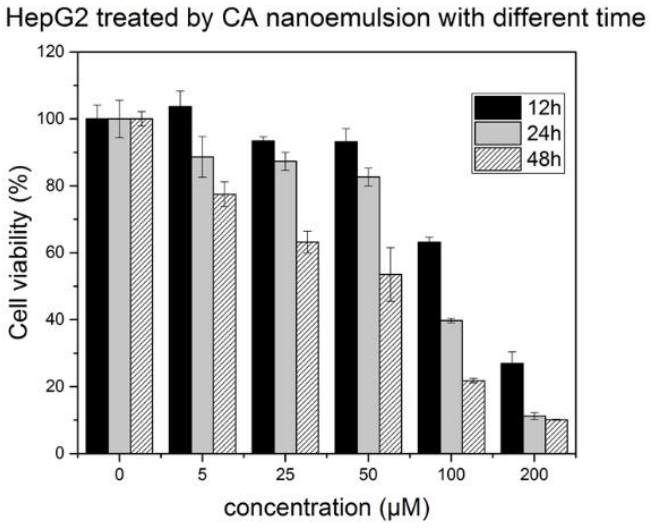


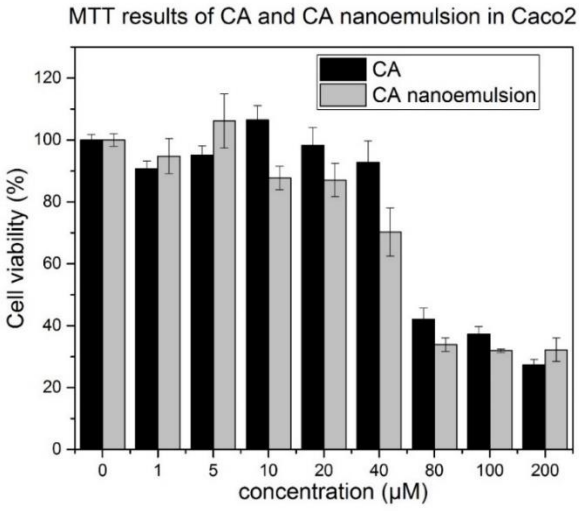

**Figure 32.** Dose-dependent and time-dependent antiproliferation activity of carnosic acid and nanoemulsion on the relative viability of HepG2 cell for different length of time with different concentrations of CA nanoemulsion. The data were calculated from six wells for each assay, expressed as mean± SD (n=6).

As shown by **Figure 32**, that the inhibition of HepG2 cell growth by CA-NE clearly dependent both on the concentration and treatment period length. For example, at concentrations of 50 μM, treat the cells for 48 hours nearly half of the cells was inhibited, while treat for 24 hours, the cell viability is around 85%, and even shorter treat time of

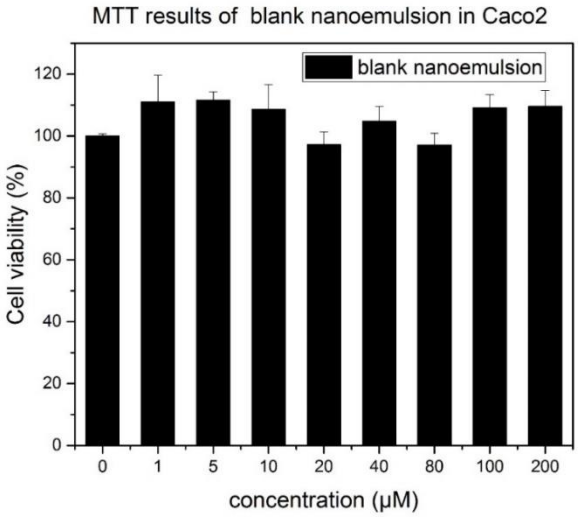
twelve hours only has an inhibition around 5% with 95% viability. This result demonstrated that we can either treat the cells with longer time or higher concentration to get the same effect depends on which is more applicable. $EC_{50}$ of the CA nanoemulsion treated cells at 12h, 24 h and 48h are 141.36 μM, 104.30 μM and 74.06 μM, respectively.

7.3.2. Anti-proliferation effect on Caco-2 and HT29 cell lines

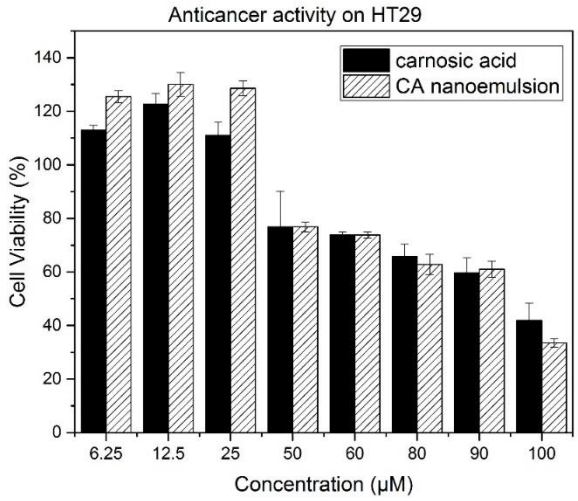
Colorectal cancer is said the third most prevalence cancer diagnosed around the world. Due to changing dietary styles, limited physical activity, as well as genetic influences, the incidence rates of colon cancer increased dramatically during the past decades.[101] Data have showed that the occurrence of colon cancer is closely correlated with the life style and most importantly the diet habit. Such that, by apply natural extracted bioactive compounds to prevent or even treat the cancer is one of the best acceptable and less costive way.[115]




(A)




(B)




(C)

**Figure 33.** MTT test on the anti-proliferation of Caco2 cells of carnosic acid and carnosic acid nanoemulsion (A) and B-NE (B). MTT study of anti-proliferation to HT29 cells of carnosic acid and carnosic acid nanoemulsion (C). The data were calculated from six wells for each assay, expressed as mean± SD (n=6).

As we can see from **Figure 33**, carnosic acid and carnosic acid nanoemulsion could inhibit the Caco2 cell showed a dependence on the concentration, CA nanoemulsion showed inhibition activity greater than carnosic acid dissolved by DMSO and blank nanoemulsion, which possesses no anticancer activity on Caco2 cells. The results demonstrated that the anti-proliferation activity on Caco2 is relied on the CA rather than other components in the CA-NE from the data. $EC_{50}$ of CA nanoemulsion is 63.00μM CA (CA in nanoemulsion), which is lower than 69.88 μM for pure CA.

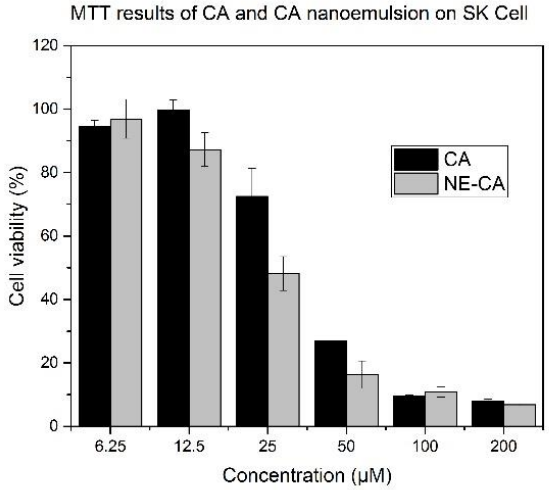
Barni et al [144] studied before of carnosic acid on the in vitro cancer cell -inhibition ability of carnosic acid using Caco-2 cell model, which reported a slightly higher $IC_{50}$ were 92.1 ± 6.4 μM. They explained this anti-proliferation activity of carnosic acid related with the apoptosis occurred in the cells. In addition, as we know, that the happening of cancer is a bundle of sophisticated and complex procedures and the carnosic acid is believed to inhibit the secretion and express of several enzymes that necessary for the cancer cell to migrate and grow.

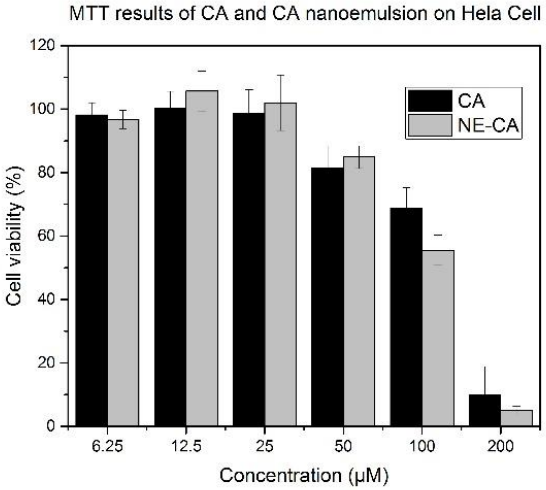
7.3.3. Anti-proliferation effect on HT29 cell line

We compared the anti-proliferation activity of HT-29 and Caco-2, as both of them origin from colon cancer with different properties. The $EC_{50}$ for HT 29 was found at 96.24 μM for pure carnosic acid, and 93.24 μM for carnosic acid nanoemulsion, so compared with Caco-2 cells, we found the nanoemulsion and pure carnosic acid are more effective on Caco-2 cells, presenting a different response in similar tissue originated cells.

