AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE INHIBITORY EFFECTS OF FREE AND ENCAPSULATED CURCUMIN ON FOODBORNE PATHOGENS

by
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AN ABSTRACT OF THE THESIS OF

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Tahini-based products are common food items throughout the Middle East while gaining popularity around the world. Consumption of such products both locally and internationally requires that they meet food safety standards along with an acceptable shelf-life. Over the past few years, several outbreaks associated with tahini-based products have been reported as well as product recalls due to confirmed contamination with *Salmonella* spp., *Listeria monocytogenes, Escherichia coli* or *Staphylococcus aureus*.

Various preservation techniques have been used including chemical and thermal processing to insure the safety of such products. However, treating tahini and tahini-based products using such methods alters the texture and quality of the final product. Moreover, given the public's growing concerns regarding the use of chemical preservatives, there has been an increased interest to explore natural additives that may contribute to preserving the quality and safety of the final product. Such natural ingredients include spices some of which have already proven to possess antimicrobial effects. Curcumin, the bioactive component of turmeric, is a natural spice that has been shown to possess a wide range of anti-microbial, anti-fungal as well as anti-cancer activities when tested both *in-vitro* and *in-vivo*. To the best of the authors' knowledge, no studies have been conducted to study the antimicrobial effect of curcumin on foodborne pathogens. Accordingly, this study aims to investigate the anti-microbial effect of Curcumin preparations, both free and encapsulated in liposomes, against *Salmonella* spp., *E. coli*, *Listeria monocytogenes* and *Staphylococcus aureus*.

Based on the results of the microbiological tests, curcumin in both forms (free and encapsulated) showed no effect on the foodborne pathogens under consideration in this study with gram-negative bacteria being more resistant than gram-positive bacteria attributed to the difference in the membrane structure of these microorganism.

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ABBREVIATIONS

U.S.	United States
CDC	Center for Disease Control
FDA	Food and Drug Administration
CFIA	Canadian Food Inspection Agency
FSVP	Foreign Supplier Verification
PVL	Panton-Valentine Leukocidin
DNA	Deoxyribose nucleic acid
HPLC	High Performance Liquid Chromatography
DMSO	Dimethyl sulfoxide
SDS	Dodecyl sulfate sodium salt
HDPB	1-hexadecyl pyridinium bromide
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
DPPC	Dipalmitoylphosphadidylcholine
ml	milliliter
PCA	Plate Count Agar
CFU	Colony Forming Unit
µl	microliter
mg	milligram
mM	millimolar
µM	micromolar
S. aureus	*Staphylococcus aureus*
E. coli	*Escherichia coli*
L. mono	*Listeria monocytogenes*
O. stamineus	*Orthosiphon stamineus*

H. pylori	*Helicobacter pylori*
B. subtilis	*Bacillus subtilis*
B. cerues	*Bacillus cereus*

CHAPTER I

INTRODUCTION

Foodborne illness is a major public health concern worldwide. Foodborne illness can be defined as: "an outcome when sources and reservoirs of causative agents (organisms) get from the source to a food that is capable of supporting the growth of the organism at a temperature range long enough to allow this organism to grow to sufficient numbers or produce sufficient toxins and consequently consumed in sufficient amounts to cause illness" (Mead, et al. 1999). An updated estimate of annual foodborne illnesses in the United States by the Centers for Disease Control and Prevention (CDC) shows that merely 48 million people get food poisoned with 128,000 hospitalized and 3000 reported dead (Olaimat and Holley, 2012). While safer foods contribute to healthier and longer lives, they save on healthcare costs and contribute to more resilient food industry, thus improving livelihoods and increasing productivity. Preserving and providing safe foods are crucial in ensuring both health and food security.

The major foodborne pathogens contributing to most outbreaks include: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*.

Staphylococcus aureus is a facultative anaerobic gram-positive cocci bacterium that reproduces asexually by binary fission. This bacterium is commonly found on the skin and hair as well as in the noses and throats of people and animals. About 25 percent of healthy people are carriers of this bacterium. *S. aureus* can cause intoxication that is when

preformed toxins present in foods are ingested. The bacteria release exotoxins into foods during cell growth and metabolism. The incubation period is usually between 1 and 6 hours and the symptoms include, nausea, vomiting, diarrhea, loss of appetite, severe abdominal cramps, and mild fever. The illness most often lasts from 28 to 48 hours. Among the bacterial pathogens, S. aureus is particularly well adapted to reduced water activity environments (Fratamico, et al. 2005). According to the International Commission on Microbiological Specifications for Foods, under optimal conditions, *S. aureus* can grow at a water activity as low as 0.83 (Beuchat, et al. 2013).

Escherichia coli are also capable of causing foodborne illness through toxic-infection. It is a facultative anaerobic gram-negative rod shaped bacterium which is commonly found in the lower intestine of warm-blooded organisms. (Fratamico, et al. 2005) Most *E. coli* strains are harmless, but some serotypes can cause serious illnesses in their hosts, and are occasionally responsible for product recalls due to food contamination. This bacterium is ingested via foods after which toxins are produced inside the intestinal tract during growth, and released into the gut attacking the epithelial cells and, in some cases, into other organs via the bloodstream. The symptoms appear between 1 and 10 days and include: severe diarrhea that is often bloody, severe abdominal pain, vomiting, and little fever. The illness lasts for a period that extends from 5 to 10 days.(Fratamico, et al. 2005)

Listeria monocytogenes is the bacterium that causes the infection listeriosis. It is a facultative anaerobic gram-positive bacterium, capable of surviving in the presence or absence of oxygen. It can grow and reproduce inside the host's cells and is one of the most virulent food-borne pathogens, with 20 to 30 % of clinical infections resulting in death. It is responsible for an estimated 1,600 illnesses and 260 deaths in the United States (U.S.)

annually. Listeriosis is the third leading cause of death among foodborne bacterial pathogens, with fatality rates exceeding even *Salmonella* . Its incubation period ranges between 3 to 70 days and the symptoms include: fever, stiff neck, confusion and weakness, as well as vomiting. (Fratamico, et al. 2005) *L. mono* is a major concern in perishable refrigerated foods, particularly those that do not receive a lethal processing treatment, as well as foods that may be contaminated after processing. The ubiquitous nature of *L. mono* and its conditions, including low temperature, pH, water activity and high NaCl content, are the main reasons for its high prevalence in many ready-to-eat foods. Most human listeriosis cases are caused by the consumption of foods that are contaminated with high levels of *L .mono*, usually greater than 3 Log(CFU)/g. (AlNabulsi, et al. 2013)

Salmonella is gram-negative facultative anaerobe that has peritrichous flagella which are highly invasive and can spread easily. It can cause an infection type of foodborne illness including Salmonellosis, a disease caused by bacteria in this genus, with more than 2,000 serotypes, among which some are pathogenic to humans. After ingestion, Salmonella invades the small intestine and colon by entering absorptive mucosal cells and mucosa associated macrophages (Fratamico, et al. 2005). Symptoms usually occur 12 to 24 hours after the ingestion of the contaminated food and mostly include: headache, fever and chills, diarrhea, cramps, vomiting and nausea. The period of illness ranges between 1 and 3 days. As a matter of fact, infected individuals can carry and shed Salmonella for months. Usually foodborne illnesses from Salmonella are linked to consumption of poultry; nonetheless, other tahini based products have proven to be frequent vehicles. (Fratamico, et al. 2005)

Tahini, a sesame seed based product, is a ready-to-eat product common in the Middle East usually stored at room temperature. It is mainly comprised of 57-65% lipids,

23-27% protein and 6-9% carbohydrates. Tahini is considered as the base ingredient in the preparation of various ready-to-eat foods including halva (sweetened tahini), dips like hummus or baba ghanoush, as well as sauces like tajin or taratoor. Tahini-based dressing (taratoor) is prepared by mixing tahini (sesame paste) with water, garlic and lemon juice (citric acid). The dressing is freshly prepared and served with various traditional foods. Due to its popularity; consumers prefer to buy the product pre-prepared if available in the market. Hence, this is only possible if taratoor is safely processed and packaged with an extended shelf life.

