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Enhancement of Antimicrobial Activity of Naturally occuring Phenolic Compounds by Nano-particle Mediated Delivery Against Listeria monocytogenes, Escherichia coli O157:H7, and Salmonella Typhimurium

Madhuram Ravichandran
University of Arkansas, Fayetteville

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Enhancement of Antimicrobial Activity of Naturally Occuring Phenolic Compounds by Nano-particle-mediated Delivery Against *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Typhimurium

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By

Madhuram Ravichandran
SASTRA University
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University of Arkansas

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_____
Dr. Navam S. Hettiarachchy
Thesis Director:

_____
Dr. Dan Davis
Committee member

_____
Dr. Luke Howard
Committee member

_____
Dr. Mahendran Mahadevan
Committee member

_____
Dr. Jackson Lay Jr.
Committee member

_____
Dr. Soo Hyoun Ahn
Ex-officio committee member

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DEDICATION

Ella pugazhum iraivanuke (Praise be to the almighty)!

My MS Thesis is completely dedicated to the Almighty, under whose skillful direction these 7 years brought in all the characters and experiences that ultimately helped to pulverize the fine layers of delusion that blocks the awareness of the presence of supreme energy. Without his vigilant protection I would be lost in this arduous and illusionary play!

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USDA-ERS – United States Department of Agriculture – Economic Research Service

CHAPTER I

INTRODUCTION

A. Foodborne Illness

The foodborne illnesses caused by pathogens pose an enormous social and economic burden in the nation amounting to over $152 billion annually (Scraff, 2010). With increasing number of recalls and outbreaks, losses associated with every major food contamination issue, more research is being directed towards finding measures to mitigate the present problem. In addition to this, there is an increasing demand for the adoption of natural and alternative ways to disinfect food products (Smid and Gorris, 1999). Several disinfection strategies are in place to control pathogens but there is a need for a system that less interacts with the food helps deliver an antimicrobial in a better way and helps sustain the antimicrobial activity over a longer period of time.

B. Plant extracts as anti-microbials

The global population has recently turned its interest over natural ingredients as an alternative for artificial chemicals and disinfectants to sanitize food products, because of their well documented no or negligible side-effects (Beuchat and Golden 1999). Plants are excellent sources of phenolic metabolites. Phenolic compounds can serve as good antioxidants, degrade bacterial cell membranes and hence have potential for antimicrobial activity (Shetty and Lin 1999). However there is very little information on other bioactivities of these individual phenolic compounds especially the antimicrobial properties that could be well exploited by the food industry as they would not only preserve the food but also add nutritional value to the food being powerful anti oxidants. Nisin is a natural membrane active bacteriocin protein and biopreservative that has the potential to improve the efficiency of antimicrobial action when

added along with the main antimicrobial like organic acids or grape seed extract (Sivarooban et al. 2008, Gadang et al. 2008).

C. Nanotechnology

Earlier studies conducted in our laboratory have confirmed the bacteriocidal nature of GSE and nisin incorporated into edible films and turkey frankfurters (Sivarooban *et al*., 2008; Sivarooban *et al*., 2007). Effectiveness of antimicrobials is greatly reduced in a food system because of the association with other food components (e.g. lipids, proteins). This has also been attributed to the alignment of antimicrobials in the hydrophobic regions of foods while bacteria are growing in areas containing water. This lack of activity may be overcome by using suitable delivery systems utilizing nanotechnology (Carnahan *et al*., 2005). Nanotechnology can be used in intervention technologies and targeted controlled delivery of antimicrobial compounds in food and nonfood microbial safety applications and found to be more potent than the parent compound delivered as such (Gaysinsky *et al.*, 2004). The polyphenolics can be attached to the cores of nanoparticles and delivered into bacterial cells.

C.1. Nanoparticles in food systems

Nanotechnology involves use of materials at an extremely small scale (1-100 nm). Nanotechnology has the potential to revolutionize agriculture and food systems. It can be used in intervention technologies and targeted and control delivery of antimicrobial compounds in food and nonfood microbial safety applications. Antimicrobial compounds can be attached to cores of nanoparticles and delivered into bacterial cells. Dimethyl sulfoxide (DMSO) is biocompatible and has been used as water-miscible solvents in food applications (Unger, 2000) including sauerkraut, tomato paste, milk, beer, coffee, tea, and alfalfa. Polyvinyl alcohol (PVA) is used as a

coating, binder, sealing and surface finishing agent in food products such as dairy-based desserts, confectionery and cereal products (Food Agriculture Organization, 2009).The PLGA-DMSO/PVA is a non-toxic system and is proven to be biocompatible in food systems (Astete and Sabliov, 2006). In this system, poly lactic-co-glycolic acid (PLGA) serves as a nanoparticle carrier, DMSO maintains miscibility of nanoparticle packaged antimicrobials with water, and PVA acts as surfactant to stabilize the organic nanoparticles.

No literature information is available on the antimicrobial activity of selected phenolic compounds, alone or in combination encapsulated into nanoparticles in food system. Our research related directly to the goal of evaluating inhibitory activity of phenolic compounds, alone or in combination released from nanoparticles. We investigated nanoparticles for their use as vehicles to maximize the effectiveness in the enhancement of microbial inhibition through fast and slow release of potent phenolic inhibitors to prevent growth and faster destruction of these pathogens if they re-contaminate raw meat and fully cooked ready-to-eat chicken meat products.

Our solution to the problem lied in not finding just a natural antimicrobial or a combination of such antimicrobials that can decontaminate the food but using a proper delivery system that could better the inhibition of the pathogens with little interaction with the food matrix, as a multiple hurdle technology.

The following specific objectives were developed to achieve our goal:

1. Investigate the antimicrobial activities of naturally occurring phenolic compounds with and without nisin against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium using direct and nano-scale delivery in broth and chicken meat system

2. Investigate the antimicrobial activities of naturally occurring phenolic compounds in combination with ethylene diamine tetraacetic acid against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium using direct and nano-scale delivery in broth and chicken meat system

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CHAPTER II

LITERATURE REVIEW

A. United States meat industry and associated outbreaks

Food-borne pathogens still continue to cause illnesses at an alarming rate of 40 million people annually in the US. In spite of all the technological advances in food processing and safety**,** the food borne illness continue to rise in number since the start of food net surveillance in 1996. Several outbreaks have been linked to *Listeria, Escherichia coli* and *Salmonella* and have necessitated food recalls. Most of these recall for contaminated foods have been related to ready to eat poultry and meat products (CDC, 2011). The prevention of food borne diseases is chiefly dependent on the precautions taken during production, processing, handling and storage. Strategies for inhibition of these pathogens are of great concern for both food industries and the consumers.

In the past decade, several foodborne outbreaks have been linked to poultry meat. Both in 2012 and 2013, *Salmonella* infections in live poultry have contributed to illnesses in the people reported to have had contact with the birds (CDC, 2013a). The serovars of *Salmonella* associated with the live poultry infections were Typhimurium, Infantis, Lille, Newport, Mbandaka, Hadar, Montevideo (CDC, 2013a). Another *Salmonella* outbreak occurred in July 2013, infecting 134 people in 13 states associated with raw chicken (CDC, 2013b).

Foodborne outbreaks and illnesses are also a huge economic and social health burden. In 2009, *Salmonella* and *Escherichia coli* O157:H7 infections costed nearly $2.6 billion and $0.4 billion respectively (USDA-ERS, 2009). The economic loss associated with *Salmonella* (non-typhoidal serotypes only) and *E. coli* O157:H7 was estimated as $ 2.8 billion by Economic Resource Service, USDA (ERS 2006). In 2004, 42,197, 2,544, and 753 incidences per 100,000 population of *Salmonella* and *Escherichia coli* O157:H7, and *Listeria monocytogenes* were reported (CDC 2006). The economic impact of foodborne illness and the short shelf life of

refrigerated meat products demand for the development of effective control of microorganisms. In the US, each year food-borne illness affect 14 million persons and cause 1,800 deaths (CDC 2003). Outbreaks of food-borne pathogens including *Listeria monocytogenes, E.coli* O157:H7 and *Salmonella* Typhimurium are of great concern to the food industry and the general public (Gennadios and Weller, 1991, Goft *et al.*, 1996).

Most of the recalls for pathogen contaminations are related to ready-to-eat (RTE) poultry and meat products. In 2005, 2.4 million of RTE meat products including chicken, turkey and beef meats were recalled due to *L. monocytogenes* contamination. Approximately 80,000 pounds of beef products were recalled for *E. coli* O157:H7 during 2005 (FSIS/USDA, 2006a). Each year, the cost of acute illness from foodborne *L. monocytogenes* alone is $2.3 billion in the United States (ERS/USDA, 2002). Moreover, each year, in the U.S., *L. monocytogenes* causes an estimated 2,500 illnesses and 500 deaths (CDC, 2003). Listeriosis has been shown to cause miscarriages and result in meningitis in patients with chronic underlying diseases (Alterkruse *et al.*, 1994). *E.coli* has been implicated as the causative agent in the outbreak of gastroenteritis. *Salmonella* can cause invasive disease or reactive arthritis (Cutter and Siragusa, 1997).

B. Methods of decontaminating raw and cooked poultry meat

In 2009, the annual per capita consumption of poultry meat in the United States was at 69.4 lb, higher than beef (58.4 lb) or pork (46.9 lb) consumption (USDA-ERS, 2012). Around the world, the value reached 30 lbs/ person/ year (The poultry site, 2012). According to the United States Department of Agriculture (USDA), there is a projected increase in poultry production and consumption through this decade (USDA, 2012). The per capita consumptions of total poultry, beef and pork in 2013are projected to be 98.5, 51.3 and 46.3 lbs respectively (USDA, 2012). A great demand exists for the production and consumption of poultry meat products.

During post-processing, mishandling and minimal processing can expose poultry meat to bacterial pathogens. Current chemical treatment of chicken carcasses has been approved by USDA-FSIS using rinse or wash with organic acids (acetic, lactic acids) or combination of trisodium phosphate with halogen compounds and hydrogen peroxide. Techniques including dip or spray treatments with hot water and other disinfectants, steam, electromagnetic radiation (UV and microwaves), high pressure processing or sonication have been adopted for reducing/eliminating bacterial pathogens from raw poultry meat (Dincer and Baysal 2004). In RTE meat processing plants, cooking chicken at 165 °F and beef at 145 °F eliminates most pathogens and only post-processing mishandling can lead to further contaminations and recalls. The common approaches to reduce/eliminate bacterial pathogens in post packaged meat involve in-package thermal pasteurization, irradiation, and formulating meat products with antimicrobial compounds (Zhu *et al*., 2005).

Current chemical disinfection methods: Commercial poultry operations utilize oxidizing agents such as hydrogen peroxide, peroxyacetic acid, propionic acids or acid peroxygen systems (Bell et al., 2002). These compounds are effective against bacteria, bacterial spores, viruses and fungi at low concentrations.

Other safer additive alternatives to combat pathogenic micro-organisms in food: Plant extracts, common culinary herbs, spices and aromatic plants have been studied for their antimicrobial properties against several foodborne pathogens (Kotzekidou *et al*., 2008; Perumalla & Hettiarachchy, 2011; Cote *et al*., 2011). The antimicrobial properties of several plant extracts such as rosemary (Pszczola 2002), grape seed (Ahn *et al.*, 2004), green tea (Kim *et al.*, 2004; Oh *et al.*, 2013), and *Gingko biloba* (Xie *et al.*, 2003) have been demonstrated in model systems. Cowan (1999) has extensively reviewed the phytochemicals that contain antimicrobial

properties. In many herbal, spice and aromatic plant extracts, the antimicrobial nature stems from the essential oils and essences inherent in them (Smith-Palmer *et al*., 1998). Essential oils are bioactive phytochemicals that can exhibit a spectrum of protective actions such as antibacterial, antifungal, antiviral, insecticidal and antioxidant agents (Delaquis *et al*., 2002; Burt, 2004; Prabuseenivasan *et al*., 2006; Friedman *et al*., 2002). The aromatic oils can be obtained as liquids from different plant parts such as leaf, bark, flowers, buds, fruits, roots, seeds, wood and herbs. Isolated compounds from plant essential oils include but not limited are carvacrol, limonene, orange terpeneless, eugenol, linalool, cinnamonaldehyde and thymol that contribute to the observed bactericidal and bacteriostatic effects (Hulin *et al*., 1998; Nannapaneni *et al*., 2008; Kalemba *et al*., 2012).

Apart from essential oils/spice-derived oils, organic acids and phenolic compounds form two other categories of natural antimicrobial systems. Organic acids such as acetic, citric, malic, lactic and tartaric acids have been applied in food systems for their preservative abilities (Doores, 2005; Eswaranandam *et al*., 2006).

C. Plant extracts: Grape seed, green tea

Grape seed and green tea extracts have been applied as preservatives in meat systems to serve as antioxidants (Rababah *et al.*, 2006) and antimicrobials (Ahn *et al.*, 2004). Apart from the properties of preserving food quality (as antioxidants) and safety (as antimicrobials), several phytochemicals with nutraceutical and health promoting potentials are inherent in plant extracts.

C.1. Grape seed extract

Grape seeds are rich in flavonoids, mainly oligomeric and polymeric proanthocyanidins. When purified, dried grape seeds are extracted using water under heat, increased pressure and/or

reduced oxygen, the proanthocyanidins are released. The aqueous proanthocyanidin extract can be filtered by ultrafiltration to remove the suspended solids and adsorbed onto a chromatographic column to isolate the proanthocyanidins. The proanthocyanidin-rich extract can be eluted from the column using ethanol and concentrated using nanofiltration and/or evaporation. The extract can be dried to remove the water and ethanol, ground and blended to produce the commercial GSE (San Joaquin valley concentrates, 2003).