7.3.4. Anti-proliferation effect on various cancer cell line

In order to analyze the anti-proliferation performance of CA and CA-NE on different types of cancer cells to predict any possible anticancer activity in the human body, several other common cancer cell lines from different organs were tested in this part by MTT assay. The cells include Hela cell from cervix, Hek from kidney, SK from liver and A549 from lung cancer cells.



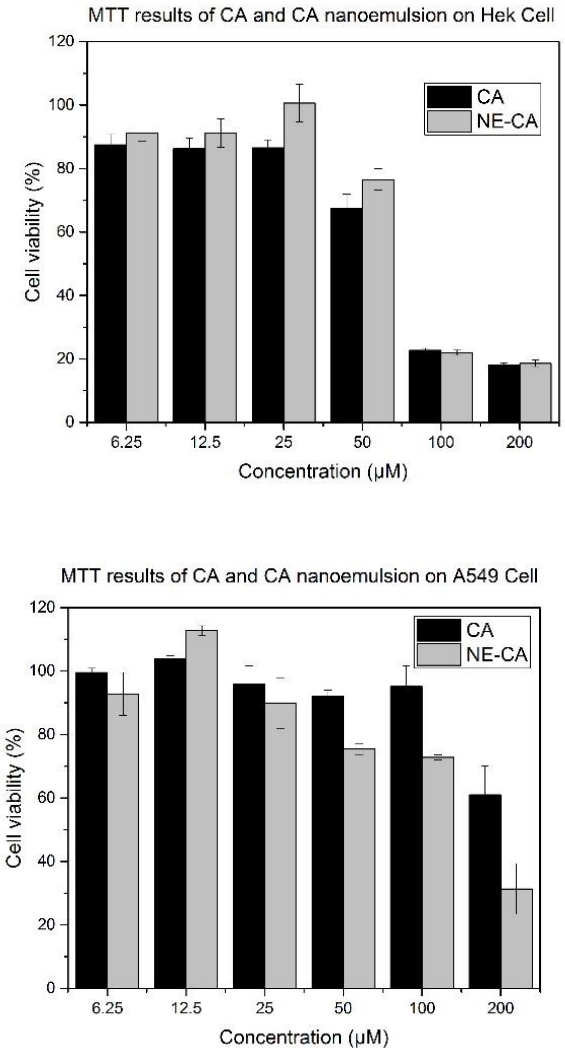



**Figure 34.** MTT on the antiproliferation on SK, Hela, Hek and A549 cells of carnosic acid and carnosic acid nanoemulsion. The data were calculated from 6 wells for each assay, expressed as mean± SD (n=6).

As we can see from **Figure 34**, different cancer cell lines exhibit different sensitivity towards CA and CA-NE treatment. With the SK being the most sensitive one, the $EC_{50}$ was 23.5 and 37.5 μM by CA and CA-NE, respectively. Followed by Hek, the kidney cancer cell, with $EC_{50}$ were 23.5 and 37.5 μM by CA and CA-NE, respectively. Among all the cells, the samples are least effective on A549, the lung cancer cells; $EC_{50}$ was more than

200 and 155.0μM by CA and CA-NE, respectively. For Hela cells, which is the cervix cancer cell, the samples showed a moderate effect, with CA-NE a little better, $EC_{50}$ were 101.1 μM of CA-NE, 131.0 μM for CA, respectively.

7.3.5. The inhibition activity studied by well diffusion

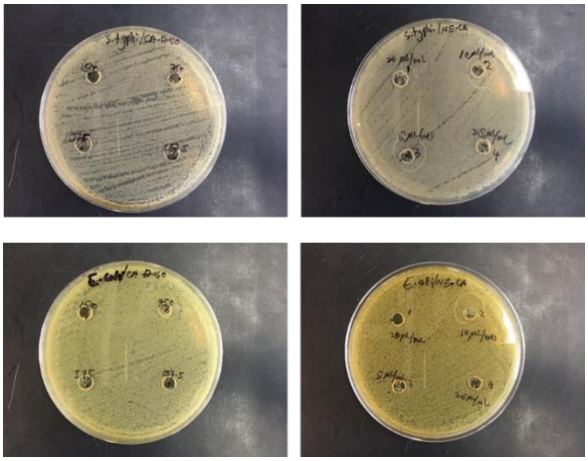


**Figure 35.** TSB diffusion of carnosic acid and CA nanoemulsion using E. coli and S. typhi as test microorganisms with the highest four concentrations, 1500 μM, 750 μM, 375 μM and 187.5 μM. The above two pictures are for S. typhi, below two are for E. coli, left one is pure CA and right one is CA nanoemulsion.

As we can see, there is no inhibition zone for the four highest concentrations for S. typhi and E. coli. Lower concentrations and blank nanoemulsion also showed no antibacterial effect on these two bacteria (picture not show here). As we can see here, carnosic acid and CA nanoemulsion are not effective on selected Gram negative bacteria by this study.

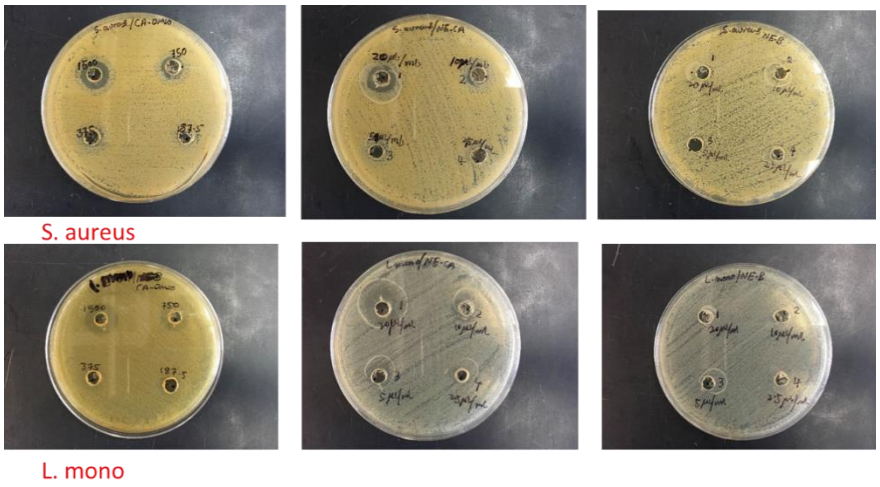



**Figure 36.** Agar well diffusion method of carnosic acid and CA nanoemulsion and blank nanoemulsion using S. aureus and L. mono as test microorganisms with the highest four concentrations, 1500 μM, 750 μM, 375 μM and 187.5 μM. The above two pictures are for S. aureus, below two are for L. mono, left two are pure CA, middle are CA nanoemulsion and right are blank nanoemulsion treated bacterial cells.

We measured the inhibition zone diameters; found it showed that the circle range is related with the concentration of carnosic acid in the CA-NE or CA as shown in **Figure 36**. In the order of 1500 μM, 750 μM, 375 μM, for L. monocytogenes: CA-NE: 9.39mm, 5.98mm, 4.88mm; CA in DMSO: 7.70mm, 7.14mm, 7.24mm; For S. aureus: CA-NE: 5.90mm, 5.14mm, 4.04mm; CA in DMSO: 6.78mm, 4.94mm, 4.43mm. From the above picture and the inhibition zone diameter, we can see clearly that both carnosic acid and carnosic acid nanoemulsion have inhibition activity on S. aureus and L. mono, which are Gram positive bacteria. The blank nanoemulsion has no effect, which means the antimicrobial effect of the carnosic acid was absolutely contributed by carnosic acid in the nanoemulsion. Even though, the measure of the inhibition zone is not a very accurate, but still the results

obtained are very producible and very efficient, thus it is widely applied by many studies for the screening and evaluation of different antimicrobial agents. It is fast and gives very direct results. The diffusion method is useful to compare the compounds or formulations with similar diffusion ability in the agar as the solvent or polarity of the compound itself will all affect the diffusion rate. For the nanoemulsion used here, the size and composition are all factors would influence the diffusion though the concentration difference is the main driving force.

