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## EVALUATION OF NATURAL ANTIMICROBIAL PHENOLIC COMPOUNDS AGAINST FOODBORNE PATHOGENS

Hayriye Cetin-Karaca  
*University of Kentucky*, hayriyekaraca@uky.edu

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EVALUATION OF NATURAL ANTIMICROBIAL PHENOLIC COMPOUNDS  
AGAINST FOODBORNE PATHOGENS

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THESIS

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A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in the  
College of Agriculture  
at the University of Kentucky

By

Hayriye Cetin-Karaca

Lexington, Kentucky

Director: Dr. Melissa Newman, Professor of Food Science

2011

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## ABSTRACT OF THESIS

### EVALUATION OF NATURAL ANTIMICROBIAL PHENOLIC COMPOUNDS AGAINST FOODBORNE PATHOGENS

Raw and processed foods are vulnerable to contamination during their production, distribution and sale. Thus, a wide variety of chemical preservatives are used in the food industry to prevent the growth of food spoilage and pathogenic bacteria. However, health and economic concerns have led to an intensive search for natural alternatives, such as plant extracts, that can safely be used as substitutes for synthetic antimicrobials and preservatives to partially or completely inhibit the growth of bacteria.

This study evaluated the antimicrobial effects of natural phenolic compounds extracted from vegetables, fruits, herbs and spices. The main objective was to determine the lowest concentration of phenolics to inhibit the visible growth of the pathogenic bacteria which is defined as the minimum inhibitory concentration (MIC).

Some of the most common Gram-positive and Gram-negative foodborne pathogens were treated with several natural phenolic compounds. Concentrations of 5, 10, 15, and 20 ppm (pH 5-6) of each compound were evaluated by broth micro-dilution method and the MICs were determined by using optical density (OD) assay.

The results demonstrated that the phenolic compounds have varying antimicrobial activities against foodborne pathogens. Natural sources of phenolic compounds contain major antibacterial components and have great potential to be used as natural antimicrobials and food preservatives.

**KEYWORDS:** Foodborne Pathogen, Phenolic Compounds, Minimum Inhibitory Concentration (MIC), Antimicrobial Activity, Bacteria

Hayriye Cetin-Karaca

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EVALUATION OF NATURAL ANTIMICROBIAL PHENOLIC COMPOUNDS  
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By

Hayriye Cetin-Karaca

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(Director of Thesis)

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(Director of Graduate Studies)

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2011

(Date)

To my parents and my husband,  
Thank you for your support and love

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# CHAPTER 1

## INTRODUCTION

A pathogen is a Greek word meaning “suffering, passion” and “I give birth to” which is an infectious agent, or more commonly referred as germ. It is a biological agent such as a virus, bacteria, prion, or fungus that causes disease to its host. Thus, pathogenic bacteria are responsible for causing bacterial infection. Although the vast majority of bacteria are harmless or even beneficial, quite a few types of them are pathogenic which can contribute to globally important diseases, such as pneumonia, and foodborne illnesses. *Salmonella*, *Listeria*, *E.coli O157:H7* and *Clostridium* are a few species of them. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis and leprosy (Microvet 2011).

Although many medical advances have been made recently to safeguard against infection by pathogens through the use of vaccination, antibiotics, and fungicide, pathogens continue to threaten human life. In entomology, pathogens are defined as one of the "Three P's" (predators, pathogens, and parasitoids) that serve as natural or introduced biological controls to suppress arthropod pest populations (Meta pathogen 2011).

Food is the ideal vehicle for the dispersion of harmful agents which can cause life threatening foodborne illnesses. There are more than 80,000 chemicals and hundreds of naturally occurring biological pathogens, toxins, heavy metals, parasites that can cause serious illnesses. Food and food products are easily accessible at multiple points in any manufacturing process while they are easily distributed over great distances resulting in a great deal of concern for widespread impact of foodborne diseases.

Foodborne disease is an increasingly serious public health problem all over the world. The main cause is determined to be microorganisms. The control of pathogens may significantly reduce the foodborne disease outbreaks (Ravi Kiran and others 2008). In recent years, polyphenols, the secondary plant metabolites, have received a great deal of attention due to their diverse biological functions. A considerable weight of evidence has been gathered suggesting that consumption of fruit and vegetables is beneficial for human health and may help in the prevention of chronic diseases (Liu 2003), because they

contain phenolic compounds (WHO/FAO 2002). Likewise, some natural substances have effective antimicrobial properties where they have been used as seasonings for centuries (Shelef 1984; Shan and others 2007a). Spices and aromatic vegetable materials have long been used in food not only for their flavor and fragrance qualities and appetizing effects but also for their preservative and medicinal properties. Since the ancient times, they have been used for preventing food spoilage and deterioration and also for extending the shelf life of foods (Shan and others 2007b). Laboratory attempts to characterize their prevention properties are dated back to the early 1900s. It has been extensively reported that the essential oils of spices have shown antimicrobial functions against foodborne pathogens (Smith and others 1998; Reichling and others 2009). Thus the secondary plant metabolites have received great attention in recent decades due to their presumed role in the cancer prevention (Kaefer and Milner 2008), as atherosclerosis preventing agents for cardiovascular diseases, and in the slowdown of the aging process (Nazzaro and others 2009). In addition, they show other beneficial biological properties, such as antimicrobial and antioxidant activities (Kanner and others 1994; Fattouch and others 2007; Geoghegan and others 2010; Korukluoglu and others 2010; Perumalla and Hettiarachchy 2011).

Because of the broad biochemical, nutritional and biological variations existing among the different cultivars/genotypes inside each species of fruit or vegetable, the identification of the best genotypes is important for breeders and consumers to have better quality products (Moreno and others 2006; Nazzaro and others 2009; Emiroglu and others 2010; Korukluoglu and others 2010).

Today, the use of plant drugs is accepted all over the world. About 57% of the top-selling prescription in the USA contains natural products or derivatives, and one out of three Americans consumes herbal drugs (Phillipson 2001; Newman and Cragg 2007). In developing countries, the use of medicinal plants has significantly increased due to the low income of the population. About 80% of the people are dependent, wholly or partially, on plant-based drugs (Kuate and others 2010). These findings explain why many research centers and universities have emphasized their search in the medicinal plant field. Herbs and spices are generally considered to be safe and proved to be effective against certain ailments. They are also extensively used, particularly, in many

Asian, African and other countries. In recent years, use of spices/herbs has been gradually increasing in developed countries (Indu and others 2006) since a number of studies linked the high consumption of vegetable and fruits to the prevention of chronic diseases (Lee and others 2003; Alviano and Alviano 2009).

Interest in the antimicrobial properties of active compounds is strengthened by the findings that they affect the behavior of pathogenic bacteria or fungi of agro-food or medical field. Indeed, their use as natural additives in food industry is increased in recent years (Nazzaro and others 2009). The antimicrobial activity of phenolics and flavonoids are also well documented (Erdemoglu and others 2007; Milovanović and others 2007; Xia and others 2011a). The mechanisms responsible for phenolic toxicity to microorganisms include: adsorption and disruption of microbial membranes, interaction with enzymes, and metal ion deprivation (Fattouch and others 2007; Xia and others 2011a).

Due to their antibacterial, antifungal and antiviral activity, phenolic compounds and antioxidant biomolecules were the subject of anti-infective research for many years (Hulin and others 1998; Suppakul and others 2003; Lai and Roy 2004; Cushnie and Lamb 2005; Fattouch and others 2007; Szabo and others 2010). These activities suggested that phenolic compounds can be used as chemotherapeutic agents, food preserving agents and disinfectants (Dorman and Deans 2000). They can affect the growth and metabolism of bacteria, activating or inhibiting the microbial growth according to their constitution and concentration (Alberto and others 2006; Nazzaro and others 2009).

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Food Safety

The food system, just like the health care system, is vulnerable to natural and artificial adulteration and contamination. Food is the ideal vehicle for the dispersion of harmful agents because of the ability to mask the harmful agents by strong flavors, strong odors, various textures or intense colors. There are more than 80,000 chemicals that can cause illnesses, given the right concentration. In addition, there are hundreds of naturally occurring biological pathogens, toxins, heavy metals, parasites, radio-isotopes, genetically engineered organisms and other potential illness-causing agents. Food and food products are easily accessible at multiple points in any manufacturing process. Because food is so easily distributed over great distances, there is increased potential for widespread impact (Sobel and Watson 2009).

Foodborne diseases, whether intentional or otherwise, can also paralyze public health services. Many countries do not have the capacity to respond to such massive emergencies. The public health services facilities for coping with these types of emergencies and for providing continuing care may be strained to the limit.

World Health Organization (WHO) identifies foodborne disease outbreaks and incidents, including those arising from natural, accidental and deliberate contamination of food, as major global public health threats in the 21<sup>st</sup> Century. These threats require urgent action, and WHO recognizes that the building of global public health security rests on solid and transparent partnerships. Full implementation of the International Health Regulations (2005), cross collaboration within governments, global cooperation in surveillance and outbreak preparedness, alert and response, open sharing of knowledge, technology and materials and capacity building in health security is necessary for Member States.

The five key principles of food safety, according to WHO are:

1. Prevent contaminating food with pathogens spreading from people, pets, and pests.
2. Separate raw and cooked foods to prevent contaminating the cooked foods.
3. Cook foods for the appropriate length of time and at the appropriate temperature to kill pathogens.
4. Store food at the proper temperature.
5. Use safe water and raw materials.

Making food safe in the first place is a major effort, involving the farm and fishery, the production plant or factory, and many other points from the farm to the table. Many different groups in public health, industry, regulatory agencies, and academia have roles to play in making the food supply less contaminated. Consumers can promote general food safety by purchasing foods that have been processed for safety. The new technologies are likely to be as important as a step forward as the pasteurization process. Milk pasteurization was a major advance in food safety that was developed 100 years ago. Buying pasteurized milk rather than raw unpasteurized milk still prevents an enormous number of foodborne diseases every day. Moreover, consuming pasteurized fruit juices and ciders prevents *E. coli* O157:H7 infections and many other diseases.

We live in a microbial world, and there are many opportunities for food to become contaminated as it is produced and prepared (CDC 2011).

- Many foodborne microbes are present in healthy animals (usually in their intestines) raised for food. Meat and poultry carcasses can become contaminated during slaughter by contact with small amounts of intestinal contents.
- Similarly, fresh fruits and vegetables can be contaminated if they are washed or irrigated with water that is contaminated with animal manure or human sewage.

- Some types of *Salmonella* can infect a hen's ovary so that the internal contents of a normal looking egg can be contaminated with *Salmonella* even before the shell is formed.
- Oysters and other filter feeding shellfish can concentrate *Vibrio* species that are naturally present in sea water, or other microorganisms that are present in human sewage dumped into the sea.

Later in food processing, other foodborne microorganisms can be introduced from infected humans who handle the food or by cross-contamination from some other raw agricultural products.

- For example, *Shigella* species, hepatitis A virus and Norwalk virus can be introduced by the unwashed hands of food handlers who are themselves infected.
- In the kitchen, microorganisms can be transferred from one food to another food by using the same knife, cutting board or other utensil to prepare both, without washing the surface or utensil in between.
- A food that is fully cooked can become re-contaminated if it touches other raw foods or drippings from raw foods that contain pathogens.

The way that food is handled after it is contaminated can also make a difference in whether or not an outbreak occurs.

- Many bacteria need to multiply to a larger number before enough are present in food to cause disease. Given warm moist conditions and sufficient supply of nutrients, one bacterium that reproduces by dividing itself every half hour can produce 17 million progeny in 12 hours.
- As a result, lightly contaminated food left out overnight can be highly infectious by the next day. If the food were refrigerated promptly, the bacteria would not multiply at all.



- In general, refrigeration or freezing prevents virtually all bacteria from growing but generally preserves them in a state of suspended animation. This general rule has a few surprising exceptions.
  - Two foodborne bacteria, *Listeria monocytogenes* and *Yersinia enterocolitica* can actually grow at refrigerator temperatures.
- High salt, high sugar or high acid levels keep bacteria from growing, which is why salted meats, jam, and pickled vegetables are traditional preserved foods.

Microorganisms may be killed by heat.

- If food is heated to an internal temperature above 160°F, or 78°C, for even a few seconds is sufficient to kill parasites, viruses or bacteria, except for the *Clostridium*, which produce heat-resistant spores.
  - *Clostridium* spores are killed only at temperatures above boiling. This is why canned foods must be cooked to a high temperature under pressure as part of the canning process.
- The toxins produced by bacteria vary in their sensitivity to heat.
  - The *staphylococcal* toxin which causes vomiting is not inactivated even if it is boiled.
  - Fortunately, the potent toxin that causes botulism is completely inactivated by boiling.

In order to reduce the burden of foodborne diseases, the food safety systems in many countries are intended to ensure the safety of the food supply. These often include safety management programs for food production, processing and distribution, which can be modified to incorporate basic consideration of food sabotage (Alvarez and others 2010). The food industry has the primary responsibility for assuring the safety of the food they produce; government agencies, working with the private sector, have regulatory and advisory responsibilities in promoting safe food practices by industry, such as good

agricultural and manufacturing practices (Sobel and Watson 2009; Ceuppens and others 2011; Gravani 2011; Nachay 2011; Toure and others 2011; Szeitz-Szabo and Farkas 2011).

In May 2010 the World Health Assembly (WHA) approved a new resolution on food safety: *Advancing food safety initiatives* (WHA 63.3). This resolution will be used to update the current WHO Global Strategy for Food Safety.

Cost provides another measure for assessing the need for action on foodborne illnesses. The U. S. Department of Agriculture's (USDA) Economic Research Service estimated the economic costs of hospitalizations and lost productivity from the five most common pathogens as \$ 6.9 billion in 2000. The greatest percent of this cost is from premature death which occurs primarily in people over age 65 for *Salmonella* and children under age five for *E. coli* O157:H7. The elderly, people with compromised immune systems, pregnant women, children and infants are most at risk of serious illness from foodborne disease. Many pathogens, including *Salmonella*, *Campylobacter* and pathogenic *E. coli* can lead to chronic illness and reduced life expectancy.

The heart of a modern food safety system lies in preventing “not merely responding” to food safety problems. Mandatory process controls, coupled with government enforced performance standards, should be the central features of a new system. The systems can be used from farm-to-table and with both domestic and imported foods.

Most foodborne illnesses are the result of contamination that occurs during production, processing, shipping or handling. These lapses result in illness, recalls, and loss of public confidence in the safety of our food supply. While in-plant and border inspection from the core of the government's food safety program, inspection is often little more than a spot check on performance. The reality is that the industry holds the key to addressing and preventing food contamination.

The safety and security of the food supply requires an integrated, system-wide approach to preventing foodborne illness, with oversight by federal food safety agencies.

Preventing food contamination can be done using programs of quality assurance and

preventive process control, such as Hazard Analysis and Critical Control Points (HACCP) that are developed by individual companies. These programs are already widely used, and they can be incorporated into food production systems at all levels.

HACCP systems are already mandated in some segments of the food supply, including seafood, juice, and all types of meat and poultry products, both raw and processed. A modern food safety system mandated by Congress requires FDA to implement HACCP or HACCP-like systems for all food processors and tie agency inspections to and audit of these systems. These industry derived programs should be coupled with performance standards, such as limits on the incidence or levels of contamination, or reductions in pathogen levels, that are established by the government. Monitoring and enforcement of the standards are key elements of inspection in a successful food safety program. This includes laboratory testing to ensure that process controls are working effectively.

Research is a vital tool in the effort to reduce the incidence of foodborne illness and is integral to the programs of all public health agencies. Research is needed to evaluate the effectiveness of control and prevention strategies and to conduct risk assessments. It is also needed to improve sanitation and food safety practices during processing. Public education is another essential component of improved food safety. Rates of illness could be reduced if food preparers and handlers were informed of risks and related safe handling practices. Educational programs that promote better understanding and practice of proper food safety techniques, such as thoroughly washing hands and cooking foods to proper temperatures, could significantly reduce foodborne illness. Programs are also needed help health professionals improve their diagnosis and treatment of food related illness and to advise individuals at special risk (DeWaal and others 2010).

## **2.2 Foodborne Diseases and Statistics**

Foodborne illness, sometimes called “foodborne disease”, “foodborne infection”, or “food poisoning”, is a common, costly-yet preventable-public health problem. Center for Disease Control (CDC) and WHO estimates that each year roughly 1 in 6 Americans (or 76 million people) get sick by consuming contaminated foods or beverages, 325,000 are

hospitalized, and 5,000 die of foodborne diseases. A 2003 World Health Organization (WHO) report concluded that about 40% of reported food poisoning outbreaks in the WHO European Region occur in private homes. Due to a wide variety of disease-causing microbes, or pathogens, poisonous chemicals, or other harmful substances having the potential to contaminate foods, there are many different foodborne infections. The 2011 CDC Estimates Report provides the most accurate picture yet of which foodborne bacteria, viruses, microbes (pathogens) are causing the most illnesses in the United States, as well as estimating the number of foodborne illnesses without a known cause.

More than 250 different foodborne diseases have been described and most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites that can be foodborne. Other diseases are poisonings, caused by harmful toxins or chemicals that have contaminated the food (i.e. poisonous mushrooms). These different diseases have many different symptoms, so there is no one "syndrome" confirming the foodborne illness. However, the microbe or toxin enters the body through the gastrointestinal tract, often causing the first symptoms there. The common symptoms in many foodborne diseases may include nausea, vomiting, abdominal cramps and diarrhea (Scallan 2010).

Many microorganisms can spread in more than one way, so we cannot always know that a disease is foodborne. The distinction matters, because public health authorities need to know how a particular disease is spreading to take the appropriate steps to stop it. For example, *Escherichia coli* O157:H7 infections can spread through contaminated food, contaminated drinking water, contaminated swimming water, and from toddler to toddler at a day care center.

According to CDC's 2011 Estimates for Foodborne Illness Report eight known pathogens account for the vast majority of illnesses, hospitalizations, and deaths. Tables 2.1- 2.3 list the top five pathogens causing illness, hospitalization, and death.

Foodborne diseases are largely preventable, though there is no simple one-step prevention measure like a vaccine. Instead, measures are needed to prevent or limit contamination all the way from the farm to the table.

- A variety of good agricultural and manufacturing practices can reduce the spread of microbes among animals and prevent the contamination of foods.
- Careful review of the whole food production process can identify the principal hazards, and the control points where contamination can be prevented, limited, or eliminated.
- A formal method for evaluating the control of risk in foods exists is called the Hazard Analysis Critical Control Point (HACCP) system, which was first developed by NASA to make sure that the food eaten by astronauts was safe. HACCP safety principles are now being applied to an increasing spectrum of foods, including meat, poultry, and seafood.

For some particularly risky foods, even the most careful hygiene and sanitation are insufficient to prevent contamination, and a definitive microbe-killing step must be included in the process.

In the end, it is up to the consumer to demand a safe food supply; up to industry to produce it; up to researchers to develop better ways of doing so; and up to government to see that it happens, to make sure it works and to identify problems still in need of solutions (CDC 2011).

### **2.3 Generally Recognized as Safe (GRAS)**

"GRAS" is an acronym for the phrase **Generally Recognized As Safe**, which is an American Food and Drug Administration (FDA) designation that a chemical or substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements. In 1958, the FDA established the "Food Additives Amendment of 1958", with a list of 700 food substances that were exempt from the new requirement that manufacturers test food additives before putting them on the market. Then, the enforcement provisions of the "Food Additives Amendment of 1958" referred to as GRAS.

Under sections 201(s) and 409 of the FFDCFA, any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive (FDA 2011).

According to the FFDCFA and FDA's implementing regulations (21 CFR 170.3 and 21 CFR 170.30), the use of a food substance may be GRAS either through scientific procedures or, for a substance used in food before 1958, through experience based on common use in food, which requires a substantial history of consumption for food use by a significant number of consumers.

General recognition of safety through scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive and ordinarily is based upon published studies, which may be corroborated by unpublished studies and other data and information (FDA 2011b). The GRAS process, when carried out with due diligence, is a robust, comprehensive, and transparent safety evaluation process. At its essence, GRAS is a scientific peer-review process that is based on widespread knowledge among a community of qualified experts and relies on publicly available scientific evidence to support reasonable certainty in the minds of these competent scientists that the substance is not harmful under its intended conditions of use (Kruger and others 2011).

Regardless of whether the use of a substance as a food additive or is GRAS, there must be evidence that the substance is safe under the conditions of its intended use. FDA has defined “safe” (21 CFR 170.3(i)) as a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use. The specific data and information that demonstrate safety depend on the characteristics of the substance, the estimated dietary intake, and the population that will consume the substance.

## 2.4. Phenolic Compounds

In the last two decades, there has been an increase in the incidence and detection of food-borne diseases together with increasing consumer demand for foods containing reduced levels of chemically-synthesized food additives. Consequently, antimicrobial compounds derived from various plants, animals and microorganisms have constituted a locus of renewed interest as potentially natural substitutes for chemical food preservatives (Roller and Lusengo 1997).

Phenolic compounds are one of the most diverse groups of secondary metabolites found in edible plants. They are found in a wide variety of fruits, vegetables, nuts, seeds, stems and flowers as well as tea, wine, propolis and honey (Table 2.5), and represent a common constituent of the human diet. In nature they are involved in plant growth and reproduction, provide resistance from pathogens and predators and protect crops from disease and pre-harvest seed germination (Ross and Kasum 2002). There are different classes of polyphenols known as tannins, lignins and flavonoids. Each class of polyphenols possesses chemical characteristics that set them apart from one another (Figure 2.1). Flavonoids are the most widely occurring polyphenol and are present in almost every form of human consumed vegetation. Dietary flavonoids have attracted interest because they have a variety of beneficial biological properties, which may play an important role in the maintenance of human health. Flavonoids are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities including anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive, anti-arthritic and antimicrobial activities.

Consumption of phenol-rich beverages, fruit and vegetables has commonly been associated to a reducing of the risk of cardiovascular diseases in epidemiological studies.

With over 9000 natural compounds identified in nature the flavonoid family is the largest group of polyphenolic compounds (Whiting 2001). Flavonoids have been found to be the most abundant polyphenols in our diets. The biosynthesis of flavonoids is stimulated by sunlight (ultraviolet radiation), so higher concentrations of flavonoids can typically be found in the outer most layers of fruits and vegetables (i.e. the skins). Flavonoids can be

divided into six subclasses according to the degree of oxidation of the oxygen heterocycle: flavones, flavonols, isoflavones, anthocyanins, flavanones and flavonols (catechins and anthocyanidins). Extraction of polyphenols can be performed using a solvent like water, hot water, methanol, methanol/formic acid, methanol/water/acetic or formic acid etc. Therefore, the total polyphenol amounts detected from the same plant and their corresponding antioxidant and antimicrobial activities may vary widely, depending on external conditions applied.

#### SUB-CLASSES OF THE COMMON FLAVONOIDS

**Flavones:** apigenin, luteolin, diosmetin

**Flavonols:** quercetin, myricetin, rutin

**Flavanones:** naringenin, hesperidin

**Catechins (flavanols):** (-) epicatechin, gallic acid, (+) catechin

**Anthocyanidins:** pelargonidin, malvidin, cyanidin

**Isoflavones:** genistein, daidzein

**Phenolic acids:** chlorogenic acid, tannic acid, ferulic acid

(Puupponen-Pimia and others 2001)

Although earlier studies were focused on the mutagenic and genotoxic activity of phenolics, later epidemiological studies have indicated that phenolic compounds may play an important role in the prevention of several common diseases, including foodborne diseases (Cueva and others 2010).

Food contamination and spoilage by microorganisms are a serious problem because they have not yet been brought under adequate control despite the new preservation techniques available. Foodborne illnesses resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. Unfortunately there is a dramatic increase throughout the world in the number of reported cases of foodborne illness. To reduce the incidence of food poisoning and spoilage by pathogenic microorganisms many synthetic chemicals were utilized. The exploration of natural antimicrobials for food preservation receives increased attention due to consumer



awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives. The use of phenolic compounds as antimicrobial agents would provide additional benefits, including dual-function effects of both preservation and delivery health benefits. Knowing the antimicrobial effect of the phenolic compounds from several kinds of edible plants on the principal pathogenic microorganisms from the different foods, it is possible to search new strategies to combine the synergic antimicrobial effects of phenolic compounds with their natural biological properties. The results may possibly permit to formulate new products to be used as food preservatives or to be included in the human diet.

#### **2.4.1. Mechanism of Action**

It was reported that an antimicrobial action of phenolic compounds was related to inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes (Moreno and others 2006). Increased membrane permeability is a major factor in the mechanism of antimicrobial action, where compounds may disrupt membranes and cause a loss of cellular integrity and eventual cell death.

Human gut microbiota plays key roles in multiple host functions, including protection against pathogenic organisms, immunomodulation, the production of essential nutrients and the degradation of xenobiotic compounds. Diet is a major factor determining the composition and evolution of the gut microbiota. In fact, the bacterial conversion of carbohydrates, proteins and non-nutritive compounds such as phenolic compounds leads to the formation of a large number of compounds that may have beneficial or adverse effects on human health (Blaut and Clavel 2007).

In general, phenolic compounds are poorly absorbed in the small intestine; it is estimated that 90-95% of dietary phenolics accumulate in the colon (Clifford 2004). In the gut, phenolics may selectively suppress or stimulate the growth of some components of

intestinal microbiota; consequently, they may influence bacterial population dynamics (Tzounis and others 2008).

However, interactions between phenolics and gastrointestinal bacteria are still poorly understood. Among the different classes of phenolic compounds, phenolic acids (i.e., benzoic, phenylacetic and phenylpropionic acids, and other aromatic acids are the predominant structures found in fecal water (Jenner and others 2005). Although some of these acids could originate directly from dietary or endogenous sources, most are thought to come from the microbial metabolism of other dietary phenolic compounds (mainly flavan-3-ols, flavonols, flavones and anthocyanins) (Aura 2008). The fecal phenolic acid profile seems to vary widely, depending on the phenolic compounds ingested.

Recently, microbial-derived phenolic acids have been implicated in providing a variety of health benefits to the host, such as the inhibition of platelet aggregation (Rechner and Kroner 2005) and antiproliferative activity in prostatic and tumoral cells (Gao and others 2006). In addition, the antimicrobial activity of specific phenolic acids towards beneficial gut bacteria and pathogens has been assessed while evaluating the antimicrobial properties of pure phenolics and plant extracts. The *Escherichia coli* species includes non-pathogenic, pathogenic and commensal bacterial strains that generally inhabit the normal human gut. *E. coli* O157:H7 is a foodborne pathogen that causes enterohemorrhagic infection and, occasionally, kidney failure. Thus, *E. coli* strains are widely used in antimicrobial screening studies, in conjunction with other beneficial and pathogenic bacteria.

Multivariate statistical analyses have been applied to confirm similarities and differences among phenolic acids based on their antimicrobial potency. In general, variations in antimicrobial activities among bacteria may reflect differences in cell surface structures between Gram-negative and Gram-positive species. *Lactobacillus spp.* and *S. aureus* (Gram-positive) appeared more susceptible to the action of phenolic acids than Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. Mutations of the *lpxC* and *tolC* genes on *E. coli* seemed to amplify the phenolic acid antimicrobial mechanisms of action against Gram-negative species.