Tahini has a long shelf-life, approximately 2 years from the production date, because of its low moisture content (less than 3%). Tahini, or its products, may be exposed to microbial contamination before, during or after processing, causing serious food safety concerns since most tahini based product are ready-to-eat and require minimal or no processing once they reach the consumer. According to Beuchat et al. (2013), mishandling and poor production of processes may also affect the hygienic and chemical quality of halva. This is of great importance since Lebanon is considered as one of the major exporters of sesame-based products including tahini and halva which have grown in popularity in the Middle East and countries such as the USA, Canada and Europe. However, Lebanese Tahini and Hummus have been recalled due to contamination/adulteration with foodborne pathogens in various countries including Australia, Canada, and the United States.(Beuchat, et al. 2013)

A survey conducted in the USA between 2007 and 2009 indicated that the average shipment prevalence of *Salmonella* in spices, including sesame seeds, was 6.6%, 4.4 times that of the regulations of FDA on imported foods, and more specifically for sesame seeds at

an even higher prevalence level of 11% (FDA 2012). In October 2011, the FDA issued a refusal report for tahini products manufactured in Lebanon due to possible Salmonella contamination (FDA 2012). On September 27, 2011, sesame seed paste containing a rare strain of Salmonella sickened 23 people in 7 states. The outbreak was identified when the District of Columbia Public Health Laboratory found that Salmonella isolated from three different patients had matching DNA fingerprints (CFIA 2012). These cases were then linked to a tahini-based product that had been imported from Lebanon.

Furthermore, *S. aureus*, *E.coli* and *Salmonella* were detected at unacceptable levels in tahini and halva samples obtained from retail markets and producers in Marmara region. Food safety issues associated with tahini have been also reported in Turkey according to (Kahraman, et al. 2010). Moreover, *S. aureus* was detected in 100% of tahini samples collected from Jordanian plants and the counts were even higher when the samples were collected from processing plants in Jordan and KDA (AlNabulsi, et al. 2013). In addition, high levels of E.coli were detected in Halva as reported by Sengun et al. (2005) (41 cfu/g). In 2008, tahini was recalled from retail markets in New Zealand due to the risk of contamination with *L. mono* without reported illness (AlNabulsi, et al. 2013).According to Kahraman et al., (2010) , 36.66 per cent (44/120) tahini halva samples were of unacceptable quality based on recommended criteria of microbiological (39 of 120, 32.5 per cent) by the Turkish Food Codex. In 2003, an outbreak of Salmonella occurred in in Montevideo involving contaminated tahini from the Middle East (OzFoodNet Working Group, 2004). In the United States of America, on September 27, 2011, 23 cases of *Salmonella enterica* serotype Bovismorbificans infections were identified among persons from seven states, with illness onset the investigation and trace back indicated that contaminated tahini used in

hummus prepared at a Mediterranean-style restaurant in DC was the source of Salmonella infections. (CDC, 2012)

Spices have long been researched and used to prevent spoilage and extend the shelf-life of foods. Many of the spices and herbs used today were known to the people of the ancient cultures throughout the world, and they were valued for their preservative and medicinal effect besides their flavor and odor qualities (Burt, 2004). How the ancient obtained their knowledge we do not know, but modern research has shown that many of these ideas are valid. According to Burt (2004), scientific experiments on the antimicrobial properties of the spices and their components have been documented in the late 19th Century and interest continues to the present. Accounting for this, spices antimicrobial activity may also be effective against food-borne pathogens (Burt, 2004). Plant extracts, including spices, are rich in phenolic secondary metabolites, and some have antibacterial activity. There were a number of studies carried on the use and effectiveness of herbal extracts for their antimicrobial activity. Among those are coriander (*Coriandrum sativum*), cinnamon (*Cinnamomum zylanicum*), clove (*Syzgium aromaticum*), tea tree oil (*Melaleuca alternifolia*), oregano (*Origanum vulgare*), sage (*Salvia officinalis*), as well as curcumin (*Curcuma longa*). The extracts of the Orthosiphon stamineus plant was tested for antimicrobial and antioxidant activity against selected food-borne bacteria *in vitro. (Ho, et al. 2010).* The inhibition observed with these *O. stamineus* extracts was comparable to the inhibition seen with the natural food preservative 5% lactic acid which is likely due to the high concentration of rosmarinic acid found in the extract. Thus, this suggests that rosmarinic acid content is closely associated with antibacterial and free radical scavenging activities of *O. stamineus* (Ho, et al. 2010). Studies have shown that Phenols, alcohols,

aldehydes, ketones, ethers and hydrocarbons have been recognized as major antimicrobial components in spices (Ceylan et al., 2004). The bactericidal effect of garlic extract was apparent within 1 h of incubation and 93% killing of *Staphylococcus epidermidis* and Salmonella typhi was achieved within 3 h. Yeasts were totally killed in 1 h by garlic extract but in 5 h with clove. (Arora et.al, 1999)

Turmeric (*Curcuma longa*) is extensively used as a spice, food preservative and coloring material in India, China and South Asia. It has been used in traditional medicine as a household remedy for various diseases. For the last few decades, extensive work has been done to establish the biological activities and pharmacological actions of turmeric and its extracts. A survey of the literature revealed several reports on volatile oil constituents, isolation and identification of curcuminoids, various biological activities and also in treating a variety of ailments and metabolic disorders. Modern interest in turmeric began in the 1970's when researchers found evidence suggesting that the herb may possess anti-inflammatory properties.

Curcumin (1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione/diferuloyl methane), the main bioactive chromophore of turmeric, has a wide spectrum of biological activities. The structure of curcumin, as indicated in Figure 1, consists of two ortho methoxylated phenols linked with a b-di-ketone function and they are all conjugated making it a potential candidate for the development of new inhibitors (Kaur, Modi et al. 2010).

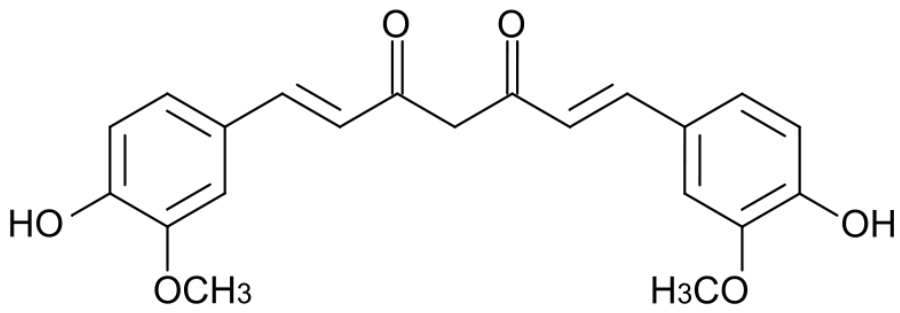

Figure 1 Structural formula of Curcumin

Recently, it has been demonstrated that curcumin inhibits polymerization of Bacillus subtilis FTsZ by perturbing the Z-ring formation and inhibits bacterial cytokinesis by inhibition FTsZ assembly (De, Kundu et al. 2009). Curcumin has also shown to prevent the growth of *Helicobacter pylori* growth, bacteria related to gastroduodenal diseases like gastritis, peptic ulcer, and gastric cancer. According to Kundu et al. (2009), the antibacterial activity of curcumin against 65 clinical isolated of *H. pylori* in vitro and during protection against *H. pylori* infection in vivo was examined showing effectiveness in inhibiting its growth in vitro irrespective of the genetic makeup of the strains (De, et al. 2009). Extensive research over the 5 decades indicates that curcumin possesses potent antioxidant, anti-inflammatory, antitumor, anti-HIV, and antimicrobial properties. It also inhibits lipid peroxidation and scavenges superoxide anion, singlet oxygen, nitric oxide and hydroxyl radicals(Basniwal, et al. 2011). Despite having multiple medicinal benefits and extremely superior safety profile, the administration of curcumin to patients has a serious practical problem. Studies have indicated that the insolubility of curcumin in water implies that a patient is required to swallow between 20g and 40g of curcumin every day in order for curcumin to exhibit the therapeutic effect in the human body (Basniwal, et al. 2011). As a result, despite the

inherent advantages of curcumin, it has never really made the journey from the kitchen shelf to the pharmacist's counter. To overcome the problems of poor solubility and low bioavailability, nanoparticle-based drug delivery approaches, in which curcumin is encapsulated in liposomes, have been reported.