7.3.6. Inactivation kinetics and Minimum inhibition concentration (MIC)

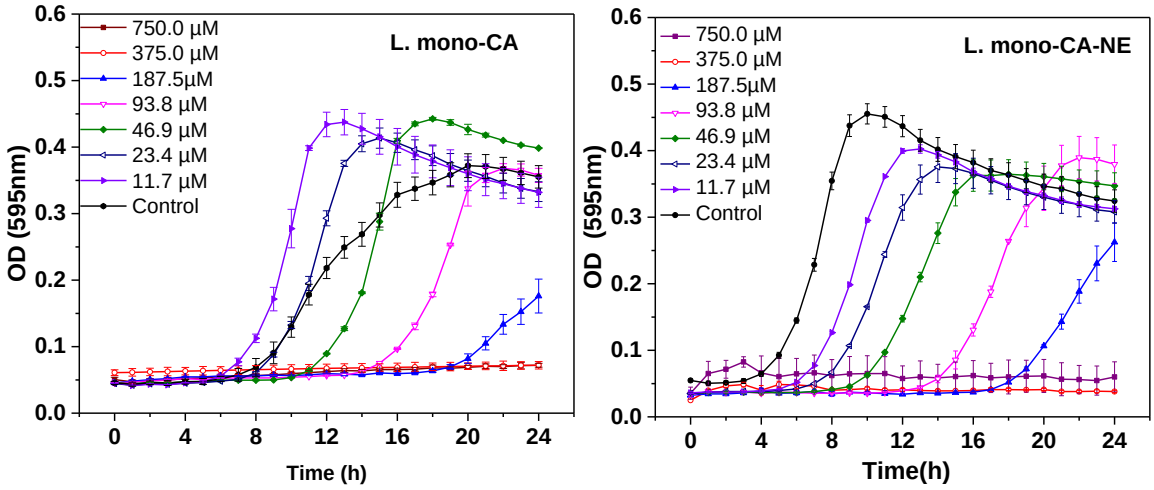



**Figure 37.** Growth curve of L. monocytogenes treated by CA and CA-NE

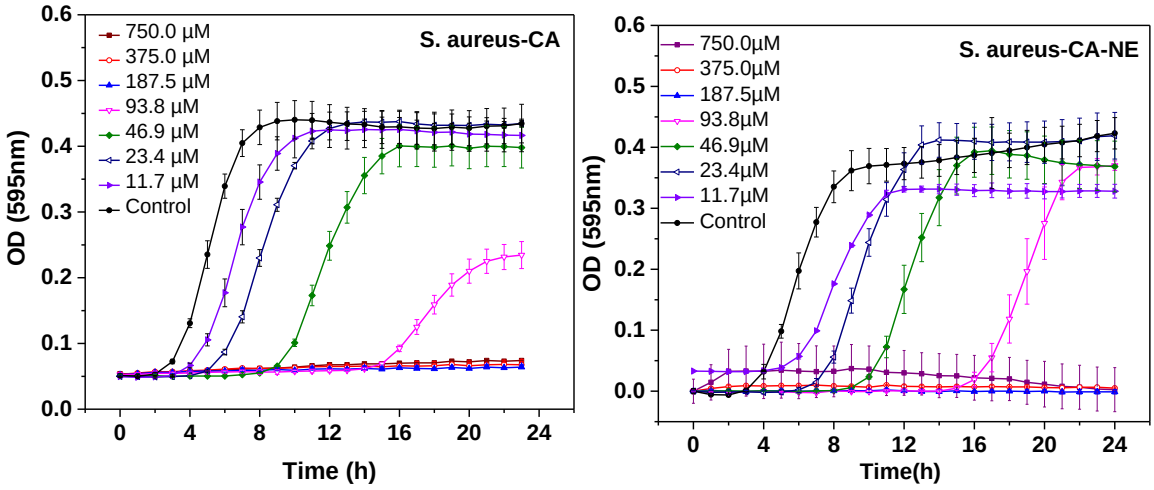



**Figure 38.** Growth curve of S. aureus treated by CA and CA-NE

**Table 9.** Summary on the antibacterial effect of carnosic acid and carnosic acid nanoemulsion

| | | Carnosic acid (CA)(µM) | CA-Nanoemulsion |
|---|---|---|---|
| Gram negative | E. coli | No effect | No effect |
| | S. typhi | No effect | No effect |
| Gram positive | L. mono | 375 | 375 |
| | S. aureus | 187.5 | 187.5 |

From the above pictures, we can see that carnosic acid and carnosic acid nanoemulsion exhibit antilisterial and antistaphylococcal effect with the minimum inhibition concentration for L. mono is 187.5µM for CA and NE-CA, for S. aureus, the MIC for CA is 93.75, for NE-CA is 187.5 µM. The test were also applied on E. coli and S. typhi, however, in coincidence with the result of Agar well diffusion, there is no inhibition for both of them by CA or CA-NE between the tested concentrations.

The mechanism of how phenolic diterpene exhibit the antibacterial effect is not thoroughly understood. It has been proposed by some studies that because the hydrophobic nature, they could insert into the bacteria membrane[145] As it would be possible for these lipophilic carnosic acid to react with the hydrophobic area of cell membrane and thus it would affect the rigidity and functioning of the cell membrane.[51-52] And also it seems pure carnosic acid and carnosic nanoemulsion has almost the same MIC, even with pure CA better, one reason may that pure carnosic acid was first dissolved by DMSO, which bring it well to the broth, came contact with the bacteria cells. From the results, we can also interpret the data from another way that the nanoemulsion we produced for carnosic acid has comparable effect of the DMSO mediated carnosic acid.

There have been quite a few studies about the difference of antibacterial ability of active compounds before and after incorporation into the delivery systems, however, there is no consensus on whether the encapsulation would increase or decrease the antibacterial activity.

Donsì et al.[146] in their research formulated a nanoemulsion system for incorporation of different essential oil and bioactives and found that the antimicrobial ability of these compounds was enhanced, thus they proposed that the nanoemulsion formulation should be capable to improve the permeation or transport of the compound encapsulated.

Other studies also suggested that after incorporated in the different carrier formulations, the stability regarding both the ability to resist different pH or ionic salts to maintain their physical and chemical stability in the environment might be greatly improved. More importantly, the encapsulation materials may also have an positive effect on the transport

and uptake of the molecules into the bacterial cells, thus improve the antibacterial activity.[147]

In another study by Salvia et al,[148] they found that after encapsulated two essential oils by nanoemulsion respectively, a more promote and improved inhibition on the bacteria growth than the conventional emulsion can be observed. The results indicated the antimicrobial enhancement is related to the smaller size of nanoemulsion for some type of essential oil, even though the type of essential oil plays a very crucial part. They proposed the more efficient release of the incorporated phenolic compounds contributed to the increased antibacterial efficacy. They also proposed that the solubilization of lipophilic compounds in the nanoemulsion would help them to interact with porins, which is a barrier for lipophilic compounds access into the cell membrane of Gram-negative bacteria. In many studies, carnosic acid was found possess a better antibacterial effect on Gram positive bacteria than the negative ones.

The antimicrobial activity was related to the carrier formulations applied for the encapsulation of the bioactive compounds. The properties of the carrier systems such as its composition, size and surface charge can all exhibit different effects. From the viewpoint of carnosic acid, the dissolving solvent or the delivery systems would affect its release and transfer rate accessing to the bacteria cells. Faster or slower release will either enhance or decrease the inhibition kinetic. In addition, the encapsulation will also affect the stability of the carnosic acid, as our preliminary results showed that the nanoemulsion could improve the physical and chemical stability of carnosic acid in the TSB culture medium. In some scenario, the bioactive compounds may have much stronger interaction with the encapsulation material than with the bacteria cell wall, thus their inhibition activity may be

undermined. One example is if chitosan is in strong interaction forces with other components in the formulation such as some nanoparticles or emulsions, such that, in the culture medium, the effective amount that would be able to adhere to the cell wall for inhibiting the cell growth is decreased. [149]

Another study[150] have shown that the MIC and MBC on their tested bacteria were enhanced by nano-encapsulation of thymol, the results indicated that the effectiveness of the bioactive compounds in the essential oil on the inhibition of the this bacteria is decreased by the encapsulation method. They proposed the reason maybe that ethyl cellulose/methylcellulose shell being non-degradable and the release of those lipophilic compounds in the nanoparticle takes priority on the inhibition of the growth of bacteria.

## 7.4. Conclusion

In this chapter, we mainly studied two areas. Firstly, by using a widely MTT assay, we tested the anti-proliferation activity of carnosic acid in either formulated or free form on different cancer cell lines. Carnosic acid dissolved by DMSO and formulated with nanoemulsion could efficiently inhibited the proliferation of HepG2, SK, HT29, Hela, Caco-2and A549 cells and the efficacy is related to the concentration of the carnosic acid and showed time-dependent when tested on HepG2 cells. We found the nanoemulsion was a little better than the DMSO dissolved carnosic acid. The possible reasons we proposed is that the nanoemulsion formulation would be able to enhance the absorption and stability of the carnosic acid into the carcinoma cells. Among all the cells, SK was the most sensitive one, with $EC_{50}$ the lowest.

The inhibition of the various cancer cells growth in our research now showed a very promising application future for carnosic acid nanoemulsion formulation. In addition, this formulation is fabricated with food grade materials, thus is more realistic to be used than the organic solvent mediated delivery forms. Based on this, it is worth to study the mechanisms involved in this process regarding to different cell lines. Thus, we proposed that our formulated CA-NE has a promising future to be used for the prevention and treatment for different cancer diseases.