Also, the number and position of substitutions in the benzene ring of the phenolic acids and the saturated side-chain length influenced the antimicrobial potential of the phenolic acids against the different microorganisms, but in different ways. Phenolic acids seemed to show greater antimicrobial potency than their corresponding precursors such as the monomers (p)-catechin, (-) epicatechin and dimers B1 and B2. Therefore, microbial transformations of dietary phenolics (flavonols, flavan-3-ols, flavones and anthocyanins) could lead to more potent microbial-inhibitory compounds (phenolic acids) that selectively influence intestinal bacteria species. These transformations could affect the diversity and metabolic activity of the intestinal microbiota, including the transformation of phenolics in the gut. Further studies taking into account the diversity and complexity of the human microbiota are required in order to confirm the potential microbiota modulating effects of phenolic acids (Cueva and others 2010).

Active compounds such as thymol, eugenol, and carvacrol have been shown to cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents of several microorganisms such as *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, *Lactobacillus sakei*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *S. aureus* (Lambert and others 2001; Gill and Holley 2006; Oussalah and others 2006; Raybaudi-Massilia and others 2006). However, Oussalah and others (2006) and Gill and Holley (2004, 2006) indicated that cinnamon oil and cinnamaldehyde produced a decrease in the intracellular ATP by ATPase activity without apparent changes on the cell membrane of *E. coli*, *E. coli* O157:H7, and *L. monocytogenes*. This fact could be attributed to interaction of cinnamaldehyde with the cell membrane, which may cause enough disruption to disperse the proton motive force by leakage of small ions but without leakage of larger cell molecules such as ATP (Raybaudi-Massilia and others 2009).

Organic acids are used in food preservation because of their effects on bacteria. The key basic principle on the mode of action of organic acids on bacteria is that non-dissociated (non-ionized) organic acids can penetrate the bacteria cell wall and disrupt the normal physiology of certain types of bacteria that we call *pH-sensitive*, meaning that they cannot tolerate a wide internal and external pH gradient (Figure 2.2). Among those

bacteria are *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*, *Listeria monocytogenes*, and *Campylobacter* species.

Upon passive diffusion of organic acids into the bacteria, where the pH is near or above neutrality, the acids will dissociate and lower the bacteria internal pH, leading to situations that will impair or stop the growth of bacteria. On the other hand, the anionic part of the organic acids that cannot escape the bacteria in its dissociated form will accumulate within the bacteria and disrupt many metabolic functions, leading to osmotic pressure increase, incompatible with the survival of the bacteria.

Essential oils, which are concentrated hydrophobic liquid containing volatile aroma compounds from plants, provide a wide research area as well. According to Burt (2004) it is most likely that the antibacterial activity of the essential oils is not attributable to one specific mechanism but to action over several specific targets in the cell. Nychas and Tassou (1999) have reported the location and mechanisms of action in the bacterial cell of bioactive plant compounds, for instance: degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins (Lambert and others 2001), leakage contents out of the cell, coagulation of cytoplasm (Burt 2004), and depletion of the proton motive force (Figure 2.3). Raybaudi-Massilia and others (2009) indicated that the mode of action of essential oils is concentration dependent, indicating that low concentrations inhibit enzymes associated with energy production, while higher amounts may precipitate proteins.

Nisin, a small, heat-stable antimicrobial peptide of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis* (Davidson and Zivanovic 2003), has shown a narrow antimicrobial spectrum, inhibiting only Gram-positive bacteria, including *Alicyclobacillus*, *Bacillus cereus*, *Brochothrix thermosphacta*, *Clostridium botulinum*, *Clostridium sporogenes*, *Desulfotomaculum*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *L. monocytogenes*, *Micrococcus*, *Pediococcus*, *Sporolactobacillus*, and *Staphylococcus*. Against bacterial spores, nisin is sporostatic rather than sporicidal. On the other hand, nisin does not generally inhibit Gram-negative bacteria, yeasts, or molds.

In vegetative cells the primary site of action for nisin is the cytoplasmic membrane where it forms pores, thus destroying membrane integrity (Figure 2.4), and acts as a voltage-dependent polarizer (Abee and others 1994; Ross and others 2003). Pore formation results in depletion of proton motive force (Raybaudi-Massilia and others 2009) and loss of cellular ions, amino acids, and ATP (Crandall and Montville 1998; Davidson and Zivanovic 2003).

#### **2.4.2. Antimicrobial Resistance**

The discovery of effective agents to prevent and treat infections caused by bacteria and other pathogenic microorganisms has been one of the most important developments of modern medicine (Thomson and others 1994b). Undertaking laboratory testing of organisms causing infections can play a role in deciding the most effective treatment options.

However, microorganisms have a short generation time -from minutes to hours- can therefore respond rapidly to changes in their environment. Thus, as antimicrobial agents are introduced into the environment, microorganisms respond to the selective pressure of these agents by becoming resistant, that is able to survive and reproduce in the presence of the agent (Aminov and Mackie 2007). In a few instances some micro-organism are naturally resistant to particular antimicrobial agents, but a more common problem is when micro-organisms that are normally susceptible to the action of particular antimicrobial agents become resistant (HPA 2011).

Multiple mechanism of acquired resistance results from changes in the cellular physiology and structure of a microorganism due to alterations in its usual genetic makeup. There are three main mechanisms whereby bacteria initially susceptible to an antimicrobial agent may acquire the ability to resist the effects of an agent (Thomson and others 1994a). These include:

1. Prevention of intracellular antimicrobial agent/drug accumulation by:
  - alterations in the bacterial outer membrane, such that the antimicrobial agent/drug no longer binds to the exterior of the microorganism
  - active transport of the antimicrobial agent/drug across the cytoplasmic membrane in the cell ceases, or
  - active efflux (pumping) of the antimicrobial agent/drug out of the cell before it can damage the micro-organism
  
2. Alteration in the antimicrobial agent/drug target (the part of the microorganism where damage is done) which leads to ineffective levels of antimicrobial agent/drug binding to or near the target site.
  
3. Production by the microorganism of an antimicrobial agent/drug inactivating enzyme that destroys or greatly diminishes the antimicrobial agent/drug's ability to kill the microorganism.

Many of these mechanisms result from genetic mutations, the acquisition of resistance genes from other microorganisms via gene transfer, and combinations of these two types of events. In some cases, the genes causing resistance can be transferred between different strains of microorganism, and when this happens the recipient organisms will also become resistant. Irrespective of how they arise, resistant microorganisms may spread and it seems likely that the extensive use of antimicrobial agents helps this process along by eliminating competing susceptible microorganisms.

The consequences of increasing resistance are enormous. Resistance leads to increased morbidity and mortality, the need to use more toxic and expensive therapies, and the need to expend ever greater resources on monitoring the development of resistance and surveillance. The origins of the current crisis in antimicrobial resistance are complex and may differ somewhat for hospital and community-acquired pathogens and for developed and underdeveloped countries. Due to the inappropriate use of antimicrobials, such as ineffective doses, for an inadequate or in appropriate length of time, antimicrobial agents can not only be ineffective, but exert strong selective pressures upon bacterial populations, favoring those organisms that are capable of resisting them (Sanders and

Sanders 1995). In addition to human uses of antimicrobial agents, there are a number of non-human applications that increases the pool of resistant organisms that may impact the clinical effectiveness of many antimicrobial agents used to treat infectious diseases of man. These applications include production of food animals (livestock, poultry), aquaculture, plant and crop protection, and food production.

It is reported that pathogenic isolates have a relatively large potential for developing antibiotic resistance advantage (Fluit and others 2000). The increase in antibiotic resistant bacteria is largely due to the widespread use of antibiotics in medicine, in animal care and in agriculture (SENTRY 1998). The problem is compounded by the lack of new antibiotics to attack bacteria in different ways to circumvent the resistant genes. Therefore, finding antimicrobial agents which are effective or might enhance the antibiotic efficacy against resistant bacteria would be an advantage (Darwish and others 2010).

### **2.4.3. Food Applications**

Concerns and potential risks regarding the use of synthetic chemical antimicrobials and antioxidants have renewed the interests of consumers using natural and safe alternatives. To address the need for natural and safe alternatives, several plant extracts have been used in the food industry for years (Biswas and others 2002). Previous studies have reported that natural plant extracts have the potential to improve the overall quality and extend the shelf life of food products (Biswas et al. 2002; Raman and others 2009; Bussmann and others 2010; Mubarak Ali and others 2011). Furthermore, they can also be used in various food model systems such as meat (turkey, beef, and chicken), seafood (Miladi and others 2010), vegetable produce (spinach), probiotics, and packaging films (Cagri and others 2001, 2003) along with other multiple hurdle technologies (bacteriocins, organic acids, temperature, and packaging) to improve the overall microbial quality and safety of the food products (Serra and others 2008; Nazzaro and others 2009; Patra and Thatoi 2011; Perumalla and Hettiarachchy 2011).

Different natural antimicrobials of animal, plant, and microbial origin, directly or indirectly added to fresh-cut fruits and fruit juices, reported effectively to reduce or inhibit pathogenic and spoilage microorganisms, thus representing a good alternative to the use of traditional antimicrobials (Raybaudi-Massilia and others 2009; Mulaudzi and others 2011). On the other hand, the addition of antimicrobials to these products without adversely affecting the sensory characteristics is still a challenge for researchers, since the concentrations that are necessary to ensure safety (up to 5 log CFU/g reductions in the most resistant pathogenic microorganism, based on USFDA (2002) regulation) of natural fruit and vegetable extracts are several times higher than those accepted by consumers from sensory point of view (Peixoto and others 2011). Therefore, new studies combining the use of antimicrobials with other methodologies of food preservation are necessary to reduce the impact of these compounds on sensory properties (Raybaudi-Massilia and others 2009).

Moreover, different studies have demonstrated the effectiveness of essential oils and their active compounds to control or inhibit the growth of pathogenic and spoilage microorganisms in both fresh-cut fruit and fruit juices. That effectiveness depended on the pH of the fruit product, kind and concentration of used essential oils or active compound, and microorganism type. In this way, Raybaudi-Massilia and others (2009) incorporated active compounds of herbs and spices into an alginate-based edible coating and applied on fresh-cut apples where they found a high effectiveness for reducing populations of inoculated *E. coli* O157:H7 during storage time.

Several agents were previously used to suppress contamination by *L. monocytogenes*, such as the addition of bio-preservatives and essential oils. Active compounds from clove oil had antimicrobial properties and were able to restrict the proliferation of *L. monocytogenes* in food products. For this reason, clove oil has good potential as an antilisterial substance in food preservation as it may be more acceptable to consumers (Miladi and others 2010). Antimicrobial and antioxidant activities of edible coatings enriched with rosemary and olive oleoresins studied *in vitro* and *in vivo*, which offered a great advantage in the prevention of bacterial population and browning reactions which typically result in quality loss in fruits and vegetables (Ponce and others 2008).



According to Ceylan and others (2004) addition of cinnamon into apple juice gradually decreased the number of *E. coli* O157:H7 at 8°C and 25°C. Cinnamon in combination with sodium benzoate or potassium sorbate reduced the number of *E. coli* O157:H7 more than 5.0 log CFU/mL during storage at 8°C for 14 days and 25°C for 3 days. In fact, cinnamon in food systems with other extrinsic and intrinsic factors (for example, acidulation with organic acids) might provide a hurdle effect to control *E. coli*.

Antibacterial activity of soy protein edible films (SPEF) incorporated with oregano or thyme essential oils were evaluated against *Escherichia coli*, *E. coli* O157:H7, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Lactobacillus plantarum* (Emiroglu and others 2010). Due to the complexity of ground beef matrix using essential oils incorporated films resulted in limited reduction of antimicrobial activity of *Pseudomonas* spp. and coliform bacteria in ground beef patties during refrigerated storage.

## **2.5 Antimicrobial Susceptibility Testing**

One of the most important tasks of the clinical microbiology laboratory is the performance of antimicrobial susceptibility tests. The goal of a susceptibility test is to predict through an *in vitro* assessment of successfully finding sensitivity or resistance to a particular antimicrobial agent.

Antimicrobial agents are chemicals that kill or inhibit the growth of micro-organisms and are used to treat microbial infections. Some are produced naturally by microbes but many are synthetic. Antimicrobials include antibiotics, antivirals, antifungals and other natural plant bioactive compounds such as phenolics. Susceptibility testing is most important with bacterial species that are not predictably susceptible to drugs and/or compounds of choice for infections such as, staphylococci, enterococci, pneumococcus, and foodborne diseases including, salmonellosis, gastroenteritis, and hemolytic-uremic syndrome. The natural history of the infection and the immune status of the patient must be taken into consideration. The pharmacological properties of the antibiotics must also be considered; ease of absorption, protein-binding, metabolism, ability to reach the site of infection, and

excretion. The most important function of a susceptibility test is the detection of clinically relevant antimicrobial resistance in etiologic agents causing an infection, including foodborne infections (Murray and others 2005).

### **2.5.1 Antibacterial Susceptibility Tests**

Antibacterial susceptibility testing may be performed by either dilution or diffusion methods. The choice of methodology is often based on many factors, including relative ease of performance, flexibility, use of automated or semi-automated devices for both identification and susceptibility testing (Murray and others 2005).

#### **2.5.1.1 Dilution Methods**

Dilution susceptibility testing methods are used to determine the minimal concentration, usually expressed in micrograms per milliliter of antimicrobial agents required to inhibit or kill a microorganism. Procedures for determining the antimicrobial inhibitory activity are carried out by either agar or broth based methods. Antimicrobial agents are usually tested as log<sub>2</sub> (twofold) serial dilutions, and the lowest concentration that inhibits visible growth of an organism is recorded as the MIC. The concentration range used may vary with the compound, organism identification, and site of infection. Generally ranges should include concentrations that allow determination of the interpretive categories such as, susceptible, intermediate, and resistant (Murray and others 2005). Serial dilutions are made of the products in bacterial growth media. The test organisms are then incorporated into the dilutions of the products, incubated, and scored for growth.

Dilution methods offer flexibility in the sense that the standard medium used to test routinely encountered microorganisms (e.g., staphylococci, members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*) may be readily supplemented or even replaced with another medium to allow accurate testing of various fastidious bacteria not reliably tested by disk diffusion. Dilution methods are also adaptable to automated

systems and they provide quantitative results (MICs in micrograms per milliliter) or category results (susceptible, intermediate, or resistant) without any need for conversion or regression lines. Mechanization and automation have made broth dilution susceptibility testing much less time consuming, and fluid systems are used almost without exception in advanced, automated tests.

The general approaches for broth methods include macro-broth dilution, in which the broth volume for each antimicrobial concentration is  $\geq 1.0$  ml contained in test tubes and micro-broth dilution, in which antimicrobial dilutions are in 0.05 to 0.1 ml volumes contained in wells of micro-titer trays. The macro-broth dilution broth method is a well-standardized and reliable reference method that is useful for research purposes, but because of the laborious nature of the procedure and the availability of more convenient dilution systems (i.e., micro dilution), this procedure is generally not useful for routine susceptibility testing.

In the present study, the micro-broth dilution testing method is performed, which has always been considered a primary laboratory approach to the study of the *in vitro* antimicrobial susceptibilities of pathogenic organisms (Shan and others 2007a). The convenience of the micro-broth dilution susceptibility testing is afforded by the availability of the testing in micro dilution trays. The inoculation and reading procedures allow relatively convenient simultaneous testing of several antimicrobial agents against individual organisms, also the results of testing may be determined by visual examination or with semi-automated or automated instrumentation (i.e., spectrophotometry) (Murray and others 2005).

#### **2.5.1.2 Disk Diffusion Testing**

Disc diffusion tests were introduced mainly because they were less cumbersome technically when large numbers of organisms were tested against many antimicrobials including antibiotics (Shan and others 2007b).

The disk diffusion method of susceptibility testing allows categorization of bacterial isolates as susceptible, resistant, or intermediate to a variety of antimicrobial agents. It is technically simple to perform and very reproducible. Moreover, it does not require any special equipment and provides category results that are easily interpreted by clinicians. The primary disadvantage of disk diffusion susceptibility testing is that, it provides a qualitative result, but a quantitative result indicating the degree of susceptibility may be desirable in most of the cases (Murray and others 2005).

### **2.5.2 Interpretation**

The qualitative results of the antibacterial susceptibility tests may be reported with appropriate corresponding interpretative categories, including susceptible, intermediate and resistant (Watase and others 2011).

Susceptible: The isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection (CLSI 2011).

Intermediate: The isolate may be inhibited by attainable concentrations of certain drugs and/or compounds, if higher dosages can be used. It also implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and beta-lactams in urine) (CLSI 2011).

Resistant: The isolates are not inhibited by the usually achievable concentrations of the antimicrobial agent with the normal dosage schedules, and/or that yield results that fall within a range where specific microbial resistance mechanisms are likely (CLSI 2011), and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies (Howard and others 1987).

### **2.5.3 Quality Control**

Quality control recommendations are designed to effectively evaluate the precision and accuracy of the dilutions and test procedures used, monitor reagent reliability, and evaluate the performance of individuals who are conducting the tests. Appropriate controls, depending on genera, must be included with every batch of MIC determinations. The tests should be performed under standardized conditions so that the results are reproducible.

### **2.6 Minimum Inhibitory Concentrations of Natural Compounds**

The MIC is defined as the lowest concentration of a drug/compound that will inhibit the visible growth of an organism *in vitro* after overnight incubation (this period is extended for organisms such as anaerobes, which require prolonged incubation for growth). Minimum inhibitory concentrations (MICs) are considered the “gold standard” for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent, to give a definitive answer when a borderline result is obtained by other methods of testing, and also to monitor the activity of new antimicrobial agents (Barros and others 2007). An MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism.

Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents (European Comm. Antimicrobial 2000). Using the MIC data, the dosage of antibiotics/atimicrobials may be refined. The MIC result is used in conjunction with therapeutic dosing information to select the most appropriate antimicrobial agent (Watase and others 2011).

In recent years, MICs have been used in Phytotherapy, the use of plants for medical purposes, which is one of the oldest practices in the world. The traditional practice, based on empirical data, is considered as folk medicine and the approach based on scientific studies aims to extract and study active components from plants. The demands for natural remedies along with the natural food additives combine phytotherapy with scientific studies. Thus, determination of the MICs has become the main factor for the scientific studies regarding the feasibility of bioactive components of the plants in industry.

The standard procedures for determination of MICs and MIC limits used by the microbiology laboratory for determining the qualitative interpretations are included either in Clinical and Laboratory Standards Institute or National Committee for Clinical Laboratory Standards.

**Table 2.1. Estimated annual number of domestically acquired, foodborne illnesses, hospitalizations, and deaths due to 31 pathogens and unspecified agents transmitted through food in United States (adapted from CDC 2011).**

<b>Foodborne Agents</b>	<b>Estimated annual number of illnesses*</b>	<b>%</b>	<b>Estimated number of hospitalizations*</b>	<b>%</b>	<b>Estimated annual number of deaths*</b>	<b>%</b>
<b>31 known pathogens</b>	9.4 million (6.6-12.7 million)	20	55,961 (39,534-75,741)	44	1,351 (712-2,268)	44
<b>Unspecified agents</b>	38.4 million (19.8-61.2 million)	80	71,878 (9,924-157,340)	56	1,686 (369-3,338)	56
<b>Total</b>	47.8 million (28.7-71.1 million)	100	127,839 (62,529-215,562)	100	3,037 (1,492-4,983)	100

\*: 90% credible interval

**Table 2.2. Top five pathogens contributing to domestically acquired foodborne illnesses (adapted from CDC 2011).**

<b>Pathogen</b>	<b>Estimated number of illnesses</b>	<b>90% Credible Interval</b>	<b>%</b>
<b>Norovirus</b>	5,461,731	3,227,0778-8,309,480	58
<b><i>Salmonella, nontyphoidal</i></b>	1,027,561	644,786-1,679,667	11
<b><i>Clostridium perfringens</i></b>	965,958	192,316-2,483,309	10
<b><i>Campylobacter spp.</i></b>	845,024	337,031-1,611,083	9
<b><i>Staphylococcus aureus</i></b>	241,148	72,341-529,417	3
<b>Subtotal</b>			91



**Table 2.3. Top five pathogens contributing to domestically acquired foodborne illnesses resulting in hospitalization (adapted from CDC 2011).**

<b>Pathogen</b>	<b>Estimated number of hospitalizations</b>	<b>90% Credible Interval</b>	<b>%</b>
<b><i>Salmonella</i>, nontyphoidal</b>	19,336	8,545-37,490	35
<b>Norovirus</b>	14,663	8,097-23,323	26
<b><i>Campylobacter spp.</i></b>	8,463	4,300-15,227	15
<b><i>Toxoplasma gondii</i></b>	4,428	3,060-7,146	8
<b><i>E. coli</i> (STEC) O157</b>	2,138	549-4,614	4
<b>Subtotal</b>			88

**Table 2.4. Top five pathogens contributing to domestically acquired foodborne illnesses resulting in death (adapted from CDC 2011).**

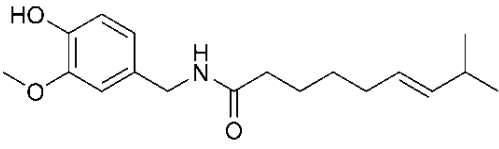
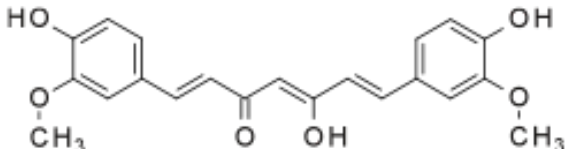
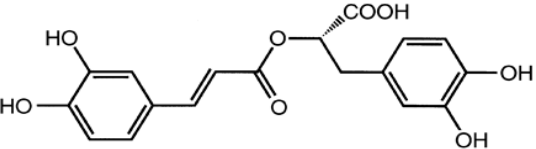
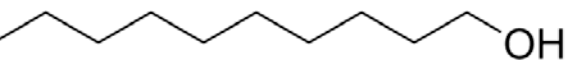
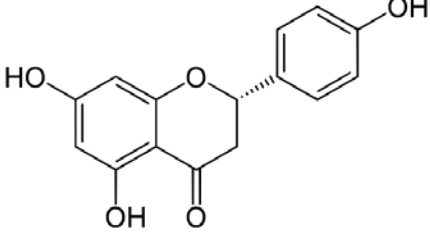
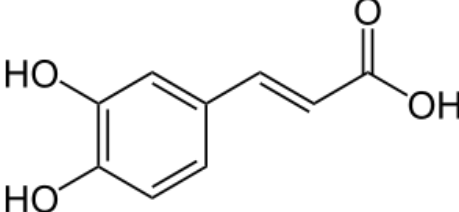
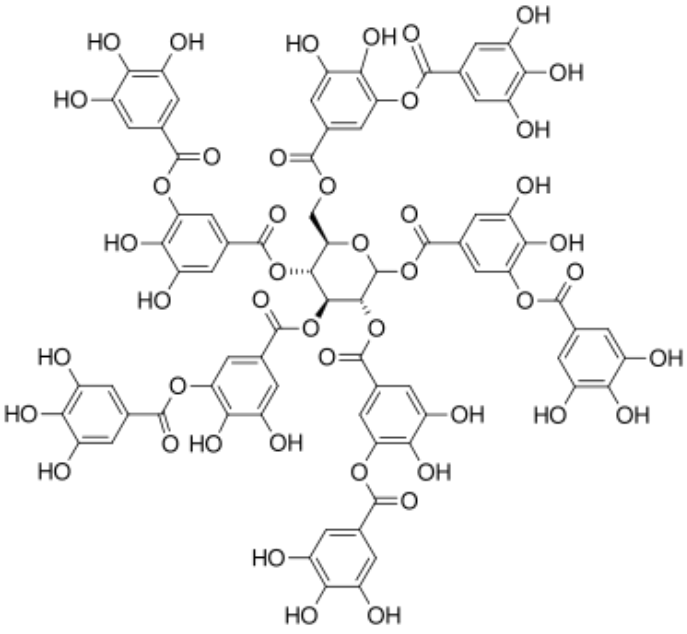
<b>Pathogen</b>	<b>Estimated number of deaths</b>	<b>90% Credible Interval</b>	<b>%</b>
<b><i>Salmonella, nontyphoidal</i></b>	378	0-1,011	28
<b><i>Toxoplasma gondii</i></b>	327	200-482	24
<b><i>Listeria monocytogenes</i></b>	255	0-733	19
<b>Norovirus</b>	149	84-237	11
<b><i>Campylobacter spp.</i></b>	76	0-332	6
<b>Subtotal</b>			88

**Table 2.5. Phenolic compounds and their natural sources.**

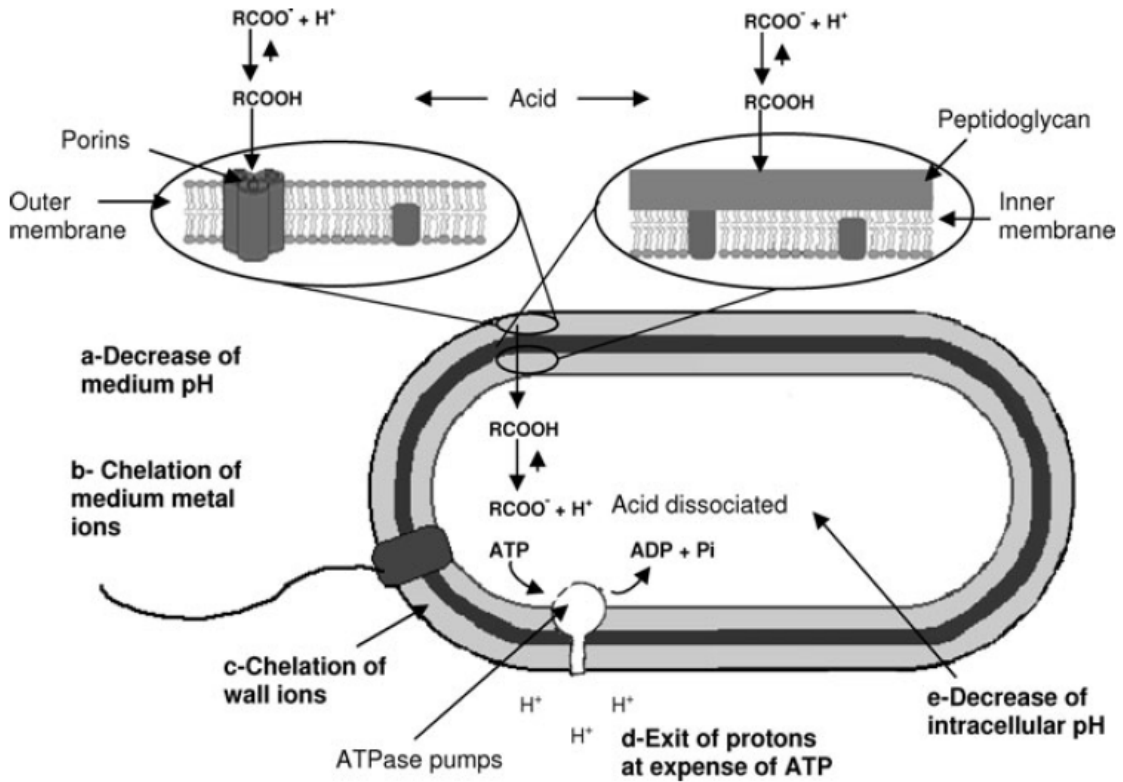
<b>Compound</b>	<b>Source</b>	<b>Reference</b>
Caffeic a.	coffee beans, white grapes, olive, spinach	(Askun and others 2009a)
Capsaicin	chili peppers (septa)	(Kachoosangi and others 2008)
Chlorogenic a.	coffee beans, cumin, bamboo, honeysuckle flower	(Xia and others 2011a)
Coumarin	tonka beans, vanilla, sweet grass, strawberries, cherries, woodruff, apricots	(Bettaieb and others; Maddox and others 2010)
Curcumin	turmeric, mustard	(Bhawana and others 2011)
Decanol	strawberries, <i>E. coli</i>	(Yu and others 2000; Jetty and others 2007)
Ellagic a.	berries, pomegranate, grape, walnut, pecan	(Duman and others 2009)
(-) Epicatechin	tea leaves, cacao beans, cinnamon stick	(Cueva and others 2010)
Eugenol	cinnamon, clove, nutmeg, bay leaf, basil	(Singh and others 2008)
Gallic a.	grapes, gallnuts, sumac, tea leaves, hops, oak bark	(Rivero-Cruz 2008; Bancirova 2010)
Myricetin	berries, grapes, walnuts	(Yao and others 2011)
Naringenin	grapefruits, oranges	(Erlund 2004; Vikram and others 2010)
<i>p</i> -Coumaric a.	peanuts, tomatoes, carrots, garlic	(Acar and others 2010)
Quercetin	onions, skins of apples, citrus fruits, cherries, fennel	(Geoghegan and others 2010)
Rosmarinic a.	rosemary leaves and flowers, thyme	(Moreno and others 2006)
Rutin	asparagus, brake fern, citrus fruits, buckwheat	(Pereira and others 2007a)
Syringic a.	acai palm trees, swiss chard, corn	(Korukluoglu and others; Kosina and others 2010)
Tannic a.	berries, nettle, tea leaves, sumac leaves, oak wood, chestnut, gallnuts	(Taguri and others 2004)
Thymol	thyme, nigella seeds (black cumin)	(Emiroglu and others 2010)
Thymoquinine	nigella seeds (black cumin)	(Salem 2005; Tanis and others 2009)
Xanthohumol	female hops flowers, dandelion, marigold	(Zanoli and Zavatti 2008)