The GSE contains a complex mixture of flavonoids (73.3-77.6% proanthocyanidins; <5.5% catechins on dry weight basis). The predominant polyphenolics in GSE are epicatechin (1,158.5 mg/100g), catechin (887.4 mg/100 g), gentisic acid (472.8 mg/100 g) and syringic acid (253.4 mg/100 g). Other foods that are rich in proanthocyanidins include chocolate, wine, apples, cherries, plums, fruit juices, beans and tea (Macheix *et al.*, 1990; Adamson *et al.*, 1999; Arts *et al.*, 2000a, 2000b; de Pascual-Teresa *et al.*, 2000; Hammerstone *et al.* , 2000; Santos-Buelga and Scalbert, 2000; Scalbert and Williamson, 2000; Teissedre and Landrault, 2000). The USDA 1994-1996 continuing survey of food intakes by individuals (USDA CSFII, 1994-1996) and the 1998 Supplemental children's survey (USDA CSFII, 1998) (USDA, 2000) estimated that the mean intake of GSE from foods was 153 mg GSE/person/day or 2.9 mg/kg body weight/day. However, from the 90 percentile intake of GSE showed an intake of 291 mg GSE/person/day or 6.09 mg/kg body weight/day, from the combined dietary intake of catechin and proanthocyanidins from natural food sources.

Green tea is a widely consumed beverage that has attracted more attention in the recent years due to its health benefits like antioxidant, antimicrobial, anticarcinogenic and anti-inflammatory properties (Perumalla and Hettiarachchy, 2011).

Antimicrobial potency of grape seed extract: Grape seed extract has shown better antimicrobial properties when used in conjunction with other antimicrobials such as nisin (Theivendran *et al.*, 2006), EDTA (Sivarooban *et al.*, 2008) and organic acids (Ganesh *et al.*, 2010; 2012). When GSE (1%) was combined with nisin (10,000 IU) in an edible coating for ready-to-eat meat products, there was a significantly better anti-listerial activity (2.8 log CFU/ml at day 28) compared to the antimicrobial effects of each compound alone (Theivendran *et al.*, 2006). Further, the combination of GSE (1%), nisin (10,000 IU/g) and EDTA (0.16%) effectively reduced *E.c*. and *S*.T. by 1.8 and 0.6 logs CFU/ml respectively (Sivarooban *et al.*, 2008).

The antimicrobial properties of GSE are linked to the phenolic compounds present in the extract, that can form phenoxyl radicals upon entering the bacterial cells. The phenoxyl radicals can bind to the cell wall components including proteins, extracellular enzymes, disrupt the OM, cause leakage of cell components and interfere with the proton motive force (Perumalla and Hettiarachchy, 2011).

C.2. Phenolic compounds

Phenolics constitute a large group of secondary plant metabolites present in natural plant extracts that are mainly responsible for the plant's defense mechanisms against microbial agents.

Phenolic compounds and their sources: Phenolic acids are the precursors of flavonols that are found in plants. The most common flavanols found in plants are catechin,,epicatechin, gallocatechin and epigallocatechin.

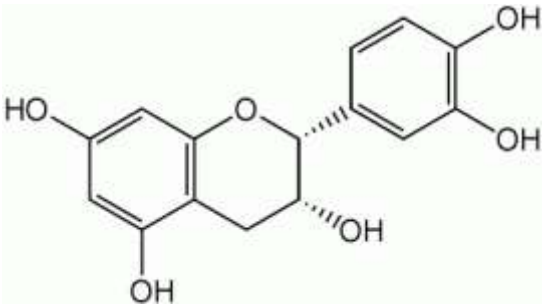


Fig 1. Epicatechin (Adapted from: commons.wikimedia.org)

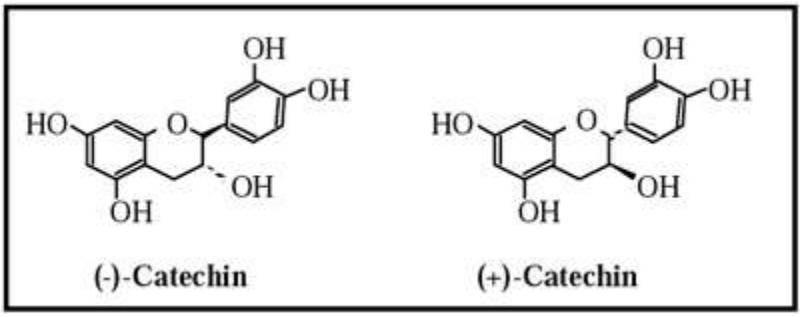


Fig 2. Catechin (Adapted from: 5e.plantphys.net)

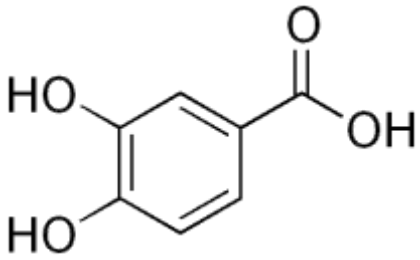


Fig 3. Protocatechuic acid (Adapted from: commons.wikimedia.org)

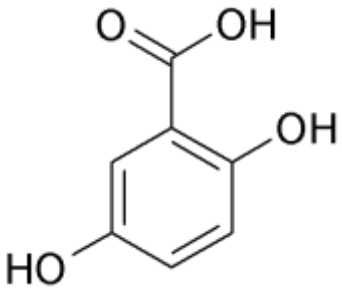


Fig 4. Gentisic acid (Adapted from: commons.wikimedia.org)

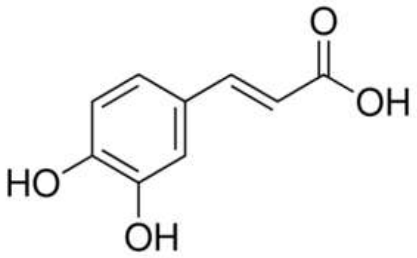


Fig 5. Caffeic acid (Adapted from: Sigma Aldrich)

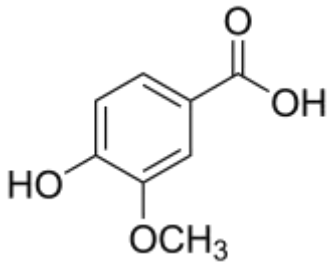


Fig 6. Vanillic acid (Adapted from: commons.wikimedia.org)

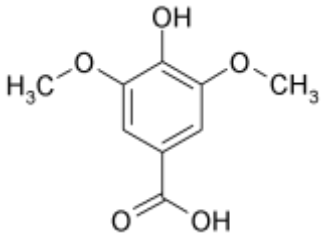


Fig 7. Syringic acid (Adapted from: commons.wikimedia.org)

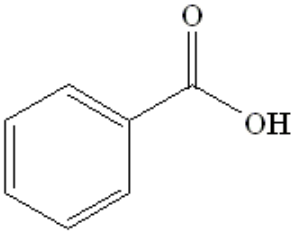


Fig 8. Benzoic acid (Adapted from: commons.wikimedia.org)

Some of the best sources of caffeic acid are white grapes, white wine, olives, olive oil, spinach, cabbage, asparagus, and coffee (Free Radic. Biol. Med. 1996;20:933-56). It also occurs in coffee, particularly in its esterified form, chlorogenic acid. Gallic acid occurs in flaxseeds and watercress. Gallnuts, sumach, tea leaves, oak bark, and many other plants, also contain gallic acid in both its free state and as part of the tannin molecule. Gentisic acid is found in Solanaceae (tomato, egg-plant, pepper) and Cucurbitaceae (melon, cucumber) family and in kiwi fruit, citrus and grapes. Vanillic acid naturally occurs in carrots, crisp-bread and red raspberry while protocatechuic acid is found in apple, tomato, red raspberry, strawberry. Epicatechin is inherent in fruits such as apricot, cherry , grapes, peaches, blackberry, and apples, along with green tea, black tea, red wine and chocolate. Beans and cranberries are sources of catechin and benzoic acid respectively (Manach et al., 2004).

Antimicrobial potency of phenolics: The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups, or through more nonspecific interactions with proteins often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface exposed adhesions, cell wall polypeptides, and membrane-bound enzymes. Phenols may also render substrates unavailable to microbes. Interactions between both lipids and membrane embedded proteins with the phenolic compound results in the destabilizing of the membrane and loss of integrity.

Benzoic acid has been evidenced to act on cell wall, penetrate and intervene in enzymatic functions at various points in citric acid cycle while vanillic acid can affect the integrity of the

cytoplasmic membrane, with the resultant loss of ion gradients, pH homeostasis and inhibition of respiratory activity.

D. Nisin

Nisin is a natural protein and a bio-preservative that does not have adverse side effects when consumed. Nisin is produced by a strain of lactic acid bacteria (lantibiotic); *Lactococcus lactis subsp. lactis*, as a 34 amino acid antimicrobial peptide. Nisin has been shown to be effective against Gram-positive bacteria such as *Listeria monocytogenes* (Benkerroum and Sandine, 1988), some Gram negative bacteria and spores of Bacilli and Clostridia (de Arauz et al., 2009). Using nisin with other phenolic compounds, alone or in combination could enhance the efficiency of these antimicrobials.

E. Ethylene diamine tetraacetic acid (EDTA)

EDTA is a chelating agent that is used in several food products to minimize oxidation and other deteriorative reactions. The outer membrane of Gram-negative bacteria (Nikaido and Vaara 1985; Gilbert *et al*. 1990) and the cell wall of mycobacteria (Trias and Benz 1994) act as permeability barriers and are responsible for the intrinsic resistance of these micro-organisms to antimicrobial compounds. Some chemical agents, such as permeabilizers (EDTA) interact with these structures (Maillard, 2002).

F. Application/ delivery methods

Effectiveness of antimicrobials is greatly reduced in a food system because of the association with other food components (e.g. lipids, proteins). This has also been attributed to the alignment of antimicrobials in the hydrophobic regions of foods while bacteria are growing in areas

containing water. This lack of activity may be overcome by using suitable delivery systems using nanotechnology (Carnahan *et al*., 2005).

F.1. Nanoparticle-mediated delivery

Nanotechnology involves use of materials at an extremely small scale (1-100 nm). Nanotechnology has the potential to revolutionize agriculture and food systems. This includes all aspects of the food chain from storage, quality monitoring, food processing and food packaging to which nanomaterials are added (Joseph and Morrison, 2006). It can be used in intervention technologies and targeted and control delivery of antimicrobial compounds in food and nonfood microbial safety applications.

G. Nanoencapsulation and Food-safety

Nanoencapsulation of drugs involves forming drug-loaded particles with diameters ranging from 1 to 100 nm. Nanoparticles are defined as solid, submicron-sized drug carriers that may or may not be biodegradable (Couvreur et al., 1995). The term nanoparticle is a collective name for both nanospheres and nanocapsules. Nanospheres have a matrix type of structure. Drugs may be absorbed at the sphere surface or encapsulated within the particle. Nanocapsules are vesicular systems in which the drug is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane. In this case the active substances are usually dissolved in the inner core but may also be adsorbed to the capsule surface (Allemann et al., 1993). Nanoparticles are receiving considerable attention for the delivery of therapeutic drugs. The submicron size of nanoparticles offers a number of distinct advantages over microparticles, including relatively higher intracellular uptake compared with microparticles (McClean et al., 1998; Soppimath et al., 2001). In terms of intestinal uptake, apart from their particle size,

nanoparticle nature and charge properties seem to influence the uptake by intestinal epithelia. Uptake of nanoparticles prepared from hydrophobic polymers seems to be higher than that of particles with more hydrophilic surfaces (Jung et al., 2000), thus more hydrophilic particles may be rapidly eliminated.

In the food safety studies, Gaysinsky *et al*. (2004) produced nanocapsules by solubilizing carvacrol and eugenol in selected food-approved surfactants and found that nanocapsules were more efficient than parent compounds in the model microbiological system. Nanotechnology has been successfully used to enhance emulsifying properties in food and controlled release in food and pharmaceutical products, and hence toxicity is not a concern (proprietary information working with a nutrition and pharmaceutical company).

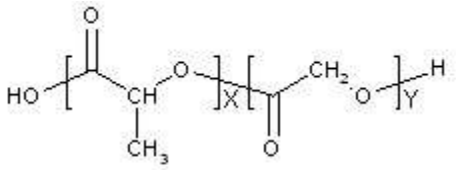
Polysaccharide-based nano-encapsulation and targeted delivery systems are envisaged to have a potential for the development of food/nutraceutical formulations, particularly the poly acids (lactic, glycolic, lactic-co-glycolic acids) and copolymers (Luykx *et al.*, 2008). Poly lactic acid and glycolic acids are used in food products as flavoring and preservative agents. This group of poly-lactics and glycolic acids has been FDA approved for application in food systems (Harris and Chess, 2003, Jain, 2000).

An experiment conducted with antimicrobial nanocapsules used terpenes at minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for three different classes of microorganisms (*Lactobacillus delbrueckii*, *Saccharomyces cerevisiae*, *Escherichia coli*). The increase of the antimicrobial activity resulted depends on the formulation and mean diameter of the delivery systems as well as on the microorganisms class. Additionally, GC–MS analysis revealed that high intensity processing for nanoemulsion

production may affect the chemical stability of several active compounds. The application of the most efficient antimicrobial nanocapsules was tested in pear and orange juices inoculated with *L. delbrueckii*. Due to the higher antimicrobial activity of the nanoencapsulated compounds, lower antimicrobial concentrations are required for a bactericidal action under accelerated aging at 32 °C, with a minimal alteration of the organoleptic properties of the juice (Donsi et al., 2011).