As the antibacterial conducted in this part, after tested on four bacteria, we can see the pure carnosic acid possess antibacterial activity mainly on Gram-positive bacterial. After incorporate it into the nanoemulsion formulation, the situation did not change. Carnosic acid and carnosic acid nanoemulsion exhibit anti-listerial and anti-staphylococcal effect with the minimum inhibition concentration for L. mono is 187.5μM for CA and NE-CA, for S. aureus, the MIC for CA is 93.75, for NE-CA is 187.5 μM. The test was also applied on E. coli and S. typhi, however, in coincidence with the result of Agar well diffusion; there is no inhibition for both of them by CA or CA-NE between the tested concentrations. The results showed the encapsulation did not have much influence on the antibacterial activity of carnosic acid. Moreover, with similar IC 50 on the tested bacteria S. aureus and Listeria monocytogenes.

# CHAPTER 8: SUMMARY AND FUTURE WORK

## 8.1. Summary of the research

As the awareness of health-promoting activity from dietary sources, such as vegetables, herbs, arise, many new nutraceuticals have been extracted and studied for their bio functions. More importantly, as the occurrence of many chronic diseases are related to the dietary habits and living habits, more and more people realized it is better to prevent the happen of these diseases from bioactive compounds. However, many of these nutraceuticals has a poor oral bioavailability because low solubility or absorption, and instability. To improve their bioavailability, we must firstly identify the problems of the specific compound we are interested. Then, we need to design the suitable solution to solve the problems.

Carnosic acid is the most powerful antioxidant from rosemary extract according to many studies, which is highly lipophilic and can hardly solubilized in aqueous phase. Until now, there is insufficient study on its bioavailability and even less research on using natural material to form oral delivery systems to improve the solubility in aqueous-rich food systems or increase the dissolution in human gastrointestinal tract.

In my research, a functional nanoemulsion containing carnosic acid was successfully fabricated. With the potential application of the nanoemulsion in food area in mind, we chose all the materials from natural sources or within the status of GRAS to formulate the nanoemulsion. As the concern of synthetic emulsifier, using natural emulsifiers such as lecithin for the emulsion preparation is favorable for health and acceptable for us.

We optimized the formulation in order to make a high carnosic acid-loaded nanoemulsion formulation with good stability and finally improved bioavailability. We finally get a nanoemulsion formulation with size around 170 nm, loading efficiency around 3.5% of carnosic acid, and stable under various conditions. Then our studies are divided mainly on two aims. Firstly, we applied both in vitro models and in vivo animal studies for the evaluation of the bioaccessibility and bioavailability of carnosic acid in nanoemulsion form compared with the unformulated one. pH-stat and TIM-1 in vitro digestion models clearly showed an obvious improvement of the bioaccessibility of carnosic acid by nanoemulsion. In vivo pharmacokinetic study using rats model further confirmed the results that nanoemulsion could efficiently improve the bioavailability of carnosic acid. Secondly, we tested the bioactivity of carnosic acid nanoemulsion regarding the cellular antioxidant, anti-inflammatory, anti-proliferation on cancer cells and anti-bacterial activity on four different food-borne bacteria. Results showed either improved or unchanged activity or even decreased activity were discussed and explained. Overall speaking, the encapsulation dose not affected much on the activity of carnosic acid, which means the formulation is suitable for carnosic acid to perform its function.

To summarize the results from current work, we can conclude that lecithin-based nanoemulsion may be used as a promising carrier for carnosic acid and has great potential to be utilized in a wide area of food industry either as a functional food or as antimicrobial agents. This work will also be a good reference for the future formulate of carrier formulations for carnosic acid and other similar compounds.

## 8.2. Future directions

Due to the limitations of time and experience on the topic, there is always more work could be done to broad the current work. Even with the majority and a relatively complete study has been done here, there are still some very interesting and meaningful projects or extensions could be done for others.

For the formulation part, more other materials from natural resources could be tested to make different emulsion systems or other delivery systems. For example, by combination of the polymers with small surfactant, the surface property would be changed, thus the formulated emulsion systems would exhibit different bioavailability properties and biofunctions. The materials could also be used for preparation of the Electrospun fibers by the help of different polymers, such as different proteins or polysaccharide. These nanofibers could be used as food packaging material. Because the antioxidant and antimicrobial activity of carnosic acid, this nanofiber containing carnosic acid is expected having a promising application in the food packaging area.

Another aspect would be doing the mechanism studies regarding the antibacterial and also meaningful to do is a thorough analysis of the pharmacokinetic and tissue distribution after the oral ingestion of the prepared nanoemulsion formulation. This would give us a whole idea of the bioavailability and possible target to perform bioactivity.

## REFERENCES


1. Siro, I.; Kapolna, E.; Kapolna, B.; Lugasi, A., Functional food. Product development, marketing and consumer acceptance--a review. *Appetite* **2008,** *51* (3), 456-67.

2. Bech-Larsen, T.; Grunert, K. G., The perceived healthiness of functional foods: A conjoint study of Danish, Finnish and American consumers' perception of functional foods. *Appetite* **2003,** *40* (1), 9-14.

3. Otles, S.; Cagindi, O., Safety Considerations of Nutraceuticals and Functional Foods. In *Novel Technologies in Food Science: Their Impact on Products, Consumer Trends and the Environment*, McElhatton, A.; do Amaral Sobral, P. J., Eds. Springer New York: New York, NY, 2012; pp 121-136.

4. Sloan, A. E., The Top Ten Functional Food Trends. *Food Technol.* **2000,** *54* (4), 33-62.

5. Sloan, A. E., Top 10 Functional Food Trends. *Food Technol.* **2018,** *72* (4), 26-43.

6. Tan, C.; Xue, J.; Abbas, S.; Feng, B.; Zhang, X.; Xia, S., Liposome as a Delivery System for Carotenoids: Comparative Antioxidant Activity of Carotenoids As Measured by Ferric Reducing Antioxidant Power, DPPH Assay and Lipid Peroxidation. *J. Agric. Food. Chem.* **2014,** *62* (28), 6726-6735.

7. Gaspar, R. B.; Nele, M.; Ferraz, H. C., Encapsulation of α-tocopherol and β-carotene in concentrated oil-in-water beverage emulsions stabilized with whey protein isolate. *J. Dispersion Sci. Technol.* **2017,** *38* (1), 89-95.

8. Kausar, H.; Saeed, S.; Mushtaq Ahmad, M.; Salam, A., STUDIES ON THE DEVELOPMENT AND STORAGE STABILITY OF CUCUMBER-MELON FUNCTIONAL DRINK. *Journal of Agricultural Research (03681157)* **2012,** *50* (2), 239-248.

9. Vijaya Kumar, B.; Vijayendra, S. V. N.; Reddy, O. V. S., Trends in dairy and non-dairy probiotic products - a review. *J. Food Sci. Technol.* **2015,** *52* (10), 6112-6124.

10. Castro, W. F.; Cruz, A. G.; Bisinotto, M. S.; Guerreiro, L. M. R.; Faria, J. A. F.; Bolini, H. M. A.; Cunha, R. L.; Deliza, R., Development of probiotic dairy beverages: Rheological properties and application of mathematical models in sensory evaluation. *J. Dairy Sci.* **2013,** *96* (1), 16-25.

11. Ribeiro, H. S.; Guerrero, J. M. M.; Briviba, K.; Rechkemmer, G.; Schuchmann, H. P.; Schubert, H., Cellular Uptake of Carotenoid-Loaded Oil-in-Water Emulsions in Colon Carcinoma Cells in Vitro. *J. Agric. Food. Chem.* **2006,** *54* (25), 9366-9369.

12. Meroni, E.; Raikos, V., Physicochemical stability, antioxidant properties and bioaccessibility of [small beta]-carotene in orange oil-in-water beverage emulsions: influence of carrier oil types. *Food Funct.* **2018,** *9* (1), 320-330.

13. Joung, H. J.; Choi, M. J.; Kim, J. T.; Park, S. H.; Park, H. J.; Shin, G. H., Development of Food-Grade Curcumin Nanoemulsion and its Potential Application to

Food Beverage System: Antioxidant Property and In Vitro Digestion. *J. Food Sci.* **2016,** *81* (3), N745-53.

14. Meroni, E.; Raikos, V., Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: Improvements in chemical stability, antioxidant activity and gastrointestinal fate of lycopene using carrier oils. *Food Res. Int.* **2018,** *106*, 439-445.

15. Brower, V., Nutraceuticals: poised for a healthy slice of the healthcare market? *Nat. Biotechnol.* **1998,** *16* (8), 728-31.

16. Editorial Board. *Food Res. Int.* **2012,** *46* (2), IFC.

17. McClements, D. J.; Li, Y., Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food Funct.* **2010,** *1* (1), 32-59.

18. Yu, H., Huang, Q. , Bioavailability and Delivery of Nutraceuticals and Functional Foods Using Nanotechnology. In *Bio-Nanotechnology*, 2013.

19. Espin, J. C.; Garcia-Conesa, M. T.; Tomas-Barberan, F. A., Nutraceuticals: facts and fiction. *Phytochemistry* **2007,** *68* (22-24), 2986-3008.