Quercetin $C_{15}H_{10}O_7$	Myricetin $C_{15}H_{10}O_8$	Rutin $C_{27}H_{30}O_{16}$
Coumarin $C_9H_6O_2$	Xanthohumol $C_{21}H_{22}O_5$	(-) Epicatechin $C_{15}H_{14}O_6$
Ellagic acid $C_{14}H_6O_8$	Eugenol $C_{10}H_{12}O_2$	Gallic acid $C_7H_6O_5$
Chlorogenic a. $C_{16}H_{18}O_9$	Thymoquinone $C_{10}H_{12}O_2$	Thymol $C_{10}H_{14}O$

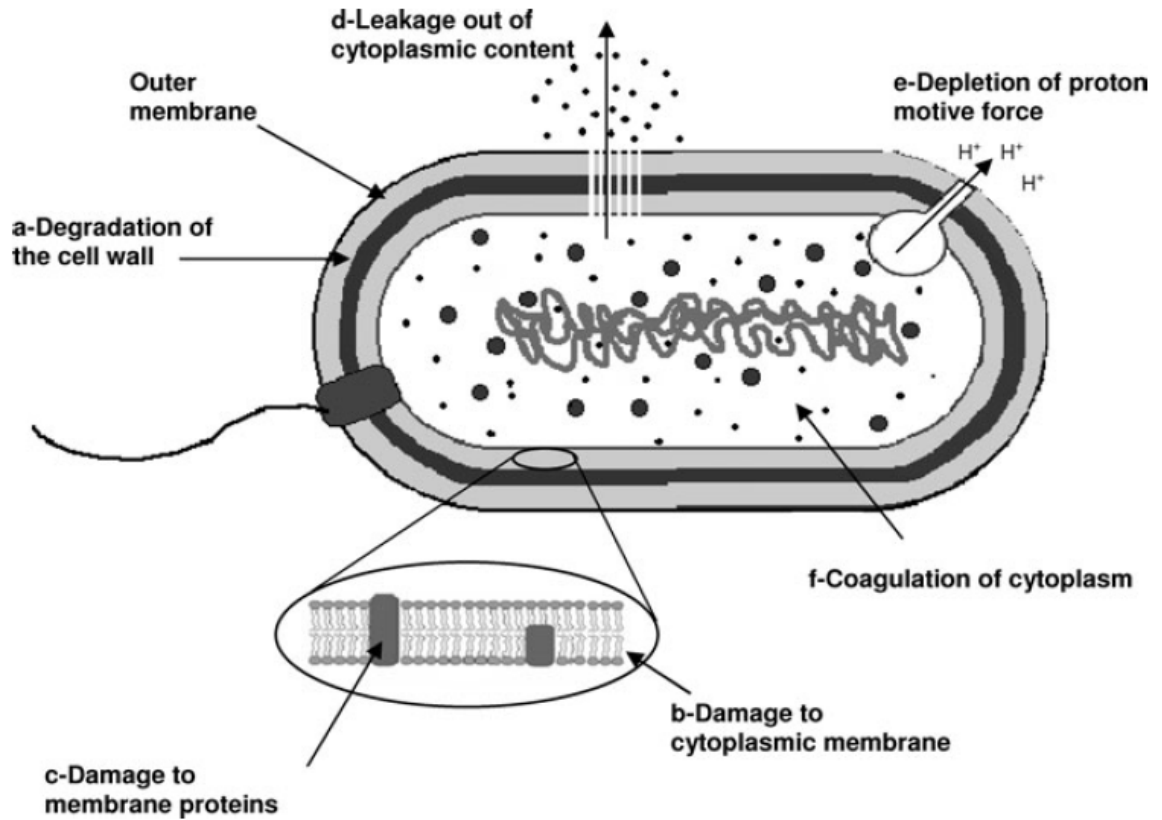
**Figure 2.1.** Chemical structures of some commonly used natural phenolic compounds.

	
Capsaicin $C_{18}H_{27}NO_3$	Curcumin $C_{21}H_{20}O_6$
	
Rosmarinic acid $C_{18}H_{16}O_8$	Decanol $C_{10}H_{22}O$
	
Naringenin $C_{15}H_{12}O_5$	Caffeic acid $C_9H_8O_4$
	
Tannic acid $C_{76}H_{52}O_{46}$	

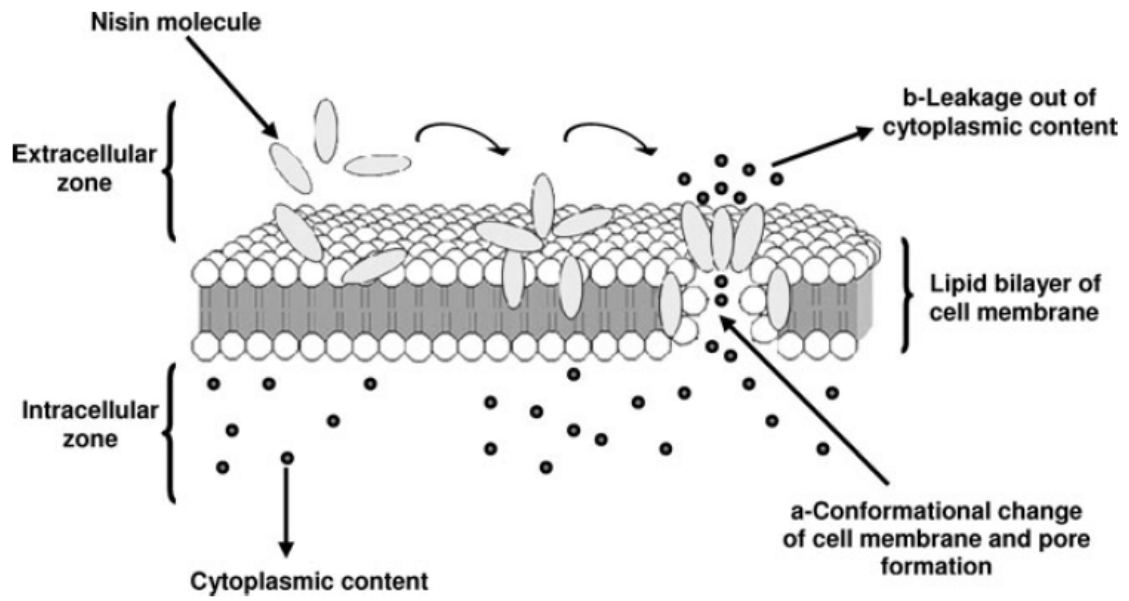
**Figure 2.1.** continues



**Figure 2.2.** Mechanisms of action of organic acids in a bacterial cell (a-e). The left figure illustrates how the organic acids can pass through the outer membrane in Gram-negative bacteria, whereas the right figure shows how they can pass through the inner membrane in Gram-positive bacteria. Adapted from Raybaudi-Massilia and others 2009.



**Figure 2.3.** Mechanisms of action of essential oils and their components in a bacterial cell (a-f). Illustrates the mode of action at the inner membrane. Adapted from Burt 2004a.



**Figure 2.4.** Mechanism of action of nisin in a bacterial cell (a-b). Adapted from Raybaudi-Massilia and others 2009.



## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Preparation of Phenolic Compounds

Several types of natural phenolic compounds extracted from herbs, spices, vegetables, and fruits are used for this study. Capsaicin, Chlorogenic acid, Coumarin, Decanol, Ellagic acid, (-) Epicatechin, Myricetin, Quercetin, Rutin, Tannic acid, Curcumin, Eugenol, Gallic acid, Rosmarinic acid, Thymol, Thymoquinine, and Xanthohumol were obtained from Sigma-Aldrich Group and stored at optimum temperature and conditions required for each compound.

Four different concentrations; 5, 10, 15 and 20 parts per million (ppm) of each compound were prepared in appropriate solvents including; Ethanol, 190 proof (95%) (Decon Laboratories, Inc.), Propylene glycol (Fisher Scientific), Dimethyl sulfoxide (DMSO) (Fisher Scientific), and ionized H<sub>2</sub>O according to the National Committee for Clinical Laboratory Standards (NCCLS 1994). Some of the solutions were heated up in a steamer (Wilmot Castle Sterogage, Co., NY, USA) including quercetin, ellagic acid, and xanthohumol, to obtain complete dissociation of the compound in the solvent. pH analysis was performed with an electronic pH meter (Accumet Basic AB15, Fisher Scientific, Fairlawn, NJ). For accurate pH measurement, pH meter was standardized through the use of pH=4.01 and pH=6.86 standard buffer solutions. The final phenolic solution was adjusted to approximately pH=5.00 to ensure the pH would not affect the bacterial growth. All solutions were filter sterilized using 0.2 µm filters (Millipore Corporation). The filter sterilized solutions were stored at 4°C in sterilized glass sealed containers until needed. All solutions were allowed to come to room temperature prior to use.

#### 3.2 Bacterial Strains, Culture Conditions and Preparation of Inoculum

Three different strains of pathogenic *Salmonella* spp., *E. coli*, *Bacillus* spp., *Listeria monocytogenes* and *Clostridium* spp. were supplied from the American Type Culture

Collection (ATCC 6051, Difco Spores, ATCC 842, ATCC 7644, UK Animal Diagnostic Lab., ATCC 49594, R. Newsome Research, ATCC 8260, ATCC 7955, F. T. Jones, ATCC 43895, ATCC 35150, UK Micro 29A, ATCC 10708, and (-) H<sub>2</sub>S). In this study, they were evaluated to determine the minimum inhibitory concentration (MIC) of several natural compounds.

Aerobic bacteria cells were grown and maintained on slants of brain-heart infusion (BHI) agar, (Difco Laboratories, Becton Dickinson and Company, USA) and stored at 4°C until needed. Anaerobic bacteria cells including *Clostridium* species were grown and maintained in Thioglycollate Medium (Difco, Becton, Dickinson and Company, USA) under anaerobic conditions and stored at 4°C until needed. Thioglycollate broth was steamed for about 15 minutes to drive off the oxygen from the media prior to each use. Prior to each study at least three consecutive transfers of the aerobic bacterial cultures were inoculated in BHI broth, (Difco Laboratories, Becton, Dickinson and Company) and anaerobic cultures were performed in Differential Reinforced Clostridial (RC) Media (Difco Laboratories, Becton, Dickinson and Company, USA) under anaerobic conditions. Incubation for *Clostridium* was grown in GasPak jars (GasPak Anaerobic System, BBL, BioQuest) along with O<sub>2</sub> scavenger BD GasPak Ez Anaerobic Container System (Becton, Dickinson and Company, Maryland, USA) to maintain the anaerobic conditions. Prior to each use, RC broth was steamed for about 15 minutes to drive off the oxygen and rapidly cooled. The cultures were incubated overnight at optimal temperatures for each bacterium. With most bacteria, an overnight incubation period is sufficient, but an additional day may be needed for the more slowly growing microorganisms, such as *Clostridium*. After a sufficient period of incubation, the inoculums were standardized according to a MacFarland 0.5 turbidity standard (10<sup>8</sup> CFU/ml) by diluting the sample (National Committee for Clinical Laboratory Standards, NCCLS, 2004). Culture growth turbidity, which indicated by the Optical Density (OD), was adjusted to a specific rate for each type of bacteria at a wavelength of 660 nm (OD<sub>660</sub>), using the spectrophotometer, (BioTek Synergy 4) to the final concentrations of approximately 10<sup>7</sup>-10<sup>8</sup> CFU/ml. After the culture growth, turbidity has been adjusted to a specific OD regarding the bacteria. For *E. coli* turbidity was adjusted to 0.02 OD whereas, it was 0.2 for *Listeria monocytogenes* and *Salmonella*. Direct culture was used without adjusting the optical

density for *Bacillus* and *Clostridium*. Once diluted, suspension of the inoculums was tested within 20-30 minutes. Cell counts were confirmed using a spiral plating method. Serial dilutions ( $10^{-3}$  and  $10^{-4}$ ) of the bacterial cultures were plated onto Plate Count Agar (PCA), (Difco Laboratories, Becton, Dickinson and Company) with the Eddy Jet spiral plater (IUL Instruments, Neutec Group Inc.) and incubated for 24 to 48 hours at appropriate incubation conditions for each type of bacteria. The bacterial counts were determined by the Flash and Go plate reader (IUL Instruments, Neutec Group Inc.).

Table 3.1 shows the optical density of initial inoculums, initial bacterial cell counts of PCA plates, and the incubation conditions for each of the bacterial strain used in this study.

### **3.3 Screening for Antimicrobial Activity**

Micro broth dilution technique of Antimicrobial Susceptibility testing is employed as outlined in the NCCLS.

#### Procedure for Aerobic Organisms:

Five ml of Mueller Hinton Broth (MHB) (Difco Laboratories, Becton, Dickinson and Company) dispensed into each test tube and sterilized in the scientific gravity sterilizer (STERIS AMSCO Century, SG-120, Thermo Scientific). One hundred  $\mu$ l of each serial solution of the compounds were dispensed into each 5 ml MHB, to obtain the final concentrations of 5, 10, 15 and 20 ppm ( $\mu$ g/L). Then, 100  $\mu$ l of the overnight culture (at least three consecutive transfers) of each bacterial strain was transferred aseptically into each MHB tube containing the specific amount of compound. Three controls were used. First, a compound and culture free, pure un-inoculated MHB was used as a blank for sterility check and adjusting the spectrophotometer. Then a compound free MHB broth (growth control), including only culture, was used as a positive control. Finally, a negative control in which 100  $\mu$ l of the solvent, either ethanol, ionized H<sub>2</sub>O, propylene glycol, or DMSO was dispensed in 5 ml of MHB, in order to determine the possible effect of the solvent on the bacterial growth performance. One hundred and fifteen  $\mu$ l of

each sample, including the blank, positive and negative controls, was dispensed to each well of a 96-well flat bottom micro-titer plate (Nalge NUNC Int., NY, USA). The final assay volume was 150  $\mu$ l. The serial concentrations of phenolic compounds were adjusted to a final assay volume of 150  $\mu$ l: 3  $\mu$ l of positive or negative control solutions plus 147  $\mu$ l of the diluted strain; 3  $\mu$ l of phenolic compound solution plus 147  $\mu$ l of the diluted strain. All treatments were carried out in duplicates for each individual strain.

After inoculation, the micro-titer plates were read immediately using a calibrated spectrophotometer (BioTek Synergy 4) at 660 nm wavelength. After getting the initial OD, the micro-titer plates were incubated at optimal temperatures for each bacterial strain. Prior to each incubation process, the samples in the micro-titer plate were shaken for 10 seconds to get a consistent homogeneity. The absorbance was read at every 12-hour intervals of total 60 hours of incubation period.

#### Procedure for Anaerobic Organisms:

Five ml of RC Media was dispensed into sterilized 10 ml serum bottles (Fisher Scientific) and covered with aluminum foil. They were autoclaved and cooled for 10-15 minutes. The oxygen free serum bottles were covered with appropriate sterile plastic stoppers (Fisher Scientific) and sealed air tight with aluminum caps (Fisher Scientific) to maintain anaerobic conditions.

One hundred  $\mu$ l of each serial solution of the compounds were released via a 1 ml syringe (Fisher Scientific) into each 5 ml RC media, to obtain the final concentrations of 5, 10, 15 and 20 ppm ( $\mu$ g/L). Then, 100  $\mu$ l of the overnight culture (at least three consecutive transfers) of each bacterial strain was transferred via a syringe into each RC media serum bottle containing the specific amount of compound. Three controls were used. First, a compound and culture free, pure un-inoculated RC media was used as a blank for sterility check and adjusting the spectrophotometer. Then a compound free RC media (growth control), including only the culture, was used as a positive control. Finally, a negative control in which 100  $\mu$ l of the solvent, either ethanol, ionized H<sub>2</sub>O, propylene glycol, or DMSO was released in 5 ml of RC media, in order to determine the possible effect of the solvent on the bacterial growth performance. One hundred and fifteen  $\mu$ l of each sample,

including the blank, positive and negative controls, was dispensed into each well of a 96-well flat bottom micro-titer plate (Nalge NUNC Int., NY, USA). The final assay volume was 150  $\mu$ l. The serial concentrations of phenolic compounds were adjusted to a final assay volume of 150  $\mu$ l: 3  $\mu$ l of positive or negative control solutions plus 147  $\mu$ l of the diluted strain; 3  $\mu$ l of phenolic compound solution plus 147  $\mu$ l of the diluted strain. All treatments were carried out in duplicates for each individual strain.

After inoculation, the micro-titer plates were read immediately using a calibrated spectrophotometer (BioTek Synergy 4) at 660 nm wavelength. In order to maintain the anaerobic conditions for *Clostridium* serum bottles were incubated and kept in the 37<sup>o</sup>C incubator during the 60 hours of incubation period. After getting the initial OD, the micro-titer plates were discarded. Prior to each screening process samples from the incubated RC media were taken via a syringe and then, dispensed into the micro-titer plates. Prior to each screening process, the samples in the micro-titer plate were shaken for 10 seconds to get a consistent homogeneity. Absorbance was read at 12-hour intervals over 60 hours of incubation. All of the micro-titer plates were discarded immediately after the screening process.

The same procedures were employed for all the compounds tested in this study. The experiments were conducted in two replicates for each individual strain.

### **3.4 Determination of Minimum Inhibitory Concentrations of Phenolic Compounds**

There are various *in vitro* techniques to test minimum inhibitory concentrations to determine antimicrobial susceptibility or resistance of individual isolates. The goal of our study was to determine whether the etiologic agent is resistant or sensitive to the natural antimicrobial agents being tested.

MIC, being the lowest concentration that inhibits visible growth of the organism was determined according to 90 % inhibition level. The antimicrobial agent has to inhibit 90 % of the visible microbial growth to be considered as the MIC of the antimicrobial agent against the organism used. Thus, the tested organism was called “susceptible (sensitive)”

to the natural antimicrobial agent used. However, when the microbial growth was not inhibited 90 %, then the organism tested was called “resistant” to the natural antimicrobial agent used (NCCLS 2004). Turbidity was compared between the control well and the test wells.

Formula:

MIC= [OD Control after incubation - OD Blank] x 90% > [OD Treatment after incubation - OD Blank]

The minimum concentration (ppm) of the natural antimicrobial compound tested, which showed less growth than 90% of the control regarding the specific bacteria was recorded as the MIC.

### **3.5 Investigation of Structural Changes via Scanning Electron Microscopy**

#### **3.5.1 Materials and Sample Preparation**

Treated and non-treated (control) bacterial cultures were incubated for 24 hours at appropriate conditions for the type of bacteria. Then, they are diluted to  $10^{-4}$  and/or  $10^{-6}$  concentrations and filtered using sterilized 0.2  $\mu\text{m}$  filters (Thermo Scientific, Nalgene, Analytical Filter, CN) to capture the bacteria for Scanning Electron Microscope (SEM) observations. Every specimen destined for conventional SEM must be dry. As living microorganisms, bacteria contain proteins and a high amount of water in their cells. It is essential to fix them to micro filters in order to preserve their structure. To prepare the samples in reasonable time, the specimens should be relatively thin (<2 mm) and only a few millimeters in dimensions (Kalab and others 2009). Large solid materials which contain bacteria on their surfaces such as contaminated meat, skin, vegetables, composted materials, or agar gel plates with bacterial colonies are first excised and trimmed to approximately 10 mm x 10 mm x 2 mm in dimensions and fixed before they are further reduced into smaller (approx. 5 mm x 5 mm x 2mm) particles.

Glutaraldehyde-Phosphate Buffer Preparation:

Pure 50 % solution of Glutaraldehyde reagent (E.M. Grade, SPI Supplies, Structure Probe, Inc., USA) was prepared with Sodium Phosphate Monobasic ( $\text{Na}_2\text{HPO}_4$ , Anhydrous, Fisher Scientific), to 6 % of final concentration.

12 ml 50 % pure Glutaraldehyde

44 ml  $\text{Na}_2\text{HPO}_4$  (0.1 M, pH 7.0)

+ 44 ml ionized  $\text{H}_2\text{O}$

---

100 ml Glutaraldehyde fixative (6%)

The filters containing thin layers of bacterial specimens were soaked in the buffered fixative, glutaraldehyde (6 %) for a minimum of 1 hour.

Fixed specimens were submerged in ionized  $\text{H}_2\text{O}$  and dehydrated for 5-10 minutes by soaking in serial dilutions of ethanol including; 20 %, 40 %, 60 %, 80 %, and 100 %, respectively.

5-10 minutes in 20 % Ethyl alcohol

5-10 minutes in 40 % Ethyl alcohol

5-10 minutes in 60 % Ethyl alcohol

5-10 minutes in 80 % Ethyl alcohol

5-10 minutes in 100 % Ethyl alcohol

5-10 minutes in 100 % Ethyl alcohol

For critical point drying, the dehydrated specimens were submerged in hexamethyldisilazane (HMDS, Sigma-Aldrich, USA) for 5 minutes. Then, the specimens were air dried under the fume hood.

The double sided adhesive conductive carbon tabs (SPI Supplies, Structure Probe, Inc., PA, USA) were placed over the specimen stubs (SPI Supplies, Structure Probe, Inc., PA,

USA). Double sided sticky tape on the face of a SEM stub facilitates the specimens' (filters with bacteria on top) positioning. Then, the specimens were cut in appropriate sizes and mounted over the carbon tabs. They were left at the desiccators overnight. Specimens can be kept at desiccators for several weeks prior to SEM observation. Specimens were prepared in two replicates for each individual strain.

#### Coating the Specimens:

Fixed and dried specimens were sputter-coated with Carbon using a plasma coating system for SEM (Hummer VI, Sputtering System by Technics). They acquire electrically conductive surfaces after they are sputter-coated to be examined efficiently by SEM.

### **3.5.2 Capturing Images with Scanning Electron Microscope**

The specimens were examined on a Scanning Electron Microscope (SEM S-800, Hitachi, Tokyo, Japan) operated at 10-20 kV accelerating voltage. Magnification and working distance were adjusted for the position and type of specimen. The captured images were analyzed using the software Evex Nanoanalysis and Digital Imaging (Evex Analytical Version 2.0.1192, 2006).

### **3.6 Statistical Analysis**

The antimicrobial activity of the phenolic compounds was subjected to General Linear Model procedure of Statistix 9.0(2008). Significance level of  $P < 0.05$  of the null hypothesis was used to determine significant variables. Difference between means was identified by use of Tukey HSD randomized complete block design between treatments and time.



**Table 3.1. Initial bacterial inoculums and bacterial cell counts, and the incubation temperatures.**

<b>Bacteria</b>	<b>Initial Count (CFU/ml)</b>	<b>Initial Inoculum (OD)</b>	<b>Incubation Temperature (°C)</b>
<i>Bacillus subtilis</i> ATCC 6051	6.025 x 10 <sup>8</sup>		
<i>Bacillus cereus</i> Difco Spores	8.465 x 10 <sup>7</sup>	Direct culture	32°C
<i>Bacillus polymyxa</i> ATCC 842	3.975 x 10 <sup>8</sup>		
<i>Listeria monocytogenes</i> ATCC 7644	1.985 x 10 <sup>8</sup>		
<i>Listeria monocytogenes</i> UK Animal Diag. Lab.	2.725 x 10 <sup>8</sup>	0.2	37°C
<i>Listeria monocytogenes</i> ATCC 49594	4.270 x 10 <sup>8</sup>		
<i>Clostridium perfringens</i> R. Newsome Research	1.590 x 10 <sup>7</sup>		
<i>Clostridium butyricum</i> ATCC 8260	1.040 x 10 <sup>7</sup>	Direct culture	37°C
<i>Clostridium sporogenes</i> ATCC 7955	1.000 x 10 <sup>7</sup>		
<i>Escherichia coli</i> F. T. Jones	3.490 x 10 <sup>7</sup>		
<i>Escherichia coli</i> O157:H7 ATCC 43895	6.165 x 10 <sup>7</sup>	0.02	37°C
<i>Escherichia coli</i> O157:H7 ATCC 35150	2.315 x 10 <sup>7</sup>		
<i>Salmonella paratyphi</i> UK Micro 29A	1.090 x 10 <sup>8</sup>		
<i>Salmonella choleraesuis</i> subsp. ATCC 10708	1.335 x 10 <sup>8</sup>	0.2	37°C
<i>Salmonella enteridis</i> (-) H <sub>2</sub> S	1.130 x 10 <sup>8</sup>		

## CHAPTER 4

### Minimum Inhibitory Concentrations of Natural Antimicrobial Phenolic Compounds

#### Derived from Herbs & Spices

##### 4.1 Introduction

In recent years, consumers have increasing concerns about the food safety to prevent and control pathogenic microorganisms in food, leading to many attempts to use natural substances of plant origin. Spices and aromatic herbs have long been used in food for not only their flavor but also for their preservative and medicinal properties (Shan and others 2007a). Many herb and spice extracts possess antimicrobial activities against a wide range of bacteria, yeasts, and molds (Shan and others 2007a; Raybaudi-Massilia and others 2009). Since the ancient times, these have been used throughout the world for preventing food spoilage and deterioration and also for extending shelf life of foods, while attempts to characterize these properties in the laboratory date back to the early 1900s.