G.1. Materials in the preparation of PLGA nanoparticles

PLGA (Poly lactic glycolic acid): PLGA or poly(lactic-co-glycolic acid) is an Food and Drug Administration (FDA) approved copolymer. PLGA is synthesized by means of random ring-opening co-polymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid.



x - Number of units of Lactic Acid
y - Number of units of Glycolic Acid

PLGA has been successful as a biodegradable polymer because it undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid. The degradation products of PLGA are lactic and glycolic acids that are formed at a very slow rate and are easily metabolized in the body via the Krebs cycle and are eliminated. Since the body effectively deals with the two monomers, there is very minimal systemic toxicity associated with using PLGA for drug delivery or biomaterial applications. PLGA has already been approved as a component of number of drug delivery systems and has a long history of safe use in humans and it has been

widely explored in several immunological studies due to their biodegradability, biocompatibility, reproducibilty and slow release characteristics in vivo (Panyam et al., 2002).

Biodegradable polylactic acid (PLA) polymer was evaluated for its application as a material for antimicrobial food packaging. PLA films were incorporated with nisin to for control of foodborne pathogens. The combination of a biopolymer and natural bacteriocin has potential for use in antimicrobial food packaging (Jin and Zhang, 2008).

Dimethyl sulfoxide (DMSO): DMSO is a highly non-toxic (Robert Vignes, 2000), 100% natural product that comes from the wood industry. DMSO is derived from trees as a manufacturing by-product from the processing of paper. Metabolites (breakdown products) of DMSO, such as the sulfide and sulfone forms, are naturally present in the human body. These are food or USP (United States Pharmacoepia) grade DMSO that are biocompatible and can be used as water-miscible solvents in drug applications (Unger, 2003).

Polyvinyl alcohol (PVA): Polyvinyl alcohol for food use is an odourless and tasteless, translucent, white or cream colored granular powder. It is soluble in water, slightly soluble in ethanol, but insoluble in other organic solvents. Polyvinyl alcohol is produced commercially from polyvinyl acetate, usually by a continuous process.

Polyvinyl alcohol has various applications in the food industries as a binding and coating agent. It is a film coating agent specially in applications where moisture barrier/ protection properties are required. As a component of tablet coating formulations intended for products including food supplement tablets, Polyvinyl alcohol protects the active ingredients from moisture, oxygen and other environmental components, while simultaneously masking their taste and odor. It allows for easy handling of finished product and facilitates ingestion and

swallowing. The viscosity of Polyvinyl alcohol allows for the application of the Polyvinyl alcohol coating agents to tablets, capsules and other forms to which film coatings are typically applied at relatively high solids contents.

Polyvinyl alcohol may be used in high moisture foods in order to retain the overall satisfactory taste, texture and quality of the foods. Confectionery products may also contain Polyvinyl alcohol in order to preserve the integrity of the moisture sensitive constituents. Use levels for polyvinyl alcohol were developed by the sponsor assuming the application of 2.3 mg PVA/cm^2 in aqueous film coatings. Maximum use levels of polyvinyl alcohol were derived for the final foods by selecting products within each food category with the greatest proportion of moisture sensitive components, estimating the surface area of those components, and assuming coating of the entire surface area with polyvinyl alcohol. Polyvinyl alcohol is used as a coating, binder, sealing and surface finishing agent in food products such as dairy-based desserts, confectionery and cereal products and dietary supplement tablets, in the range of 0.2–1.8% by weight.

Water solutions of Polyvinyl alcohol are also stable. Under intended conditions of use and storage there would be negligible interaction between Polyvinyl alcohol and food constituents. Polyvinyl alcohol (PVA) is a preferred material for the film because it is non-toxic and medically safe to use internally. PVA comes in different grades that can be classified as cold water soluble (dissolves from 40-212 °F.), intermediate dissolving (110-212 °F), fully hydrolyzed (140-212 °F.), and superhydrolyzed (180-212 °F.) (Taylor and Verger, 2009)

H. Developments in nanotechnology: Antimicrobial agents

In the non-food application studies, Sondi and Salopek-Sondi (2004) reported that *E. coli* cells damaged by silver nanoparticles accumulated in the bacterial membrane, showed pit formation in the cell membrane, and increased permeability which resulted in cell death. Sambhy *et al*. (2006) reported that silver bromide nanoparticle/polymer composites had the antimicrobial activity (evaluated by zone of inhibition) against gram-positive *Bacillus cereus* and *Staphylococcus aureus* and gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. Ren et al. (2009) investigated the antimicrobial effect of copper oxide nanoparticles against methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* at MBC between 100-5000 µg/ml. Nanocomposite films have been developed with the addition of cellulose nanocrystals and silver nanoparticles into PLA matrix that demonstrated anti-*S. aureus* and *E.coli* activities (Fortunati et al., 2012).

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CHAPTER III

ENHANCEMENT OF ANTIMICROBIAL ACTIVITIES OF NATURALLY OCCURRING PHENOLIC COMPOUNDS BY NANOSCALE DELIVERY AGAINST *LISTERIA MONOCYTOGENES, ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* TYPHIMURIUM IN BROTH AND CHICKEN MEAT SYSTEM

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Abstract

Phenolic compounds commonly occurring in fruits, vegetables and tea, were studied for their effects on *Listeria monocytogenes* (*L.m.*), *Escherichia coli* O157:H7 (*E.c.*) and *Salmonella* Typhimurium (*S.*T.) in brain heart infusion broth (BHI) and meat system. Incubated at 37°C for 72 h in BHI, gentistic, benzoic and vanillic acids inhibited *L.m.*, *E.c.* and *S.*T. at 5000 μg/ml by 2.8 to 3.0 log CFU/ml, 2.8 to 3.0 log CFU/ml and 2.7 to 2.9 log CFU/ml respectively. Encapsulation of benzoic acid (1100 μg/ml) in polylactic-co-glycolic acid nanoparticles inhibited 6.5 log CFU/ml of *L.m.* and *S.*T., and 6.0 log CFU/ml of *E.c.* at 48 h. In raw and cooked chicken meat systems, nanoparticle delivery of benzoic acid was effective against *S.*T. and *L.m.* (1.0 and 1.6 log CFU/g reduction of S.T. and 1.1 and 3.2 log CFU/g reduction of *L.m.* compared to 1.2 log CFU/g without nanoparticles on the days 9 and 14 of storage respectively). These findings demonstrate the efficacy of phenolics on pathogen reduction delivered by nanoparticles and their potential for commercial food safety applications.

Keywords: Phenolics, L. monocytogenes, E. coli O157:H7*, S. Typhimurium, Nanoparticles, antimicrobial*

Practical applications

Nanotechnology is an emerging and promising technology that has been advocated for the delivery of antimicrobial phenolic compound extracts to effectively inhibit foodborne pathogens. The method improves the rate of inhibition compared to conventional delivery and retains the antimicrobial efficacy for a longer time. This hurdle technology using natural antimicrobials (phenolic compounds) and nanoparticle-mediated delivery system can effectively decontaminate foodborne pathogens and improve food safety. Phenolic compounds can be used as natural and safer alternatives to chemical disinfectants in food systems and delivered using nanoparticles to better control pathogens for commercial food safety applications.

Introduction

The global population has recently turned its interest towards natural ingredients as alternatives for artificial chemicals to satisfy their needs in a wide range of applications such as food preservation, nutraceuticals, and alternative medicine (Kannan *et al.* 2008).

Foodborne pathogens cause significant economic losses for the food industry (ERS 2009). Despite the stringent regulatory systems on food processing and preservation, foodborne illnesses have been estimated to affect about 76 million persons, causing 325,000 hospitalizations and 5,000 deaths annually in the U.S. (Mead *et al.* 1999). *Salmonella*, *Listeria*, *E. coli* O157:H7 continue to be some of the major foodborne pathogens. Common food vehicles for such contamination include processed and/or raw foods from the stage of production until distribution. Therefore, use of novel and efficient antimicrobials to preserve these foods is attractive. There has been increasing evidence on the antimicrobial activities of the extracts from culinary ingredients such as green tea, grape seed and spices against foodborne pathogens (Sivarooban *et al.* 2008a, 2008b; Heinonen 2007; Nychas 1995). Among the different bioactive substances present in green tea and grape seed, phenolic compounds possess both antimicrobial and antioxidant activities (Hinneburg *et al.* 2006, Rababah *et al.* 2004).

Phenol and its derivatives have been well known for their antimicrobial activities. Naturally occurring polyphenolics are phenol derivatives including phenolic acids, anthocyanins, flavonols and flavan-3-ols that elicit strong antioxidative properties (Raccach 2007; Sengul *et al.* 2009). Grape seed and green tea extracts are rich sources of such polyphenolic compounds. Previous studies in our laboratory have confirmed the bactericidal nature of grape seed extract and nisin incorporated into edible films and turkey frankfurters (Sivarooban *et al.* 2007; 2008b). Cloudberry, strawberry and raspberry extracts have been examined by Puuponen-Pimia *et al.*

(2001) and reported to strongly inhibit *Salmonella* Typhimurium in liquid culture. Their antimicrobial properties were attributed to their phenolic constitution.

However there is very little information on the potential use of the individual phenolic compounds that could be exploited by the food industry for use as natural value-added preservatives and antioxidants in the food. Furthermore, the loss of activity observed generally in the use of such compounds in food matrices is due to their interaction with food components (e.g. lipids, proteins) and hence there is also a need for efficient antimicrobial delivery systems (Devlieghere et al., 2004).

With increasing awareness of the interaction of nanoscopic, microscopic and macroscopic particles, nanoparticle mediated delivery of the antimicrobial compounds would serve as a new potential delivery system (McClements *et al.* 2009). Nanotechnology can be used in intervention technologies and targeted controlled delivery of antimicrobial compounds in food and nonfood microbial safety applications (Kriegel *et al.* 2008; Bouwmeester *et al.* 2009; Chen *et al.* 2009)**.** Antimicrobial compounds can be attached to the cores of nanoparticles and delivered into bacterial cells (Gaysinky *et al.* 2004).

Thus this study aims at identification of the minimum inhibitory concentrations of phenolic compounds, fabrication of the nanoparticles, packaging of these phenolic compounds and investigating their antimicrobial properties upon release from such nanoparticles.

Materials and Methods

Bacterial culture preparation

Frozen stock cultures of *Listeria monocytogenes* V7 serotype (1/2a) (US-FDA) (*L.m.*), *Escherichia coli* O157:H7 (C7929) (*E.c.*) from apple cider and *Salmonella* Typhimurium (ATCC 14028) (*S.*T.) from pools of heart and liver of four week old chickens) were obtained from the Center for Food Safety culture collection at the University of Arkansas. For each bacterial culture, an inoculum containing 10 µl of frozen stock culture (-80 °C) of *L.m.*/*E.c.*/*S.*T. was added to 10 ml of brain heart infusion broth (BHI) (Becton Dickinson Company, Sparks, MD, U.S.A.) and maintaining them at 37 °C for 24 h with 200-rpm agitation using a New Brunswick Scientific (Edison, NJ, U.S.A.) agitating incubator. Before use in the experiment, the first-day culture was diluted six-fold with sterile BHI broth.

Evaluation of antimicrobial activities of individual phenolic compounds

a. Minimum inhibitory concentration (MIC) determination of phenolic compounds without nisin on *L. monocytogenes* / *E. coli* O157:H7/ *S.* Typhimurium in BHI broth

Brain heart infusion broth (100 µl) was transferred into sterile 96-well microtiter plate (Sigma Aldrich Corp., St. Louis, MO, U.S.A.). Phenolic compounds (Epicatechin, catechin, protocatechuic, caffeic, syringic, vanillic, gallic, gentisic, benzoic acids) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Stock solutions of individual phenolic compounds (20 mg/ml) were prepared in BHI broth. Serial dilution of the pure phenolic compound (stock solution) was carried out using BHI to obtain concentrations of 0, 78, 156, 312, 625, 1250, 2500 and 5000 µg/ml in the wells. One hundred microliters of the second-day culture of *L.m.*/*E.c.*/*S.*T. (7.0 log CFU/ml) was added to the wells containing BHI supplemented with the pure phenolic compounds (Total test volume of 200 µl) individually. Negative controls of BHI without the respective organism and phenolic compounds were included to detect any cross contamination from one well to the other during shaking or handling of plates. A column of BHI wells with

70% ethanol instead of the phenolic compound inoculated with the pathogens served as positive controls. The microtiter plate was incubated at 37 °C for 72 h (progress until the stationary phase of growth). Growth curves were monitored by obtaining absorbance readings at 595 nm after 3, 6, 9, 12, 24, 48 and 72 h using a microplate reader (Bio Rad Model 3550, Bio Rad laboratories, Hercules, CA, U.S.A.). Experiments were performed in triplicates, and the MIC was determined as the lowest concentration of phenolic compound that inhibited growth with an absorbance value <0.05 at 595 nm (Takarada et al. 2004).

b. Bacterial plating at 0 and 72 h

To estimate the extent of growth inhibition of *L.m.*/*E.c.*/*S.*T. by each phenolic compound, 100 µl of the incubated sample mixture from the wells was plated onto *Listeria* selective agar/ MacConkey sorbitol agar with Cefixime tellurite supplement/ XLT4 agar accordingly. MacConkey sorbitol agar base, Cefixime tellurite supplement, XLT4 (xylose lysine tergitol 4) agar base and XLT4 supplement were purchased from Becton Dickinson Microbiology Systems (Becton Dickinson Company, Sparks, MD, U.S.A.). The Listeria Selective Agar (Oxford formulation) was purchased from EM Science (EM Industries, Gibbstown, NJ, U.S.A.). Any viable and injured bacterial cells were enumerated as colonies after incubation of the plates at 37 °C for 48 h.

Evaluation of combined effects of phenolic compounds without nisin on *L.m.*/*E.c.*/*S.*T. in BHI media

The phenolics that were effective in the above study were combined and used in equal proportions (2500 µg/ml) to obtain a total phenolic concentration of 5000 µg/ml in the wells. Cultures of *L.m.*/*E.c.*/*S.*T. (100 µl) (prepared as described before) were added to the respective

wells and incubated at 37 °C. The growth phase and viability of bacteria were monitored as described in a and b during 72 h.