20. Porter, C. J.; Trevaskis, N. L.; Charman, W. N., Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. *Nat. Rev. Drug Discov.* **2007,** *6* (3), 231-48.

21. Faridi Esfanjani, A.; Assadpour, E.; Jafari, S. M., Improving the bioavailability of phenolic compounds by loading them within lipid-based nanocarriers. *Trends Food Sci. Technol.* **2018,** *76*, 56-66.

22. Johnson, L. R. G., T. A., Gastrointestinal physiology. *Elsevier Philadelphia* **2007**.

23. Dahan, A.; Miller, J. M., The Solubility–Permeability Interplay and Its Implications in Formulation Design and Development for Poorly Soluble Drugs. *The AAPS Journal* **2012,** *14* (2), 244-251.

24. McClements, D. J.; Li, F.; Xiao, H., The Nutraceutical Bioavailability Classification Scheme: Classifying Nutraceuticals According to Factors Limiting their Oral Bioavailability. *Annu. Rev. Food Sci. Technol.* **2015,** *6* (1), 299-327.

25. Dempe, J. S.; Scheerle, R. K.; Pfeiffer, E.; Metzler, M., Metabolism and permeability of curcumin in cultured Caco-2 cells. *Mol. Nutr. Food Res.* **2013,** *57* (9), 1543-9.

26. Monagas, M.; Urpi-Sarda, M.; Sanchez-Patan, F.; Llorach, R.; Garrido, I.; Gomez-Cordoves, C.; Andres-Lacueva, C.; Bartolome, B., Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010,** *1* (3), 233-253.

27. Kosińska, A.; Andlauer, W., Modulation of tight junction integrity by food components. *Food Res. Int.* **2013,** *54* (1), 951-960.

28. Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J., Nanoemulsion delivery systems: Influence of carrier oil on β-carotene bioaccessibility. *Food Chem.* **2012,** *135* (3), 1440-1447.

29. Ting, Y.; Zhao, Q.; Xia, C.; Huang, Q., Using in Vitro and in Vivo Models To Evaluate the Oral Bioavailability of Nutraceuticals. *J. Agric. Food. Chem.* **2015,** *63* (5), 1332-1338.

30. McClements, D. J.; Li, Y., Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food Funct.* **2010,** *1* (1), 32-59.

31. Zhao, Q. Design of functional beverage emulsion systems for improved citral stability and coenzyme Q10 bioavailability. 2016.

32. Yang, X.; Tian, H.; Ho, C.-T.; Huang, Q., Stability of Citral in Emulsions Coated with Cationic Biopolymer Layers. *J. Agric. Food. Chem.* **2012,** *60* (1), 402-409.

33. McClements, D. J.; Decker, E. A.; Park, Y.; Weiss, J., Designing Food Structure to Control Stability, Digestion, Release and Absorption of Lipophilic Food Components. *Food Biophys.* **2008,** *3* (2), 219-228.

34. Ting, Y.; Jiang, Y.; Lan, Y.; Xia, C.; Lin, Z.; Rogers, M. A.; Huang, Q., Viscoelastic Emulsion Improved the Bioaccessibility and Oral Bioavailability of Crystalline Compound: A Mechanistic Study Using in Vitro and in Vivo Models. *Mol. Pharm.* **2015,** *12* (7), 2229-2236.

35. Mosgaard, M. D.; Sassene, P.; Mu, H.; Rades, T.; Müllertz, A., Development of a high-throughput in vitro intestinal lipolysis model for rapid screening of lipid-based drug delivery systems. *Eur. J. Pharm. Biopharm.* **2015,** *94*, 493-500.

36. Speranza, A.; Corradini, M. G.; Hartman, T. G.; Ribnicky, D.; Oren, A.; Rogers, M. A., Influence of Emulsifier Structure on Lipid Bioaccessibility in Oil–Water Nanoemulsions. *J. Agric. Food. Chem.* **2013,** *61* (26), 6505-6515.

37. Anson, N. M.; Selinheimo, E.; Havenaar, R.; Aura, A. M.; Mattila, I.; Lehtinen, P.; Bast, A.; Poutanen, K.; Haenen, G. R., Bioprocessing of wheat bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. *J. Agric. Food. Chem.* **2009,** *57* (14), 6148-55.

38. Maathuis, A. J.; van den Heuvel, E. G.; Schoterman, M. H.; Venema, K., Galacto-oligosaccharides have prebiotic activity in a dynamic in vitro colon model using a (13)C-labeling technique. *J. Nutr.* **2012,** *142* (7), 1205-12.

39. Maier-Salamon, A.; Hagenauer, B.; Wirth, M.; Gabor, F.; Szekeres, T.; Jäger, W., Increased Transport of Resveratrol Across Monolayers of the Human Intestinal Caco-2 Cells is Mediated by Inhibition and Saturation of Metabolites. *Pharm. Res.* **2006,** *23* (9), 2107-2115.

40. Walgren, R. A.; Karnaky, K. J.; Lindenmayer, G. E.; Walle, T., Efflux of Dietary Flavonoid Quercetin 4′-β-Glucoside across Human Intestinal Caco-2 Cell Monolayers by Apical Multidrug Resistance-Associated Protein-2. *J. Pharmacol. Exp. Ther.* **2000,** *294* (3), 830-836.

41. Pérez-Sánchez, A.; Borrás-Linares, I.; Barrajón-Catalán, E.; Arráez-Román, D.; González-Álvarez, I.; Ibáñez, E.; Segura-Carretero, A.; Bermejo, M.; Micol, V., Evaluation of the intestinal permeability of rosemary (Rosmarinus officinalis L.) extract polyphenols and terpenoids in Caco-2 cell monolayers. *PLoS One* **2017,** *12* (2), e0172063.

42. Gagnon, M.; Zihler Berner, A.; Chervet, N.; Chassard, C.; Lacroix, C., Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion. *J. Microbiol. Methods* **2013,** *94* (3), 274-9.

43. Pan, F.; Han, L.; Zhang, Y.; Yu, Y.; Liu, J., Optimization of Caco-2 and HT29 co-culture in vitro cell models for permeability studies. *Int. J. Food Sci. Nutr.* **2015,** *66* (6), 680-685.

44. Jordán, M. J.; Lax, V.; Rota, M. C.; Lorán, S.; Sotomayor, J. A., Relevance of Carnosic Acid, Carnosol, and Rosmarinic Acid Concentrations in the in Vitro Antioxidant and Antimicrobial Activities of Rosmarinus officinalis (L.) Methanolic Extracts. *J. Agric. Food. Chem.* **2012,** *60* (38), 9603-9608.

45. Cattaneo, L.; Cicconi, R.; Mignogna, G.; Giorgi, A.; Mattei, M.; Graziani, G.; Ferracane, R.; Grosso, A.; Aducci, P.; Schininà, M. E.; Marra, M., Anti-Proliferative Effect of Rosmarinus officinalis L. Extract on Human Melanoma A375 Cells. *PLoS One* **2015,** *10* (7), e0132439.

46. Maione, F.; Cantone, V.; Pace, S.; Chini, M. G.; Bisio, A.; Romussi, G.; Pieretti, S.; Werz, O.; Koeberle, A.; Mascolo, N.; Bifulco, G., Anti-inflammatory and analgesic activity of carnosol and carnosic acid in vivo and in vitro and in silico analysis of their target interactions. *Br. J. Pharmacol.* **2017,** *174* (11), 1497-1508.

47. Calabrese, V.; Scapagnini, G.; Catalano, C.; Dinotta, F.; Geraci, D.; Morganti, P., Biochemical studies of a natural antioxidant isolated from rosemary and its application in cosmetic dermatology. *Int. J. Tissue React.* **2000,** *22* (1), 5-13.

48. Steiner, M.; Priel, I.; Giat, J.; Levy, J.; Sharoni, Y.; Danilenko, M., Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Nutr. Cancer* **2001,** *41* (1-2), 135-44.

49. Danilenko, M.; Wang, Q.; Wang, X.; Levy, J.; Sharoni, Y.; Studzinski, G. P., Carnosic acid potentiates the antioxidant and prodifferentiation effects of 1alpha,25-dihydroxyvitamin D3 in leukemia cells but does not promote elevation of basal levels of intracellular calcium. *Cancer Res.* **2003,** *63* (6), 1325-32.

50. Kuo, C.-F.; Su, J.-D.; Chiu, C.-H.; Peng, C.-C.; Chang, C.-H.; Sung, T.-Y.; Huang, S.-H.; Lee, W.-C.; Chyau, C.-C., Anti-Inflammatory Effects of Supercritical Carbon Dioxide Extract and Its Isolated Carnosic Acid from Rosmarinus officinalis Leaves. *J. Agric. Food. Chem.* **2011,** *59* (8), 3674-3685.