Recent research studies revealed that active compounds such as thymol (obtained from thyme) and eugenol (obtained from cinnamon and clove) disrupts the cellular membrane, eventually causing the death of several microorganisms such as *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella enteridis* (Raybaudi-Massilia and others 2009). Eugenol was found to inactivate the pathogenic microorganisms including; *E. coli* O157:H7 and *L. monocytogenes* with 4 to 6 log CFU/ml reductions when applied in pasteurized apple juice. Dorman and Deans (2000) reported that thymol has greater inhibitory activity against Gram-positive organisms such as *Bacillus subtilis* and *Clostridium sporogenes*. Moreno and others (2006) determined the antimicrobial activity of rosmarinic acid which showed inhibitory activity for neither *E. coli* nor *B. subtilis*. Xanthohumol reported to be highly effective against Gram-positive and some certain Gram-negative bacteria (Natarajan and others 2008). Some clinical *in vitro* studies suggest that xanthohumol has a potential antibacterial, antifungal, and chemo-preventive activity (Zanoli and Zavatti 2008).

The purpose of this study was to determine the antimicrobial activity and the MIC of the phenolic compounds derived from herbs and spices for both Gram-positive and Gram-negative foodborne pathogens. Antimicrobial Susceptibility Testing Method was employed to investigate inhibition and control the growth of aerobic foodborne pathogens in Mueller Hinton Broth and anaerobic pathogens in Reinforced Clostridial Broth.

## 4.2 Results and Discussion

Antimicrobial characteristics of phenolic compounds obtained from herbs and spices, including curcumin, eugenol, gallic acid, rosmarinic acid, thymol, thymoquinine, and xanthohumol were investigated against foodborne pathogens (NCCLS 2004). The phenolic compounds were found to be effective for inhibiting the growth of various Gram-positive bacterial species including *Bacillus* (Table 4.1), *Listeria monocytogenes* (Table 4.2), *Clostridium* (Table 4.3), and Gram-negative bacteria such as *Escherichia coli* (Table 4.4) and *Salmonella* species (Table 4.5).

### Curcumin

The results of this study for *B. subtilis* ATCC 6051 (Appendix Figure 1) are consistent with the previous studies done with curcumin, which is found to be antimicrobial (Bhawana and others 2011). Inhibition of *B. subtilis* Difco Spores was observed at 24 hours at higher concentrations of curcumin (15 ppm). *B. cereus* (Figure 4.1) and *B. polymyxa* ATCC 842 (Figure 4.2) showed high sensitivity with the MICs of 15 and 5ppm, respectively. After 24 hours of incubation they recovered.

Curcumin showed no antimicrobial activity against *L. monocytogenes* ATCC 7644 (LM1) and *L. monocytogenes* UK Animal Diagnostic Lab. (UK ADL (LM3)), which were observed to be highly resistant. However, it relatively inhibited the growth of *L. monocytogenes* ATCC 49594 (LM2) (Figure 4.3) after 60 hours of incubation, where the MIC of curcumin was determined to be 20 ppm.

All three strains of *Clostridium*, *C. perfringens* R. Newsome Research (RNR (C1)), *C. butyricum* ATCC 8260 (C2), and *C. sporogenes* ATCC 7955 (C3) were observed to have

relatively close antimicrobial sensitivity when tested with curcumin, with the MICs of 5 ppm following 60 hours of incubation. The Figure 4.4 shows the average of antimicrobial activities of curcumin when tested against those three strains of *Clostridium*.

Curcumin has been previously observed to have antimicrobial activity against *E. coli* species (Bhawana and others 2011). *E. coli* O157:H7 ATCC 43895 (EC2) showed no antimicrobial activity with curcumin exposure during the entire 60 hours of incubation period. We showed varying degrees of antimicrobial activity against non-pathogenic *E. coli* F. T. Jones (FTJ (EC1)) (Figure 4.5), and *E. coli* O157:H7 ATCC 35150 (EC3) (Appendix Figure 2) with MICs of <20 ppm.

*S. paratyphi* UK Micro 29A (S1) (Appendix Figure 3), *S. choleraesuis subsp.* ATCC 10708 (S2) (Figure 4.6) and *S. enteridis* (-) H<sub>2</sub>S (S3) (Appendix Figure 4) were observed to be sensitive against curcumin after 60 hours of incubation. The MIC of curcumin was determined as <20 ppm for those species of *Salmonella*. Also, *S. choleraesuis subsp.* demonstrated the highest antimicrobial sensitivity during the entire incubation period.

## **Eugenol**

MIC of eugenol was determined to be 15 ppm when tested against *B. cereus* following 60 hours of incubation time (Figure 4.8). *B. subtilis* (Figure 4.7) and *B. polymyxa* (Appendix Figure 5) showed a similar antimicrobial activity with MIC of 20 ppm, immediately following exposure. However *B. subtilis* recovered resistance against eugenol following 36 hours of incubation. Previous studies also denote eugenol being effective against inhibiting the growth of *B. subtilis* (Saei-Dehkordi and others 2010).

Eugenol was observed to have a high antimicrobial activity when it was tested against LM1 (Appendix Figure 6), LM2 (Appendix Figure 7) and LM3 (Appendix Figure 8). MICs of eugenol against those *L. monocytogenes* strains were determined to be <10 ppm after 60 hours of incubation period. To some extent this was consistent with previous studies on antibacterial activity of eugenol, which was found to be effective to inhibit the growth of *L. monocytogenes* after being incorporated into alginate-based edible coatings (Raybaudi-Massilia and others 2009). Eugenol, extracted from clove, was also reported

being highly effective in reducing the proliferation of *L. monocytogenes* on the surface of fresh lettuce (Kim and others 2011).

*C. butyricum* and *C. sporogenes* were observed to be highly resistant against eugenol during the entire 60 hour incubation period. However, *C. perfringens* (Figure 4.9) was determined to be sensitive to <20 ppm eugenol, following 60 hours of incubation.

All *E. coli* strains tested with eugenol were observed to have high degrees of antimicrobial activity following 60 hours of incubation, with resulting MICs of 20 ppm. The average antimicrobial activities of the three strains of EC1, EC2, and EC3 were shown in Figure 4.10. These eugenol results were also consistent with previous studies, where eugenol was found to be highly antimicrobial against *E. coli* after being incorporated into alginate-based edible coatings (Raybaudi-Massilia and others 2009). *In vitro* studies of *E. coli* O157:H7 also confirmed eugenol's high antimicrobial activity (Saei-Dehkordi and others 2010). According to Kim and others (2011) eugenol, from clove found to be highly effective against reducing the population of *E. coli* O157:H7 on the surface of fresh lettuce.

Eugenol was observed to be very effective against inhibiting the growth of *Salmonella* species during 60 hours of incubation. Previous studies confirm these results, where eugenol was found to be highly effective in reducing the population of *Salmonella* species on fresh lettuce (Kim and others 2011). The MICs of eugenol against *S. paratyphi* (Appendix Figure 9), *S. choleraesuis subsp.* (Figure 4.11) and *S. enteridis* (Figure 4.12) were determined as 15 ppm, after 60 hours.

### **Gallic acid**

Both *B. cereus* and *B. polymyxa* were highly resistance against gallic acid. Previous studies reported *B. cereus* isolates to be sensitive to gallic acid (Askun and others 2009a). On the other hand, MIC of *B. subtilis* (Figure 4.13) was determined to be 10 ppm during the entire 60 hours of incubation.

Gallic acid was observed to be effective in inhibiting the growth of LM2 (Appendix Figure 10) and LM3 (Appendix Figure 11) following 60 hours of incubation, with MIC

of <10 ppm. However, LM1 showed a very high resistance against gallic acid during the entire 60 hours of incubation period.

When treated with gallic acid, all three strains of *Clostridium* were observed to be very sensitive, having similar antimicrobial activities after the incubation period of 60 hours. Figure 4.14 shows the average antimicrobial activity of *C. perfringens*, *C. butyricum* and *C. sporogenes*. The MIC of gallic acid was determined as 5 ppm for all of the three strains of *Clostridium*.

Gallic acid was reported to be highly antimicrobial against Gram-negative pathogens (Askun and others 2009a) including the phytopathogen *Xylella fastidiosa* (Maddox and others 2010). All of the three strains of *E. coli* were observed to have very similar antimicrobial activity against gallic acid, with MIC of 15, after 60 hours of incubation. Figure 4.15 shows the average degrees of antimicrobial sensitivity of EC1, EC2, and EC3.

*Salmonella* species tested against gallic acid were observed to have varying degrees of antimicrobial activity. *S. paratyphi* (Appendix Figure 12) was determined to be sensitive until 36 hours of incubation period and then, it became resistant at 15 ppm. *S. enteridis* (Figure 4.16) showed a similar antimicrobial sensitivity after 60 hours of incubation when treated with gallic acid at 15 ppm. However, *S. cholerasuis subsp.* was observed to be highly resistant when treated and incubated with gallic acid.

### **Rosmarinic acid**

All three strains of *Bacillus* showed varying degrees of antimicrobial sensitivity when they were treated and incubated with rosmarinic acid for 60 hours. *B. cereus* demonstrated high antimicrobial susceptibility confirming the previous reported results with rosmarinic acid (Askun and others 2009a). MICs were determined for *B. subtilis* (Appendix Figure 13), *B. cereus* (Figure 4.17), and *B. polymyxa* (Appendix Figure 14) as 5, 10, and 15 ppm, respectively. The previous studies show that rosmarinic acid did not inhibit the growth of *B. subtilis* (Moreno and others 2006). However, in this study, *B. subtilis* was found to be highly sensitive against rosmarinic acid.

Rosmarinic acid was observed to have no antimicrobial activity against *Listeria monocytogenes* strains tested, LM1, LM2, and LM3, during the 60 hour incubation period.

*C. butyricum* (Figure 4.18) and *C. sporogenes* (Figure 4.19) tested against rosmarinic acid were determined to have a very similar sensitivity, with MICs of <20 ppm, but with different incubation times. *C. butyricum* showed consistent antimicrobial sensitivity until the end of 60 hours incubation, while *C. sporogenes* regained its resistance after 48 hours. However, rosmarinic acid was found not to be effective against inhibiting the growth of *C. perfringens*.

Gram-negative pathogens were previously reported to be highly susceptible to rosmarinic acid (Askun and others 2009a). Distinctly, in this study *E. coli* strains EC1, EC2, and EC3 were observed to be highly resistant to rosmarinic acid during the 60 hours of incubation. Therefore these findings validated that the previous studies on effectiveness of rosmarinic acid on *E. coli* are inconsistent (Moreno and others 2006).

*Salmonella* species were observed to have high degrees of antimicrobial sensitivity when treated with rosmarinic acid. The MICs of rosmarinic acid for *S. paratyphi* (Figure 4.20), *S. choleraesuis subsp.* (Figure 4.21) and *S. enteridis* (Appendix Figure 15) were determined as <20 ppm after the 60 hours of incubation.

### **Thymol**

All of the three strains of *Bacillus* were highly sensitive to thymol after 60 hours incubation. *B. subtilis* was previously reported to be highly susceptible to thymol (Salem 2005; Saei-Dehkordi and others 2010). MIC of thymol was determined as <20 ppm against *B. subtilis* (Appendix Figure 16), *B. cereus* (Figure 4.22) and *B. polymyxa* (Figure 4.23). Consistently, previous reports also show the susceptibility of *B. cereus* to thymol at 25 mg/ml (Tanis and others 2009).

Thymol was previously reported to have antimicrobial activity against *Listeria* (Tanis and others 2009). However in this study, thymol was observed to have no antimicrobial

activity against *L. monocytogenes* strains tested, LM1, LM2, and LM3, during the 60 hours of incubation period.

The three strains of *Clostridium*, *C. perfringens*, *C. butyricum*, and *C. sporogenes* tested against thymol were determined to have very similar sensitivity, with MIC of 5 ppm following 60 hours of incubation time. Figure 4.24 shows the average antimicrobial activity of thymol against the *Clostridium* species tested.

Thymol was previously reported to not have antimicrobial activity against *E. coli*, when suspended with H<sub>2</sub>O (Bakathir and Abbas 2011). In this study, thymol concentration with ethanol was used and a consistent antimicrobial activity was observed among the three treated strains of *E. coli*, following 60 hours of incubation period. Previous reports are inconsistent with the results of this study for *E. coli* O157:H7 being highly susceptible to thymol (Saei-Dehkordi and others 2010). The MIC was determined as 15 ppm and Figure 4.25 shows the average antimicrobial activity of EC1, EC2, and EC3 together.

*S. paratyphi* (Appendix Figure 17) and *S. choleraesuis subsp.* (Appendix Figure 18) were observed to be sensitive against thymol, with the MIC of 15 ppm following 60 hours of incubation period. However, *S. enteridis* was found to be consistently resistant to thymol during the entire incubation period.

### **Thymoquinine**

*Bacillus* species were found to have varying degrees of antimicrobial sensitivity during the whole 60 hours of incubation period, when tested against thymoquinine. Previous studies reported thymoquinine to be effective against several *Bacillus* species (Salem 2005; Salem and Hossain 2000) including *B. cereus* (Tanis and others 2009) with MIC of <27 mm in diameter (highly active). MICs of *B. subtilis* (Appendix Figure 19), *B. cereus* (Figure 4.26), and *B. polymyxa* (Appendix Figure 20) were determined as <15 ppm.

Thymoquinine was observed to have a consistent antimicrobial activity among all the three strains of *L. monocytogenes*, which are consistent with previous *Listeria* studies with thymoquinine (Tanis and others 2009). The MIC for all strains was determined as 10 ppm. Appendix Figure 21 shows the average antimicrobial activity of LM1, LM2, and



LM3. After 60 hours of incubation LM1 and LM3 were observed to be very sensitive, whereas LM2 became resistant to thymoquinine after being incubated for 12 hours.

*C. perfringens* (Figure 4.27) and *C. sporogenes* (Figure 4.28) were observed to have similar antimicrobial sensitivity to thymoquinine during the entire incubation period. The MICs for *C. perfringens* and *C. sporogenes* were determined to be <10 ppm following 60 hours of incubation.

In previous studies, thymoquinine was not found to be effective against *E. coli* when suspended with H<sub>2</sub>O (Bakathir and Abbas 2011). However, in this study using ethanol as the diluents for thymoquinine was highly effective. It was determined to have a MIC of 20 ppm against non-pathogenic EC1, EC2, and EC3 following 60 hours of incubation. Figure 4.29 depicts the average of consistent antimicrobial activity for the three *E. coli* strains.

*S. paratyphi* (Appendix Figure 22), *S. choleraesuis subsp.* (Figure 4.30) and *S. enteridis* (Figure 4.31) were observed to have a similar antimicrobial activity after being treated and incubated for 60 hours with thymoquinine. The MICs were determined as <20 ppm.

### **Xanthohumol**

Xanthohumol was shown to have high antimicrobial activity against *B. subtilis* (Figure 4.32), and *B. polymyxa* (Appendix Figure 4.23) when used at 5 ppm concentration. *B. cereus* (Figure 4.33) also showed antimicrobial sensitivity to Xanthohumol, with the MIC of 20 ppm at 60 hours of incubation.

All three strains of *Listeria* were observed to be highly susceptible when treated with xanthohumol. MICs of LM1, LM2 and LM3 were determined as 5 ppm after 60 hours of incubation. Figure 4.34 depicts the average antimicrobial activity of xanthohumol against *L. monocytogenes*.

Xanthohumol was determined to have consistently high antimicrobial activity against three species of *Clostridium*; *C. perfringens*, *C. butyricum* and *C. sporogenes*. MICs of those three strains were determined to be 5 ppm after 60 hours of incubation period.

Figure 4.35 depicts the average antimicrobial activity of xanthohumol against *Clostridium* species.

Previous studies suggest that xanthohumol has no antimicrobial activity against *E. coli* (Zanoli and Zavatti 2008). Contrary, *E. coli* strains used in this study were found to have varying degrees of antimicrobial sensitivity during the 60 hour incubation period, when tested against xanthohumol. EC1 (Figure 4.36) and EC2 (Appendix Figure 24) were observed to be sensitive when treated with xanthohumol, with concentrations of 15 ppm. EC3 showed high resistance during the whole incubation period.

*S. enteridis* was found to be very resistant against xanthohumol, showing no growth inhibition during the 60 hours of incubation period. However, *S. paratyphi* (Appendix Figure 25) and *S. cholerasuis subsps.* (Appendix Figure 26) were observed to be sensitive, following 60 hours. MICs of xanthohumol for *S. paratyphi* and *S. cholerasuis subsps.* were determined as 20 and 15 ppm, respectively.

### **4.3 Conclusion**

The results revealed that the tested phenolic compounds have varying antimicrobial effects against pathogenic bacteria. Generally, Gram-negative bacteria (*E. coli* and *Salmonella*) Gram-positives (*Bacilli*, *Listeria monocytogenes*, and *Clostridium*) were observed to have different degrees of antimicrobial susceptibility following 60 hours of incubation. These variations may reflect the differences in cell surface structures between Gram-negative and Gram-positive bacteria. In particular, the outer membrane of Gram-negative bacteria functions as a preventive barrier against hydrophobic compounds (Puupponen-Pimia and others 2001). Some of the results were not consistent with previous studies on antibacterial activity of the bioactive compounds of cinnamon stick (Shan and others 2007a). The reason for that may be using different sources of phenolic compounds and/or different bacterial species, which affect the bacterial response to the antimicrobials.

Different strains of the same family showed varying degrees of antimicrobial susceptibility, with different concentrations of phenolic compounds. Thus, each phenolic compound had different MICs for each bacterial strain. Figure 4.6 and Figure 4.7 depict the summary of MICs of both Gram-positive and Gram-negative bacteria for 60 hours of incubation period. However, curcumin, gallic acid, thymol, and xanthohumol had the same MICs (<5 ppm) for all strains of *Clostridium*. Thymoquinine had MIC of 10 ppm for all strains of *Listeria monocytogenes*. Moreover, all three strains of *E. coli*, including non-pathogenic *E. coli* and *E. coli* O157:H7 showed consistent antimicrobial susceptibility against eugenol, gallic acid, thymol, and thymoquinine, with the MICs of 20, 15, 15, and 20 ppm, respectively.

Overall, thymoquinine was found to be the most effective phenolic compound against both Gram-positive and Gram-negative pathogens. The highest concentration of thymoquinine used for the antimicrobial affect was 20 ppm. The investigated strength of antimicrobial activity was followed by eugenol, xanthohumol, curcumin, gallic acid, rosmarinic acid and thymol, respectively. This study provides long term storage food safety results for foodborne pathogens. Previous studies only established MICs for 24 hour incubation periods, while in this study, we determined the MICs after the incubation period of 60 hours. Thus, the antimicrobial affects of these natural phenolic compounds understood more efficiently when they are exposed to long term storage in food and food applications. We were also able to compare the antimicrobial activity of the phenolics between 24 hours and 60 hours of incubation. Table 4.8 and Table 4.9 depict the MICs of phenolics for both Gram-positive and Gram-negative pathogens after 24 hours of incubation. The results suggest that the sensitivity of the pathogenic bacteria to phenolic compounds depends on bacterial species and polyphenol structure of the phenolics. Incubation period is another factor affecting the bacterial growth, where some of the sensitive pathogens recover and become resistant after 24 hours of incubation.

**Table 4.1. Minimum Inhibitory Concentrations of Phenolic Compounds Derived from Herbs and Spices against *Bacillus* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	Solvent	pH	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco S.	<i>B. polymyxa</i> ATCC 842	<i>Bacillus</i> spp.
Curcumin	ETOH	5.54	< 15.0	< 15.0	< 5.0	< 15.0
Eugenol	ETOH	5.54	< 20.0	< 15.0	< 20.0	< 20.0
Gallic acid	H <sub>2</sub> O	5.14	< 10.0	NA	NA	< 10.0
Rosmarinic a.	ETOH	5.08	< 15.0	< 10.0	< 15.0	< 15.0
Thymol	ETOH	5.46	< 20.0	< 15.0	< 10.0	< 20.0
Thymoquinine	DMSO	5.59	< 5.0	< 15.0	< 10.0	< 15.0
Xanthohumol	ETOH	8.7	< 5.0	< 20.0	< 5.0	< 20.0

NA: not active

**Table 4.2. Minimum Inhibitory Concentrations of Phenolic Compounds Derived from Herbs and Spices against *Listeria monocytogenes* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	Solvent	pH	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	LM spp.
Curcumin	ETOH	5.54	NA	< 20.0	NA	< 20.0
Eugenol	ETOH	5.54	< 5.0	< 10.0	< 5.0	< 10.0
Gallic acid	H <sub>2</sub> O	5.14	NA	< 5.0	<10.0	< 10.0
Rosmarinic a.	ETOH	5.08	NA	NA	NA	NA
Thymol	ETOH	5.46	NA	NA	NA	NA
Thymoquinine	DMSO	5.59	< 10.0	< 10.0*	< 10.0	< 10.0
Xanthohumol	ETOH	8.7	NA	< 5.0	< 5.0	< 5.0

NA: not active

\*: Incubation period before 24 hours

**Table 4.3. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Herbs and Spices against *Clostridium* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	C1 RNR	C2 ATCC 8260	C3 ATCC 7955	<i>Clostridium</i> species
Curcumin	5.54	ETOH	< 5.0	< 5.0	< 5.0	< 5.0
Eugenol	5.54	ETOH	< 20.0	NA	NA	< 20.0
Gallic acid	5.14	H <sub>2</sub> O	< 5.0	< 5.0	< 5.0	< 5.0
Rosmarinic a.	5.08	ETOH	NA	< 20.0	< 10.0	< 20.0
Thymol	5.46	ETOH	< 5.0	< 5.0	< 5.0	< 5.0
Thymoquinine	5.59	DMSO	< 10.0	NA	< 10.0	< 10.0
Xanthohumol	8.7	ETOH	< 5.0	< 5.0	< 5.0	< 5.0

NA: not active

**Table 4.4. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Herbs and Spices against *Escherichia coli* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	EC1 FTJ	EC2 ATCC 43895	EC3 ATCC 35150	<i>E. coli</i> species
Curcumin	5.54	ETOH	< 20.0	NA	< 5.0	< 20.0
Eugenol	5.54	ETOH	< 20.0	< 20.0	< 20.0	< 20.0
Gallic acid	5.14	H <sub>2</sub> O	< 15.0	< 15.0	< 15.0	< 15.0
Rosmarinic a.	5.08	ETOH	NA	NA	NA	NA
Thymol	5.46	ETOH	< 15.0	< 15.0	< 15.0	< 15.0
Thymoquinine	5.59	DMSO	< 20.0	< 20.0	< 20.0	< 20.0
Xanthohumol	8.7	ETOH	< 15.0	< 15.0	NA	< 15.0

NA: not active

**Table 4.5. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Herbs and Spices against *Salmonella* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	S1 UKM 29A	S2 ATCC 10708	S3 (-) H <sub>2</sub> S	<i>Salmonella</i> species
Curcumin	5.54	ETOH	< 5.0	< 15.0	< 20.0	< 20.0
Eugenol	5.54	ETOH	< 15.0	< 15.0	< 15.0	< 15.0
Gallic acid	5.14	H <sub>2</sub> O	< 15.0	NA	< 15.0	< 15.0
Rosmarinic a.	5.08	ETOH	< 15.0	< 20.0	< 15.0	< 20.0
Thymol	5.46	ETOH	< 15.0	< 15.0	NA	< 15.0
Thymoquinine	5.59	DMSO	< 20.0	< 15.0	< 20.0	< 20.0
Xanthohumol	8.7	ETOH	< 20.0	< 15.0	NA	< 20.0

NA: not active

**Table 4.6. MICs (ppm) of Phenolic Compounds Derived from Herbs and Spices for Gram-positive bacteria.**

Gram-positive bacteria										
Compound	pH	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco Spores	<i>B. polymyxa</i> ATCC 842	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	C1 RNR	C2 ATCC 8260	C3 ATCC 7955
Curcumin	5.54	< 15.0	< 15.0	< 5.0	NA	< 20.0	NA	< 5.0	< 5.0	< 5.0
Eugenol	5.54	< 20.0	< 15.0	< 20.0	< 5.0	< 10.0	< 5.0	< 20.0	NA	NA
Gallic a.	5.14	< 10.0	NA	NA	NA	< 5.0	<10.0	< 5.0	< 5.0	< 5.0
Rosmarinic a.	5.08	< 15.0	< 10.0	< 15.0	NA	NA	NA	NA	< 20.0	< 10.0
Thymol	5.46	< 20.0	< 15.0	< 10.0	NA	NA	NA	< 5.0	< 5.0	< 5.0
Thymoquinine	5.59	< 5.0	< 15.0	< 10.0	< 10.0	< 10.0*	< 10.0	< 10.0	NA	< 10.0
Xanthohumol	8.7	< 5.0	< 20.0	< 5.0	NA	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0

NA: not active, \*: Incubation period before 24 hours

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**Table 4.7. MICs (ppm) of Phenolic Compounds Derived from Herbs and Spices for Gram-negative bacteria.**

Gram-negative bacteria							
Compound	pH	<i>E. coli</i> FTJ	<i>E. coli</i> 0157:H7 ATCC 43895	<i>E. coli</i> 0157:H7 ATCC 35150	<i>S. paratyphi</i> UK Micro 29A	<i>S. cholerasuis subsp.</i> ATCC 10708	<i>S. enteridis</i> (-) H <sub>2</sub> S
Curcumin	5.54	< 20.0	NA	< 5.0	< 5.0	< 15.0	< 20.0
Eugenol	5.54	< 20.0	< 20.0	< 20.0	< 15.0	< 15.0	< 15.0
Gallic a.	5.14	< 15.0	< 15.0	< 15.0	< 15.0	NA	< 15.0
Rosmarinic a.	5.08	NA	NA	NA	< 15.0	< 20.0	< 15.0
Thymol	5.46	< 15.0	< 15.0	< 15.0	< 15.0	< 15.0	NA
Thymoquinine	5.59	< 20.0	< 20.0	< 20.0	< 20.0	< 15.0	< 20.0
Xanthohumol	8.7	< 15.0	< 15.0	NA	< 20.0	< 15.0	NA

NA: not active

**Table 4.8. MICs (ppm) of Phenolic Compounds Derived from Herbs and Spices for Gram-positive bacteria (after 24 hours).**

Gram-positive bacteria										
Compound	pH	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco Spores	<i>B. polymyxa</i> ATCC 842	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	C1 RNR	C2 ATCC 8260	C3 ATCC 7955
Curcumin	5.54	< 15.0	< 15.0	< 5.0	NA	< 20.0	NA	< 5.0	< 10.0	< 15.0
Eugenol	5.54	NA	< 15.0	< 20.0	< 5.0	< 20.0	< 5.0	< 20.0	NA	NA
Gallic a.	5.14	< 15.0	NA	NA	NA	< 20.0	NA	< 10.0	< 15.0	NA
Rosmarinic a.	5.08	< 5.0	< 20.0	NA	NA	NA	NA	NA	< 10.0	< 10.0
Thymol	5.46	< 15.0	< 15.0	< 20.0	NA	< 15.0	NA	< 5.0	< 20.0	< 5.0
Thymoquinine	5.59	< 5.0	< 15.0	< 10.0	NA	< 20.0	NA	< 15.0	< 20.0	< 5.0
Xanthohumol	8.7	< 5.0	< 10.0	< 5.0	< 10.0	< 10.0	< 10.0	< 5.0	< 5.0	< 5.0