Potential vehicles are liposomes that are small artificial vesicles of spherical shape with a membrane composed of phospholipid bilayer. They are widely used as carriers, especially in their application to topical delivery for a variety of drugs, because of their small size, biodegradability, hydrophobic and hydrophilic character and low toxicity. Results from several studies demonstrate that liposomes have the potential to enhance drug penetration into the skin, improve therapeutic effectiveness, reduce serious side effects, and act as local depots for the sustained release of drugs (Chen, et al. 2012). Liposome-encapsulated antimicrobial agents have been successfully used in humans and/or in animal models against protozoal, fungal, and bacterial infections (Beaulac, et al. 1998).In vitro bactericidal efficacy of sub-MIC concentrations of liposome-encapsulated antibiotic against Gram-negative and Gram-positive bacteria was determined in the Faculty of Medicine in the University of Montreal (Beaulac, et al. 1998). Such experimental studies have been performed with rigid liposomes, composed primarily of natural phospholipids and cholesterol yielding promising result through which direct interaction of liposome and bacteria was obtained by a fusion process (Beaulac, et al. 1998). Furthermore, clinical experience was obtained with liposomal preparations of amphotericin B in the treatment of systemic fungal diseases in cancer patients; these preparations were shown to be effective and very well-tolerated (Coune 1988).

Likewise, more studies were carried in an attempt to study the effect of curcumin encapsulation in liposomes' efficiency on different aspects. A study done in the Academy of Chinese Medicine investigated the in vitro skin permeation and in vivo antineoplastic effect of curcumin by using liposomes as the transdermal drug-delivery system (Chen, et al. 2012). Soybean phospholipids, egg yolk phospholipids, and hydrogenated soybean phospholipids were selected for the preparation of different kinds of phospholipids composed of curcumin-loaded liposomes and the results suggested that liposomes would be promising transdermal carriers for curcumin in cancer treatment (Chen, et al. 2012).Accordingly, similar studies were performed to assess the efficiency of liposomal curcumin nanoparticles to treat prostate cancer (Thangapazham, et al. 2008).The level of curcumin and its metabolites in portal and peripheral blood, bile and liver tissues was measured by High Performance Liquid Chromatography (HPLC) and was found to be so low that it was unlikely to exert pharmacological activity in those human tissues. This is why, according to Thangapazham et al. (2008), it is critical for a drug to reach the infection site and attain a pharmacologically desired concentration to be considered a potential drug candidate. Thangapazhm et al. (2008) suggested the use of liposomes as a delivery model for curcumin since the results from the cell proliferation assays provide strong evidence for liposomes as effective nano-delivery vehicles that increase the bio-activity of curcumin.

Interpreting all of the valid information related to curcumin efficiency drives the attention towards the encapsulation of curcumin liposomes in order to assess its effectiveness against foodborne pathogens that contaminate the Middle Eastern products, including tahini. There is not a lot of research done on this aspect so far. A study that focuses on the antibiotic activity of microcapsule curcumin against foodborne pathogens

and spoilage microorganisms' revealed inhibitory effect with varying sensitivities of one species compared to the others (Wang, et al. 2009). The inhibitory activity of microcapsule curcumin was evaluated against *Y. enterocolitica, B. subtilis, B. cerues, A. niger, P. notatum* and *S. cerevisiae* and showed that microcapsule curcumin kept the broad spectrum inhibitory activity of the free curcumin after microencapsulation process and high spray-drying process (Wang, et al. 2009).

In order to include liposomes in food products, the ultimate way would be to test the effect of encapsulated curcumin in the food package while maintaining quality and minimizing processing. This directed research towards the use of packaging to provide the intended safety and quality. One option is to use antimicrobial curcumin with packaging to extend the shelf-life of foods and reduce the risk from pathogens. Antimicrobial packaging is a form of active packaging that acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Appendini and Hotchkiss 2002). Therefore, the targeted microorganisms and the food composition must be considered in antimicrobial packaging. In a study done to assess the antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties revealed a greater antimicrobial activity against *S. aureus*, and *E.coli* as compared to *P. aureginosa* and *L. plantarum* in growth media (Emiroğlu, et al. 2010). It is thus essential to investigate curcumin as the antimicrobial agent in packaging in order to target the foodborne pathogens that contaminate tahini and its products.

With a heat sensitive product like tahini-based taratoor, where its natural qualities and freshness are to be retained with minimal processing and without additives, it is, thus, essential to investigate a novel method. Hence, the aim of this study was to investigate

curcumin as the antimicrobial agent with the liposomal encapsulation, and to assess its effect against the main pathogens: *Salmonella spp., E.coli, L. mono* and *S. aureus* that may contaminate the product.

CHAPTER II

MATERIALS AND METHODS

A. Bacterial suspension

A total of 10 bacterial strains were used in this study isolated from various food products. These strains include: two *E. coli* strains E1 and E2 isolated from meat, three *Salmonella* strains S1, S2 and S3 isolated from chicken, four *S. aureus* strains St1, St2, St3 and St4 isolated from milk, and one *L. mono* strain L1 isolated from milk. To test a suspension, a sterile loop was used to streak the isolate on a Plate Count agar (PCA) (356-4475)[1]. The plate was then incubated for 24-48hrs at 37°C, after which 4 to 5 well-isolated colonies of the same morphological type were transformed and suspended in 5ml Brain heart infusion broth (BHI) (356-4014)[1] and consequently incubated at 37°C for 18-24hrs.

[1] Bio-Rad Laboratories 2000 Alfred Nobel Drive, Hercules, CA 94547

B. Preparation of liposomes free from curcumin

1mM concentration of liposome was obtained by dissolving 3.67mg of Dipalmitoylphosphadidylcholine (DPPC) (850-355P)[2] membrane in 2.5ml of chloroform (166-252-500)[3] and 2.5ml of methanol (268-280-025)[3]. The solvent was evaporated on a rotary evaporator at 60 °C and then hydrated at 37 °C for 35 minutes in a neutral buffer solution (pH=7) of 5ml total volume with continuous vortex (Basniwal, et al. 2011).