51. Souza, A. B.; de Souza, M. G.; Moreira, M. A.; Moreira, M. R.; Furtado, N. A.; Martins, C. H.; Bastos, J. K.; dos Santos, R. A.; Heleno, V. C.; Ambrosio, S. R.; Veneziani, R. C., Antimicrobial evaluation of diterpenes from Copaifera langsdorffii oleoresin against periodontal anaerobic bacteria. *Molecules* **2011,** *16* (11), 9611-9.

52. Ojeda-Sana, A. M.; Repetto, V.; Moreno, S., Carnosic acid is an efflux pumps modulator by dissipation of the membrane potential in Enterococcus faecalis and Staphylococcus aureus. *World J. Microbiol. Biotechnol.* **2013,** *29* (1), 137-44.

53. Richheimer, S. L.; Bernart, M. W.; King, G. A.; Kent, M. C.; Beiley, D. T., Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. *Journal of the American Oil Chemists' Society* **1996,** *73* (4), 507-514.

54. Song, Y.; Yan, H.; Chen, J.; Wang, Y.; Jiang, Y.; Tu, P., Characterization of in vitro and in vivo metabolites of carnosic acid, a natural antioxidant, by high performance liquid chromatography coupled with tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2014,** *89*, 183-96.

55. Doolaege, E. H.; Raes, K.; De Vos, F.; Verhe, R.; De Smet, S., Absorption, distribution and elimination of carnosic acid, a natural antioxidant from Rosmarinus officinalis, in rats. *Plant Foods Hum. Nutr.* **2011,** *66* (2), 196-202.

56. Yan, H.; Wang, L.; Li, X.; Yu, C.; Zhang, K.; Jiang, Y.; Wu, L.; Lu, W.; Tu, P., High-performance liquid chromatography method for determination of carnosic acid in rat plasma and its application to pharmacokinetic study. *Biomed. Chromatogr.* **2009,** *23* (7), 776-81.

57. Wang, L.; Gan, C.; Wang, Z.; Liu, L.; Gao, M.; Li, Q.; Yang, C., Determination and Pharmacokinetic Study of Three Diterpenes in Rat Plasma by UHPLC-ESI-MS/MS after Oral Administration of Rosmarinus officinalis L. Extract. *Molecules* **2017,** *22* (6), 934.

58. Vaka, S. R. K.; Shivakumar, H. N.; Repka, M. A.; Murthy, S. N., Formulation and evaluation of carnosic acid nanoparticulate system for upregulation of neurotrophins in the brain upon intranasal administration. *J. Drug Target.* **2013,** *21* (1), 44-53.

59. Vaka, S. R. K.; Murthy, S. N.; Repka, M. A.; Nagy, T., Upregulation of Endogenous Neurotrophin Levels in the Brain by Intranasal Administration of Carnosic Acid. *J. Pharm. Sci.* **2011,** *100* (8), 3139-3145.

60. RUSSO, E.; BISIO, A.; ROMUSSI, G.; PARODI, B.; CAVIGLIOLI, G.; MINUTO, G.; CAFAGGI, S., Microspheres Containing Carnosic Acid for the Treatment of Basil Fungal Diseases. *Sci. Pharm.* **2009,** *77* (7), 236.

61. Ubbink, J.; Krüger, J., Physical approaches for the delivery of active ingredients in foods. *Trends Food Sci. Technol.* **2006,** *17* (5), 244-254.

62. Augustin, M. A.; Sanguansri, L., Challenges and solutions to incorporation of nutraceuticals in foods. *Annu. Rev. Food Sci. Technol.* **2015,** *6*, 463-77.

63. Xiao, J.; Li, C.; Huang, Q., Kafirin Nanoparticle-Stabilized Pickering Emulsions as Oral Delivery Vehicles: Physicochemical Stability and in Vitro Digestion Profile. *J. Agric. Food. Chem.* **2015,** *63* (47), 10263-10270.

64. Lu, X.; Xiao, J.; Huang, Q., Pickering emulsions stabilized by media-milled starch particles. *Food Res. Int.* **2018,** *105*, 140-149.

65. Wang, L.-J.; Hu, Y.-Q.; Yin, S.-W.; Yang, X.-Q.; Lai, F.-R.; Wang, S.-Q., Fabrication and Characterization of Antioxidant Pickering Emulsions Stabilized by Zein/Chitosan Complex Particles (ZCPs). *J. Agric. Food. Chem.* **2015,** *63* (9), 2514-2524.

66. McClements, D. J., Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter* **2012,** *8* (6), 1719-1729.

67. Jaiswal, M.; Dudhe, R.; Sharma, P. K., Nanoemulsion: an advanced mode of drug delivery system. *3 Biotech* **2015,** *5* (2), 123-127.

68. Bali, V.; Ali, M.; Ali, J., Study of surfactant combinations and development of a novel nanoemulsion for minimising variations in bioavailability of ezetimibe. *Colloids Surf. B. Biointerfaces* **2010,** *76* (2), 410-420.

69. Jo, Y.-J.; Kwon, Y.-J., Characterization of β-carotene nanoemulsions prepared by microfluidization technique. *Food Sci. Biotechnol.* **2014,** *23* (1), 107-113.

70. Sari, T. P.; Mann, B.; Kumar, R.; Singh, R. R. B.; Sharma, R.; Bhardwaj, M.; Athira, S., Preparation and characterization of nanoemulsion encapsulating curcumin. *Food Hydrocolloids* **2015,** *43*, 540-546.

71. Belhaj, N.; Dupuis, F.; Arab-Tehrany, E.; Denis, F. M.; Paris, C.; Lartaud, I.; Linder, M., Formulation, characterization and pharmacokinetic studies of coenzyme Q(1)(0) PUFA's nanoemulsions. *Eur. J. Pharm. Sci.* **2012,** *47* (2), 305-12.

72. Yukuyama, M. N.; Ghisleni, D. D. M.; Pinto, T. J. A.; Bou-Chacra, N. A., Nanoemulsion: process selection and application in cosmetics – a review. *Int. J. Cosmet. Sci.* **2016,** *38* (1), 13-24.

73. Forgiarini, A.; Esquena, J.; González, C.; Solans, C., Formation of Nano-emulsions by Low-Energy Emulsification Methods at Constant Temperature. *Langmuir* **2001,** *17* (7), 2076-2083.

74. Usón, N.; Garcia, M. J.; Solans, C., Formation of water-in-oil (W/O) nano-emulsions in a water/mixed non-ionic surfactant/oil systems prepared by a low-energy emulsification method. *Colloids Surf. Physicochem. Eng. Aspects* **2004,** *250* (1), 415-421.

75. Izquierdo, P.; Esquena, J.; Tadros, T. F.; Dederen, C.; Garcia, M. J.; Azemar, N.; Solans, C., Formation and Stability of Nano-Emulsions Prepared Using the Phase Inversion Temperature Method. *Langmuir* **2002,** *18* (1), 26-30.

76. Ting, Y. Application of emulsion-based delivery system to enhance bioavailability and efficacy of tangeretin. 2014.

77. Weiss, J. Emulsion Processing-Homogenization. http://people.umass.edu/mcclemen/FoodEmulsions2008/Presentations(PDF)/(5)Emulsion_Formation.pdf.

78. McClements, D.; Rao, J., *Food-Grade Nanoemulsions: Formulation, Fabrication, Properties, Performance, Biological Fate, and Potential Toxicity*. 2011; Vol. 51, p 285-330.

79. Oh, D. H.; Balakrishnan, P.; Oh, Y. K.; Kim, D. D.; Yong, C. S.; Choi, H. G., Effect of process parameters on nanoemulsion droplet size and distribution in SPG membrane emulsification. *Int. J. Pharm.* **2011,** *404* (1-2), 191-7.

80. Laouini, A.; Fessi, H.; Charcosset, C., Membrane emulsification: A promising alternative for vitamin E encapsulation within nano-emulsion. *J. Membr. Sci.* **2012,** *423-424*, 85-96.

81. Li, P. H.; Chiang, B. H., Process optimization and stability of d-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. *Ultrason. Sonochem.* **2012,** *19* (1), 192-197.

82. Bueschelberger, H., Lecithins. In *Emulsifiers in Food Technology*, Whitehurst, R. J., Ed. 2007.

83. Bylaite, E.; Nylander, T.; Venskutonis, R.; Jönsson, B., Emulsification of caraway essential oil in water by lecithin and β-lactoglobulin: emulsion stability and properties of the formed oil–aqueous interface. *Colloids Surf. B. Biointerfaces* **2001,** *20* (4), 327-340.

84. Iwata, T.; Kimura, Y.; Tsutsumi, K.; Furukawa, Y.; Kimura, S., The effect of various phospholipids on plasma lipoproteins and liver lipids in hypercholesterolemic rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* **1993,** *39* (1), 63-71.

85. Fernstrom, J. D., Can nutrient supplements modify brain function? *The American Journal of Clinical Nutrition* **2000,** *71* (6), 1669S-1673S.

86. Xiao, J.; Shi, C.; Zheng, H.; Shi, Z.; Jiang, D.; Li, Y.; Huang, Q., Kafirin Protein Based Electrospun Fibers with Tunable Mechanical Property, Wettability, and Release Profile. *J. Agric. Food. Chem.* **2016,** *64* (16), 3226-3233.