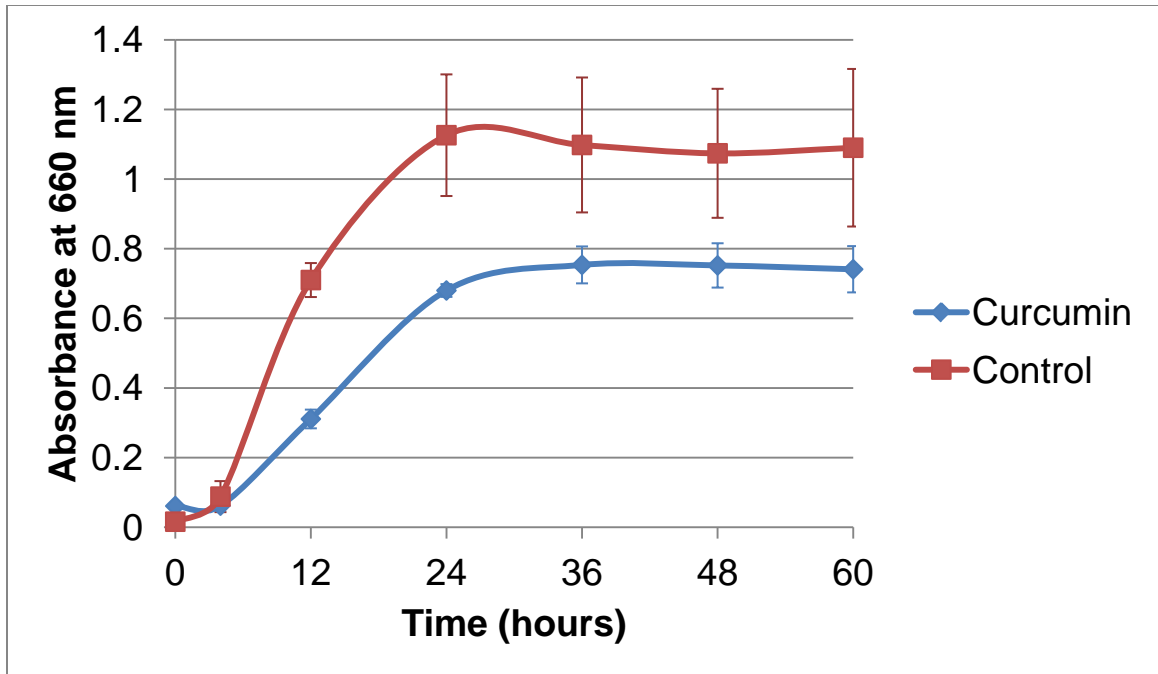
NA: not active

**Table 4.9. MICs (ppm) of Phenolic Compounds Derived from Herbs and Spices for Gram-negative bacteria (after 24 hours).**

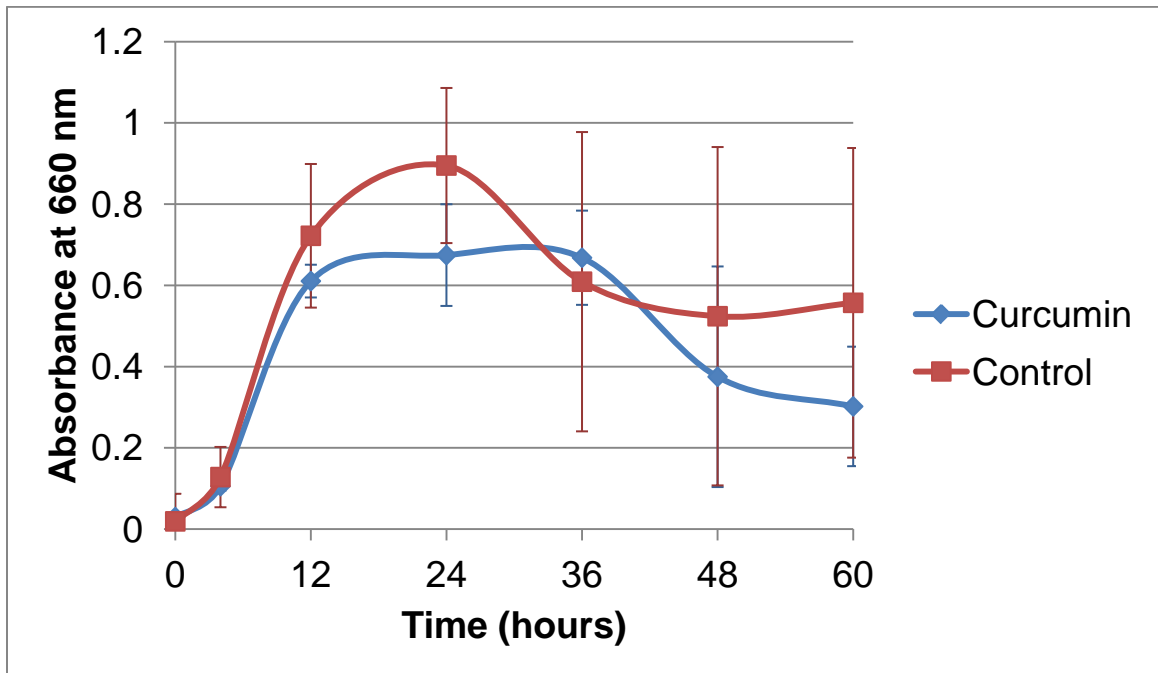
Gram-negative bacteria							
Compound	pH	<i>E. coli</i> FTJ	<i>E. coli</i> 0157:H7 ATCC 43895	<i>E. coli</i> 0157:H7 ATCC 35150	<i>S. paratyphi</i> UK Micro 29A	<i>S. cholerasuis subsp.</i> ATCC 10708	<i>S. enteridis</i> (-) H <sub>2</sub> S
Curcumin	5.54	< 20.0	NA	< 5.0	< 15.0	< 15.0	< 5.0
Eugenol	5.54	< 20.0	< 20.0	< 20.0	< 20.0	< 20.0	< 15.0
Gallic a.	5.14	< 10.0	NA	< 20.0	NA	NA	< 15.0
Rosmarinic a.	5.08	NA	NA	NA	NA	NA	< 15.0
Thymol	5.46	< 20.0	< 15.0	< 15.0	NA	NA	NA
Thymoquinine	5.59	NA	NA	NA	NA	< 5.0	< 20.0
Xanthohumol	8.7	< 5.0	< 10.0	NA	NA	< 20.0	NA

NA: not active

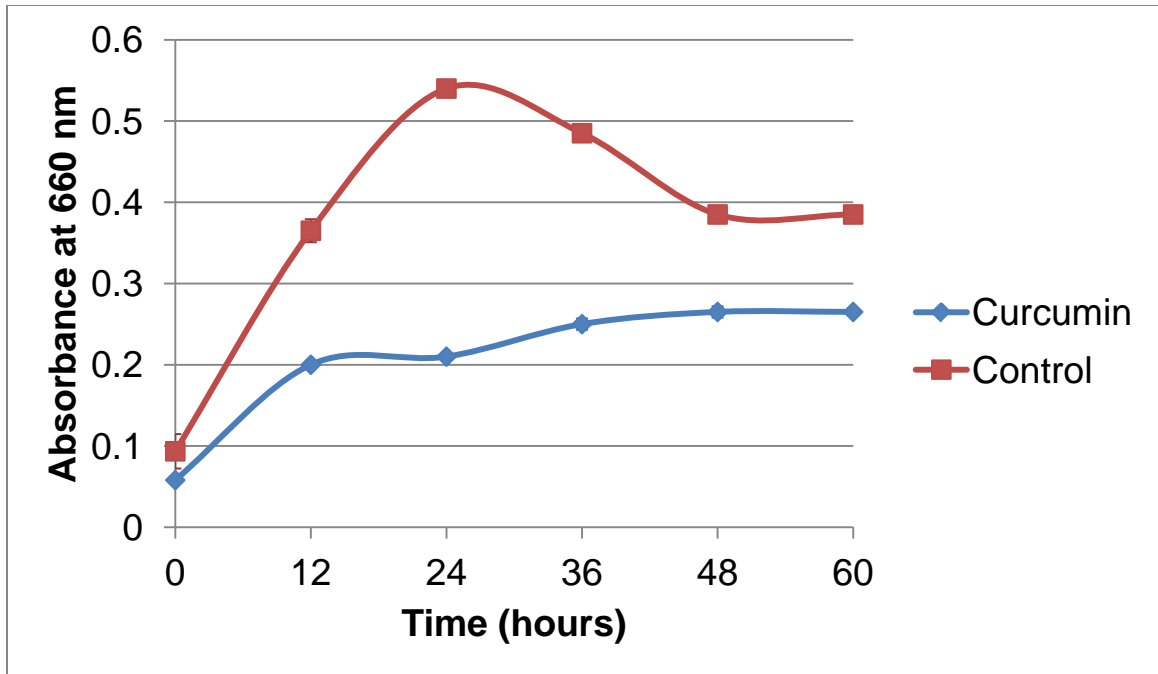




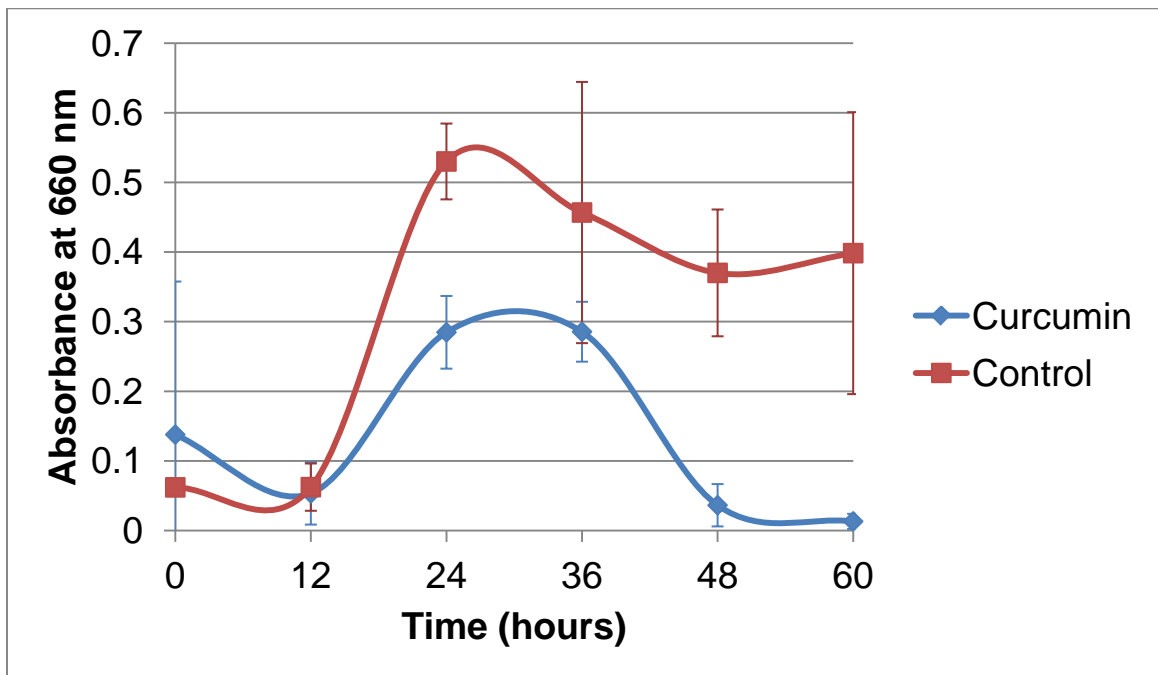
**Figure 4.1.** The antimicrobial activity of curcumin at MIC of 15 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



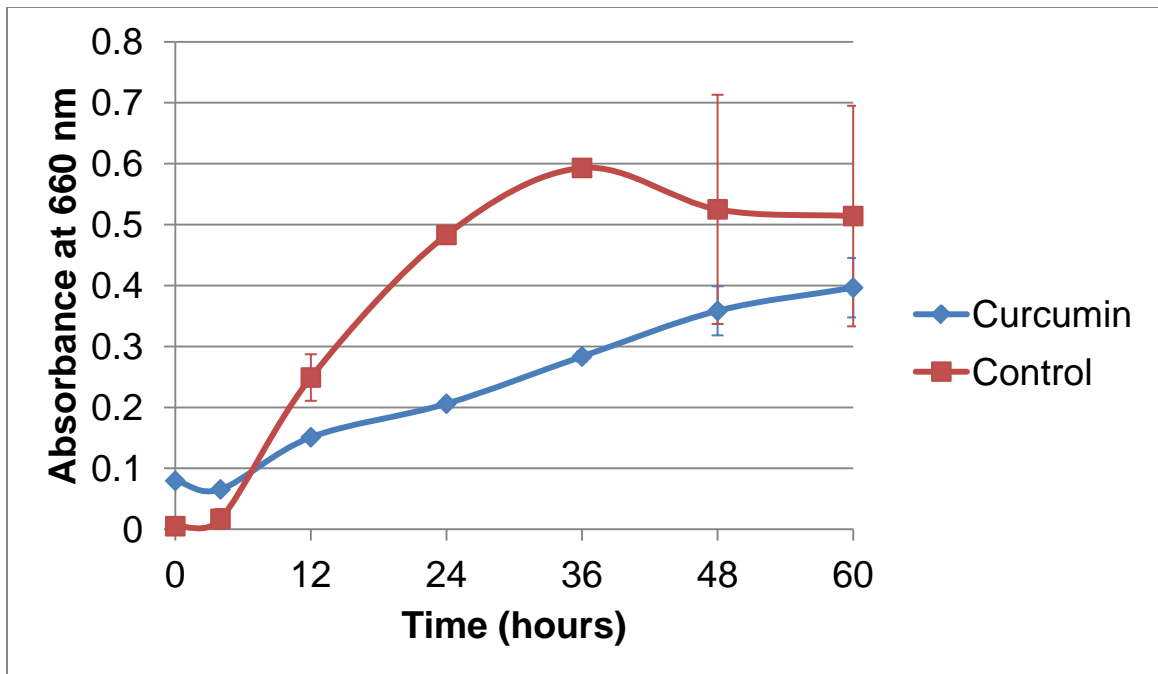
**Figure 4.2.** The antimicrobial activity of curcumin at MIC of 5 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



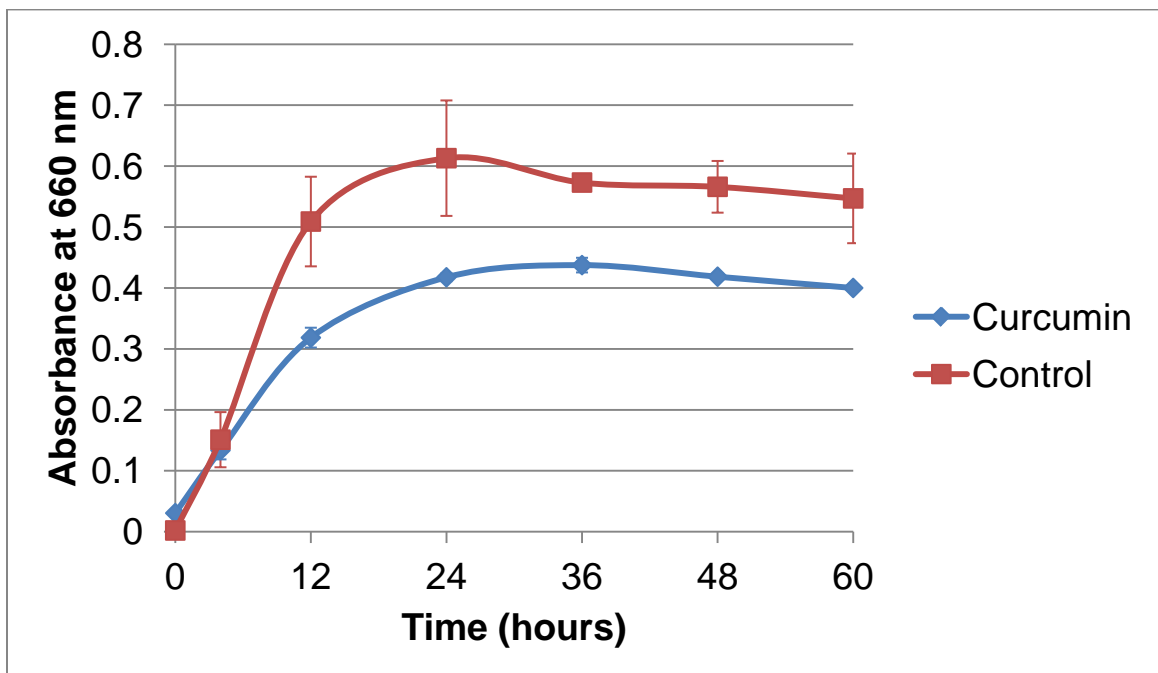
**Figure 4.3.** The antimicrobial activity of curcumin at MIC of 20 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



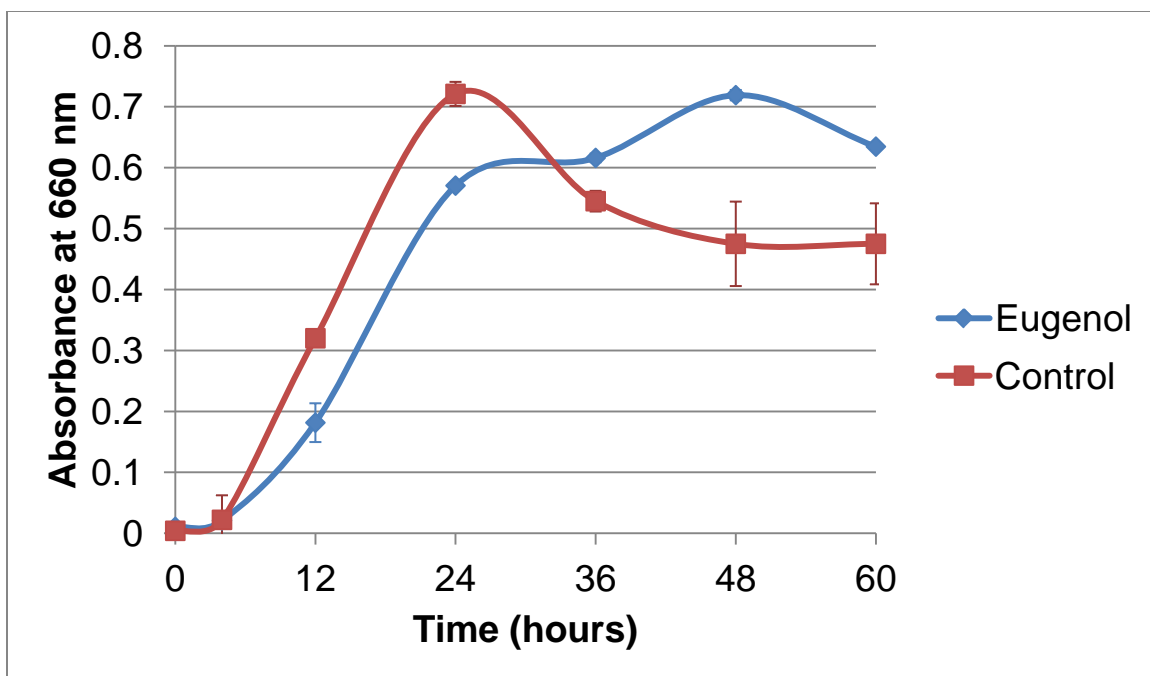
**Figure 4.4.** The average antimicrobial activity of curcumin at MIC of 5 ppm against *C. perfringens*, RNR, *C. butyricum*, ATCC 8260, and *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



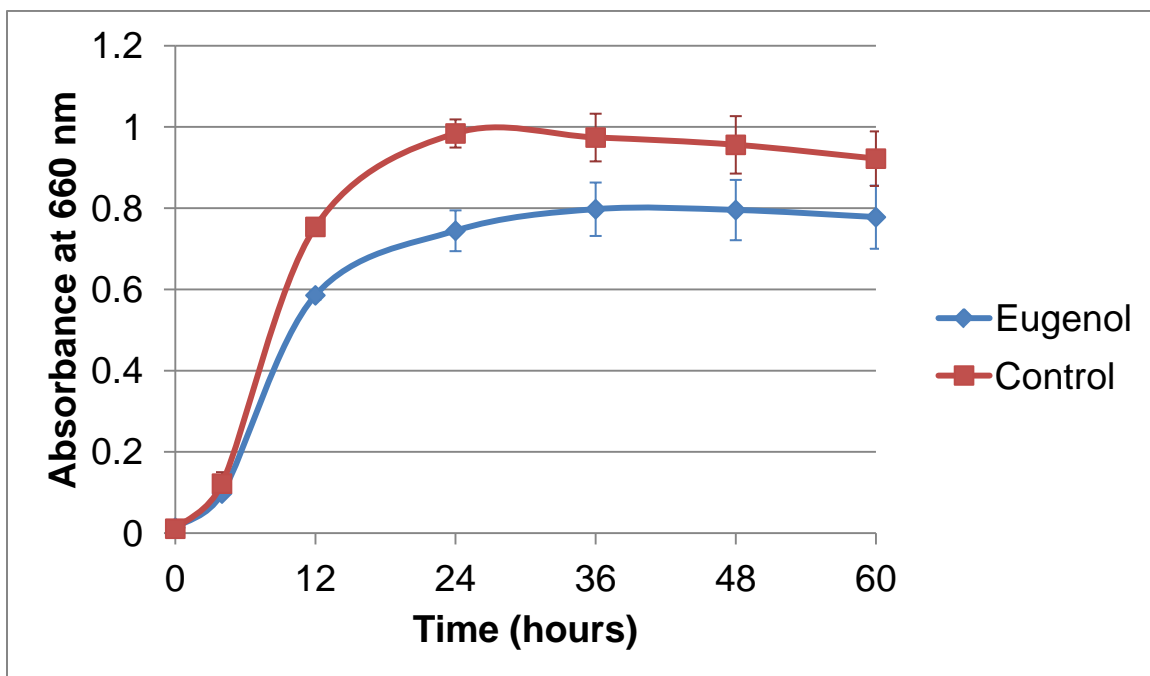
**Figure 4.5.** The antimicrobial activity of curcumin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



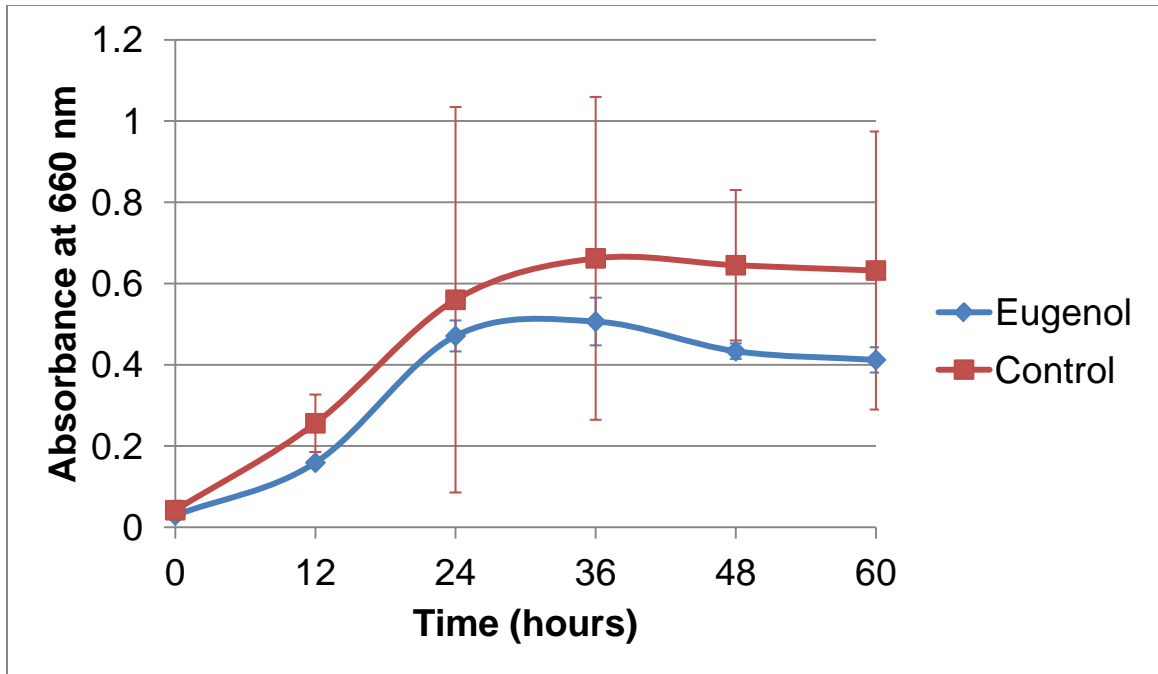
**Figure 4.6.** The antimicrobial activity of curcumin at MIC of 15 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



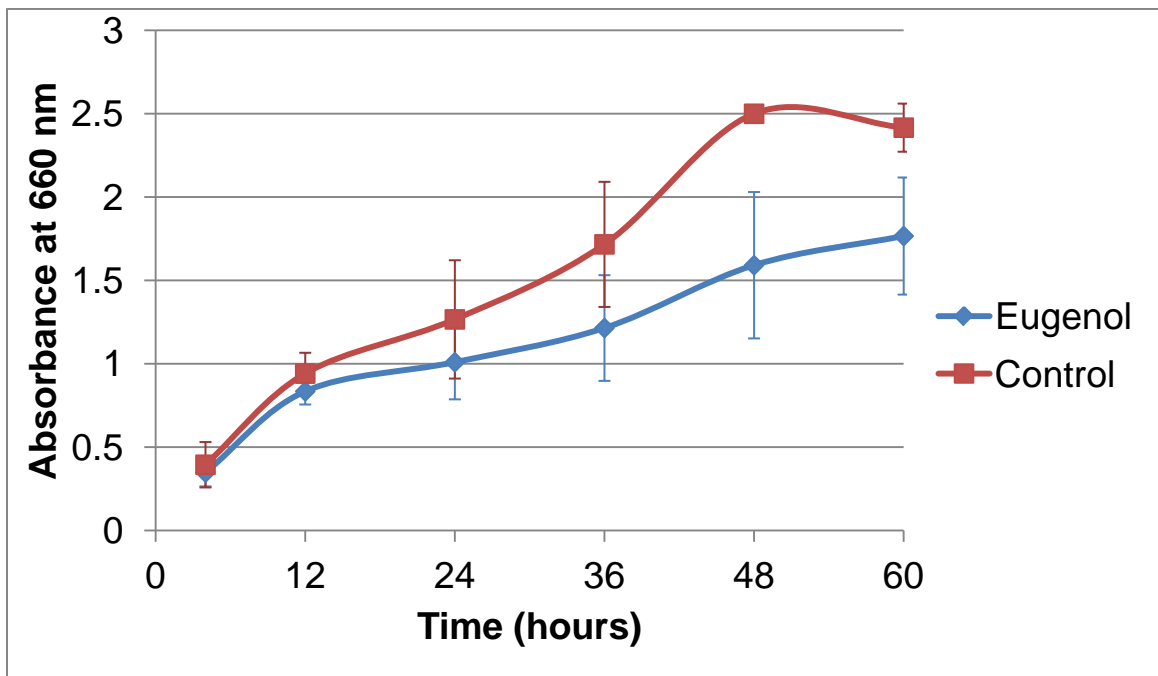
**Figure 4.7.** The antimicrobial activity of eugenol at MIC of 20 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