C. Preparation of curcumin encapsulated in liposomes:

1. Curcumin Encapsulated in Liposomes

DPPC membrane was added to a specific volume of curcumin (218-858-0100)[3] in ethanol depending on the concentration prepared. Using this method, different concentrations of curcumin in liposomes were prepared for different purposes. The curcumin was obtained from a 5mM stock obtained by dissolving 36.838mg of powder curcumin in 20 ml of methanol. Seven different concentrations of curcumin in liposomes were prepared:

[2] Avanti®, Polar lipids, inc.
[3] Acros Organics-New Jersey, USA

1. 3.67 mg of DPPC was dissolved in 2.5ml of each of chloroform and ethanol and 5µl of curcumin, taken from the curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1mM and that of curcumin 5µM.
2. 3.67mg DPPC was dissolved in 2.5ml of each of chloroform and methanol and 10µl of curcumin, taken from the 5 mM curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1mM and that of curcumin 10µM.
3. 3.67mg DPPC was dissolved in 2.5ml of each of chloroform and methanol and 20µl of curcumin, taken from the 5 mM curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1mM and that of curcumin 20µM.
4. 3.67mg DPPC was dissolved in 2.5ml of each of chloroform and methanol and 50µl of curcumin, taken from the 5 mM curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1mM and that of curcumin 50µM.
5. 3.67mg of DPPC was dissolved in 2.5ml of each of chloroform and methanol and 100µl of curcumin, taken from the 5 mM curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1mM and that of curcumin 100µM.
6. 3.67mg of DPPC was dissolved in 2.5 ml of each of chloroform and methanol and 150 µl of curcumin, taken from the 5 Mm curcumin stock previously prepared, were added. The resultant concentration of DPPC was 1 mM and that of curcumin 150 µM.
7. 7.52mg of DPPC was dissolved in 2.5 ml of each of chloroform and methanol and 1ml of curcumin, taken from the 5 mM curcumin stock previously prepared, were added. The resultant concentration of DPPC was 2 mM and that of curcumin 1 mM

Then, the prepared solutions were evaporated on rotary evaporator at 60 °C, and then hydrated at 37 °C for 35 minutes in 5ml buffer solution with continuous vortex. The buffer solutions used were of three different pH mediums: pH=4.7, pH= 7 and pH= 8.5 depending on the objective of each experiment performed.

2. Curcumin and cholesterol encapsulated in liposomes

3.67 mg of DPPC, 5 µl of curcumin in ethanol (obtained from 5 mM stock solution), and0.5mlof cholesterol (247-1)[4] (20%) which was taken from prepared 2 mM cholesterol stock, were dissolved in equal amounts of chloroform and methanol to make up a total volume of 5ml. Then the solvent was evaporated on rotary evaporator at 60 °C, and then hydrated at 37 °C for 35 minutes in a 5 ml neutral buffer solution with continuous vortex (Basniwal, Buttar et al. 2011). The obtained liposomal suspension had a concentration of 1 mM of DPPC, 5 µM of curcumin and 0.2 mM of cholesterol.

[4] E.Merck AG.Darmstadt-Germany

D. Preparation of penicillin encapsulated in liposomes

Another control solution prepared was the encapsulated penicillin (704-206)[5] in liposomes. 3.76 mg DPPC membrane was added to 1.4 µl of penicillin taken from a previously prepared stock of 336 mM penicillin dissolved in distilled water. The 2 (DPPC and penicillin) were dissolved in 5 ml of chloroform and methanol equally. The solvent was evaporated on rotary evaporator at 60 °C, and then hydrated at 37 °C for 35 minutes in 5 ml neutral buffer solution with continuous vortex. The obtained concentration of penicillin in 1 mM DPPC membrane was 100 µM.

E. Preparation of free curcumin

Curcumin suspensions were dissolved and tested in different solutions throughout experiment. Different concentrations of curcumin were prepared as follow:

1. 36.838 mg of pure curcumin was dissolved in 5 ml ethanol (120592)[6] to obtain an overall stock concentration of 5 mM curcumin.

5 Panpharma S.A- France
6 Sigma-Aldrich-China

2. Curcumin was dissolved in dimethyl sulfoxide (DMSO) (311-10P-26)[3] to prepare various concentrations of curcumin. A higher concentration of 5mM stock of curcumin in DMSO was prepared by dissolving 36.838 mg of curcumin in the solvent. To prepare 5 , 10, 15, 25, 50, 70, 100, 1000, 1500, and 2000 µM, 5 , 10, 15, 25, 50, 70, 100, 1000, 1500, and 2000 µL of stock was respectively pipetted into 5 ml DMSO.
3. 1.84 mg of curcumin and 1.43 mg of piperine (101-131-038)[6] (spice) were dissolved in DMSO to obtain a final concentration of 1mM of each in 5 ml total volume solution.
4. 1.84 mg of curcumin was dissolved in different solvents to get a final concentration of 1 mM. The solvents prepared are: Dodecyl Benzene-sulfonic acid (DBSA) (325-910-250)[3], Dodecyl sulfate sodium salt (SDS) (419-530-00)[3], and 1-hexadecylpyridinium bromide (HDPB) (411-370-100)[3].

F. Preparation of free penicillin

A concentration of 100 µM penicillin was obtained from a 336 mM stock solution of penicillin. The solution was prepared by pipetting 1.4 µL of penicillin and making up the volume of solution to 5 ml by adding distilled water.

G. Antibacterial Sensitivity test

The antibacterial sensitivity test was be performed as described by the Clinical and Laboratory Standards Institute (Cockerill, et al. 2012).

1. Disk Diffusion

A pure suspension of each grown microorganism was poured on the surface of prepared agar plates. The agar used was Plate Count Agar. The experiment was performed on duplicate plates. Sterile filter paper discs (6-mm diameter) were applied and 0.1ml of pure curcumin preparations was pipetted. Each disk is pressed down to ensure complete contact with the agar surface. The disks are placed evenly so that they are no closer than 24 mm from center to center in order to avoid overlapping zones. The plates were then be maintained for 2 hours at room temperature and inverted to be placed in an incubator at 37°C for 24-48 hours afterwards. Standard antimicrobial discs (30 mg), penicillin, were included for comparison and as controls.

2. Well diffusion

The bacterial strains were inoculated with 1ml of an adjusted bacterial suspension of 10^6 CFU/ml within PCA prior to solidifying. The plates were then torn with sterile tips.

50 µl of different solutions were pipetted in the each hole. The plates were incubated at 37°C for 24 hrs. The diameter of the zones of inhibition was measured with a ruler and compared to the nearest millimeter. The average of zone diameter of duplicates plates was calculated.

H. Antibacterial Susceptibility Test

1. Determination of the Minimum Inhibitory Concentration (MIC)

The samples were analyzed for MIC according to the Clinical Laboratory Standards Institute. Double serial dilutions were prepared from a higher concentration of 1mg/mL of pure curcumin to a lower dilution in a series of test tubes. The prepared solutions had curcumin concentrations of: 10 µg/ml, 20 µg/ml, 30 µg/ml, 50 µg/ml, and 100 µg/ml in DMSO. 2 ml of each concentration of emulsion curcumin was pipetted into sterile test tubes. The test tubes then inoculated with 0.2 ml of the bacterial suspension being tested and incubated overnight at 37 °C. The tubes were inspected for growth (turbidity) using Turner Ultra Violet Visible Scanning Spectrophotometer (Model No.SM1102050-33) before incubation (at time 0) and after 24 hrs. of incubation at a constant wavelength of 540 nm. Negative and positive controls were also prepared and tested spectroscopically. The negative control consisted of the concentrations of curcumin without bacteria, while the positive control consisted of 0% curcumin solution inoculated with the bacterial suspension. The Minimum Inhibitory Concentration (MIC) was regarded as the lowest concentration in the series of dilutions, which did not permit the growth of the susceptible bacteria. This was repeated for each of the 4 pathogens; *Salmonella* spp., *E.coli, Listeria monocytogenes* and *S. aureus* (Cockerill, et al. 2012).