87. Xiao, J.; Wang, X. a.; Perez Gonzalez, A. J.; Huang, Q., Kafirin nanoparticles-stabilized Pickering emulsions: Microstructure and rheological behavior. *Food Hydrocolloids* **2016,** *54*, 30-39.

88. Birtić, S.; Dussort, P.; Pierre, F.-X.; Bily, A. C.; Roller, M., Carnosic acid. *Phytochemistry* **2015,** *115*, 9-19.

89. Thorsen, M. A.; Hildebrandt, K. S., Quantitative determination of phenolic diterpenes in rosemary extracts: Aspects of accurate quantification. *J. Chromatogr. A* **2003,** *995* (1), 119-125.

90. Razboršek, M. I.; Ivanović, M., Stability studies and determination of carnosic acid and its oxidative degradation products by gas chromatography–mass spectrometry. *Int. J. Mass spectrom.* **2016,** *407*, 29-39.

91. Cuvelier, M. E.; Richard, H.; Berset, C., Characterization of two new carnosic acid derivatives by spectrophotometry, mass spectrometry and electrochemical detection. *2nd International Electronic Conference on Synthetic Organic Chemistry (ECSOC-2)* **1998**, 1-30.

92. Lawrence, M. J.; Rees, G. D., Microemulsion-based media as novel drug delivery systems. *Adv. Drug Del. Rev.* **2000,** *45* (1), 89-121.

93. Narang, A. S.; Delmarre, D.; Gao, D., Stable drug encapsulation in micelles and microemulsions. *Int. J. Pharm.* **2007,** *345* (1), 9-25.

94. Yi, J.; Zhang, Y.; Liang, R.; Zhong, F.; Ma, J., Beta-Carotene Chemical Stability in Nanoemulsions Was Improved by Stabilized with Beta-Lactoglobulin–Catechin Conjugates through Free Radical Method. *J. Agric. Food. Chem.* **2015,** *63* (1), 297-303.

95. Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J., Physical and chemical stability of beta-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chem.* **2012,** *132* (3), 1221-1229.

96. Jaworska, M.; Sikora, E.; Ogonowski, J., Rheological Properties of Nanoemulsions Stabilized by Polysorbate 80. *Chemical Engineering & Technology* **2015,** *38* (8), 1469-1476.

97. Yu, H.; Huang, Q., Improving the Oral Bioavailability of Curcumin Using Novel Organogel-Based Nanoemulsions. *J. Agric. Food. Chem.* **2012,** *60* (21), 5373-5379.

98. Speranza, A.; Corradini, M. G.; Hartman, T. G.; Ribnicky, D.; Oren, A.; Rogers, M. A., Influence of emulsifier structure on lipid bioaccessibility in oil-water nanoemulsions. *J. Agric. Food. Chem.* **2013,** *61* (26), 6505-15.

99. Trevaskis, N. L.; Charman, W. N.; Porter, C. J., Lipid-based delivery systems and intestinal lymphatic drug transport: a mechanistic update. *Adv Drug Deliv Rev* **2008,** *60* (6), 702-16.

100. Dahan, A.; Hoffman, A., Rationalizing the selection of oral lipid based drug delivery systems by an in vitro dynamic lipolysis model for improved oral bioavailability of poorly water soluble drugs. *J. Controlled Release* **2008,** *129* (1), 1-10.

101. McClements, D. J.; Xiao, H., Potential biological fate of ingested nanoemulsions: influence of particle characteristics. *Food Funct.* **2012,** *3* (3), 202-20.

102. Ting, Y.; Jiang, Y.; Ho, C.-T.; Huang, Q., Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. *J. Funct. Foods* **2014,** *7*, 112-128.

103. Dahan, A.; Hoffman, A., Use of a dynamic in vitro lipolysis model to rationalize oral formulation development for poor water soluble drugs: correlation with in vivo data and the relationship to intra-enterocyte processes in rats. *Pharm. Res.* **2006,** *23* (9), 2165-74.

104. Huang, Q.; Yu, H.; Ru, Q., Bioavailability and Delivery of Nutraceuticals Using Nanotechnology. *J. Food Sci.* **2010,** *75* (1), R50-R57.

105. Wang, L.; Gan, C.; Wang, Z.; Liu, L.; Gao, M.; Li, Q.; Yang, C., Determination and Pharmacokinetic Study of Three Diterpenes in Rat Plasma by UHPLC-ESI-MS/MS after Oral Administration of Rosmarinus officinalis L. Extract. *Molecules* **2017,** *22* (6).

106. Young, I. S.; Woodside, J. V., Antioxidants in health and disease. *J. Clin. Pathol.* **2001,** *54* (3), 176.

107. de Sá Junior, P. L.; Câmara, D. A. D.; Porcacchia, A. S.; Fonseca, P. M. M.; Jorge, S. D.; Araldi, R. P.; Ferreira, A. K., The Roles of ROS in Cancer Heterogeneity and Therapy. *Oxid. Med. Cell. Longev.* **2017,** *2017*, 2467940.

108. Manoharan, S.; Guillemin, G. J.; Abiramasundari, R. S.; Essa, M. M.; Akbar, M.; Akbar, M. D., The Role of Reactive Oxygen Species in the Pathogenesis of Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease: A Mini Review. *Oxid. Med. Cell. Longev.* **2016,** *2016*, 8590578.

109. Chen, X.; Guo, C.; Kong, J., Oxidative stress in neurodegenerative diseases. *Neural Regeneration Research* **2012,** *7* (5), 376-385.

110. Zhang, H.; Tsao, R., Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Current Opinion in Food Science* **2016,** *8*, 33-42.

111. He, Y.; Yue, Y.; Zheng, X.; Zhang, K.; Chen, S.; Du, Z., Curcumin, Inflammation, and Chronic Diseases: How Are They Linked? *Molecules* **2015,** *20* (5), 9183.

112. Wang, P. Y.; Fang, J. C.; Gao, Z. H.; Zhang, C.; Xie, S. Y., Higher intake of fruits, vegetables or their fiber reduces the risk of type 2 diabetes: A meta-analysis. *Journal of Diabetes Investigation* **2016,** *7* (1), 56-69.

113. AGGARWAL, B. B.; BHARDWAJ, A.; AGGARWAL, R. S.; SEERAM, N. P.; SHISHODIA, S.; TAKADA, Y., Role of Resveratrol in Prevention and Therapy of Cancer: Preclinical and Clinical Studies. *Anticancer Res.* **2004,** *24* (5A), 2783-2840.

114. Salminen, A.; Lehtonen, M.; Suuronen, T.; Kaarniranta, K.; Huuskonen, J., Terpenoids: natural inhibitors of NF-κB signaling with anti-inflammatory and anticancer potential. *Cell. Mol. Life Sci.* **2008,** *65* (19), 2979-2999.

115. Barni, M. V.; Carlini, M. J.; Cafferata, E. G.; Puricelli, L.; Moreno, S., Carnosic acid inhibits the proliferation and migration capacity of human colorectal cancer cells. *Oncol. Rep.* **2012,** *27* (4), 1041-1048.

116. Dickmann, L. J.; Vandenbrink, B. M.; Lin, Y. S., In vitro hepatotoxicity and cytochrome P450 induction and inhibition characteristics of carnosic acid, a dietary supplement with antiadipogenic properties. *Drug Metab. Disposition* **2012,** *40* (7), 1263-1267.

117. Ou, J.; Huang, J.; Zhao, D.; Du, B.; Wang, M., Protective effect of rosmarinic acid and carnosic acid against streptozotocin-induced oxidation, glycation, inflammation and microbiota imbalance in diabetic rats. *Food Funct.* **2018,** *9* (2), 851-860.

118. Ting, Y.; Chiou, Y.-S.; Pan, M.-H.; Ho, C.-T.; Huang, Q., *In vitro and in vivo anti-cancer activity of tangeretin against colorectal cancer was enhanced by emulsion-based delivery system*. 2015; Vol. 15.

119. Zhao, L.; Wei, Y.; Huang, Y.; He, B.; Zhou, Y.; Fu, J., Nanoemulsion improves the oral bioavailability of baicalin in rats: in vitro and in vivo evaluation. *International journal of nanomedicine* **2013,** *8*, 3769-79.

120. Rashidi, L.; Vasheghani-Farahani, E.; Soleimani, M.; Atashi, A.; Rostami, K.; Gangi, F.; Fallahpour, M.; Tahouri, M. T., A cellular uptake and cytotoxicity properties study of gallic acid-loaded mesoporous silica nanoparticles on Caco-2 cells. *J. Nanopart. Res.* **2014,** *16* (3), 2285.

121. Wolfe, K. L.; Liu, R. H., Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J. Agric. Food. Chem.* **2007,** *55* (22), 8896-8907.