Evaluation of antimicrobial activity of individual phenolics with nisin against *L.m.*/*E.c.*/*S.*T. in BHI media

To investigate if the addition of nisin along with phenolic compounds in media improved the inhibitory properties, the following procedure was conducted. A commercial sample of nisin, Nisaplin, was purchased from Alpin & Barrett Ltd. (Trowbridge, Wilts., U.K.). As nisin could lose its activity during storage, the activity of nisin was determined before the study using the agar overlay method (Lungu and Johnson 2006). Based on the activity (3200 AU/ml), different amounts of Nisaplin (0, 20, 40, 60, 80 and 100 mg) were measured and added to autoclaved 20 ml bottles. Five milliliters of diluted second day culture of *L.m.*/*E.c.*/*S.*T. and 5 ml of BHI were added to each of the bottles. The individual phenolic compounds were added to achieve a final concentration of 5000 µg/ml in each of the bottles while the final nisin concentrations used were 0, 640 AU/ml, 1280 AU/ml, 1920 AU/ml, 2560 AU/ml and 3200 AU/ml respectively. The sample bottles were placed at 37 °C in a 200-rpm agitating incubator and 100 µl of sample was plated every 0, 6, 12, 24, 48 and 72 h on appropriate selective agar plates with proper dilutions.

Nanoencapsulation of phenolics and test for antimicrobial activities

a. Preparation of nanoparticles and packaging of phenolics

Nanoparticles were prepared on the day of the study using the nanoprecipitation method of Murakami *et al.* (2000) with modifications. Approximately 0.75 g of poly lactic glycolic acid (PLGA) was dissolved in 15.0 ml dimethyl sulfoxide (DMSO) (5% w/v). Phenolics were dissolved at MIC (5000 µg/ml) in the PLGA-DMSO solution. This constituted the organic phase.

Kuraray polyvinyl alcohol (PVA) (0.05 g) was dissolved in 10 ml of deionized water (0.5%) which formed the aqueous phase. PLGA used was a polymer with 50/50 molar ratio of polylactic and polyglycolic acid (Resomer RG 503H) purchased from Boehringer Ingelheim Chemicals, Inc. (Petersburg, VA, U.S.A.), PVA from Kuraray, NY, U.S.A. and DMSO from Fisher Scientific, NJ, U.S.A.). The organic phase was injected using a syringe (3cc, 23 G1 syringe and precisionglide needle, Becton Dickinson & Co., Franklin Lakes, NJ, U.S.A.) at the rate of 1 ml/min into a continuously stirring (on a RT 10 power IKAMAG® magnetic stirrer with speed set at 6 or approximately 720 rpm) aqueous phase. Due to insolubility of organic phase containing phenolics and PLGA in water, nanoparticles were formed on contact with the aqueous phase. The particles sizes were measured using particle size analyzer and the nanoparticles were stored at 4 °C until use. The particle size analyzer (Model BI-9000AT Digital Correlator, Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.) was used at Dr. Surendra Singh's laboratory at Department of Physics (University of Arkansas, Fayetteville, AR).

b. Evaluation of antimicrobial activities of nanoparticles containing phenolics.

Sterile BHI and bacterial cultures (*L.m.*/*E.c.*/*S.*T.) were added in equal proportions to culture tubes (polystyrene culture tubes 17x100mm, 14 ml, VWR). Nanoparticles containing phenolics were added to the samples to deliver 1100 and 5000 µg/ml respectively. The tubes were kept at 37 °C in a 200-rpm agitating incubator and 100 µl of the sample was removed at 0, 24 and 48 h for plating onto respective agar plates for enumerating the surviving pathogens.

Antimicrobial activities of phenolics and nanoparticles in meat system.

a. Evaluation of antimicrobial activities of nanoparticles containing phenolics in raw and cooked chicken meat system.

The phenolic compound (benzoic acid) that showed the highest antimicrobial activity against the pathogens was chosen, packaged in PLGA nanoparticles and tested in raw and cooked chicken meat system. Deboned fresh chicken breast meat (Tyson Foods Inc., Springdale, AR, U.S.A.) was kept frozen and only before the study, the meat was thawed at 45 °F for 8-12 h and cut into 2.5-cm × 2.5-cm pieces. For cooked chicken meat, half the number of samples were cooked to 165 ^{o}F for 15 minutes (Paster, 2007). The cooked pieces were allowed to cool and stored in zip lock bags at 4 ^{o}C until inoculation. The cooked as well as noncooked chicken pieces were injected with nanoparticles containing benzoic acid (5000 µg/ml) or controls/ PLGA nanoparticles and then surface inoculated with approximately 10^4 CFU/ml (higher than the general bacterial contamination of 10^2 or 10^3 CFU/ml that can occur during storage or processing of chicken meat) of *S.*T. and *L.m.* (Lang *et al.*, 2004a, 2004b). Inoculated pieces were allowed to dry for a minute under Biosafety Class II laminar hood. Since some of the antimicrobial could get washed off by the surface inoculation step, the nanoparticles containing antimicrobials were re-administered using surface application. The treated meat samples were stored at 4 °C.

b. Sampling and plating of meat samples.

Three inoculated raw or cooked chicken pieces on a particular sampling day were transferred to Whirl Pack bags in triplicates and were tested for survivors. Phosphate buffer saline (PBS at pH 7.0) was added to the bags to make a 10 fold dilution and stomached for 2 min. Stomached sample was serially diluted with PBS and surface plated in duplicate onto selective agar plates for the enumeration. Plates were incubated at 37 ^{o}C for 24 h and then counted for colonies.

Statistical analysis

All values are reported as means of three determinations from triplicate experiments with standard error of means. Analysis of variance (ANOVA) was conducted using the JMP 7.0 Statistical Analysis System (SAS Inst. Inc., Cary, NC, U.S.A.). The least significant difference (LSD) procedure (Student t-test) was used to compare the means and significant mean differences among the treatments at $p < 0.05$.

Results

Minimum inhibitory concentration (MIC) determination of phenolic compounds without nisin against *L.m.*/*E.c.*/*S.*T. in BHI broth

The antimicrobial effects of the individual phenolics (5000 µg/ml) against the three pathogens are shown in Table 1 at 72 h incubation at 37 °C. Of the nine phenolic compounds tested, five (protocatechuic, gentisic, benzoic, vanillic and syringic acids) demonstrated antimicrobial activities against *L.m.* and three (gentisic, benzoic and vanillic acids) against *E.c.* and *S.*T. The MIC of these phenolic compoundswere determined to be 5000 µg/ml. This was the minimum concentration at which no growth or absorbance ≤ 0.05 was observed. *S.*T. and *E.c.*, were effectively inhibited by gentisic, benzoic and vanillic acids (2.7 to 3.0 log CFU/ml reduction) while *L.m.* was inhibited by protocatechuic, gentisic, benzoic, vanillic and syringic acids (2.8 to 3.0 log CFU/ml reduction) at 5000 µg/ml (MIC) after 72 h incubation. The other phenolic treatments effected lower log reductions of approximately 0 to 1.5 log CFU/ml at the end of 72 h.

Evaluation of combined effects of phenolic compounds without nisin against *L.m.*/*E.c.*/*S.*T. in BHI media

The combination of the gentisic, benzoic and vanillic acids was investigated for their enhanced antimicrobial properties against *L.m.*/*E.c.*/*S.*T. The effects of treatment with individual and combinations of phenolic compounds (gentisic, benzoic and vanillic acids) during 72 h of incubation of the pathogens in BHI broth at 37 °C are depicted in Table 1. Gentisic-benzoic acid combination (2500 µg/ml each) and gentisic-benzoic-vanillic acid combinations (2500 µg/ml each) inhibited *L.m.* by 1.8 log CFU/ml each at 72 h. These were lower than the log reductions caused by the individual phenolic compounds (2.8 to 3.0 log CFU/ml). The combinations of vanillic and benzoic acid (2500 µg/ml each) had the highest inhibitory effect on *E.c.* and reduced it by 3.1 log CFU/ml but not significantly different when compared to the reduction caused by their individual treatments (3.0 and 2.8 log CFU/ml of *E.c.*). Similarly, the gentisic-benzoic acid combination (2500 µg/ml each) exhibited a 3.0 log CFU/ml reduction of *S.*T., which was not significantly different from individual inhibitory properties of each compound (2.8 and 2.9 log CFU/ml reductions respectively) (Table 1). Observed from growth phase curves, the other combinations of phenolic compounds showed early inhibition at 12 h but did not prevent the growth of the surviving pathogens (data not shown).

Evaluation of antimicrobial activity of individual phenolics with nisin against *L.m.*/*E.c.*/*S.*T. in BHI media

Antimicrobial activities of nisin (3200 AU/ml) combined with phenolic compounds (2500 µg/ml) against the three pathogens after 72 h have been shown in Table 1. The combination of nisin with phenolic compounds showed 2.9 to 3.3 log CFU/ml reduction of *L.m.*, although there was not a statistically significant change in log reduction with the individual phenolic treatment in cultures of *L.m.* ($p>0.05$).

Nisin by itself caused an almost undetectable to at most a 1.0 log CFU/ml inhibition of *E.c.* and *S.*T., respectively. Combinations of nisin with phenolics yielded minimal log reductions of the two pathogens (0.8 to 1.3 log CFU/ml reduction of *E.c.* and 1.0 to 2.5 log CFU/ml reduction of *S.*T.). Characteristically there was a lower inhibition by phenolic compounds of *E.c* and *S.*T. when combined with nisin (3200 AU/ml) than when used individually and were statistically non-significant.

Evaluation of antimicrobial activities of PLGA nanoparticles containing phenolics

Gentisic, benzoic, vanillic, protocatechuic and syringic acids were packaged into PLGA nanoparticles and the sizes were measured to be 145 to 273 nm (Table 2). Nanoparticles containing phenolics showed higher log reductions of all three pathogens compared to that by the same concentration of individual phenolics at 1100 µg/ml.

L.m. was inhibited by 2.3 and 6.5 log CFU/ml at 24 and 48 h by benzoic acid packaged in nanoparticles at 1100 µg/ml (Table 3). Protocatechuic and benzoic acid packaged in nanoparticles inhibited *E.c.* by 6.2 and 6.0 log CFU/ml respectively at 48 h incubation while they caused only 1.4 and 1.5 log CFU/ml reduction of *E.c.* respectively when used directly (Table 4).

At 1100 µg/ml concentration, benzoic acid in nanoparticles provided a 6.5 log CFU/ml reduction of *S.*T. compared to the control with PLGA nanoparticles (1.9 log CFU/ml) and other phenolics (2.1 to 3.9 log CFU/ml) at 48 h (Table 5). Direct use of the phenolics at 1100 µg/ml inhibited *S.*T. by only 0 to 0.5 log CFU/ml at 48 h and required 5000 µg/ml and incubation for 72 h to cause 1.6 to 2.9 log CFU/ml reductions (Table 1).

Evaluation of antimicrobial activities of nanoparticles containing phenolics in raw and cooked chicken meat system.

Treatment of benzoic acid nanoparticles (5000 µg/ml) in raw and cooked chicken meat system showed 1.1 and 3.2 log CFU/g reduction of *L.m.* on the 9th and 14th days of storage at 4 °C respectively (Table 6). The same treatment inhibited *S.*T. by 1.0 and 1.6 log CFU/g on raw and cooked chicken on the 9th and 14th days of storage at 4 °C respectively (Table 6).

Discussion

Phenolic compounds as effective antimicrobials

Several mechanisms have been proposed for cellular toxicity caused by diffusion of phenolic compounds. These include enzyme inhibition, nonspecific interactions with the proteins, membrane destabilization, loss of integrity and leakage of cell constituents (Mason and Wasserman 1987; Schulz *et al.* 1992; Amarowicz *et al.* 1999; Stammati *et al.* 1999; Johnston *et al.* 2003). Benzoic acid has been proposed to act on cell wall, as well as penetrate and intervene in enzymatic functions at various points in citric acid cycle (Luck and Jager 1997). Vanillin and gallic acid moieties have been found to affect the integrity of the cytoplasmic membrane, cause morphological changes, with the resultant loss of ion gradients, pH homeostasis and inhibition of respiratory activity (Fitzgerald *et al.* 2004; Hattori *et al.* 1990; Ikigai *et al.* 1993).

In our study, gram-positive bacteria (*Listeria*) were found to be more sensitive to phenolics than gram-negative bacteria (*E.c.* and *S.*T.) which are protected by an outer membrane of lipopolysaccharides. Gram-negative bacteria such as *S.*T. possess outer membranes that can prevent the penetration of hydrophobic compounds (Helander *et al.* 1998). Ramos-Nino *et al.* (1996) showed that lipophilicity and degree of ionization of the phenolic acid molecule determined the extent of its anti-*Listerial* activity. The inner and outer surfaces of the bacterial cell membrane are hydrophilic, whereas the interior is hydrophobic, so lipophilicity of a

compound affects its movement into the membrane lipid bilayer portion (McDonnell 2007; Sikkema *et al.* 1995).

The molecular weights of the phenolic compounds tested were epicatechin and catechin (290.3 g/mol), protocatechuic and gentisic acid (154.12 g/mol), benzoic acid (122.12 g/mol), vanillic acid (168.15 g/mol), caffeic acid (180.17 g/mol) and syringic acid (198.17 g/mol) respectively. Smaller hydrophobic acid molecules can penetrate better than larger molecules which explained the higher inhibitory activities of benzoic, gentisic and protocatechuic acids against pathogens (Brul and Coote 1999). Higher molecular weight flavanols like epicatechin and catechin can precipitate in the medium during incubation and render reduced efficacy. The early inhibition of pathogens by phenolic compounds and their poor antimicrobial activities under later conditions could have been due to the loss of activity of the phenolics when combined or the resistance developed by microbial cells against phenolics during their growth phase (Gilbert *et al.*, 1990; Wen *et al.*, 2003).