2. Determination of the Minimum Bactericidal Concentration (MBC)

The MBC were determined according to the standards of the Clinical Laboratory Standards Institute. A volume of 0.1 ml subcultures was taken from the tubes which did not

yield any visible turbidity in the MIC assay and grown on freshly prepared PCA plates in duplicates. After 24 hours of incubation at 37°C, the Minimum Bactericidal Concentration (MBC) was considered as the lowest concentration of the test solution that allowed for less than 0.1% of the original inoculum to grow on the surface of the agar plates. Test solutions were tested in duplicates for each experiment (Cockerill, et al. 2012).

CHAPTER III

RESULTS AND DISCUSSION

A. Liposomes

1. Disk Diffusion Method

Table 1 Disk diffusion test results for encapsulated curcumin as compared to Penicillin and liposome controls

Bacteria	Solution	Diameter Zone(mm)
L1	Penicillin Susceptibility Disks	15.5
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
St1	Penicillin Susceptibility Disks	31.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
St2	Penicillin Susceptibility Disks	21.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
St3	Penicillin Susceptibility Disks	15.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
St4	Penicillin Susceptibility Disks	13.5
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
E1	Penicillin Susceptibility Disks	18.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
E2	Penicillin Susceptibility Disks	18.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
S1	Penicillin Susceptibility Disks	15.0

	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
S2	Penicillin Susceptibility Disks	19.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0
S3	Penicillin Susceptibility Disks	18.0
	Liposome (1mM)	0.0
	Encapsulated Curcumin (5 µM) in Liposome (1mM)	0.0

DPPC liposomes were incapable of inhibiting the present microorganisms since the zone diameter was zero for all bacterial strains when compared to varying inhibition zone of penicillin standard disks ranging between 15 and 31 mm (Table 1). However, this cannot be applied on curcumin encapsulated in liposomes and there was a need to test higher concentrations of encapsulated curcumin.

2. Well diffusion

Table 2: Well diffusion tests for all isolates using different solutions at various pH values

Concentrations	pH	Diameter Zone (mm)
DPPC 1Mm with curcumin in ethanol 5 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
DPPC 1 mM with curcumin in ethanol 10 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
DPPC1mM with curcumin in ethanol 20 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0

DPPC 1 mM with curcumin in ethanol 50 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
DPPC 1 mM with curcumin in ethanol 100 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
DPPC 1 mM with curcumin in ethanol 150 µM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
DPPC 2 Mm with curcumin in ethanol 1 mM	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0
1mM DPPC, 10 µM curcumin, and 20 % cholesterol	Acidic (pH=4.7)	0.0
	Neutral (pH=7.0)	0.0
	Basic (pH=8.5)	0.0

Different concentrations of curcumin in liposomes were tested (Table 2).At each step, a control of DPPC liposomes having the same concentration of the tested encapsulated curcumin was set as a control. Despite this, there was no inhibition diameter zone recorded for all isolates tested.

Since the pH medium can highly affect the liposomal suspension, other pH mediums, besides the neutral chemical medium, were tested. Acidic (pH=4.7), and basic (pH=8.5) chemical mediums were used as the buffer solution of the encapsulated curcumin. In fact, results of a study done to analyze the effects of pH and intra-liposomal buffer strength on the rate of liposome content release and intracellular drug delivery suggested that the pH titration behavior of liposome entrapped drug molecules must be considered in

the design of liposome-mediated delivery systems that enter cells by endocytosis (Burt 2004). Where the drug is only permeable at low pH, selective intracellular unloading can be engineered by entrapping the drug within the liposome at high pH. In contrast, where the drug is more membrane permeable at high, cell specific unloading can be achieved by entrapping the agent within the liposome in a strongly buffered acidic medium. The reduced pH within the endosome will then promote the diffusional release of both types of drug molecules (Burt 2004). The tested pH mediums did not alter the results of the experiment at all.

To make sure that curcumin was being incorporated into the membrane, a slight difference in the liposomal preparation was altered, where encapsulated curcumin in liposomes were prepared by incorporating cholesterol to the suspension (Table 2). Even though studies have proven that liposomal formulations that include cholesterol make the liposomes rigid and strong and thus the drugs entrapped in these rigid liposomes are more effective than free drugs in vivo (Beaulac, et al. 1998), in this study, the incorporation of cholesterol to the liposomal suspension did not enhance the results of the bacterial strains being studied.

Taking all of this into consideration, it was clear that liposomes were not being effective against the bacterial suspensions tested. There must be, therefore, a problem in the liposome-curcumin suspension. This comes with contradictions with some research done in this field. A study done to assess the antibiotic activity of micro-capsuled curcumin against foodborne pathogens showed that when compared to free curcumin, although after the microencapsulation process, the microcapsule curcumin still had effective inhibition activity (Wang, et al. 2009). However, to obtain these results, the curcumin was dissolved in acetone

and encapsulated in starch (Wang, et al. 2009). To ensure that the liposome encapsulation was altering the antimicrobial activity of curcumin, encapsulated penicillin in liposome was tested.

B. Encapsulated penicillin in liposome

Table 3: Effect of encapsulation of penicillin in liposomes on the inhibition zone diameter using the well diffusion

Bacterial Strains	Inhibition Zone Diameter of Free Penicillin (mm)	Inhibition Zone Diameter of Encapsulated Penicillin (mm)
St1	18.0	14.5
St2	36.0	26.5
St3	21.0	16.0
St4	21.0	15.0
L1	26.0	13.5

The results summarized in Table 3 show that free penicillin yields more inhibitory effect on the bacterial strains compared to encapsulated penicillin. For example, the measured inhibition zone diameter for St1 was 18mm and decreased to 14.5 mm when the penicillin was entrapped in the liposome. St2 was inhibited by free penicillin with an inhibition zone diameter of 36 mm making it a susceptible microorganism to this suspension, while this inhibition zone diameter decreased to 26.6mm when the penicillin was encapsulated in liposome.

The antibacterial assay studies revealed that the therapeutic efficacy of curcumin significantly enhanced upon nanoparticle formation of curcumin. It was quite an unusual finding that an aqueous dispersion of nano-curcumin had more effective antimicrobial

activity than the solution of normal curcumin in DMSO (Basniwal, et al. 2011). Meanwhile, the delivery of curcumin in liposome as an anticancer drug to treat osteosarcoma (OS) and breast cancer leading to effective results was considered as an *in vivo* study (Dhule, et al. 2012). However this work showed that the *in vitro* liposomal encapsulation method of curcumin was ineffective against the tested bacterial strains. Therefore, free curcumin had to be tested for its inhibitory effect against the tested foodborne pathogens.

C. Free curcumin

1. Well diffusion

Being an insoluble component in water, curcumin had to be dissolved in another solvent that yields no inhibitory effect. Accordingly, well diffusion has proved to be more precise than disk diffusion method. Therefore, the experiments were performed in the well diffusion method. Different solvents were thus prepared and tested (Table 4). For each tested solvent dissolving curcumin, a control of the solvent alone was used. The final concentration of curcumin in the solution was 1mM. Results were obtained in the well diffusion method.