122. Zhang, T. T.; Lu, C. L.; Jiang, J. G.; Wang, M.; Wang, D. M.; Zhu, W., Bioactivities and extraction optimization of crude polysaccharides from the fruits and leaves of Rubus chingii Hu. *Carbohydr. Polym.* **2015,** *130*, 307-15.

123. Heo, W.; Kim, J. H.; Pan, J. H.; Kim, Y. J., Lecithin-Based Nano-emulsification Improves the Bioavailability of Conjugated Linoleic Acid. *J. Agric. Food. Chem.* **2016,** *64* (6), 1355-1360.

124. Jordan, M. J.; Lax, V.; Rota, M. C.; Loran, S.; Sotomayor, J. A., Relevance of carnosic acid, carnosol, and rosmarinic acid concentrations in the in vitro antioxidant and antimicrobial activities of Rosmarinus officinalis (L.) methanolic extracts. *J. Agric. Food. Chem.* **2012,** *60* (38), 9603-8.

125. Jones, D. P., Redefining oxidative stress. *Antioxid. Redox Signal.* **2006,** *8* (9-10), 1865-79.

126. Chen, X.; Zou, L. Q.; Niu, J.; Liu, W.; Peng, S. F.; Liu, C. M., The Stability, Sustained Release and Cellular Antioxidant Activity of Curcumin Nanoliposomes. *Molecules* **2015,** *20* (8), 14293-311.

127. Hu, B.; Ting, Y.; Zeng, X.; Huang, Q., Bioactive Peptides/Chitosan Nanoparticles Enhance Cellular Antioxidant Activity of (−)-Epigallocatechin-3-gallate. *J. Agric. Food. Chem.* **2013,** *61* (4), 875-881.

128. Park, J. S.; Han, T. H.; Lee, K. Y.; Han, S. S.; Hwang, J. J.; Moon, D. H.; Kim, S. Y.; Cho, Y. W., N-acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: Endocytosis, exocytosis and drug release. *J. Controlled Release* **2006,** *115* (1), 37-45.

129. Fan, Y.; Zhang, Y.; Yokoyama, W.; Yi, J., Endocytosis of Corn Oil-Caseinate Emulsions In Vitro: Impacts of Droplet Sizes. *Nanomaterials (Basel, Switzerland)* **2017,** *7* (11).

130. Sun, X.; Li, F.; Wang, Y.; Liang, W., Cellular uptake and elimination of lipophilic drug delivered by nanocarriers. *Pharmazie* **2010,** *65* (10), 737-42.

131. Song, S.-M.; Ham, Y.-M.; Ko, Y.-J.; Ko, E.-Y.; Oh, D.-J.; Kim, C.-S.; Kim, D.; Kim, K.-N.; Yoon, W.-J., Anti-inflammatory activities of the products of supercritical fluid extraction from Litsea japonica fruit in RAW 264.7 cells. *J. Funct. Foods* **2016,** *22*, 44-51.

132. Liao, W.; Liu, Z.; Zhang, T.; Sun, S.; Ye, J.; Li, Z.; Mao, L.; Ren, J., Enhancement of Anti-Inflammatory Properties of Nobiletin in Macrophages by a Nano-Emulsion Preparation. *J. Agric. Food. Chem.* **2018,** *66* (1), 91-98.

133. Jovanovic, D. V.; Di Battista, J. A.; Martel-Pelletier, J.; Jolicoeur, F. C.; He, Y.; Zhang, M.; Mineau, F.; Pelletier, J.-P., IL-17 Stimulates the Production and Expression of Proinflammatory Cytokines, IL-β and TNF-α, by Human Macrophages. *The Journal of Immunology* **1998,** *160* (7), 3513-3521.

134. Park, K.-I.; Kang, S.-R.; Park, H.-S.; Lee, D. H.; Nagappan, A.; Kim, J. A.; Shin, S. C.; Kim, E. H.; Lee, W. S.; Chung, H.-J.; An, S. J.; Kim, G. S., Regulation of Proinflammatory Mediators via NF-κB and p38 MAPK-Dependent Mechanisms in RAW 264.7 Macrophages by Polyphenol Components Isolated from Korea Lonicera japonica THUNB. *Evidence-based Complementary and Alternative Medicine : eCAM* **2012,** *2012*, 828521.

135. Jurjus, A.; Eid, A.; Al Kattar, S.; Zeenny, M. N.; Gerges-Geagea, A.; Haydar, H.; Hilal, A.; Oueidat, D.; Matar, M.; Tawilah, J.; Hussein, I. H.; Schembri-Wismayer, P.; Cappello, F.; Tomasello, G.; Leone, A.; Jurjus, R. A., Inflammatory bowel disease, colorectal cancer and type 2 diabetes mellitus: The links. *BBA Clinical* **2016,** *5*, 16-24.

136. Lala, R. R.; Awari, N. G., Nanoemulsion-based gel formulations of COX-2 inhibitors for enhanced efficacy in inflammatory conditions. *Applied Nanoscience* **2014,** *4* (2), 143-151.

137. Young, N. A.; Bruss, M. S.; Gardner, M.; Willis, W. L.; Mo, X.; Valiente, G. R.; Cao, Y.; Liu, Z.; Jarjour, W. N.; Wu, L.-C., Oral Administration of Nano-Emulsion Curcumin in Mice Suppresses Inflammatory-Induced NFκB Signaling and Macrophage Migration. *PLoS One* **2014,** *9* (11), e111559.

138. Ghosh, V.; Mukherjee, A.; Chandrasekaran, N., Eugenol-loaded antimicrobial nanoemulsion preserves fruit juice against, microbial spoilage. *Colloids Surf. B. Biointerfaces* **2014,** *114*, 392-397.

139. Moreno, S.; Scheyer, T.; Romano, C. S.; Vojnov, A. A., Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radical Res.* **2006,** *40* (2), 223-231.

140. Balouiri, M.; Sadiki, M.; Ibnsouda, S. K., Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **2016,** *6* (2), 71-79.

141. Yesil-Celiktas, O.; Sevimli, C.; Bedir, E.; Vardar-Sukan, F., Inhibitory Effects of Rosemary Extracts, Carnosic Acid and Rosmarinic Acid on the Growth of Various Human Cancer Cell Lines. *Plant Foods Hum. Nutr.* **2010,** *65* (2), 158-163.

142. Moore, J.; Yousef, M.; Tsiani, E., Anticancer Effects of Rosemary (Rosmarinus officinalis L.) Extract and Rosemary Extract Polyphenols. *Nutrients* **2016,** *8* (11), 731.

143. Ghouri, Y. A.; Mian, I.; Rowe, J. H., Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. *J. Carcinog.* **2017,** *16*, 1.

144. Barni, M. V.; Carlini, M. J.; Cafferata, E. G.; Puricelli, L.; Moreno, S., Carnosic acid inhibits the proliferation and migration capacity of human colorectal cancer cells. *Oncol. Rep.* **2012,** *27* (4), 1041-8.

145. Cowan, M. M., Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999,** *12* (4), 564-82.

146. Donsì, F.; Annunziata, M.; Sessa, M.; Ferrari, G., Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT - Food Science and Technology* **2011,** *44* (9), 1908-1914.

147. Donsi, F.; Annunziata, M.; Vincensi, M.; Ferrari, G., Design of nanoemulsion-based delivery systems of natural antimicrobials: effect of the emulsifier. *J. Biotechnol.* **2012,** *159* (4), 342-50.

148. Salvia-Trujillo, L.; Rojas-Graü, A.; Soliva-Fortuny, R.; Martín-Belloso, O., Physicochemical characterization and antimicrobial activity of food-grade emulsions and nanoemulsions incorporating essential oils. *Food Hydrocolloids* **2015,** *43*, 547-556.

149. Xue, J.; Michael Davidson, P.; Zhong, Q., Antimicrobial activity of thyme oil co-nanoemulsified with sodium caseinate and lecithin. *Int. J. Food Microbiol.* **2015,** *210*, 1-8.

150. Wattanasatcha, A.; Rengpipat, S.; Wanichwecharungruang, S., Thymol nanospheres as an effective anti-bacterial agent. *Int. J. Pharm.* **2012,** *434* (1), 360-365.

# APPENDIX: LIST OF ABBREVIATIONS

| | |
|---|---|
| **ACN** | Acetonitrile |
| **ABAP** | 2,2'-azobis(2-amidinopropane) dihydrochloride |
| **AUC** | Area under the curve |
| **CA** | Carnosic acid |
| **CAA** | Cellular Antioxidant Assay |
| **$C_{max}$** | Maximum concentration |
| **DMSO** | Dimethyl Sulfoxide |
| **FBS** | Fetal Bovine Serum |
| **GRAS** | Generally Recognized As Safe |
| **MCT** | Medium Chain Triglyceride |
| **MTT** | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide |
| **Na TDC** | Sodium Taurodeoxycholate |
| **PC** | Phosphatidylcholine |
| **S.D.** | Standard Deviation |
| **TNF-α** | Tumor Necrosis Factor - α |
| **TSB** | Tryptic soy broth |
| **$T_{max}$** | Maximum time |