In the studies with nisin, *L.m.* being a gram-positive organism was more sensitive to nisin and phenolic compounds than *E.c.* or *S.*T. Both nisin and phenolics act on the cell membranes but nisin permeabilizes and contrasts from the diffusing nature of phenolics (Sivarooban *et al.* 2007). Electrostatic interactions between nisin and the negatively charged phospholipids are involved in the anti-listerial effect (Driessen *et al.* 1995; Crandall and Montville 1998). The reduced potency of nisin when added with phenolics could be attributed to binding of polyphenols with the bacteriocin, a peptide (Knoll *et al.* 2008). This would inhibit the phenolic acid diffusion into cells with only the free phenolic compounds contributing to the observed log reduction. This could be verified by separating the free and nisin-bound phenolics by

chromatographic techniques (such as, reverse phase HPLC) and observing their individual diffusion and activity using microscopic methods.

The distinguishable property of nisin between the two major classes of bacteria arose from the lipopolysaccharide layer in gram-negatives (*E.c.*, *S.*T.) that made nisin impermeable and diminished their nisin sensitivity (Jay *et al.* 2005; Bechaut and Golden 1989; Montville *et al.* 2007; Sivarooban *et al.* 2007, 2008a). Thus nisin did not improve the penetration or inhibition of *E.c.* and *S.*T. by phenolics while *L.m.* was inhibited. Our results with the inefficiency of phenolics and nisin against *S.*T. were in accordance with those of Sivarooban *et al.* (2008b) and Gadang *et al.* (2008) who tested the combined mixtures of nisin with grape seed extract (source of phenolics) on *S.*T.

Higher concentration of phenolics or metal chelators (EDTA) and a longer time of incubation with nisin may be required to destabilize the outer membrane of *E.c.* and *S.*T. and to increase sensitivity to nisin respectively. Higher inhibition by nisin-phenolic combinations in *S.*T. than *E.c.* could be due to differences in cell wall compositions and lipopolysaccarides, different modes of action of the phenolics and nisin on cell membrane components.

Nanoparticle mediated delivery for phenolic compounds

PLGA nanoparticles formed a highly effective delivery system for the hydrophobic phenolic compounds. The larger surface area of nanoparticles could have caused better distribution and potency of packaged phenolic molecules (Redhead *et al.* 2001). The higher inhibitory activity of benzoic acid observed with nanoparticles could have been due to better dissolution in the organic phase and their delivery by nanoparticles compared to direct use. Protocatechuic and benzoic acid are more hydrophobic than other phenolics which could have

allowed easier penetration of these phenolics through cell membrane with lipopolysaccharides and further enhanced their activities when delivered by nanoparticles than when used directly.

Phenolics when tested alone exhibited mild inhibitory effects. These responses could have been due to time dependent losses of potency of phenolics and lower concentration of phenolic compounds than their MIC. At 48 h, the phenolics in nanoparticles continued to effectively inhibit pathogens and to a higher extent than the individual phenolics at the same concentration (1100 µg/ml).

The antimicrobial efficacy of the nanoparticles containing phenolics increased during their incubation between 24 to 48 h while phenolics alone did not show significant increase in antimicrobial activity between 24 and 48 h. Studies of Zhong and Jin (2009) and Bezemer *et al.* (2000) have demonstrated the slow, controlled release and sustained efficacy of lysozyme delivered by nanoparticles and polyethylene glycol/ polybutylene terephthalate matrices over a longer period of time. The interaction of phenolics with medium components probably caused a loss of potency with time that necessitated application of higher concentrations of individual phenolics (Lapidot *et al.* 2002). When packaged in nanoparticles, these phenolics would be protected from the media components, reducing the undesired interactions and thus retaining the potency of phenolic compounds.

Evaluation of antimicrobial activities of nanoparticles containing phenolics in raw and cooked chicken meat system.

Log reductions of 3.2 and 3.5 log CFU/g of *L.m.* were observed in cooked chicken on day 14 as compared to 1.2 and 1.6 log CFU/g of *S.*T. The higher efficiency of the phenolics in *L.m.* compared to *S.*T. could be due to the gram-positive nature of *L.m.* lacking the outer membrane

and an easier penetration of the nanoparticles and phenolics. The enhanced inhibition in cooked meat samples versus the raw meat samples indicated the absence of normal microflora (destroyed during cooking) which could have interfered with the inhibitory properties of the nanoparticles. Food system is a complex matrix with several components when compared to the simple BHI medium. Hence, the inhibition of pathogens was lower in the food compared to the same observed in broth study. But, the current study has shown a promising potency of phenolics delivered by nanoparticles. Furthermore, a better understanding of the antimicrobial properties and the observed differences in meat system can be obtained from future studies of release kinetics of phenolics from nanoparticles and their action.

To summarize, individually gentisic, benzoic and vanillic acids were effective against *S.*T., *E.c.* and *L.m.* (2.8 to 3.0 log CFU/ml reductions each). When packaged and delivered by nanoparticles, they caused pathogen inhibition (6.0 to 6.5 log CFU/ml) at a much lower concentration (1100 µg/ml) than when used individually (5000 µg/ml). These results suggest that nanoparticles can be used as a novel delivery system for phenolic compounds at levels lower than originally required for enhanced antimicrobial efficacy. Research is in progress to study the antimicrobial release kinetics from nanoparticles, nanoparticle morphology and their inhibitory properties in model food systems. This would make them effective food preservatives for commercial food safety applications.

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Table 1. Reductions in viabilities of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium by phenolics (5000 µg/ml) alone and in combinations (2500 µg/ml each) with and without nisin (3200 AU/ml) in BHI media after 72 h incubation at 37 °C

Treatment in 96-well plate	Reduction in logs (CFU/ml)[a,c]					
	L.m.		*E.c.*		*S.*T.	
	Without nisin	*With nisin*	*Without nisin*	*With nisin*	*Without nisin*	*With nisin*
Control (No phenolics/ nisin)	0 ± 0.0 C[b]	0 ± 0.0D	0 ± 0.0 D	0 ± 0.0 C	0 ± 0.0 C	0 ± 0.0 E
Control (Nisin without phenolics)	-	1.1 ± 0.2C	-	0.2 ± 0.2 B,C	-	1.0 ± 0.4 D
Protocatechuic acid	2.8 ± 0.3A	2.9 ± 0A	2.4 ± 0.6 A,B,C	1.2 ± 0.5 A	1.6 ± 0.8B	1.3 ± 0.3 C,D
Gentisic acid	2.8 ± 0.0A	3.3 ± 0.3A	2.8 ± 0.3 A,B,C	1.0 ± 0.2 A,B	2.8 ± 0.2A	1.2 ± 0.3 C,D
Benzoic acid	2.8 ± 0.2A	2.9 ± 0.3A	2.8 ± 0.1 A,B,C	1.2 ± 0.0 A	2.9 ± 0.5A	1.4 ± 0.3 B,C,D
Vanillic acid	3.0 ± 0.4A	3.1 ± 0.1A	3.0 ± 0.1A,B	1.0 ± 0.6 A,B	2.7 ± 0.1A	1.6 ± 0.2 A,B,C,D
Syringic acid	3.0 ± 0.2A	2.9 ± 0.0A	2.0 ± 0.6 C	0.9 ± 0.2A,B,C	1.0 ± 0.4B	1.1 ± 0.3 C,D
Gentisic + Vanillic acid	1.6 ± 0.1B	1.8 ± 0.3B	2.0 ± 0.1 B,C	0.8 ± 0.3A,B,C	2.7 ± 0.2A	2.0 ± 0.0 A,B,C
Gentisic + Benzoic acid	1.8 ± 0.3B	2.0 ± 0.4B	2.4 ± 0.2A,B,C	1.1 ± 0.5 A,B	3.0 ± 0.0A	2.5 ± 0.5 A
Vanillic + Benzoic acid	1.6 ± 0.1B	1.7 ± 0.0B	3.1 ± 0.5 A	1.3 ± 0.2 A	2.8 ± 0.3A	2.3 ± 0.1 A,B
Gentisic + Vanillic + Benzoic acids	1.8 ± 0.4B	2.1 ± 0.1B	2.3 ± 0.3A,B,C	1.1 ± 0.2 A,B	2.8 ± 0.2A	1.9 ± 0.5 A,B,C,D

[a]Log reduction of treatments = (Log number of control – Log number of treatment) ± SEM (Standard error of mean); [b]The values with same uppercase letters within a column were not statistically significant at p>0.05; [c]Log number of *L.m*, *E.c.*, *S.*T. at 0 h = 7.0 ± 0.2 log CFU/ml; 7.2 ± 0.2 log CFU/ml; 7.0 ± 0.1 log CFU/ml. Nisin concentration=3200AU/ml.

Table 2. Sizes of PLGA nanoparticles containing phenolic compounds

Phenolic compound	Nanoparticle diameter (nm)
Control (No phenolics)	145.0 ± 0.8[a]
Protocatechuic acid	180.5 ± 0.5
Gentisic acid	273.2 ± 1.6
Benzoic acid	168.9 ± 0.5
Vanillic acid	187.4 ± 1.2
Syringic acid	168.2 ± 0.8

[a]Values have been expressed as mean diameter ± standard error of the mean.

Table 3. Reduction in viability of *L. monocytogenes* by phenolics alone and when packaged within nanoparticles in BHI media after 24 and 48 h at 37 °C

Treatment in 96-well plate with phenolics alone/ in nanoparticles (1100 µg/ml)	Reduction in logs (CFU/ml) [a]			
	Phenolics (24 h) (Control)	Phenolics in Nanoparticles (24 h)	Phenolics (48 h) (Control)	Phenolics in Nanoparticles (48 h)
Control (No phenolics)	0 ± 0.0 A[b]	1.8 ± 0.2 B	0 ± 0.0 B	2.3 ± 0.1 C
Protocatechuic acid	0.7 ± 0.1 A	2.4 ± 0.3 B	0.6 ± 0.7 A,B	3.1 ± 0.1 C
Gentisic acid	1.0 ± 0.2 A	2.9 ± 0.2 A,B	1.0 ± 0.0 A,B	5.6 ± 0.3 A,B
Benzoic acid	0.9 ± 0.3 A	2.3 ± 0.2 A	1.0 ± 0.2 A	6.5 ± 0.4 A
Vanillic acid	0.8 ± 0.4 A	1.8 ± 0.1 A,B	0.6 ± 0.2 A,B	4.8 ± 0.0 B
Syringic acid	0.9 ± 0.6 A	1.6 ± 0.6 A,B	0.5 ± 0.4 A,B	5.5 ± 0.4 B

[a]Log reduction = (Log number of control – Log number of treatment) ± SEM (Standard error of mean); [b] the values with the same uppercase letters within a column were not statistically significant at $p<0.05$; Log number at 0 h = 8.9 ± 0.2 log CFU/ml

Table 4. Reduction in viability of *E. coli* O157:H7 by phenolics alone and when packaged within nanoparticles BHI media after 24 and 48 h at 37 °C

Treatment in 96-well plate with phenolics alone/ in nanoparticles (1100 µg/ml)	Reduction in logs (CFU/ml) [a]			
	Phenolics (24 h) (Control)	Phenolics in Nanoparticles (24 h)	Phenolics (48 h) (Control)	Phenolics in Nanoparticles (48 h)
Control (No phenolics)	0 ± 0.0 B[b]	2.0 ± 0.0 C	0 ± 0.0 B	2.5 ± 0.3 D
Protocatechuic acid	0.6 ± 0.1 A	4.8 ± 0.1 A	1.4 ± 0.4 A	6.2 ± 0.3 A
Gentisic acid	0.4 ± 0.2 A	1.7 ± 0.3 A,B	1.2 ± 0.2 A	5.2 ± 0.2 B,C
Benzoic acid	0.5 ± 0.2 A	2.0 ± 0.3 A	1.5 ± 0.1 A	6.0 ± 0.0 A,B
Vanillic acid	0.8 ± 0.5 A,B	2.5 ± 0.0 A,B	1.3 ± 0.3 A	5.2 ± 0.0 B,C
Syringic acid	0.6 ± 0.1 A	0 ± 0.0 B,C	1.3 ± 0.4 A	4.8 ± 0.1 C

[a]Log reduction = (Log number of control – Log number of treatment) ± SEM (Standard error of mean); [b]The values with the same uppercase letters within a column were not statistically significant at $p<0.05$; Log number at 0 h = 9.4 ± 0.4 log CFU/ml

Table 5. Reduction in viability of S. Typhimurium by phenolics alone and when packaged within nanoparticles BHI media after 48 h at 37 °C

Treatment in 96-well plate with phenolics alone/ in nanoparticles (1100 µg/ml)	Reduction in logs (CFU/ml) [a]			
	Phenolics (24 h) (Control)	Phenolics in Nanoparticles (24 h)	Phenolics (48 h) (Control)	Phenolics in Nanoparticles (48 h)
Control (No phenolics)	0 ± 0.1 B[b]	1.7 ± 0.7 B	0 ± 0.1 C	1.9 ± 0.1 D
Protocatechuic acid	0 ± 0.1 A	1.3 ± 0.0 B,C	0 ± 0.1 C	3.1 ± 0.1 B,C
Gentisic acid	0.5 ± 0.2 A	1.5 ± 0.1 B,C	0.1 ± 0.1A	3.9 ± 0.1 B
Benzoic acid	0.5 ± 0.2 A	2.9 ± 0.1A	0.5 ± 0.0 C	6.5 ± 0.7 A
Vanillic acid	0.4 ± 0.0 A	1.3 ± 0.0 B,C	0.1 ± 0.1 B,C	2.3 ± 0.2 C,D
Syringic acid	0 ± 0.1 A	0.4 ± 0.0 C	0.2 ± 0.1 A,B	2.1 ± 0.1 C,D

[a]Log reduction = (Log number of control – Log number of treatment) ± SEM (Standard error of mean); [b]The values with the same uppercase letters within a column were not statistically significant at $p<0.05$; Log number at 0 h = 9.3 ± 0.3 log CFU/ml

Table 6. Reduction in logs of L.monocytogenes and S. Typhimurium by benzoic acid alone and packaged within nanoparticles in raw and cooked chicken during 14 day storage at 4 °C

Treatment	Reduction in logs of *L.m.* (log CFU/g)							Reduction in logs of *S.*T. (log CFU/g)						
	Cooked meat				Raw meat			Cooked meat				Raw meat		
	Day 3	Day 6	Day 9	Day 14	Day 3	Day 6	Day 9	Day 3	Day 6	Day 9	Day 14	Day 3	Day 6	Day 9
Control (No treatment)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control (PLGA nanoparticles)	2.0	1.2	2.1	2.1	0.5	0.8	0.8	0.9	0.9	1.3	0.6	0.4	1.2	0.8
Benzoic acid (5000 µg/ml)	2.0	1.9	**3.0**	**3.5**	0.8	0.2	1.2	1.7	1.3	1.0	1.2	1.1	1.4	1.2
Benzoic acid nanoparticles (5000 µg/ml)	1.7	1.8	2.9	**3.2**	0.6	0.2	1.1	1.8	1.4	0.7	1.6	1.1	1.7	1.0

*Log reduction = (Log number of control – Log number of treatment); Log number at 0 h in raw chicken were 5.9 ± 0.1 log CFU/g of *L.m.* and 5.3 ± 0.0 log CFU/g of S.T. Log number at 0 h in cooked chicken were 5.9 ± 0.2 log CFU/g of *L.m.* and 5.3 ± 0.1 log CFU/g of S.T. ** There were no log reductions for the control (no treatment) samples.