Table 4: Inhibition zone diameter of free curcumin using four different solvents

	Inhibition Zone Diameter (mm)							
Bacterial Strains	DMSO	Curcumin in DMSO	DBSA	Curcumin in DBSA	Solvent SDS	Curcumin in SDS	Solvent HDPB	Curcumin in HDPB
St1	0.0	10.5	12.0	14.5	12.0	12.0	12.5	14.0
St2	0.0	13.5	21.5	22.5	14.0	14.0	14.0	14.5
St3	0.0	14.0	13.0	13.0	12.0	14.0	13.0	19.0
St4	0.0	14.0	14.0	19.0	13.0	15.0	16.0	16.0
S1	0.0	0.0	13.0	13.0	12.0	12.0	14.0	14.0
S2	0.0	0.0	12.5	12.5	13.5	13.5	13.0	13.0
S3	0.0	0.0	14.0	14.0	15.0	15.0	10.0	10.0
L1	0.0	14.0	15.5	16.0	10.0	12.0	10.0	11.0
E1	0.0	0.0	12.0	12.0	10.0	10.0	10.5	10.5
E2	0.0	0.0	11.0	11.0	11.0	11.0	13.0	13.0

All bacterial suspensions tested showed no inhibition when DMSO was tested alone. However, other solvents showed inhibitory effect when tested alone (Table 4). For instance, St1 had an inhibition diameter zone of 12 mm in DBSA, St4 had an inhibition zone diameter of 13 mm in DBSA, L1 had 10mm diameter inhibition zone in SDS, and St3 had 13 mm zone in HDPB. This implies that the solvents tested had antibacterial effect prior to dissolving curcumin in them. This can be explained by the fact that these three solvents are surfactants and have proven to have antimicrobial effects. The inhibition zone diameter of solvent 1 is zero in all the bacterial suspensions tested. Since we needed a solvent that can dissolve curcumin without itself being inhibitory, curcumin solution in DMSO was used. Visual results of different solutions in well diffusion method are shown in the Appendix 1.

The inhibitory effect of curcumin dissolved in DMSO is shown in Table 4. The inhibition zone diameter of E1, E2, S1, S2, and S3 is zero. This means that these pathogens do not exhibit any inhibition when curcumin is applied. On the contrary, the remaining microorganisms showed varying inhibition zone diameters. While St1 had an inhibition zone diameter of 10.5 mm and St2 that of 13.5 mm, the other bacterial strains including St3, St4 and L1 had the same inhibition zone of 14 mm. The results implied that curcumin had an inhibitory effect against these bacterial strains. This means that St1, St2, St3, St4 and L1 have higher sensitivity toward curcumin as compared to S1, S2, S3, E1 and E2. St1, St2, St3, St4 (*Staphylococcus aureus*) strains, and L1 (*Listeria monocytogenes*) are gram-positive bacteria that showed inhibition while S1, S2, S3 (*Salmonella*) strains and E1 and E2 (*E. coli*) strains, are gram-negative bacteria that did not yield any inhibition zone diameter probably due to the outer phospholipidic membrane that leads to different types of interactions when encountered with curcumin. This coincides with different studies that have proven that gram positive bacteria contain an outer peptidoglycan layer which is an ineffective permeability barrier; the resistance of gram-negative bacteria towards curcumin could be due to the outer phospholipidic membrane carrying the structural lipopolysaccharide components, which makes it impermeable to curcumin (Wang, et al. 2009). When free curcumin was tested against two gram-positive and two gram-negative bacteria in India, results indicated that the selected gram-positive bacteria had higher sensitivity than the selected gram-negative ones (Basniwal, et al. 2011). Moreover, in vitro studies done on essential oils against bacterial suspensions demonstrated that gram-negative organisms are slightly less susceptible than gram-positive bacteria (Burt 2004). Another study including *E. coli* strain and *S. aureus* strain proved that the antibacterial activity of

curcumin was more pronounced against gram-positive bacteria than gram-negative bacteria (Wang, et al. 2009).

The same inhibition zone diameter was obtained when changing concentrations of curcumin dissolved in DMSO. All concentrations of curcumin ; 5 µl, 10 µl, 15 µl, 25 µl, 50 µl, 70 µl, 100 µl, 1.5 mM, and 2.5 mM; yielded the same inhibition zone diameter as compared to 1mM curcumin in DMSO as represented in Figure 2.

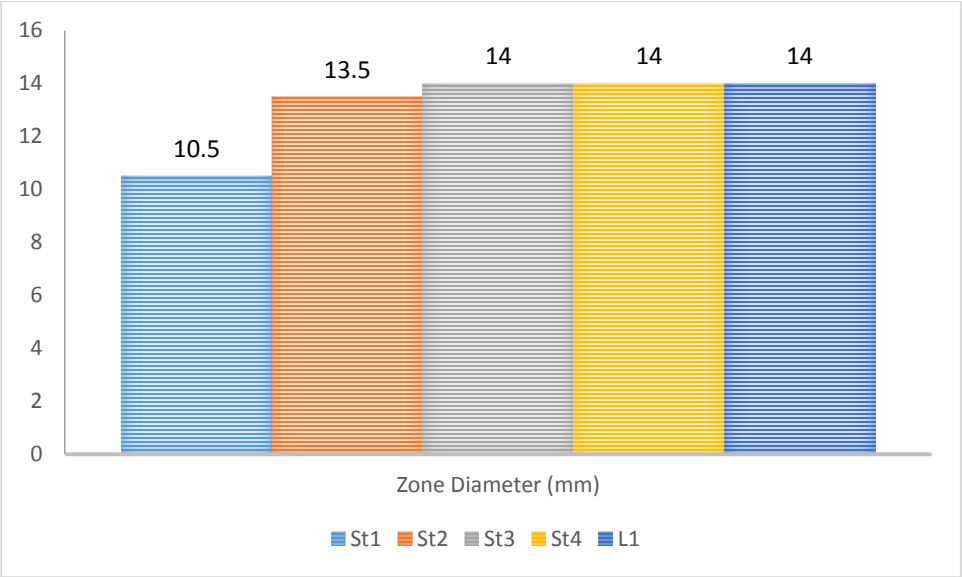

Figure 2: Inhibition zone diameter for curcumin dissolved in DMSO

According to the Clinical and Laboratory Standards institute, the zone diameter value is used to determine whether the tested bacteria are susceptible, intermediate, or resistant against a given antimicrobial agent. The effect of an antimicrobial agent against a given factor is divided into categories that vary according to the zone diameter. The standards are represented by table 5 (Cockerill, et al. 2012).

Table 5: Standard categories based on the inhibitory zone diameter for antimicrobial agents

Category	Zone diameter (mm)
Susceptible	≥20
Intermediate	15-19
Resistant	≤14

By comparing our results (Figure 4) and the standards in Table 5, the bacterial strains St1, St2, St3, St4 and L1 have an inhibition diameter zone less than or equivalent to 14 which is compatible with the resistant range. The "resistant" category implies that isolates are not inhibited by the usually achievable concentrations of Curcumin. A study performed on the medicinal plant *Curcuma longa* examining its biological effect against *S. aureus* showed a weak inhibition (23.25%) (Khattak, et al. 2005). Moreover, S. aureus was proved to be inhibited by *Curcuma malabarica* but not by *Curcuma zedoaria* in a study carried by the Center Tuber Crops Research Institute (Wilson, et al. 2005). The findings of Wilson et al. support the idea that not all curcumin extracts have the antimicrobial capacity. *Curcuma longa*, the curcumin used in this study, might also be ineffective against *S. aureus* like *Curcuma zedeoria.* These studies highly validate the obtained experimental results. Although curcumin has shown diameter zone values in well diffusion method as an antibacterial agent, yet the inhibition is not enough in affecting the bacterial strains. This is why these strains are said to be resistant against free curcumin.

According to Khan et al., (2006), piperine, a trans-trans isomer of 1-piperoyl-piperidine, in combination with ciprofloxacin markedly reduced the MICs and mutation prevention concentration of ciprofloxacin for *Staphylococcus aureus* (Khan, Mirza et al. 2006). This drives us to wonder whether Piperine is capable of doing the same when

incorporated with curcumin in DPPC membrane liposome. However, when 1Mm of curcumin and 1mM of piperine in DMSO was tested for greater inhibition zone diameter, the results revealed that curcumin with Piperine had same inhibitory effect as curcumin alone.