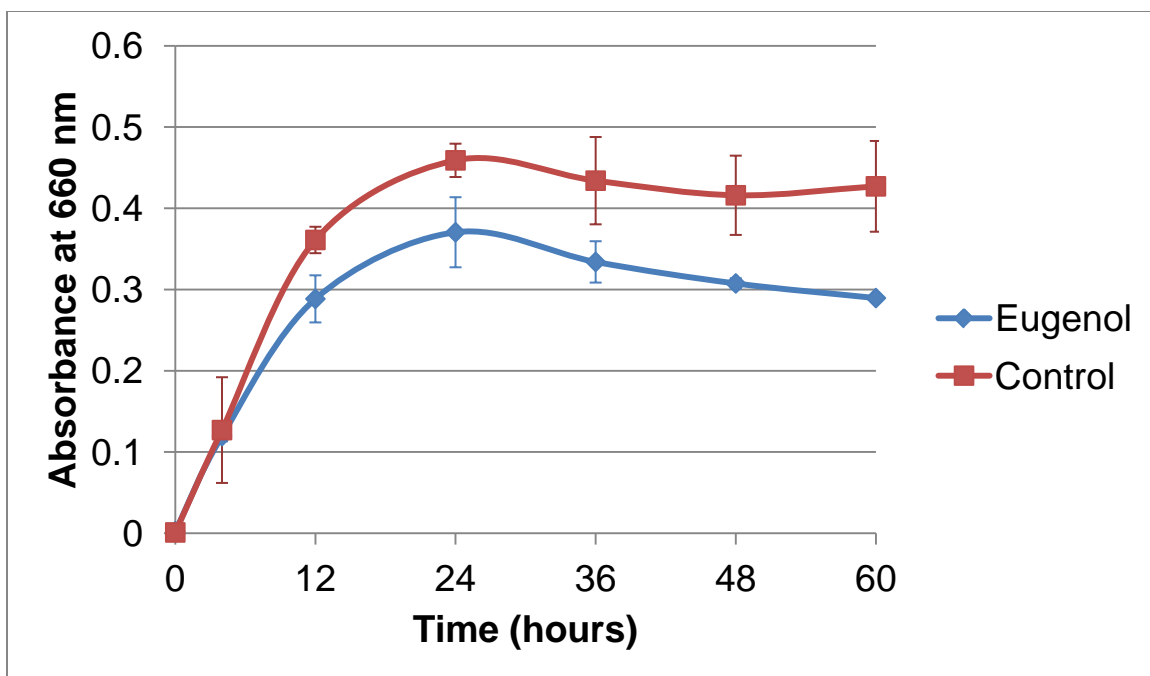
**Figure 4.8.** The antimicrobial activity of eugenol at MIC of 15 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



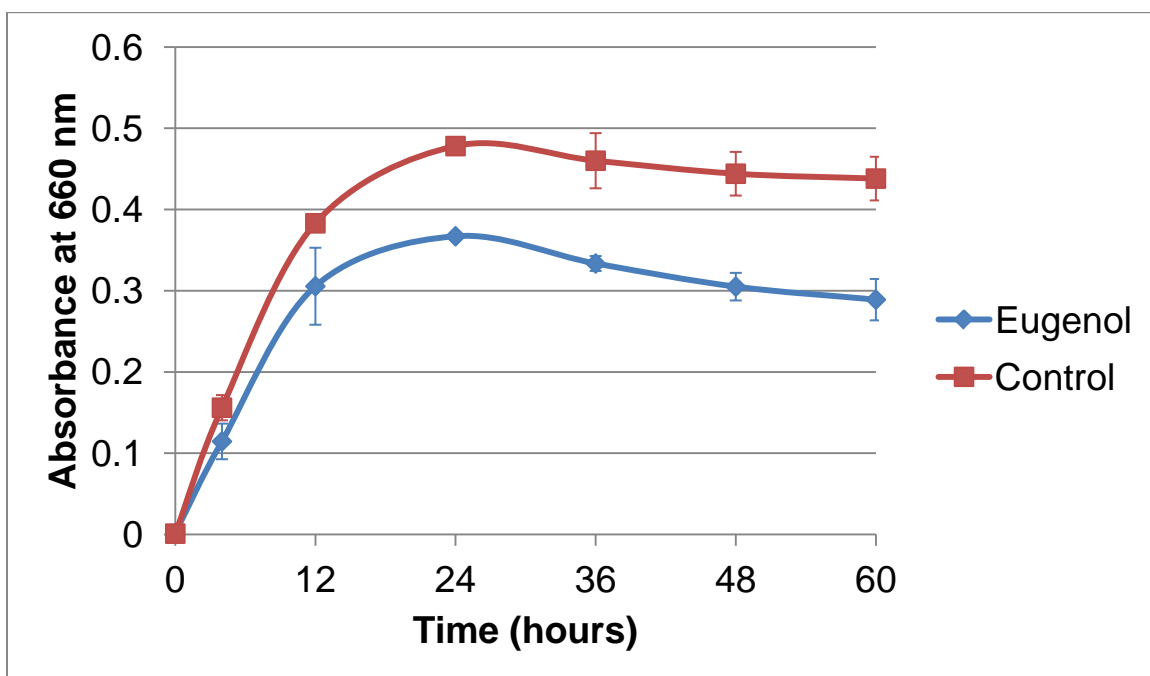
**Figure 4.9.** The antimicrobial activity of eugenol at MIC of 20 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .



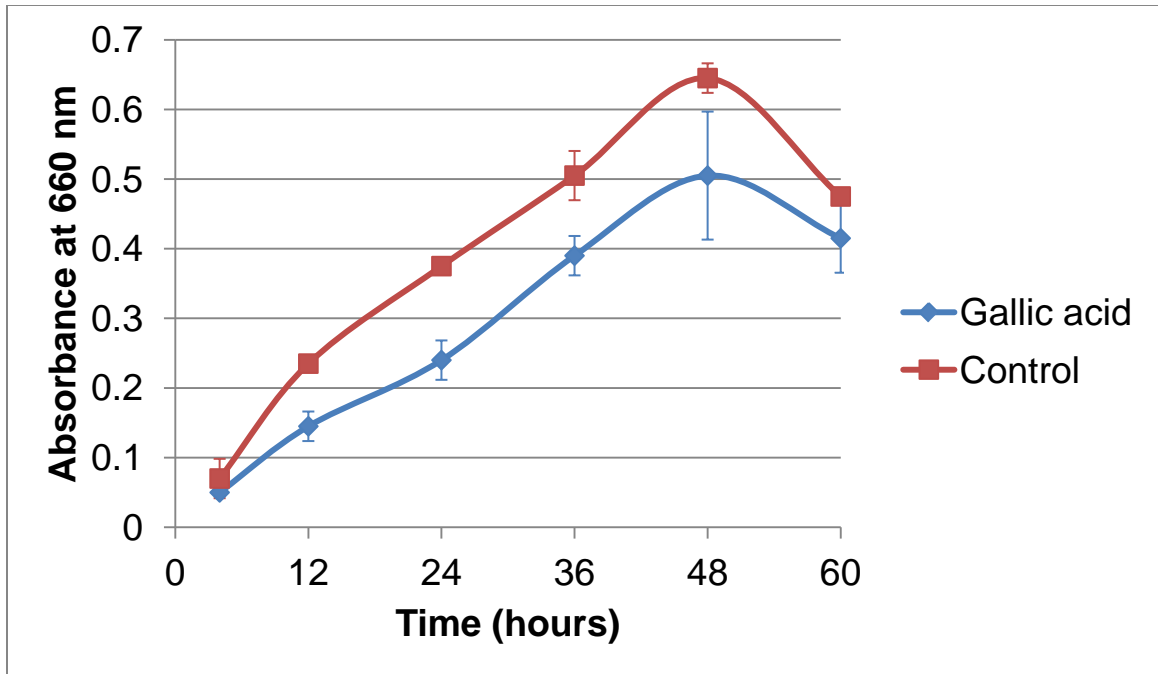
**Figure 4.10.** The average antimicrobial activity of eugenol at MIC of 20 ppm against *E. coli*, FTJ, *E. coli* O157:H7, ATCC 43895 and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



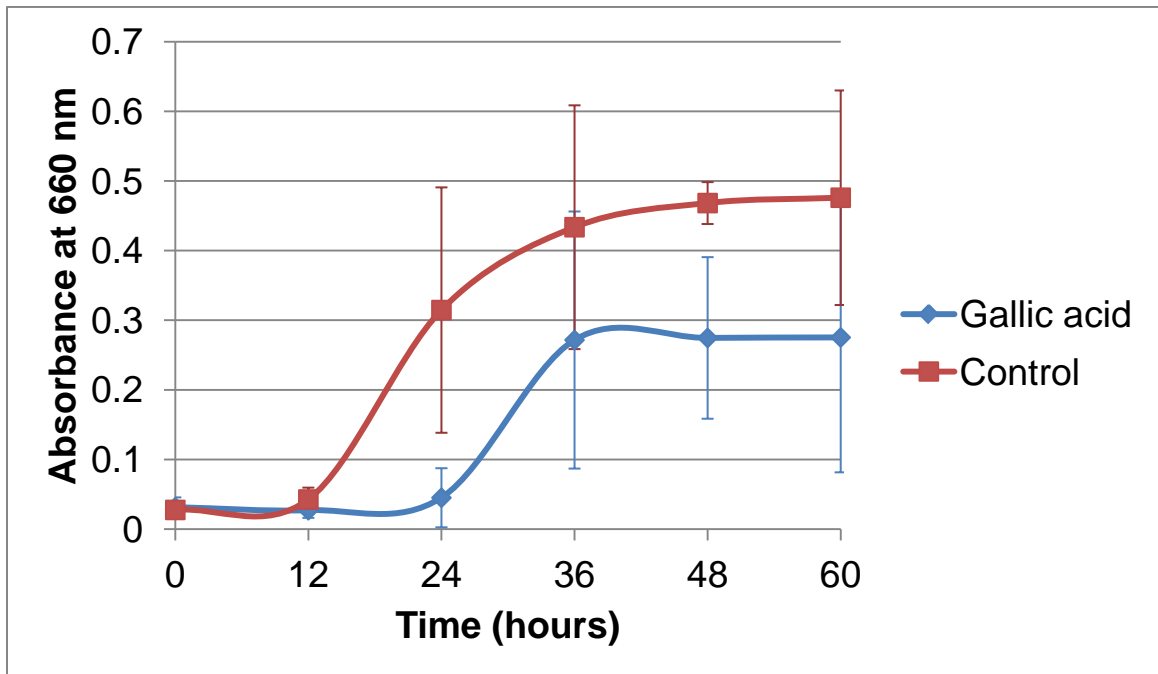
**Figure 4.11.** The antimicrobial activity of eugenol at MIC of 15 ppm against *S. choleraesuis subsps.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



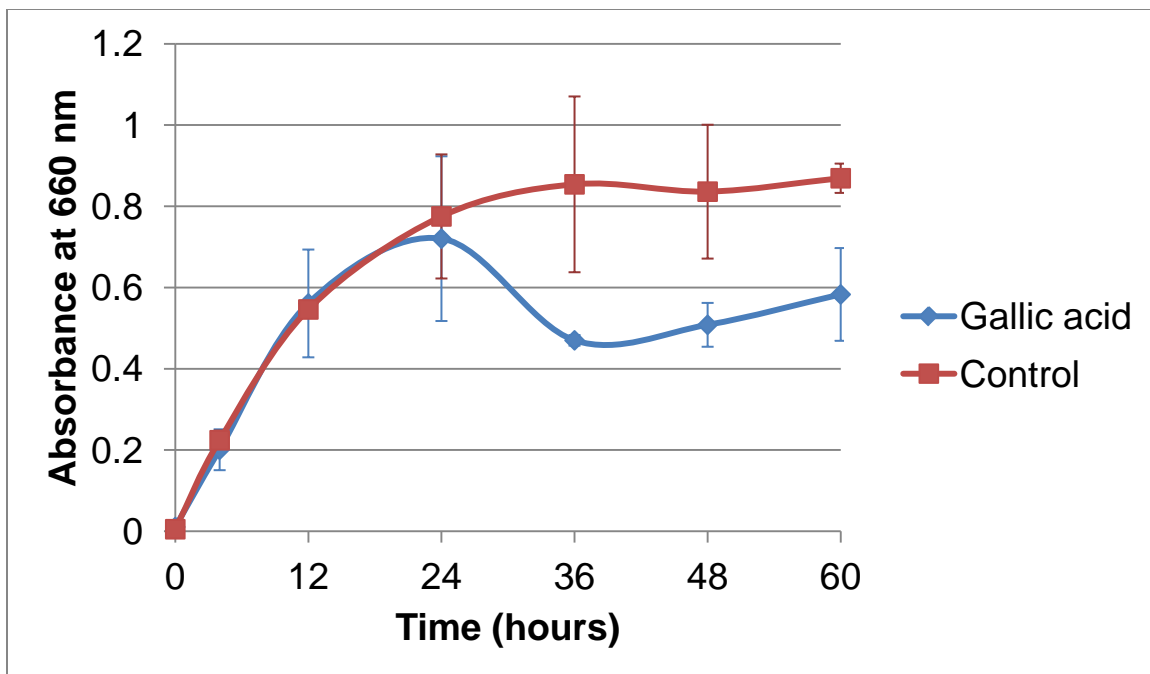
**Figure 4.12.** The antimicrobial activity of eugenol at MIC of 15 ppm against *S. enteridis*, (-)  $H_2S$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .



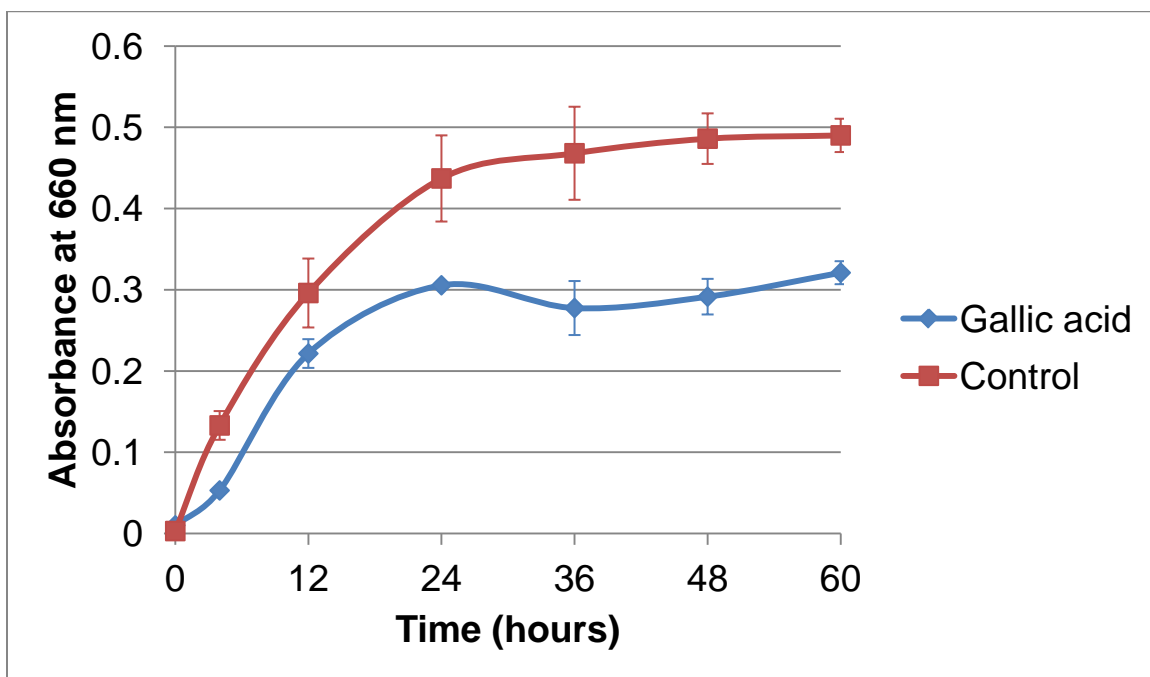
**Figure 4.13.** The antimicrobial activity of gallic acid at MIC of 10 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.14.** The average antimicrobial activity of gallic acid at MIC of 5 ppm against *C. perfringens*, RNR, *C. butyricum*, ATCC 8260, and *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .

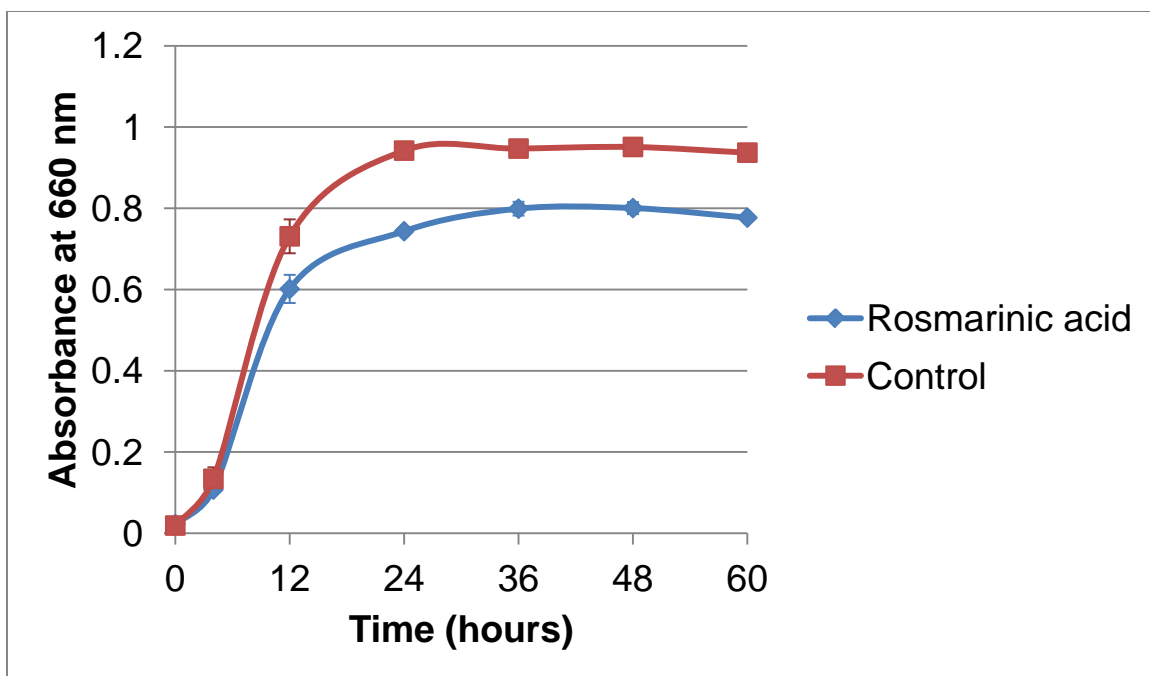


**Figure 4.15.** The average antimicrobial activity of gallic acid at MIC of 15 ppm against *E. coli*, FTJ, *E. coli* O157:H7, ATCC 43895, and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .

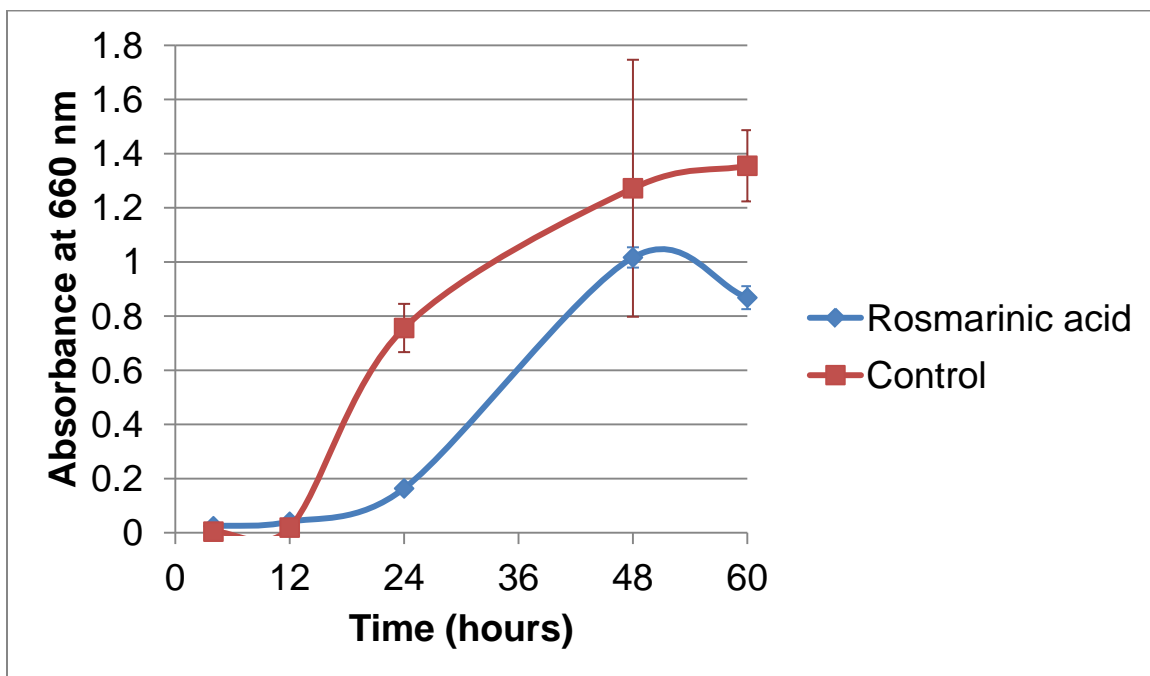


**Figure 4.16.** The antimicrobial activity of gallic acid at MIC of 15 ppm against *S. enteridis*, (-)  $H_2S$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .

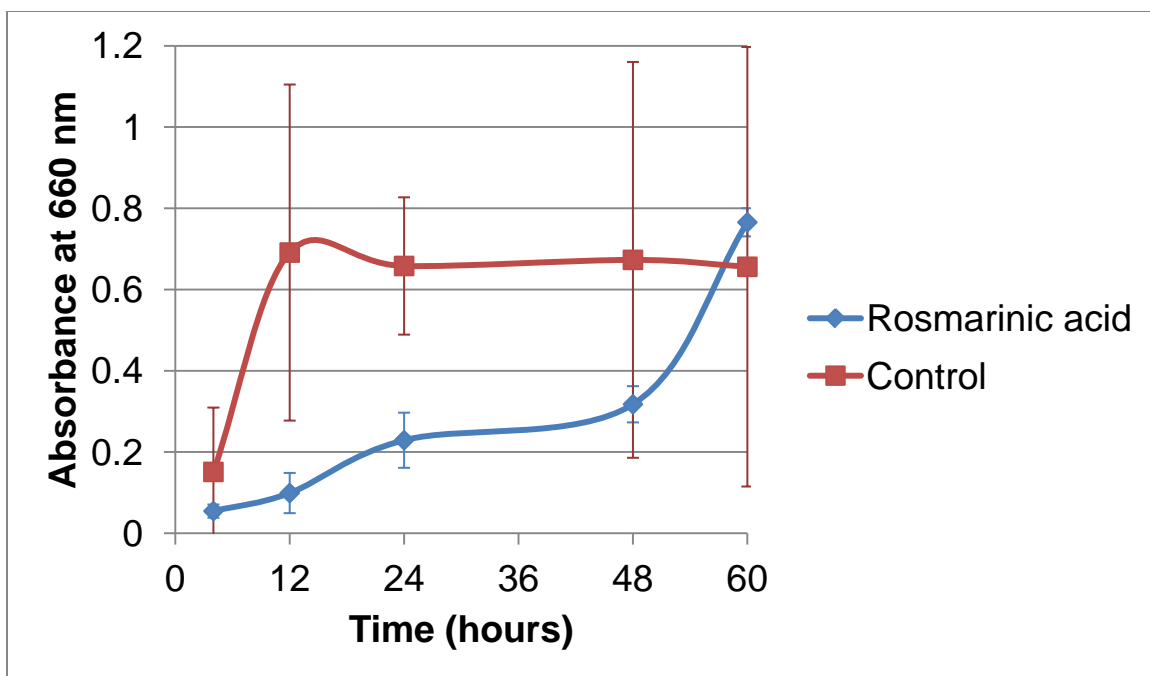




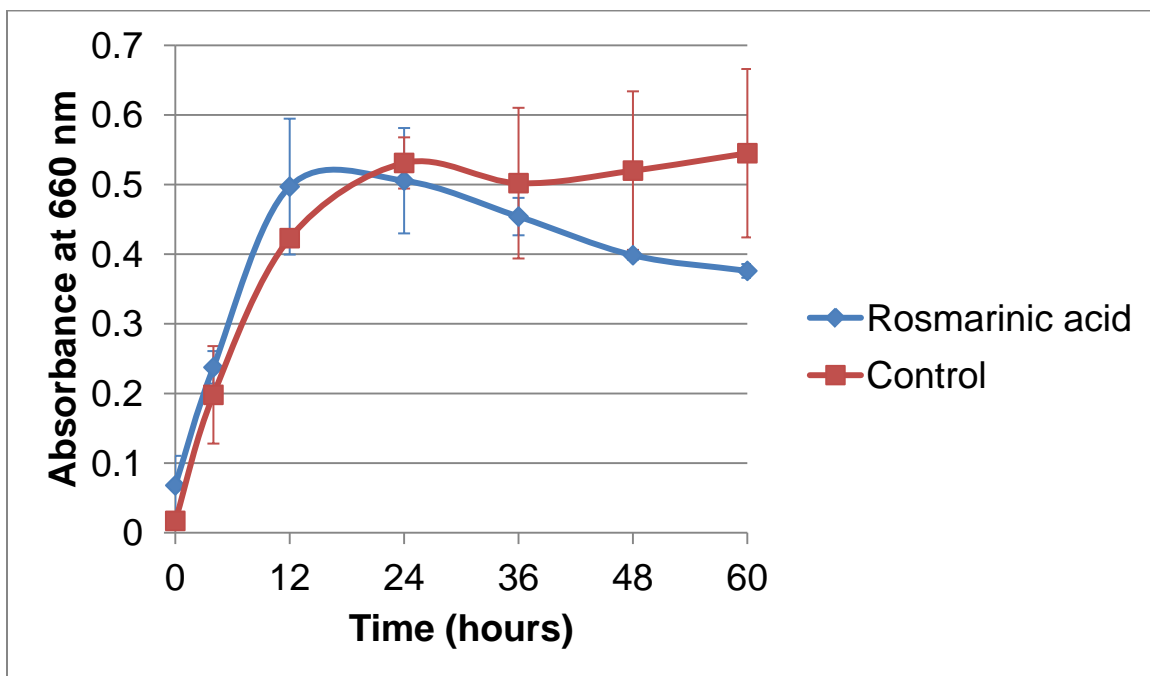
**Figure 4.17.** The antimicrobial activity of rosmarinic acid at MIC of 10 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