CHAPTER IV

ANTIMICROBIAL EFFECTS OF NANOPARTICLE MEDIATED DELIVERY OF PHENOLIC COMPOUNDS IN COMBINATION WITH ETHYLENE DIAMINE TETRAACETIC ACID ON LISTERIA MONOCYTOGENES, ESCHERICHIA COLI O157:H7 AND SALMONELLA TYPHIMURIUM IN BROTH AND CHICKEN MEAT SYSTEMS

The manuscript of this chapter will potentially be submitted to Food Control []

Abstract

Food-borne illnesses pose a serious public health concern. Recurring recalls, due to contamination of food products, demand critical antimicrobial strategies to decontaminate the food through its procession from farm to fork. In our study, we combined the effectiveness of naturally occurring phenolic compounds with/ without ethylenediamine tetraacetic acid (EDTA), and nanoparticle delivery to improve the antimicrobial potential of the compounds against *Listeria monocytogenes (L.m.), Salmonella* Typhimurium (*S.*T.), and *Escherichia coli* O157:H7 (*E.c.*), in brain heart infusion broth (BHI) and chicken breast meat systems. Encapsulation of benzoic acid (1100 µg/ml) and EDTA (200 µg/ml) in polylactic-co-glycolic acid nanoparticles inhibited the growth by 6.6 log CFU/ml of *L.m.*, 6.2 log CFU/ml of *E.c.*, and 6.8 log CFU/ml of *S.*T., at 48 h in broth system. Treatment of phenolics with EDTA (200 µg/ml) enhanced inhibition of *S.*T. and *E.c.* (3.0-3.8 log CFU/ml reductions of each), compared to their direct use (2.7-3.0 log CFU/ml reductions) in chicken meat system. This research will serve as a multiple hurdle technology to control measures for bacteria in commercial food safety applications and potentially reducing the associated economic losses.

Keywords: Nanoparticles, phenolic compounds, *L. monocytogenes*, *E. coli* O157:H7, *S.* Typhimurium, EDTA

Highlights

- Natural phenolics have antimicrobial property against major food borne pathigens.
- These phenolics packaged in PLGA nanoparticles show enhanced antimicrobial effect.
- Nanoparticle delivery is a promising delivery system to package antimicrobials.
- Nanoparticle delivery in food systems requires further research.

1. Introduction

Nanotechnology involves the use of materials at an extremely small scale (~1-100 nm) and the emerging technology has the potential to revolutionize agriculture and food systems. It can be used in intervention technologies for targeted and control delivery of antimicrobial compounds in food and nonfood microbial safety applications.

Outbreaks of food-borne pathogens including *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* Typhimurium, are of great concern to the food industry and the general public (Mead and others 1999, CDC 2003, CDC 2009). Most of the recalls for pathogen contaminations are related to ready-to-eat (RTE) poultry and meat products. The economic impact of food-borne illness and the short shelf life of refrigerated meat products demand the development of effective control of microorganisms.

The global population has recently turned its interest over to natural ingredients as an alternative for artificial chemicals to satisfy their health needs, because of their no or negligible side-effects. The use of natural ingredients in many applications like food preservation, pharmaceuticals, nutraceuticals, and alternative medicine has been of great interest (Beuchat and Golden 1999).

Plants are excellent sources of phenolic metabolites. Phenolic compounds can serve as good antioxidants, degrade bacterial cell membranes and hence have potential for antimicrobial activity (Shetty and Lin 1999). However, there is very little information on other bioactivities of these phenolic compounds in combination with chelators such as ethylene diamine tetra acetic acid (EDTA) especially the antimicrobial properties that could be well exploited by the food

industry as they would not only preserve the food but also add nutritional value to the food being powerful anti-oxidants. Ethylenediamine tetra aceticacid is a chelator and biopreservative that has the potential to improve the efficiency of antimicrobial action when added along with the main antimicrobial like organic acids or grape seed extract (Sivarooban and others 2008, Gadang and others 2008).

In this study, we investigated the effects of phenolic compounds for antimicrobial activities with and without EDTA against food-borne pathogens in BHI medium. To improve and sustain inhibition by phenolics, novel delivery systems using nanoparticles were investigated. The nanoparticles were characterized using particle size analyzer and scanning electron microscopy. The antimicrobial activities of phenolic compounds packaged in Polylactic glycolic acid (PLGA) nanoparticles were evaluated against *L.m.*, and *S.*T. in broth culture, and cooked chicken meat model system.

2. Materials & Methods

2.1 Working bacterial culture preparation

Frozen stock (at – 80 ^{o}C) of *S.*T. (ATCC 14028) (Dr. Ricke's laboratory, Center for Food safety and Quality research laboratory at the University of Arkansas, Fayetteville, AR., U.S.A.) was transferred into 10 ml brain heart infusion (BHI) broth (Becton Dickinson Microbiology Systems, Sparks, MD., U.S.A.) using a sterile inoculation loop and were maintained at 37 ^{o}C in an agitating incubator (New Brunswick Scientific, Edison, NJ., U.S.A.) for 24 h. On the second day, 10 µl of the bacterial suspension was transferred into 10 ml BHI broth and incubated at 37 ^{o}C. After 24 h, the bacterial culture was diluted with BHI. The same procedure was repeated for working cultures of *E.c.* (C7929) and *L.m.* V7 serotype (1/2a).

2.2 Evaluation of antimicrobial activities of individual phenolic compounds with and without EDTA on *L.m.* / *E.c.*/ *S.*T. in BHI broth

About 100 µl of BHI was transferred into sterile 96-well microtiter plate. The test solutions of compounds/ EDTA were prepared freshly on the day of the study. Stock solutions of individual phenolic compounds (20 mg/ml) (Eprotocatechuic, syringic, vanillic, gentisic, benzoic acids) (Sigma chemicals, St. Louis, Mo., U.S.A.) were prepared in BHI broth. Serial dilution of the pure phenolic compound (stock solution) was carried out using BHI to obtain concentrations of 5000 µg/ml (minimum inhibitory concentration deduced from our prior studies; Ravichandran and others (2011)) in the wells. Ethylene diamine tetraacetic acid (EDTA) (Sigma chemicals, St. Louis, MO., U.S.A.) was added at concentrations of 0, 100, 200, and 300 µg/ml in the wells. One hundred microliters of *L.m.*/*E.c.*/*S.*T. culture (7.0 log CFU/ml) was added to the wells containing BHI supplemented with the phenolics and EDTA (Total test volume of 200 µl) individually. Negative controls of BHI without the respective organism and phenolics-EDTA were included to detect any cross contamination from one well to the other during shaking or handling of plates. The microtiter plate was incubated at 37 °C for 72 h (progress until the stationary phase of growth). Growth curves were monitored by absorbance measurements at 595 nm after 3, 6, 9, 12, 24, 48 and 72 h using a microplate reader (Bio-Rad model 3550, Bio-Rad laboratories, Hercules, CA, U.S.A.). Experiments were performed in triplicates, and the minimum inhibitory concentration (MIC) of phenolic compound in combination with EDTA was determined as the lowest concentration of antimicrobial that inhibited growth with an absorbance value <0.05 at 595 nm (Takarada and others, 2004).

About 20 µl of the samples were plated onto Listeria Selective Agar (Oxford formulation) with Listeria supplement, Mac Conkey sorbitol agar with Oxoid Cefixime tellurite

supplement (EM Science, Gibbstown, NJ., U.S.A.), and XLT4 agar with supplement (Becton Dickinson Microbiology Systems, Sparks, Md., U.S.A.), for *L.m.*, *E.c.*, and *S.*T., respectively.

2.3 Preparation of Nanoparticles and Packaging of Phenolics.

Poly lactic glycolic acid (PLGA) (0.75 g) was dissolved in 15 mL DiMethylSulphoxide (DMSO) (5% w/v). Phenolics (gentisic, benzoic, vanillic, protocatechuic, and syringic acids) were dissolved at MIC concentrations (5000 μg/ml) with or without EDTA (200 μg/ml, observed from above experiments in a 96-well plate) in the PLGA-DMSO solution. This constituted the organic phase. Kuray polyvinyl alcohol (PVA) (0.05 g) was dissolved in 10 mL of deionized water (0.5%), which formed the aqueous phase. The organic phase was injected slowly using a syringe into a continuously stirring aqueous phase. Due to the insolubility of the organic phase containing phenolics-EDTA and PLGA in water, nanoparticles were formed upon contact with the aqueous phase. The particles sizes were measured using a particle size analyzer (Dr. Singh's laboratory at Department of Physics, University of Arkansas, Fayetteville, AR., U.S.A.) and the nanoparticles were stored at 4°C. The nanoparticles were observed using a scanning electron microscope (SEM), FEI Titan 80-300 (EDAX Detector, 80-300 kV), working with a post-column Gatan Imaging Filter (GIF) 865ER with sub-eV resolution (Institute of Nanoscience and engineering, University of Arkansas, Fayetteville, A.R., U.S.A.). The samples were prepared by adding the nanoparticles to a microscope holder using a conducting carbon strip.

2.4 Evaluation of antimicrobial activities of nanoparticles containing phenolics (with/ without EDTA) in BHI broth.

Working bacterial cultures of *L.m.*, *E.c.* and *S.*T. were prepared as described earlier. Sterile brain heart infusion broth and bacterial cultures were added in equal proportions to

culture tubes. Nanoparticles containing phenolics and EDTA were added to the samples to deliver 1100 and 5000 µg/ml. The tubes were incubated at 37°C in a shaker and 100 µl were removed at regular intervals of 0, 24 and 48 h, plated on respective agar plates to determine the surviving log numbers of pathogens.

2.5 Evaluation of antimicrobial activities of nanoparticles containing phenolics (with/ without EDTA) and comparison with the effectiveness of nanoparticles containing organic acid-plant extract in inoculated chicken meat

As *L.m.* and *S.*T. are the pathogens most frequently associated with chicken meat recalls and outbreaks, those two pathogens were chosen for this study. Deboned fresh chicken breast meat (Tyson foods, Springdale, A.R.) was kept frozen and before the study, the meat was thawed at 45 °F for 8-12 h and cut into 2.5-cm × 2.5-cm pieces. The meat was cooked to 74 ^{o}C for 15 minutes (Paster 2007). The cooked pieces were allowed to cool and stored in zip lock bags at 4 ^{o}C until inoculation. The total number of cooked meat samples required was determined from (Number of treatments x Three replicates x Five days of plating x Two pathogens).

The most effective treatment from the previous broth studies (Benzoic acid with/ without EDTA, containing nanoparticles) was attempted in the meat model system. The cooked chicken pieces were injected with nanoparticles containing benzoic acid along with EDTA or control solutions to deliver effective concentrations or control PLGA nanoparticles. These pre-treated chicken pieces were then surface inoculated using micropipette with approximately 10^4 CFU/ml (higher than the general bacterial contamination of 10^2 or 10^3 CFU/ml that can occur during storage or processing of chicken meat) of *S.*T. and *L.m.* (Lang and others, 2004a and 2004b). Inoculated pieces were allowed to dry for a minute under a biosafety II laminar cabinet. The treated meat samples were stored at 4 °C.

Bacterial count at day zero was used to determine the effectiveness of nanoparticles on initial inhibition/killing of *S*.T. and *L.m.* Bacterial count during storage was used to determine the effectiveness of nanoparticles in controlling bacterial growth and was conducted on days 3, 6, 9, and 15 for cooked chicken. Triplicates of inoculated cooked chicken pieces on a particular sampling day were transferred to Whirl Pack bags and tested for survivors. Phosphate buffer saline (PBS at pH 7.0) was added to the bags to make a 10 fold dilution and stomached for 2 min. Stomached sample was serially diluted with PBS and surface plated in duplicates onto selective agar plates for the enumeration (Xylose Lysine Tergitol 4 agar for *S*.T., Oxford Listeria agar for *L.m.*). The plates were incubated at 37 ^{o}C for 24 h and then counted for colonies.

2.6 Statistical analysis

All experiments were conducted in triplicates and statistical analysis was performed using JMP Pro 9 software (SAS Inst., Cary, NC., U.S.A.). The results were analyzed using one-way analysis of variance (ANOVA) and significantly different means ($P<0.05$) separated using the Student's T-test.