Since curcumin itself is not capable of inhibiting the foodborne pathogens being studied, it is predictable that when the curcumin is encapsulated, it would be more difficult for the suspension to diffuse and possess an antimicrobial effect. The curcumin is thus entrapped in the liposomal suspension rather than being able to diffuse and interact with the bacterial strains to inhibit their growth. It is supposed that curcumin breaks the peptidoglycan layer and penetrate inside the cell, thereby causing disruption of the structures of cell organelles and killing the cell through lysis (Basniwal, et al. 2011). This comes in disagreement with previous studies done on foodborne pathogens. Previous studies carried out on *B. subtilis* have shown that the mechanism of antibacterial activity of curcumin involves perturbing the GTPase activity of FtsZ and protofilaments, which are known to play a critical role in bacterial cytokinesis (De, et al. 2009). The tested microorganisms in this study are most probably unaffected by this mechanism and are capable of being resistant to curcumin.

2. MIC

Due to the fact the MIC and MBC determination are more specific and accurate methods in susceptibility testing than well diffusion, further experiments were carried to determine the MIC and MBC of St1, St2, St3, St4 and L1.

Spectrophotometer readings of different concentrations of emulsion curcumin at λ= 540 nm which is the negative control were recorded as shown in Table 6. The negative control represents different concentration of curcumin dissolved in DMSO without any bacterial suspension involved. Table 6 shows how the absorbance in spectrophotometer decreases as the concentration of curcumin prepared decreases.

Table 6: Negative control of different curcumin concentrations

Curcumin concentration in µg/ml	Absorbance
100 µg/ml	2.500
50 µg/ml	1.245
30 µg/ml	0.640
20 µg/ml	0.320
10 µg/ml	0.132
0 µg/ml	0.024

Table 7 Spectrophotometer readings for the different concentrations of emulsion curcumin inoculated with bacterial strains at λ= 540 nm before and after incubation

Strain		100 µg/ml	50 µg/ml	30 µg/ml	20 µg/ml	10 µg/ml	0 µg/ml
St1	Before	2.300	1.157	0.438	0.238	0.194	0.023
	After	2.096	1.059	0.742	0.528	0.274	0.051
St2	Before	2.275	1.293	0.472	0.332	0.155	0.025
	After	1.848	0.900	0.760	0.569	0.287	0.049
St3	Before	2.348	1.238	0.435	0.219	0.235	0.021
	After	1.760	0.921	0.768	0.493	0.246	0.044
St4	Before	2.422	1.269	0.455	0.223	0.196	0.032
	After	1.996	1.041	0.733	0.329	0.263	0.045
L1	Before	2.520	1.222	0.638	0.445	0.233	0.021
	After	2.168	1.046	0.799	0.512	0.259	0.052

According to Table 7, there was normal growth of the five bacterial strains St1, St2, St3, St4 and L1 in 30 µg/ml, 20 µg/ml, 10µg/ml and 0 µg/ml of curcumin; this was determined by the increase in the spectrophotometric readings after incubation as compared to the readings before incubation. Thus, these concentrations permit the growth of the susceptible bacteria. The MIC test allowed inhibition of bacterial growth from 50 µg/ml curcumin concentration to 100 µg/ml curcumin concentrations for all strains of bacteria, as indicated by the decrease in spectrophotometer readings after 24 hours of incubation (Table 7).

According to the Clinical and Laboratory Standards institute, the MIC value can also be used to determine whether the tested bacteria are susceptible, intermediate, or resistant against a given antimicrobial agent. The standards are summarized by Table 8 (Cockerill, et al. 2012).

Table 8 Standard categories for MIC

Category	MIC (µg/ml)
Susceptible	≤4
Intermediate	8-16
Resistant	≥32

Since the MIC of the bacterial strains which was recorded as 50 µg/ml is greater than 32 µg/ml, thus the strains are resistant to the antimicrobial agent being tested, curcumin (Lalitha 2004).

The results of the inhibition zones for both well diffusion and MIC are linked together as shown in Figure 3.

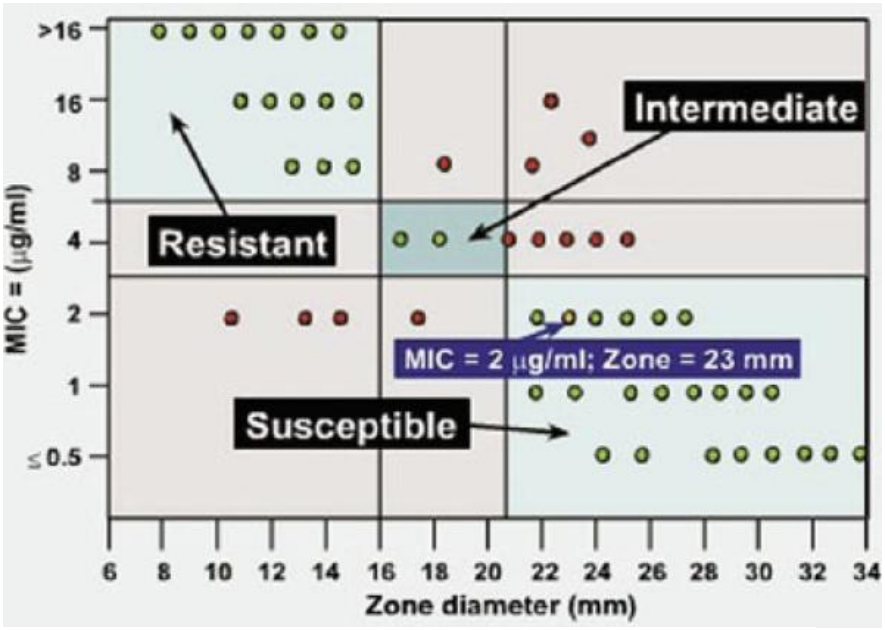

Figure 3: Scatter gram relating MIC and well diffusion

Curcumin, the antimicrobial agent being tested, exhibits a maximum diameter of inhibition of 14 mm and an MIC value of 50 µg/ml and according to the scatter gram (Figure 3 both values fall within the resistant region. Therefore, the tested microorganisms are resistant to curcumin.

3. MBC

The Minimum Bactericidal Concentration test showed bacterial growth on all plates as recorded in Table 9, because none St1, St2, St3, St4 and L1 of the plates had 10 colonies count or less. We can thereby infer that the concentrations of curcumin tested were not effective enough to kill the bacteria.

Table 9: Plate count for the Minimum Bactericidal Concentration tests

Strain	50 µg/ml	100 µg/ml
St1	TNTC	11
	TNTC	10
St2	TNTC	26
	TNTC	11
St3	TNTC	TNTC
	TNTC	TNTC
St4	TNTC	TNTC
	TNTC	TNTC
L1	TNTC	84
	TNTC	82

It is important to note that the readings of the curcumin concentrations inoculated with bacteria that exhibited inhibition after incubation (50 µg/ml and 100 µg/ml) were less than their respective readings of the curcumin without bacteria (negative controls) (Table 7). This indicates that the curcumin was used up in the process of inhibition, or that it lessened in amount over time.

A variety of methods have been reported in this study for testing the antimicrobial activity of a spice, curcumin. However, the degree of observed microbial inhibition, depending on the method employed to test for antimicrobial activity, was variable among the ten selected bacterial strains. Microorganisms differ in their resistance to a given antimicrobial agent and the findings of the study revealed that all foodborne pathogens being tested were resistant to curcumin. Gram negative bacteria are more resistant than gram-positive bacteria.