3. Results and Discussion

3.1 Evaluation of antimicrobial activities of individual phenolic compounds with and without EDTA on *L.m.* / *E.c.*/ *S*.T. in BHI broth

Ravichandran and others (2011) showed the antimicrobial properties of protocatechuic, gentisic, benzoic, vanillic, and syringic acids, with an MIC of 5000 μg/ml against *L.m.*, *E.c.*, and *S*.T. The aim of this study was to evaluate the use of EDTA to observe any improvement in the antimicrobial potencies of phenolic compounds on the pathogens. Tables 1a, 1b, and 1c, show the results from this study. Of the phenolic compounds tested, gentisic, benzoic, and vanillic acids, along with EDTA, worked effectively against *L.m.*, *E.c.*, and *S*.T. The use of EDTA alone

(100-300 μg/ml) showed 0.7-1.6, 1.8-2.1, and 1.5-1.8 logs CFU/ml reductions of *L.m.*, *E.c.*, and *S*.T., respectively at 72 h. Parente and others, (1998) observed similar properties of EDTA when used alone in broth medium against *L.m.*, >370 μg/ml was required to significantly inhibit the pathogen. According to Gill and Holley (2003), the MIC of EDTA against *L.m.*, *S*.T., and *Escherichia coli* were 250, >500 and 1000 μg/ml respectively. Thus, our results were consistent with other studies, demonstrating that the use of EDTA alone was not sufficient enough to cause a high inhibition of the pathogens.

The addition of EDTA along with phenolics increased the log reduction to a statistically insignificant extent in the case of L.m. (~0.1-0.2 log CFU/ml) while offering more significant reductions of *E.c.* and *S*.T. (~0.3-0.9 log CFU/ml). Beyond 200 μg/ml of EDTA, there was not any significant increase in the inhibitory potency of the combined addition of phenolic compound and EDTA in all three pathogens. Benzoic acid (5000 μg/ml) and EDTA (200 μg/ml) combination increased the log reduction of the pathogens from 2.8-3.0 log CFU/ml to 3.4-3.8 log CFU/ml in *E.c.* and *S*.T.

Phenolic compounds functioned as antimicrobial agents by diffusing into the cells and initiating several cell damaging processes by non-specific interactions with proteins, enzyme inhibition and causing leakage of cell constituents (Johnston and others, 2003). However, the outer membranes of gram negative bacteria such as *Salmonella* and *E. coli* contain a lipid bilayer structure composed of lipopolysaccharides and proteins and an inner layer of phospholipids. This lipid bilayer can act as a potential barrier to antimicrobials compounds (Cohen, 2011). Increasing the permeability of the outer layer using different compounds can improve disruption of the bacterial cells using antimicrobials (Vaara, 1992), including phenolic compounds.

Ethylenediamine tetraacetic acid is a metal chelator that has been found to be effective against Gram negative bacteria. The agent functioned by chelating divalent metal ions such as Ca++ and Mg++ from the binding sites on lipopolysaccharide (LPS) membrane of Gram negative bacteria, thereby causing the release of LPS from the outer layers of the cell and cellular destabilization (Leive, 1965). Further, EDTA was also found to increase the cell permeability to hydrophobic compounds (Nikaido and Vaara, 1985). As EDTA predominantly worked on gram negative cell membranes, the increase in antimicrobial properties of phenolic compounds was observed in *S.*T. and *E.c.*, while *L.m.*, being gram positive, did not have any enhancement in inhibition with EDTA.

However, even without the EDTA, the phenolic compounds were more sensitive in *L.m.*, in comparison to *E.c.* and *S.*T. This could have been due to the absence of the outer membrane in gram positive bacteria (*L.m.*) that caused easier diffusion of phenolics into the cells.

3.2 Preparation of Nanoparticles and Packaging of phenolics.

The size of nanoparticles containing the various phenolic compounds and EDTA using the particle size analyzer is shown in Table 2. Their sizes ranged from 140-270 nm. The size distribution of the PLGA nanoparticles have been influenced by several factors including the technique used for the nanoparticle synthesis and parameters like PLGA molecular mass, the addition of active components, surfactants and other additives (Barrat and others , 2000, Astete and Sabliov, 2006).

The development of scanning electron microscopy has advanced the understanding of nanostructures (the topography and composition) formed by various different methods using different materials. The typical morphology of PLGA nanoparticles encapsulating polyphenolic

compounds formed by nanoprecipitation was spherical in shape with rough and rounded surface possessing pores of varying size (Figure 1) shows the typical structure of PLGA nanoparticles containing phenolic compounds, deposited on a carbon strip, observed under SEM.

3.3 Evaluation of antimicrobial activities of nanoparticles containing phenolics in BHI system.

The inhibitory activities of phenolics and EDTA packaged in nanoparticles against *L.m.*, *E.c.* and *S.*T. are shown in Tables 3a, 3b and 3c respectively. There was a significant increase in the antimicrobial properties of nanoparticles containing the phenolics-EDTA than when used directly in the medium. Nanoparticles containing phenolics showed higher log reductions of all three pathogens compared to that by the same concentration of individual phenolics at 1100 µg/ml (Ravichandran and others, 2011), possibly due to minimal interactions of phenolics with BHI medium components. Further, a larger surface area of nanoparticles caused better distribution and potency of every packaged phenolic molecule. Owing to the success of using nanoparticles as effective delivery systems in our earlier studies, the same method reported was used in this study to package phenolic compounds and EDTA.

From Table 3a, *L.m.* was inhibited by 4.3 and 6.6 log CFU/ml with benzoic acid-EDTA combination in nanoparticles, compared to 2.3 and 6.5 log CFU/ml at 24 and 48 h with benzoic acid alone at 1100 µg/ml in nanoparticles, respectively. A similar increase in potency at 24 h was observed with *E.c.* and *S.*T. as well. Benzoic acid-EDTA in nanoparticles showed 3.7 and 6.2 log CFU/ml reduction of *E.c*. at 24 and 48 h, compared to 2.0 and 6.0 log CFU/ml without EDTA in nanoparticles, respectively (Table 3b). Similarly, the benzoic acid-EDTA treatment caused 3.7 and 6.8 log CFU/ml reduction of *S.*T., compared to 2.9 and 6.5 log CFU/ml reduction, without

EDTA in nanoparticles respectively. A reason for the higher potency at 24 h with EDTA compared to 48 h could have been due to altered release characteristics of benzoic acid in the presence of EDTA from nanoparticles. Benzoic acid is regarded as a natural antimicrobial, and the higher inhibitory activity observed with nanoparticles was due to better dissolution and sustained delivery by nanoparticles compared to direct delivery.

Direct use of phenolics and EDTA in broth medium was comparatively less effective than in nanoparticles. There may have been time dependent potency losses of phenolics. At 48 h, the phenolics-EDTA in nanoparticles continued to effectively inhibit pathogens to a higher extent than the phenolics-EDTA at a much higher concentration when used directly. Sustained release of phenolics over a longer period of time could explain the observed effect. The interaction of phenolics with medium components caused a loss of potency with time that necessitated higher concentrations of individual phenolics. When packaged in nanoparticles, these undesired interactions of phenolics were prevented, enhanced distribution throughout medium ensured reach of every phenolic molecule to bacterial cells. Differences in lipopolysaccharides and cell wall compositions between *S.*T. and *E.c.* suggest the lower efficacy of the same phenolic between the pathogens (Jay 2000).

3.4 Evaluation of antimicrobial activities of nanoparticles containing phenolics (with/ without EDTA) in inoculated chicken meat system

The inhibitory effects of benzoic acid and EDTA alone and packaged in nanoparticles on *L.m.* and *S.*T. in cooked chicken were determined. Treatment of benzoic acid nanoparticles (5000 μg/ml) in cooked chicken meat system showed 2.9 log CFU/g reduction of *L.m.* on the 9^{th} day of storage at 4 °C. The same treatment inhibited *S.*T. by 0.7 log CFU/g on cooked chicken on the 9^{th} day of storage at 4 °C (Ravichandran and others, 2011). The combination of benzoic acid and

EDTA inhibited *S*.T. and *L.m.* by 3.0 and 4.1 log CFU/g respectively on day 9 of storage compared to 1.2 and 1.1 log CFU/g reduction of the pathogens by direct treatment on cooked chicken meat.

Benzoic acid is regarded as a natural antimicrobial and the higher inhibitory activity observed with nanoparticles was due to better dissolution and sustained delivery by nanoparticles compared to direct delivery. Ravichandran and others studied the efficacy and enhancement of pathogens reduction by nanoparticle-encapsulated polyphenolics in brain heart infusion broth model system. The interaction of polyphenolic compounds with medium components could have caused a loss of potency with time that necessitated higher concentrations when individual polyphenolics were used. When packaged in nanoparticles, these undesired interactions of polyphenolics could have been prevented, and possible enhanced distribution throughout medium ensured reach of every polyphenolic molecule to bacterial cells. Further PLGA nanoparticles by themselves are able to inhibit the three pathogen by approximately 2 log CFU/ml that is in accordance with previous studies (Muñoz-Bonilla and Fernández-García, 2012) and further makes PLGA nanoparticles as suitable polymeric nanoparticle delivery vehicles for anti-microbial compounds. The increased anti-microbial effect seen with PLGA nanoparticle-phenolic combination could be because of the fact that PLGA nanoparticles could strongly bind to the cell membrane and/or be taken up by the cells by various cellular motifs (Verma and Stellaci, 2012). The extent of binding, which depends on various factors including pH and compound encapsulated, has been shown to influence the cellular uptake of the PLGA nanoparticle (Vasir et al. 2012)

The higher anti-microbial efficacy of the nano-particles against *L.m.* compared to *S.*T. could be due to the gram-positive nature of *L.m.*, which lacks the outer membrane, and easier reach of the nanoparticles (Jay and others, 2005).

Benzoic acid is highly hydrophobic and of small molecular weight (154.1 g/mol) and this could have allowed easy penetration of the phenolic compound through the cell membrane with lipopolysaccharides and enhanced its activities when delivered by nanoparticles versus direct use.

4. Conclusion

Gentisic, benzoic, and vanillic acids (5000 µg/ml), were effective along with EDTA (200 µg/ml) against *S.*T., *E.c.,* and *L.m.* (3.0-3.8 log CFU/ml reductions each). The packaging of phenolics and EDTA in nanoparticles improved the inhibition of pathogens to 6.0-6.8 log CFU/ml at a much lower concentration (1100 µg/ml) than when used individually (5000 µg/ml). Nanoparticle-mediated delivery can enhance inhibition of food-borne pathogens at lower phenolic acid concentrations (0.5 %). The activity of benzoic acid and EDTA improved when delivered by nanoparticle from 0.7 to 3.0 and 2.9 to 4.1 against *S.*T. and *L.m.* respectively. These results suggest that benzoic acid delivered as nanoparticles has the potential to serve as antimicrobials even without the addition of EDTA to improve safety of poultry meat.

These results suggest that naturally occurring phenolic compounds that are present in grape seed as well as other extracts delivered by nanoparticles have the potential to serve as more effective antimicrobials.

5. References

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Table 1a: Reduction in viabilities of *L. monocytogenes* by phenolics (5000 µg/ml) alone and in combinations with EDTA (100, 200 and 300 µg/ml) in BHI media after 72 h incubation at 37 °C

Treatment	EDTA (µg/ml)			
	0	100	200	300
Control (No phenolics/ EDTA)	0 ± 0 D	0 ± 0 D	0 ± 0 D	0 ± 0 D
EDTA	-	0.7 ± 0.1 CD	1.3 ± 0.1 BC	1.6 ± 0.1 B
Pr (5000 ug/ml)	2.8 ± 0.3 A	2.9 ± 0.7 A	2.9 ± 0.1 A	2.9 ± 0.2 A
Ge (5000 ug/ml)	2.8 ± 0.0 A	2.9 ± 0.1A	3.0 ± 0.2 A	2.9 ± 0.2 A
Be (5000 ug/ml)	2.8 ± 0.2 A	2.9 ± 0.1 A	3.0 ± 0.3 A	2.9 ± 0.3 A
Van (5000 ug/ml)	3.0 ± 0.4 A	3.0 ± 0.4 A	3.2 ± 0.1 A	3.1 ± 0.1 A
Syr (5000 ug/ml)	3.0 ± 0.2 A	2.6 ± 0.0 A	3.1 ± 0.4 A	3.1 ± 0.1 A

Log reduction = (Log number of control – Log number of treatment); Values with same uppercase letters are not significantly different at $p>0.05$; Log number at 0 h = 7.0 ± 0.2 log CFU/ml

Table 1b: Reduction in viabilities of E. coli O157:H7 by phenolics (5000 µg/ml) alone and in combinations with EDTA (100, 200 and 300 µg/ml) in BHI media after 72 h incubation at 37 °C

Treatment	EDTA (µg/ml)			
	0	100	200	300
Control (No phenolics/ EDTA)	0 ± 0 J	0 ± 0 J	0 ± 0 J	0 ± 0 J
EDTA	-	2.1 ± 0.1 G-I	1.8 ± 0.4 I	1.9 ± 0.6 I
Pr (5000 ug/ml)	2.4 ± 0.6 E-I	2.4 ± 0.2 D-I	2.6 ± 0.2 A-I	2.5 ± 0.1 C-I
Ge (5000 ug/ml)	2.8 ± 0.3 A-G	3.0 ± 0.0 A-F	3.3 ± 0.1 AB	3.3 ± 0.0 A-D
Be (5000 ug/ml)	2.8 ± 0.1 A-G	2.8 ± 0.3 A-H	3.4 ± 0.2 A	3.3 ± 0.0 A-C
Van (5000 ug/ml)	3.0 ± 0.1 A-F	2.7 ± 0.4 A-H	3.1 ± 0.1 A-E	3.2 ± 0.1 A-D
Syr (5000 ug/ml)	2.0 ± 0.6 HI	2.5 ± 0.2 B-I	2.3 ± 0.2 F-I	2.2 ± 0.2 F-I

Log reduction = (Log number of control – Log number of treatment); Values with same uppercase letters are not significantly different at $p>0.05$; Log number at 0 h = 7.2 ± 0.2 log CFU/ml

Table 1c: Reduction in viabilities of *S.* Typhimurium by phenolics (5000 µg/ml) alone and in combinations with EDTA (100, 200 and 300 µg/ml) in BHI media after 72 h incubation at 37 °C

Treatment	EDTA (µg/ml)			
	0	100	200	300
Control (No phenolics/ EDTA)	0 ± 0 H	0 ± 0 H	0 ± 0 H	0 ± 0 H
EDTA	-	1.8 ± 0.0 FG	1.5 ± 0.1 FG	1.8 ± 0.2 FG
Pr (5000 ug/ml)	1.6 ± 0.8 FG	1.6 ± 0.2 FG	1.8 ± 0.1 FG	1.9 ± 0.2 F
Ge (5000 ug/ml)	2.8 ± 0.2 DE	2.9 ± 0.1 C-E	3.5 ± 0.1 A-D	3.6 ± 0.2 A-C
Be (5000 ug/ml)	2.9 ± 0.5 C-E	3.0 ± 0.1 B-E	3.8 ± 0.1 A	3.7 ± 0.3 AB
Van (5000 ug/ml)	2.7 ± 0.1 E	2.9 ± 0.3 C-E	2.9 ± 0.2 DE	2.6 ± 0.1 E
Syr (5000 ug/ml)	1.1 ± 0.4 G	1.5 ± 0.3 FG	1.5 ± 0.2 FG	1.8 ± 0.0 FG

Log reduction = (Log number of control – Log number of treatment); Values with same uppercase letters are not significantly different at $p>0.05$; Log number at 0 h = 7.1 ± 0.1 log CFU/ml

Table 2: Sizes of nanoparticles packaged with phenolic compounds measured by a particle size analyzer

Nanoparticle with/ without phenolics and EDTA (200 μg/ml)	Particle diameter (nm)*
Particles with no phenolics/ EDTA	147.0 ± 0.3
PLGA nanoparticles with vanillic acid and EDTA	178.4 ± 1.7
PLGA nanoparticles with gentisic acid and EDTA	245.2 ± 0.6
PLGA nanoparticles with benzoic acid and EDTA	138.7 ± 1.5
PLGA nanoparticles with protocatechuic acid and EDTA	160.5 ± 1.6
PLGA nanoparticles with syringic acid and EDTA	181.6 ± 1.5

*Particle diameters have been expressed as mean diameters ± standard error of the mean from triplicate analysis, PLGA: Poly lactic glycolic acid

Table 3a: Reductions in logs of *L. monocytogenes* by phenolics alone and packaged with/ without EDTA within nanoparticles in BHI media after 24 and 48 h

Treatment in 96-well plate	Reduction in logs of *L.m.* (CFU/ml)*					
	Phenolics (24 h) (Control)**	Phenolics in Nanoparticles (24 h)**	Phenolics + EDTA Nanoparticles (24 h)	Phenolics (48 h) (Control)**	Phenolics in Nanoparticles (48 h)**	Phenolics + EDTA Nanoparticles (48 h)
Control (No phenolics)	0 ± 0.0 L	1.8 ± 0.2 H,I	1.5 ± 0.3 H-K	0 ± 0.1 L	2.3 ± 0.1 G,H	2.3 ± 0.2 G,H
Protocatechuic acid	0.7 ± 0.1 J-L	2.4 ± 0.3 G,H	3.5 ± 0.2 E,F	0.6 ± 0.7 J-L	3.1 ± 0.1 F,G	5.0 ± 0.3 C,D
Gentisic acid	1.0 ± 0.2 I-L	2.9 ± 0.2 F,G	3.6 ± 0.6 E,F	1.0 ± 0.0 I-K	**5.6 ± 0.3 B,C**	**5.7 ± 0.2 B,C**
Benzoic acid	0.9 ± 0.3 I-L	2.3 ± 0.2 G,H	4.3 ± 0.5 D,E	1.0 ± 0.2 I-K	**6.5 ± 0.4 B**	**6.6 ± 0.2 B**
Vanillic acid	0.8 ± 0.4 J-L	1.8 ± 0.1 H,I	3.2 ± 0.6 F,G	0.6 ± 0.2 K,L	4.8 ± 0.0 C,D	5.0 ± 0.1 C,D
Syringic acid	0.9 ± 0.6 I-L	1.6 ± 0.6 H-J	10.7 ± 0.4 A	0.5 ± 0.4 K,L	5.5 ± 0.4 C	3.6 ± 0.6 E,F

*Log reduction = (Log number of control – Log number of treatment); Values with same letters are not significantly different at $p>0.05$; Log number at 0 h = 8.9 ± 0.2 log CFU/ml. Concentration of phenolics and EDTA was 1100 µg/ml and 200 µg/ml respectively.**Adapted from Ravichandran and others (2011)

Table 3b: Reduction in logs of E. coli O157:H7 by phenolics alone and packaged with/ without EDTA within nanoparticles BHI media after 24 and 48 h

Treatment in 96-well plate	Reduction in logs of *E.c.* (CFU/ml)*					
	Phenolics (24 h) (Control)**	Phenolics in Nanoparticles (24 h)**	Phenolics + EDTA Nanoparticles (24 h)	Phenolics (48 h) (Control)**	Phenolics in Nanoparticles (48 h)**	Phenolics + EDTA Nanoparticles (48 h)
Control (No phenolics)	0 ± 0.1 O	2.0 ± 0.0 G-I	0.6 ± 0.1 M-O	0 ± 0.0 O	2.5 ± 0.3 F-H	2.6 ± 0.2 F,G
Protocatechuic acid	0.6 ± 0.1 M-O	4.8 ± 0.1 C,D	3.0 ± 0.2 F	1.4 ± 0.4 J-L	**6.2 ± 0.3 A**	**6.4 ± 0.3 A**
Gentisic acid	0.4 ± 0.2 N,O	1.7 ± 0.3 I-K	2.9 ± 0.2 F	1.2 ± 0.2 K-M	5.2 ± 0.2 C	6.0 ± 0.4 A
Benzoic acid	0.5 ± 0.2 M-O	2.0 ± 0.3 H-J	3.7 ± 0.0 E	1.5 ± 0.1 I-K	**6.0 ± 0.0 A,B**	**6.2 ± 0.1 A**
Vanillic acid	0.8 ± 0.5 L-N	2.5 ± 0.0 F-H	2.7 ± 0.1 F	1.3 ± 0.3 K,L	5.2 ± 0.0 C	5.4 ± 0.1 B,C
Syringic acid	0.6 ± 0.1 M-O	0 ± 0.0 O	0.6 ± 0.1 M-O	1.3 ± 0.4 J-L	4.8 ± 0.1 C,D	4.3 ± 0.3 D

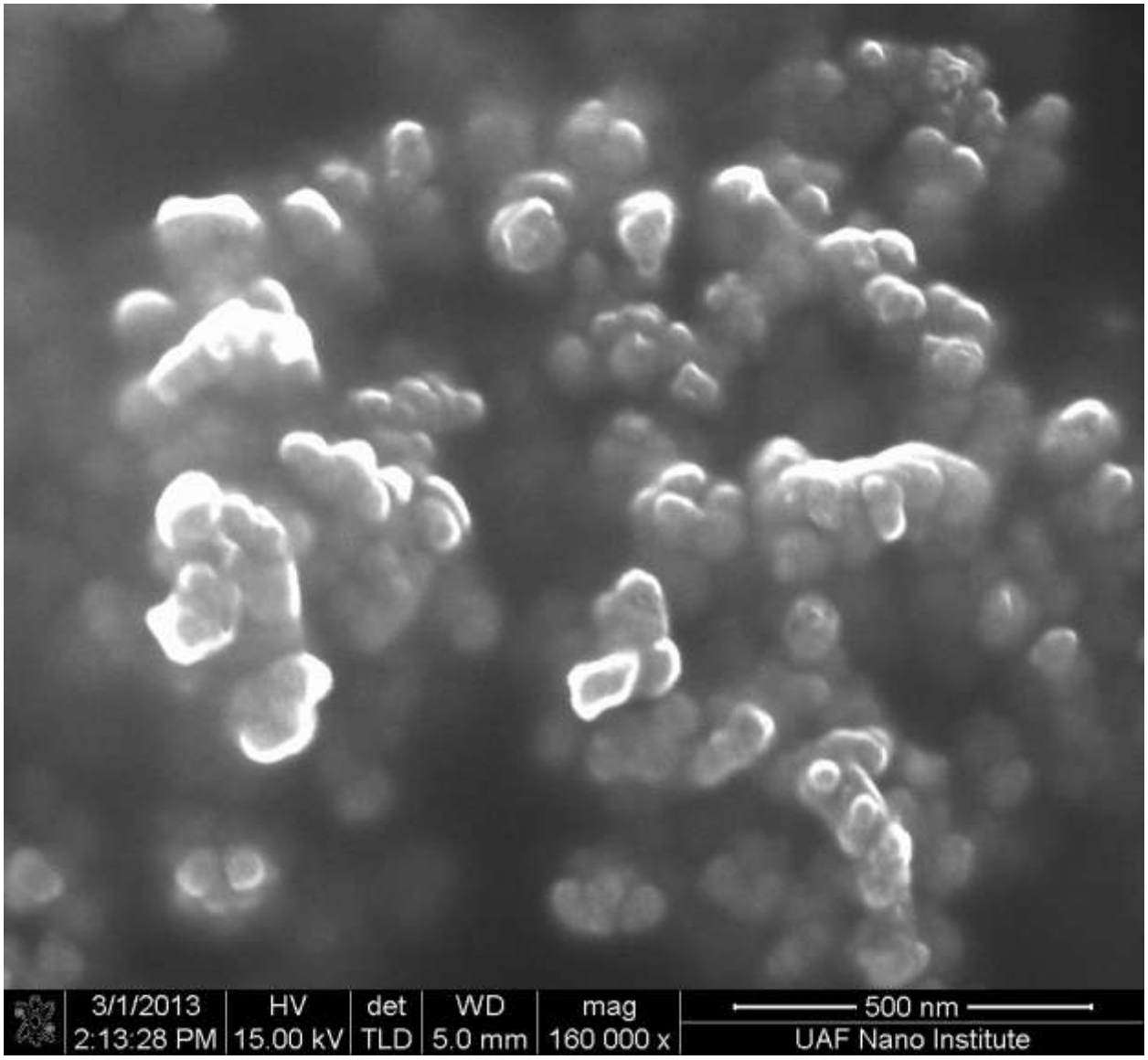
*Log reduction = (Log number of control – Log number of treatment; Values with same letters are not significantly different at $p>0.05$; Log number at 0 h = 9.4 ± 0.4 log CFU/ml. Concentration of phenolics and EDTA was 1100 µg/ml and 200 µg/ml respectively. **Adapted from Ravichandran and others (2011)

Table 3c: Reduction in logs of S. Typhimurium by phenolics alone and packaged with/ without EDTA within nanoparticles in BHI media at 24 and 48 h

Treatment in 96-well plate	Reduction in logs of *S.T.* (CFU/ml)*					
	Phenolics (1100 µg/ml) (24 h) (Control)**	Phenolics in Nanoparticles (1100 µg/ml) (24 h)**	Phenolics (1100 µg/ml) + EDTA (200 µg/ml) Nanoparticles (1100 µg/ml) (24 h)	Phenolics (1100 µg/ml) (48 h) (Control)**	Phenolics in Nanoparticles (1100 µg/ml) (48 h)**	Phenolics (1100 µg/ml) + EDTA (200 µg/ml) Nanoparticles (1100 µg/ml) (48 h)
Control (No phenolics)	0 ± 0.1 N	1.7 ± 0.7 H-K	0.9 ± 0.1 LM	0 ± 0.1 N	1.9 ± 0.1 G-J	2.0 ± 0.4 G-I
Protocatechuic acid	0 ± 0.1 N	1.3 ± 0.0 J-L	1.5 ± 0.1 I-L	0 ± 0.1 N	3.1 ± 0.1 C-E	3.5 ± 0.2 B-D
Gentisic acid	0.5 ± 0.2 MN	1.5 ± 0.1 I-L	2.0 ± 0.0 G-I	0.1 ± 0.1 N	3.9 ± 0.1 B	4.1 ± 0.1 B
Benzoic acid	0.5 ± 0.2 MN	2.9 ± 0.1D-F	3.7 ± 0.2 BC	0.5 ± 0.0 MN	**6.5 ± 0.7 A**	**6.8 ± 0.2 A**
Vanillic acid	0.4 ± 0.0 MN	1.3 ± 0.0 KL	0.4 ± 0.0 MN	0.1 ± 0.1 N	2.3 ± 0.2 F-H	3.0 ± 0.5 C-E
Syringic acid	0.4 ± 0.0 MN	0.4 ± 0.0 MN	0.0 ± 0.0 N	0.2 ± 0.1 N	2.1 ± 0.1 G-I	2.6 ± 0.1 E-G

*Log reduction = (Log number of control – Log number of treatment); Values with same letters are not significantly different at $p>0.05$; Log number at 0 h = 9.3 ± 0.3 log CFU/ml. **Adapted from Ravichandran and others (2011)

Fig 1: Scanning electron Microscope image of PLGA nanoparticles



CHAPTER V

OVERALL CONCLUSION

Conclusion

These results suggest that naturally occurring polyphenolic compounds from grape seed as well as other extracts have the potential to be used as food preservatives or natural and safe substitutes to chemical disinfectants in food systems to better control pathogens. These findings demonstrate the efficacy and enhancement of pathogens reduction by nanoparticles and have the potential for commercial food safety applications. The findings of this research will serve as a multiple hurdle technology with the application of nanotechnology and antimicrobials as control measures for bacteria. This could considerably lead to reduced economic loss associated with food borne illnesses in the US and promote a more natural and potent alternative to the use of chemicals as antimicrobials in food systems.