The results obtained in the tests could be due to various reasons. The lack of diffusion of the curcumin, due to the physical barrier present in the agar, in the well diffusion test could be a factor behind the observations of resistant category of bacterial strains. However, in the MIC test, the curcumin was in direct contact with the bacteria, and still the growth of the bacterial strains was detected. The MBC test indicated that the specific concentrations 50 µg/ml and 100 µg/ml were not bactericidal, so higher concentrations of curcumin should be used in order to kill the bacteria. Moreover, nutrients present in curcumin may stimulate growth and biochemical activities of microorganisms. Therefore, the concentrations of curcumin that could be added to food ingredients to inhibit microbial growth are generally too low to prevent foodborne illness by microorganisms.

CHAPTER VI

CONCLUSION

Many studies on curcumin show that it exhibits a wide range of anti-microbial and anti-fungal activities when tested *in vivo*. On the other hand, the results obtained in this study reveal that when encapsulated in liposome to improve its solubility, curcumin possessed no inhibitory effects on foodborne pathogens including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella spp*. and *Listeria monocytogenes*, while free curcumin dissolved in DMSO, a solvent with no antimicrobial effect, had some activity on the selected foodborne pathogens.

The effect of free curcumin on the foodborne pathogens varied between gram-negative and gram-positive bacteria. Gram-negative pathogens appeared to be more resistant than gram-positive which may be attributed to the presence of an outer phospholipidic membrane altering the permeability of the cells. Moreover, the maximum inhibition zone diameter was 14 mm using the well diffusion antibacterial testing whereas the MIC value was 50 µg/l for free curcumin. Based on these results for MBC and MIC, curcumin is considered to have low antimicrobial effects on the pathogens in question and thus curcumin in low concentrations may not be considered as an antimicrobial agent.

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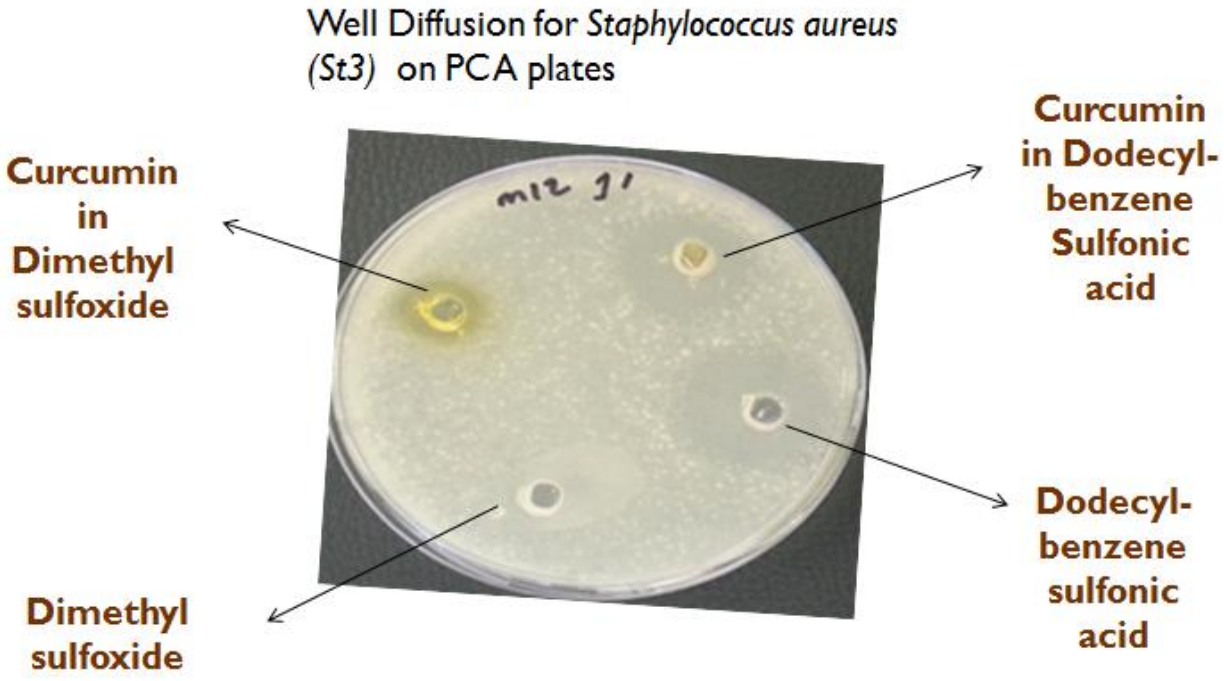
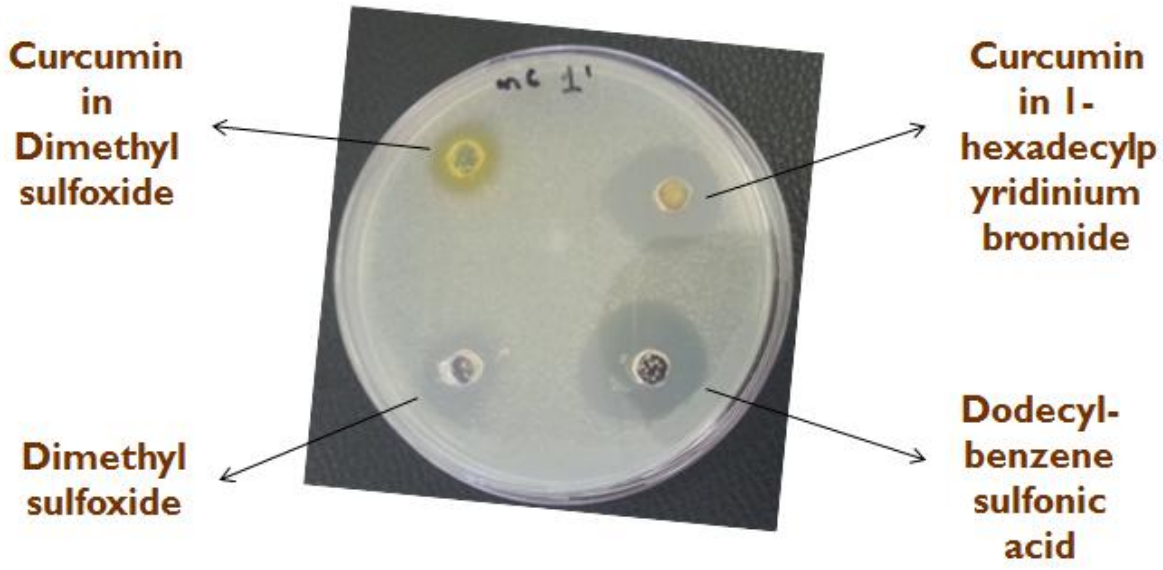
Appendix 1

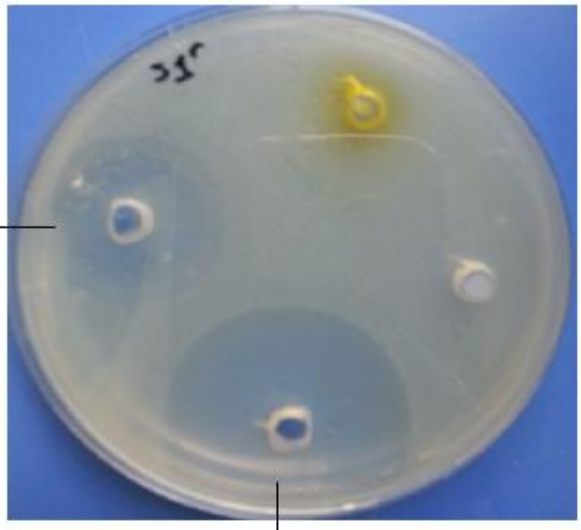
Visual results of different solutions in well diffusion method



Well Diffusion for *Staphylococcus aureus (St2)* on PCA plates



Well Diffusion for *Salmonella spp. S1* on PCA plates



Encapsulated Penicillin In Liposome

Free Penicillin