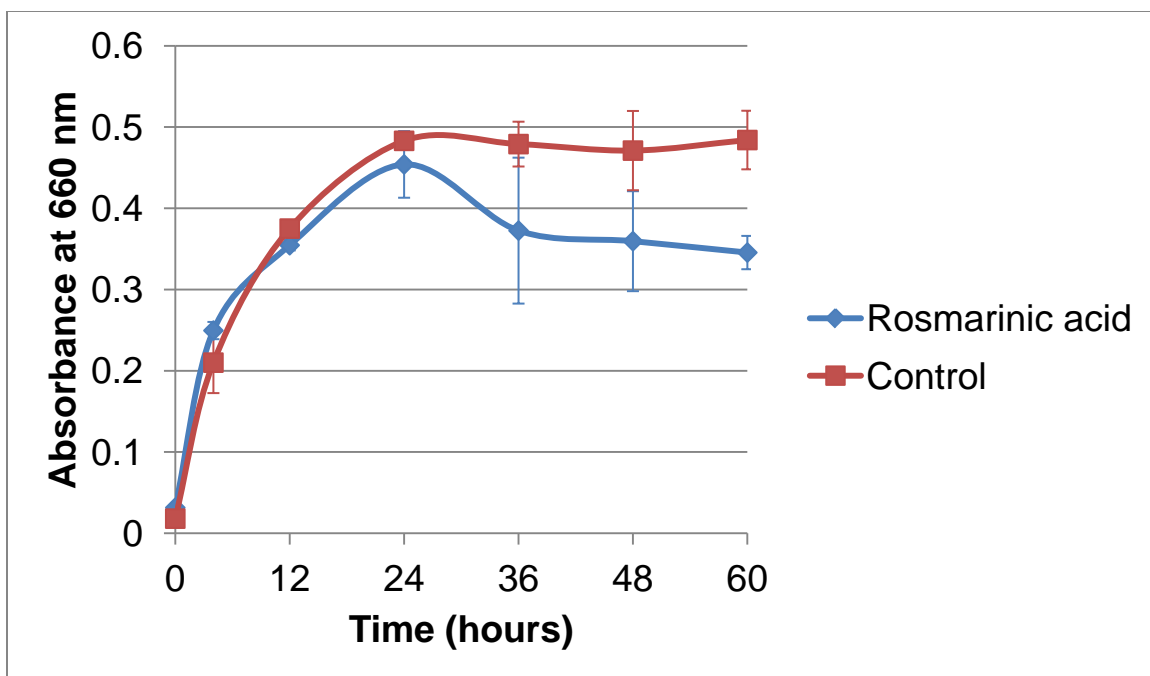
**Figure 4.18.** The antimicrobial activity of rosmarinic acid at MIC of 20 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



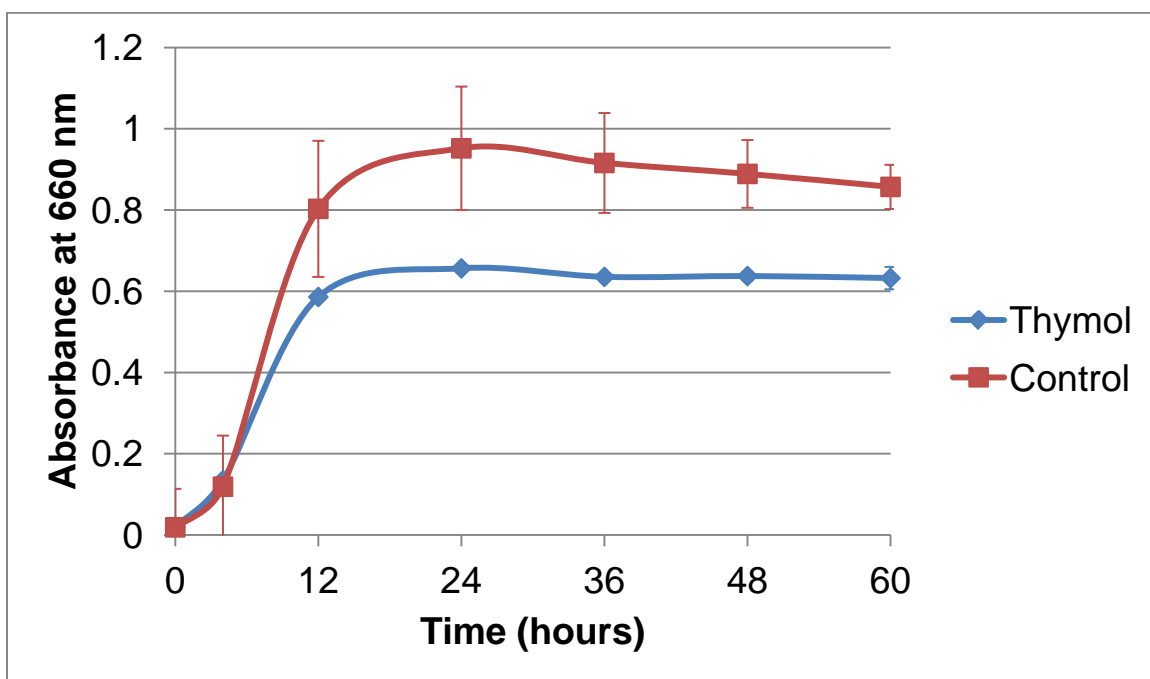
**Figure 4.19.** The antimicrobial activity of rosmarinic acid at MIC of 10 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



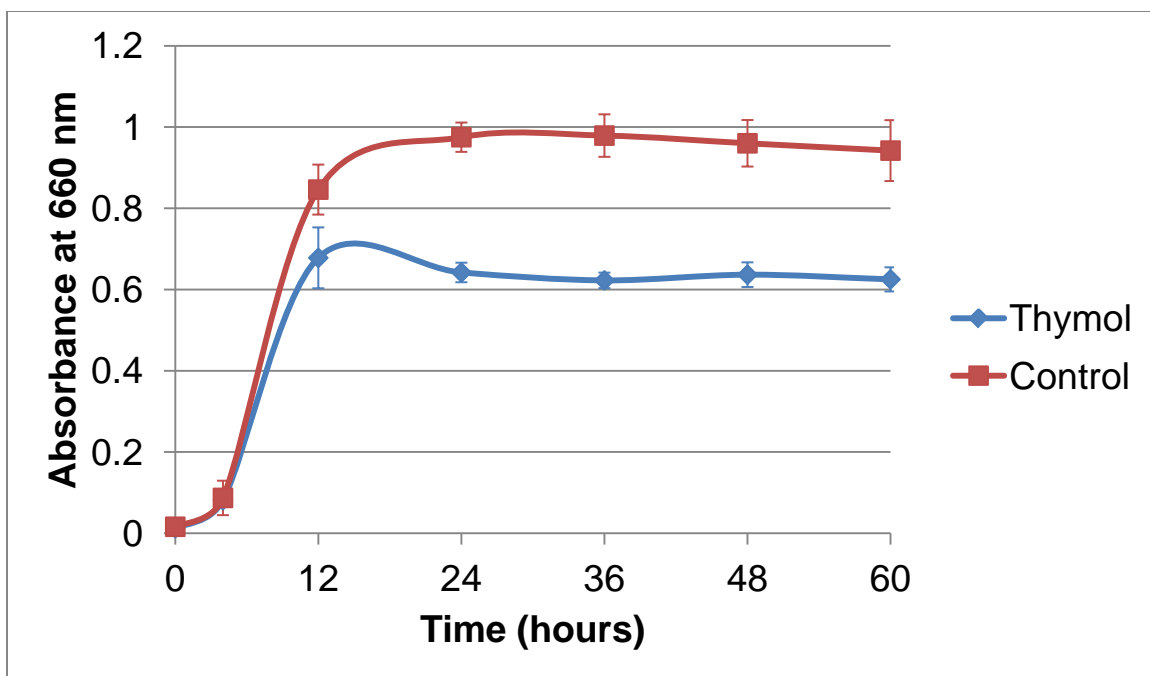
**Figure 4.20.** The antimicrobial activity of rosmarinic acid at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



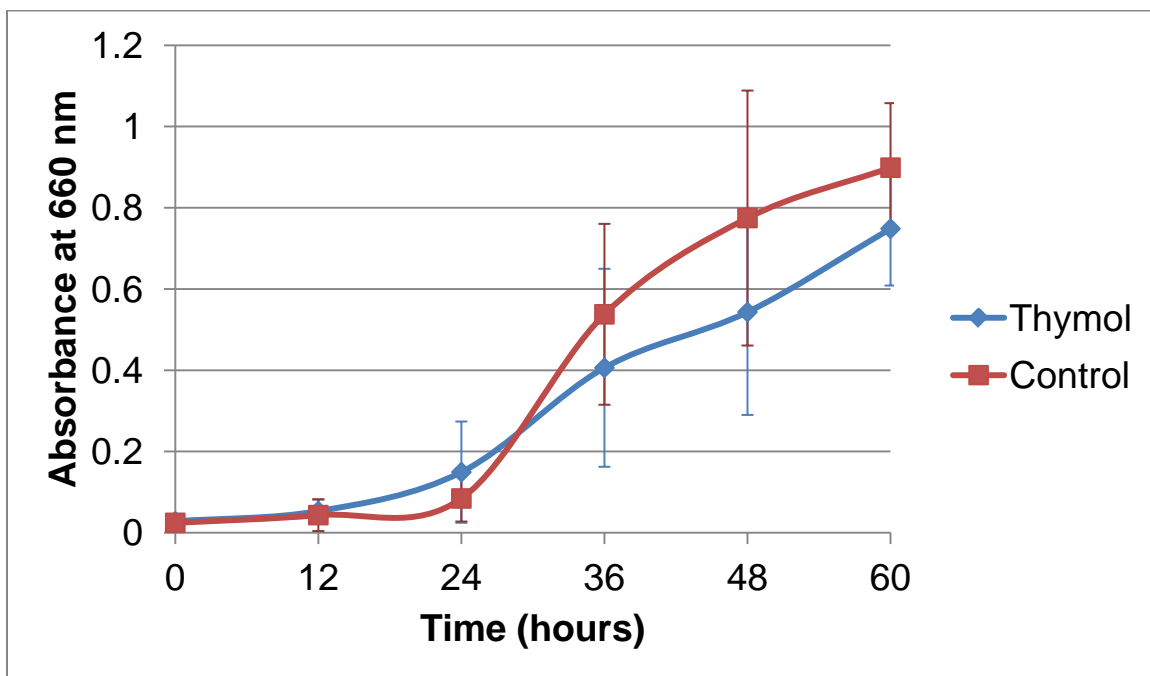
**Figure 4.21.** The antimicrobial activity of rosmarinic acid at MIC of 20 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



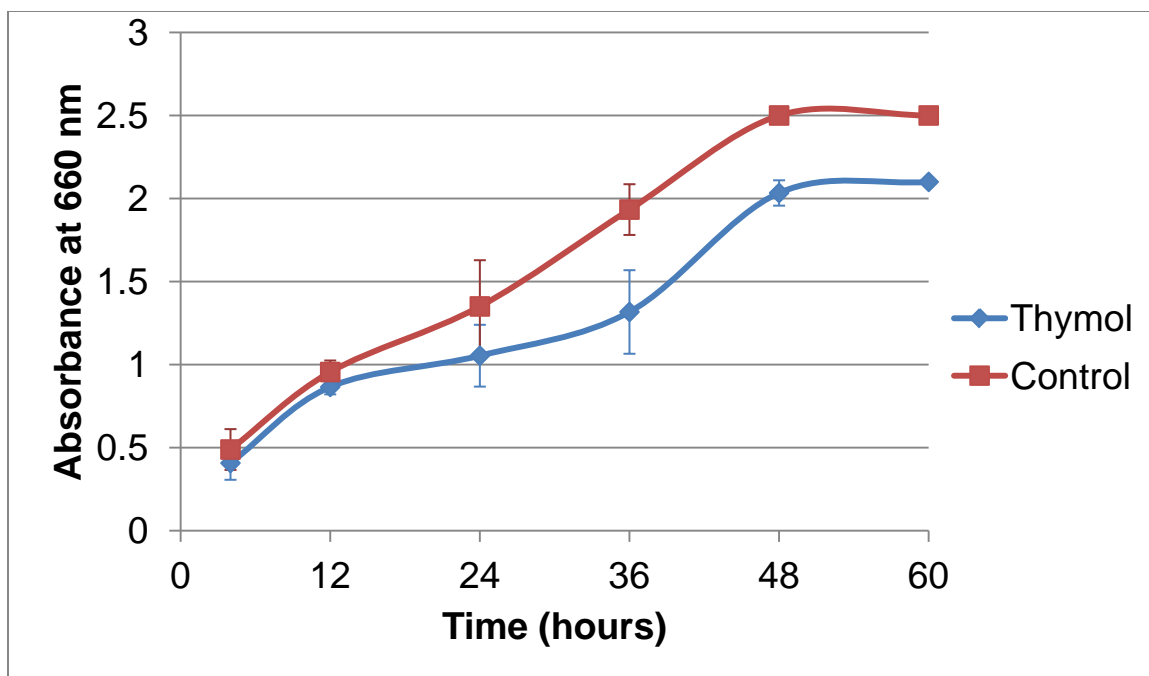
**Figure 4.22.** The antimicrobial activity of thymol at MIC of 15 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



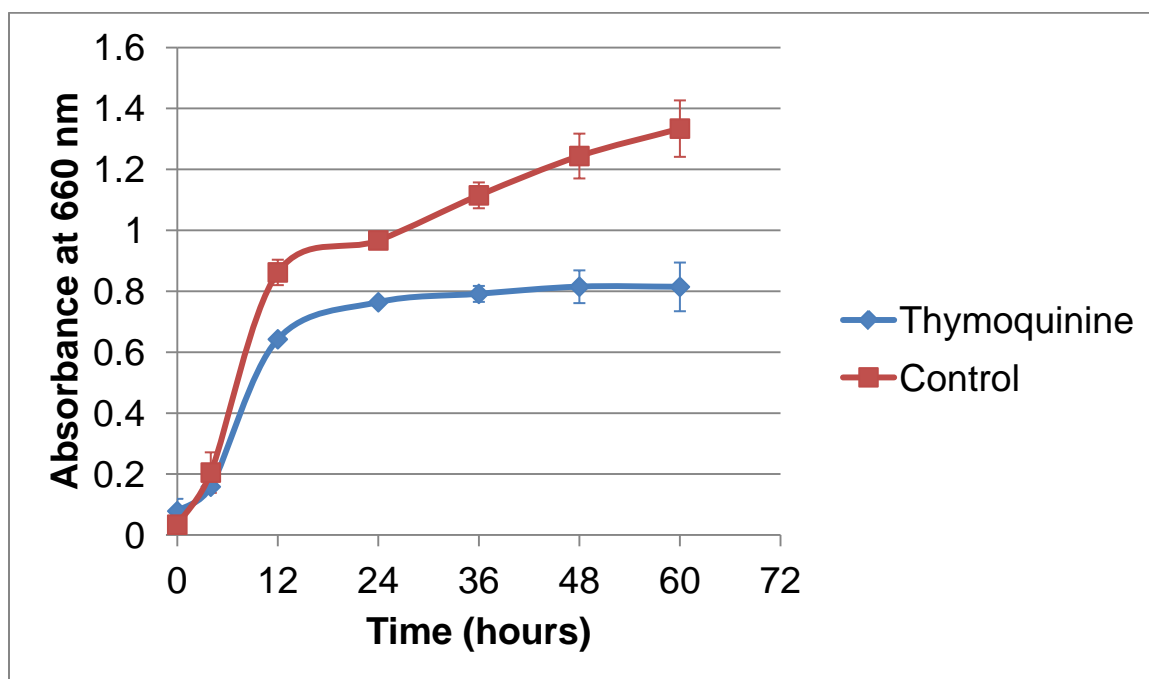
**Figure 4.23.** The antimicrobial activity of thymol at MIC of 10 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



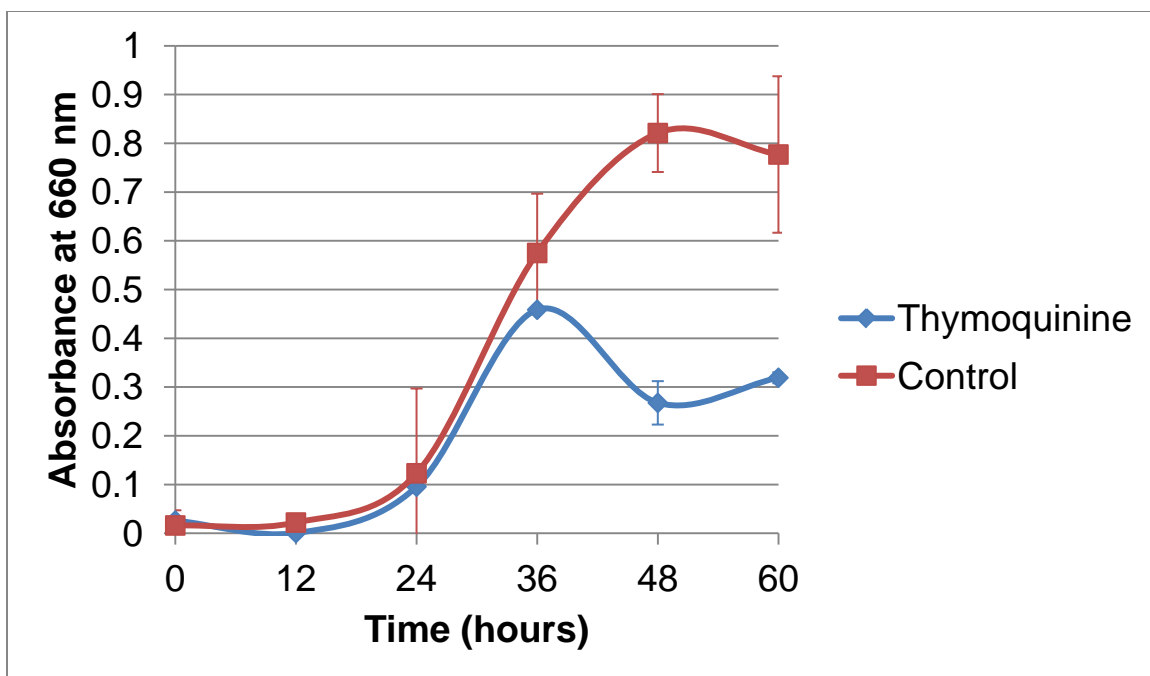
**Figure 4.24.** The average antimicrobial activity of thymol at MIC of 5 ppm against *C. perfringens*, RNR, *C. cholerasuis subsp.*, ATCC 8260 and *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



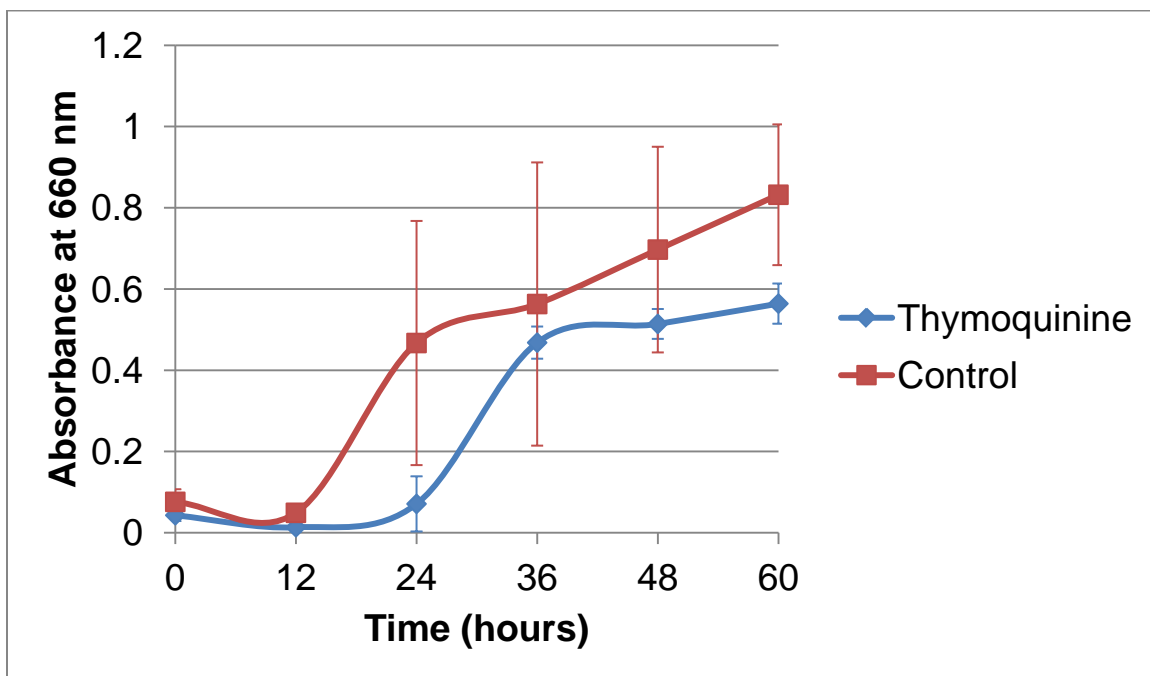
**Figure 4.25.** The average antimicrobial activity of thymol at MIC of 15 ppm against *E.coli*, FTJ, *E. coli* O157:H7, ATCC 43985 and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



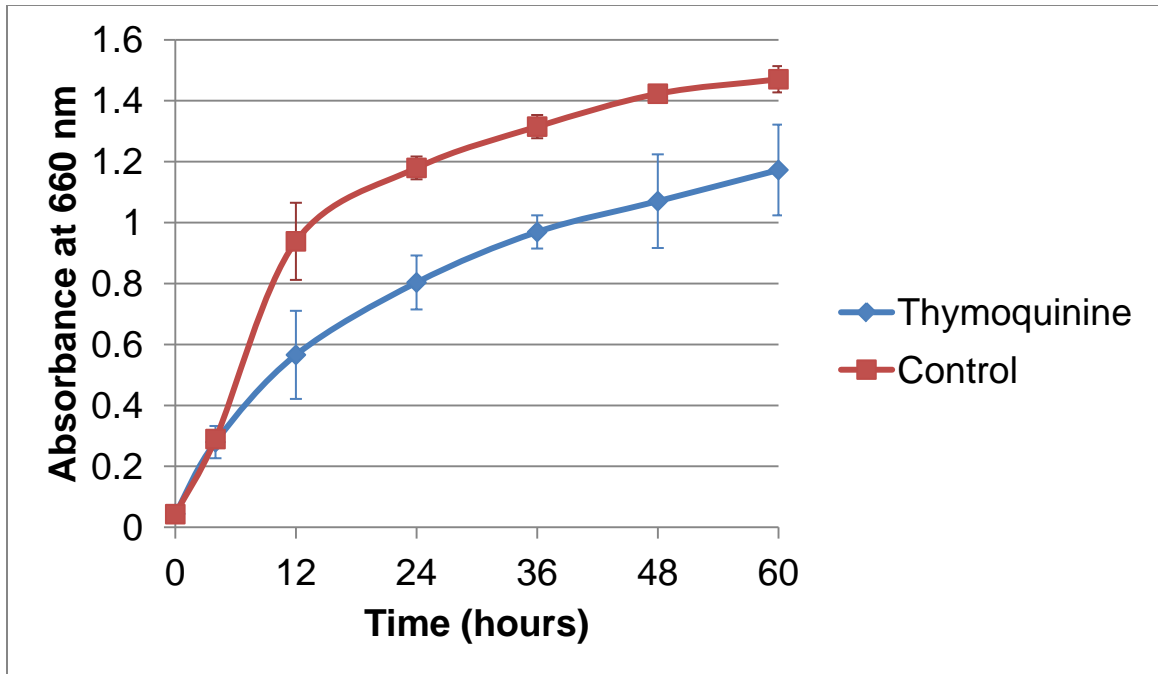
**Figure 4.26.** The antimicrobial activity of thymoquinine at MIC of 15 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



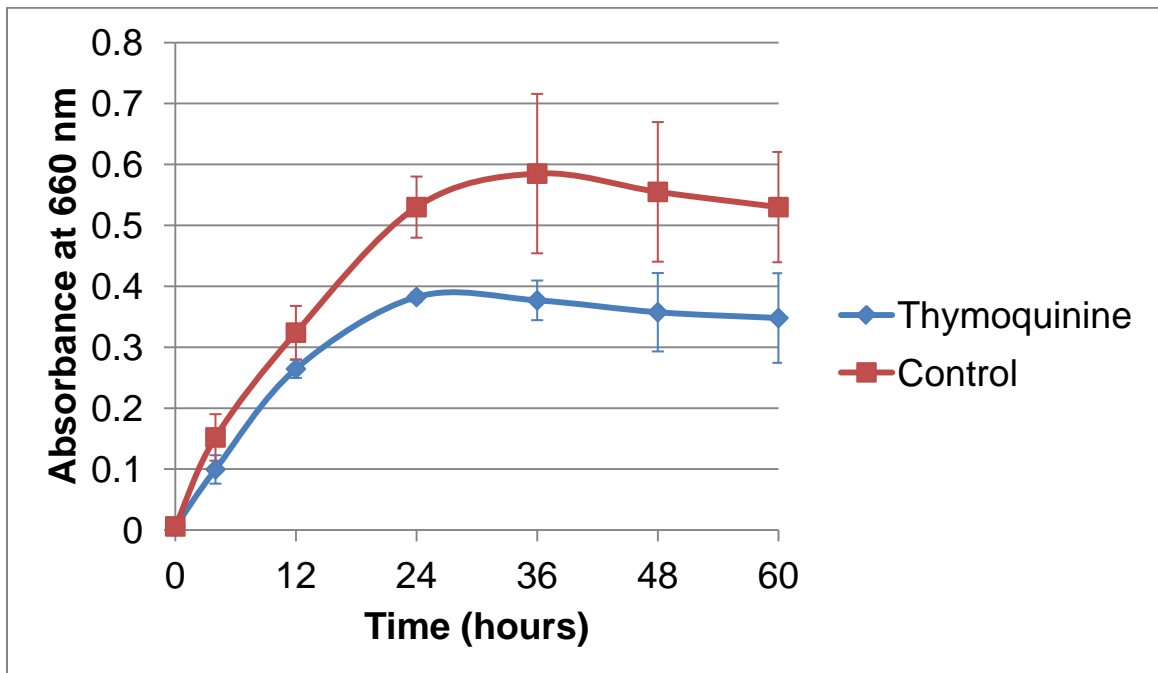
**Figure 4.27.** The antimicrobial activity of thymoquinine at MIC of 10 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .



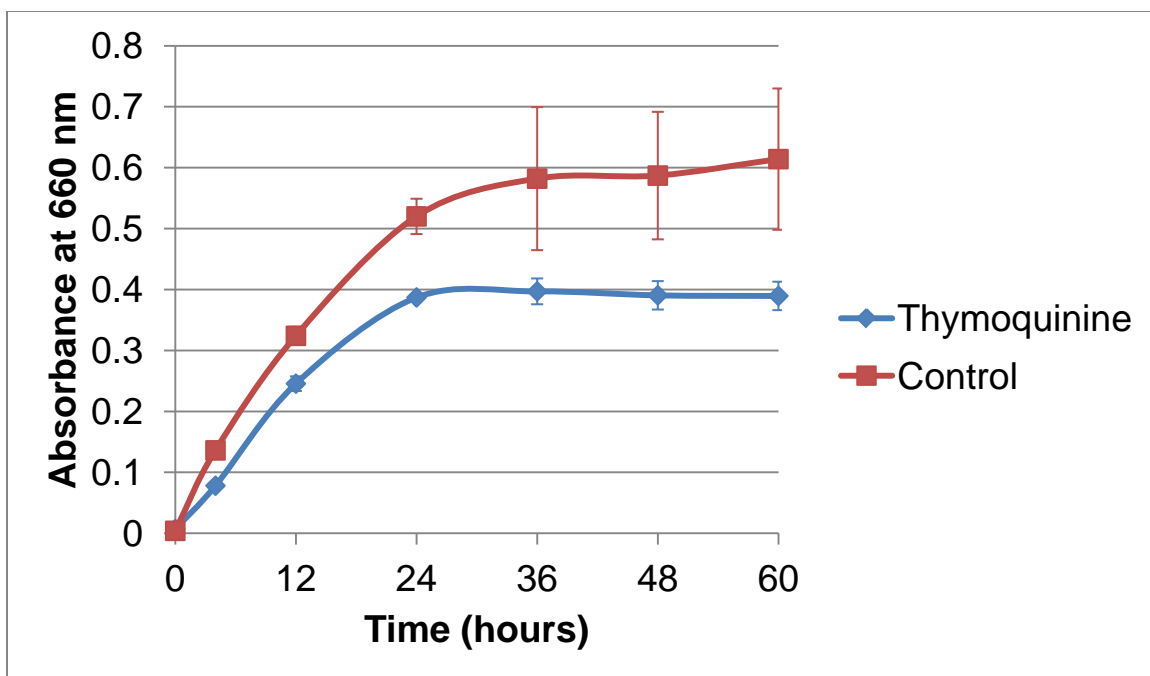
**Figure 4.28.** The antimicrobial activity of thymoquinine at MIC of 10 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



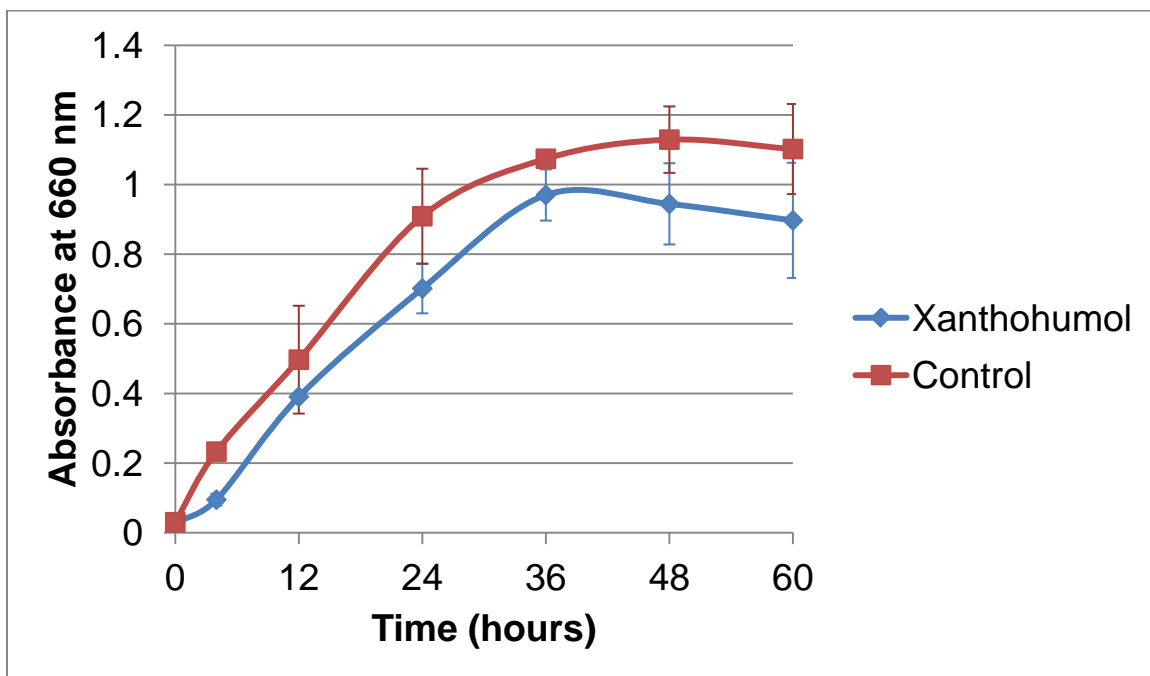
**Figure 4.29.** The average antimicrobial activity of thymoquinine at MIC of 5 ppm against *E. coli*, FTJ, *E. coli* O157:H7, ATCC 438954 and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.30.** The antimicrobial activity of thymoquinine at MIC of 15 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .

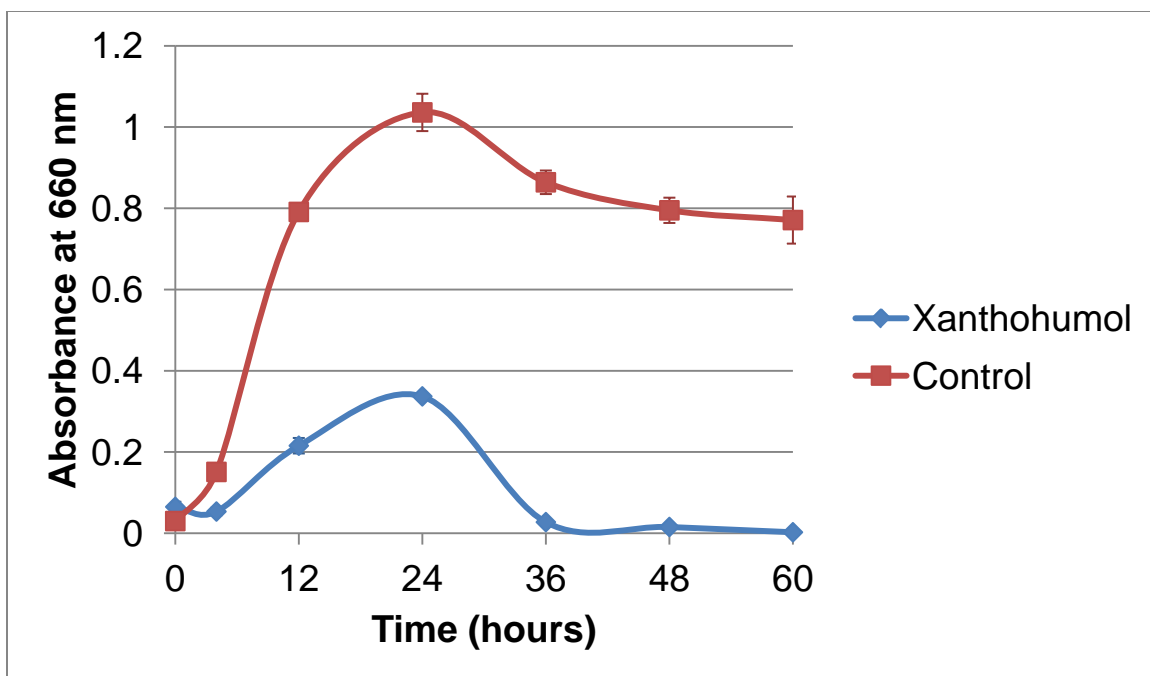


**Figure 4.31.** The antimicrobial activity of thymoquinine at MIC of 20 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.

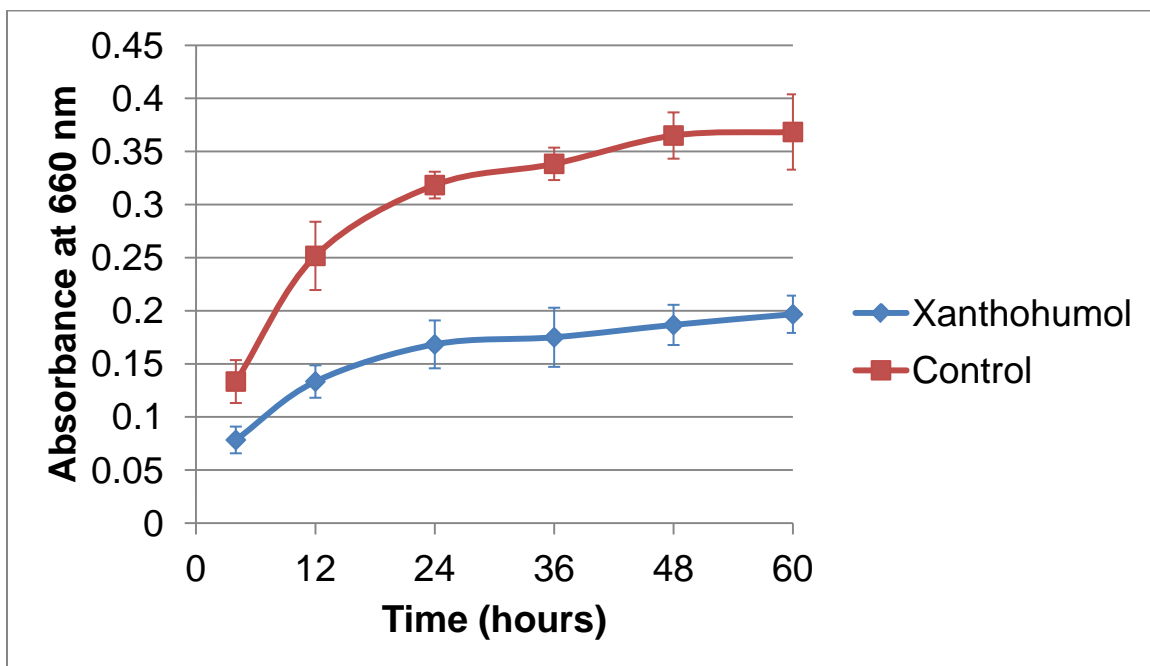


**Figure 4.32.** The antimicrobial activity of xanthohumol at MIC of 5 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.

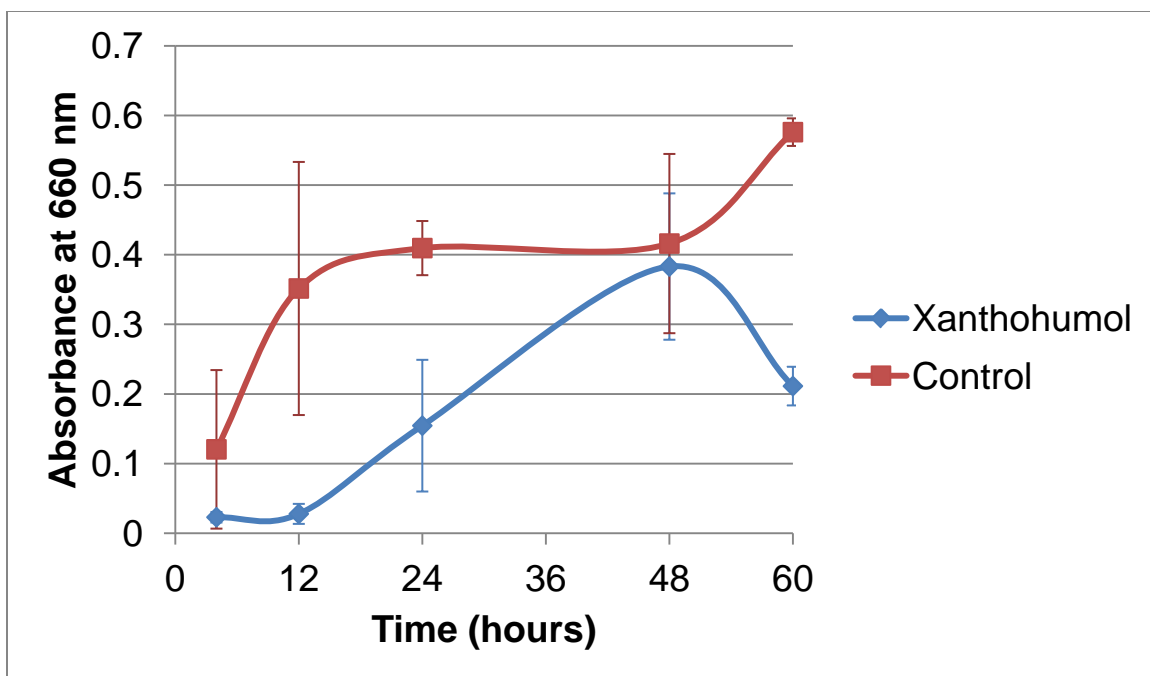




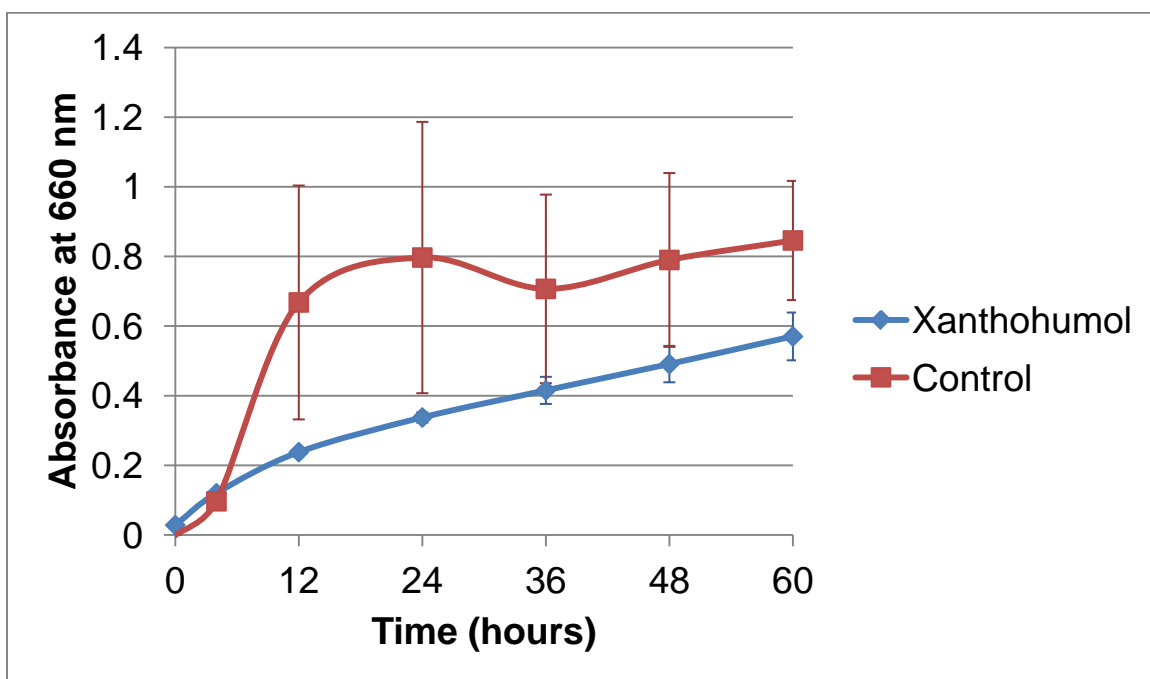
**Figure 4.33.** The antimicrobial activity of xanthohumol at MIC of 20 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.34.** The average antimicrobial activity of xanthohumol at MIC of 5 ppm against *L. monocytogenes*, ATCC 7644, *L. monocytogenes*, UK ADL and *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.35.** The average antimicrobial activity of xanthohumol at MIC of 5ppm against *C. perfringens*, RNR, *C. butyricum*, ATCC 8260 and *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.36.** The antimicrobial activity of xanthohumol at MIC of 15 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .

## CHAPTER 5

### Minimum Inhibitory Concentrations of Natural Antimicrobial Phenolic Compounds Extracted from Vegetable and Fruits

#### 5.1 Introduction

Results from refereed literature indicate that vegetables and fruits have varying degrees of antibacterial activities on common potential pathogens, including antibiotic-resistant strains (Lee and others 2003). To address the need for natural and safe alternatives, several vegetable and fruit extracts are being used in the food industry, which are natural sources of antioxidant and antimicrobial compounds (Perumalla and Hettiarachchy 2011). These extracts have the potential to improve the overall quality and extend the shelf life of food products.

The biological properties of extracts (antimicrobial, antioxidant, anticancer, anti-inflammatory, among other properties) obtained from several parts of vegetables and fruits were reported in several previous studies (Kanner and others 1994; Gordon 1996; Kris-Etherton and others 2002; Stark and Madar 2002; Miguel and others 2010). Due to such properties, the extracts have been used in therapeutics, such as in the prevention of infection, inflammation, cancer, among other applications. Recently, there is a search for new compounds (Carluccio and others 2003) to study for other applications such as preservation and other food safety purposes in food industry as well as novel biotechnological techniques (Miguel and others 2010).

Studies conducted thus far (including human, animal, *in vivo* and *in vitro*) have demonstrated that olive oil phenolic compounds have positive effects on various physiological biomarkers (Owen and others 2000; Waterman and Lockwood 2007), implicating phenolic compounds as partially responsible for health benefits associated with the Mediterranean diet (Martin-Moreno and others 1994; Visioli and Galli 2002; Visioli and Bernardini 2011). Furthermore, olive oil phenolic compounds have been shown to be highly bioavailable (Carluccio and others 2003), reinforcing their potential health promoting properties (Cicerale and others 2010).

The purpose of this study was to determine the antimicrobial activities and the MICs of the natural phenolic compounds extracted from vegetables and fruits were evaluated for both Gram-positive and Gram-negative foodborne pathogens. Antimicrobial Susceptibility Testing Method was conducted to investigate the growth inhibition of aerobic foodborne pathogens in Mueller Hinton Broth and anaerobic pathogens in Reinforced Clostridial Broth.

## 5.2 Results and Discussion

Antimicrobial characteristics of phenolic compounds obtained from fruits and vegetables, including caffeic acid, capsaicin, chlorogenic acid, coumarin, decanol, ellagic acid, (-) epicatechin, myricetin, naringenin, p-coumaric acid, quercetin, rutin, syringic acid, and tannic acid were investigated against foodborne pathogens (NCCLS 2004). In the present study tested phenolic compounds were found to be significantly effective against inhibiting the growth of both Gram-positive bacteria including *Bacillus* (Table 5.1), *Listeria monocytogenes* (Table 5.2), *Clostridium* (Table 5.3), and Gram-negative bacteria such as *Escherichia coli* (Table 5.4) and *Salmonella* (Table 5.5) species.

### Capsaicin

Capsaicin showed varying degrees of antimicrobial activity when it was tested against various species of foodborne pathogens.

The three strains of *Bacillus*; *B. subtilis* (Figure 5.1), *B. cereus* (Appendix Figure 27), and *B. polymyxa* (Figure 5.2) showed varying degrees of antimicrobial sensitivity against capsaicin. Growth of *B. subtilis* and *B. cereus* were inhibited following 60 hours of incubation when used at 15 ppm (15 µg/l) and 15 ppm, respectively. However, *B. polymyxa* at 20 ppm concentration showed inhibition until 36 hours of incubation and became resistant afterwards. Confirming the findings of this study, (Nazzaro and others 2009) reported that capsaicin extracted from sweet pepper (*Capsicum annuum* L.) had antibacterial properties for proliferating *B. cereus*.

Capsaicin was observed to have a high antimicrobial activity when it was tested against LM2 (Appendix Figure 28) and LM3 (Figure 5.3). MICs of capsaicin against LM2 and LM3 were determined to be <20 ppm following 60 hours of incubation period. However, LM1 was determined to be highly resistant to capsaicin following 60 hours of incubation period.

The three species of *Clostridium*; C1, C2, and C3 tested against capsaicin, were determined to have very close and consistent sensitivity, with MIC of 5 ppm following 60 hours of incubation time. Figure 5.4 depicts the average antimicrobial activity of capsaicin against the three species of *Clostridium* tested.

When *Escherichia coli* strains were tested with capsaicin, varying degrees of antimicrobial activity was observed after 60 hours of incubation. The previous studies also reported capsaicin, extracted from sweet pepper (*Capsicum annuum* L.), being highly effective for inhibition of *E. coli* strains (Nazzaro and others 2009). Confirming these results, non-pathogenic *E. coli*, EC1 (Appendix Figure 29) and *E. coli* O157:H7, EC2 (Figure 5.5) were observed to have antimicrobial sensitivity against capsaicin with the MIC of 20 ppm. However, EC3 was observed to be highly resistant against capsaicin.

*Salmonella* species, S1, S2 and S3 treated with capsaicin showed similar degrees of antimicrobial sensitivity following the 60 hours of incubation. The MICs of capsaicin against *S. paratyphi*, *S. cholerasuis subsp.*, and *S. enteridis* were determined as 20 pmm. Figure 5.6 depicts the average antimicrobial activity of capsaicin against those *Salmonella* species.

### **Chlorogenic acid**

Chlorogenic acid showed varying degrees of antimicrobial activity when it was tested against various strains of foodborne pathogens.

According to the previous studies, chlorogenic acid extracted from *Helichrysum* (Asteraceae) was reported to have antimicrobial activity against *B. subtilis* and *B. cereus* (Albayrak and others 2010). In this study, the two strains of *Bacillus*; *B. cereus* (Figure 5.7), and *B. polymyxa* (Appendix Figure 30) showed similar antimicrobial susceptibility

to chlorogenic acid. However, *B. subtilis* showed no inhibition during the entire incubation period. The MICs of *B. cereus* and *B. polymyxa* were determined to be 10 ppm.

Chlorogenic acid obtained from carrot extracts was reported to be highly antimicrobial when tested against *L. monocytogenes* (Babic and others 1994). Similarly, in this study it was observed to have highly consistent antimicrobial activity against three strains of *L. monocytogenes*. MICs of chlorogenic acid against LM1, LM2 and LM3 were determined to be 10 ppm following 60 hours of incubation period. Appendix Figure 31 shows the average antimicrobial activity of chlorogenic acid throughout the incubation period.

The two strains of *Clostridium*; *C. butyricum* (Figure 5.8), and *C. sporogenes* (Figure 5.9) tested against chlorogenic acid were determined to have similar antimicrobial sensitivity when used at <20 ppm. However, *C. perfringens* was observed to be very resistant against chlorogenic acid, showing no antimicrobial susceptibility at all.

When *E. coli* species were tested with chlorogenic acid, different degrees of antimicrobial activity was observed following 60 hours of incubation. Previous studies reported that chlorogenic acid extracted from different type of plants have high antimicrobial activity against some strains of *E. coli* (Babic and others 1994; Albayrak and others 2010; Xia and others 2011b). EC1 (Appendix Figure 32) and EC3 (Figure 5.10) were observed to have high antimicrobial sensitivity against chlorogenic acid with the MIC of <15 ppm. However, EC2 showed very high resistance against chlorogenic acid during the entire incubation period, showing no inhibition at all.

*Salmonella* species; *S. paratyphi*, *S. cholerasuis* subsp., and *S. enteridis* treated with chlorogenic acid showed consistently similar antimicrobial susceptibility following the 60 hours of incubation. The MICs of chlorogenic acid against S1, S2 and S3 were determined as 10 ppm. Figure 5.11 depicts the average antimicrobial activity of chlorogenic acid for *Salmonella* strains at the 60 hours of incubation period.

## Coumarin

*B. subtilis* (Appendix Figure 34), *B. cereus* (Appendix Figure 35), and *B. polymyxa* (Appendix Figure 36) showed varying degrees of antimicrobial susceptibility when tested against coumarin. However, *B. polymyxa* was observed to gain resistance after 24 hours of incubation. The MICs of coumarin for *B. subtilis* and *B. polymyxa* were determined as 20 and 15 ppm, respectively. *B. cereus* showed the highest antimicrobial susceptibility against coumarin, with the MIC of 5 ppm following 60 hours of incubation.

Coumarin showed similar antimicrobial activity against LM1 (Appendix Figure 37), LM2 (Appendix Figure 38) and LM3 (Appendix Figure 39) but at different incubation stages. LM1 and LM2 were sensitive against coumarin with MIC of 5 ppm, consistently being inhibited following 60 hours. LM3 was sensitive until 36 hours of incubation with MIC of 15 ppm and it gained resistance afterwards.

The two strains of *Clostridium*, *C. butyricum* (Figure 5.12), and *C. sporogenes* (Figure 5.13) were observed to have antimicrobial sensitivity when tested against coumarin, following 60 hours of incubation. However, *C. perfringens* showed no antimicrobial sensitivity to coumarin, being very resistant during the entire incubation period. MICs of coumarin for *C. butyricum* and *C. sporogenes* were determined as <10 ppm.

Previous studies reported coumarin being highly antimicrobial especially for Gram-negative bacteria including *E. coli* and *Salmonella* species (Maddox and others 2010). Confirming those reports, coumarin was observed to inhibit the growth of EC1 (Appendix Figure 40) following 60 hours of incubation period, with the MIC of 20 ppm. It was observed not to be effective when used at decreasing concentrations. However, after being treated with coumarin neither EC2 nor EC3 observed to be sensitive during the entire 60 hours of incubation period.

*S. paratyphi* (Appendix Figure 41), *S. choleraesuis subsp.* (Appendix Figure 42) and *S. enteridis* (Figure 5.14) were observed to be consistently sensitive against coumarin following 60 hours of incubation. The MICs of coumarin were determined as <15 ppm for all of those strains.

## Decanol

All of the *Bacillus* strains were observed to be sensitive at varying degrees against decanol. *B. subtilis* (Figure 5.15), *B. cereus* (Figure 5.16) and *B. polymyxa* (Figure 5.17) showed high antimicrobial susceptibility with MIC of <10 ppm, after 60, 24 and 24 hours of incubation, respectively. *B. cereus* and *B. polymyxa* were observed to gain resistance after 24 hours incubation.

Decanol was observed to be very effective against inhibiting the growth of LM2 (Figure 5.18) and LM3 (Appendix Figure 43) following 60 hours of incubation, with 20 ppm concentrations. However, LM1 showed very high resistance against decanol during the entire incubation period. Most of the results were confirmed by the previous studies done by (Elgaali and Newman 2005), where decanol was also found to have antimicrobial activity for several strains of *L. monocytogenes*.

When treated with decanol two strains of *Clostridium* were observed to have similar antimicrobial susceptibility at different incubation stages. MICs of decanol for *C. perfringens* (Appendix Figure 44) and *C. butyricum* (Figure 5.19) were determined to be 5 ppm after 36 and 60 hours of incubation period, respectively. *C. perfringens* showed resistance after 36 hours of incubation while *C. sporogenes* was observed to be highly resistant against decanol during the entire incubation period of 60 hours.

Decanol was observed to have consistently high antimicrobial activity against EC1 (Figure 5.20), EC2 and EC3 (Figure 5.21) when used at <10 ppm concentrations, following 60 hours of incubation.

*Salmonella* species tested against decanol were observed to have varying degrees of antimicrobial susceptibility. *S. paratyphi* (Figure 45), *S. cholerasuis subsp.* (Figure 46) and *S. enteridis* (Figure 5.22) were determined to be sensitive to decanol during the entire incubation period, when used at <15 ppm concentrations.



## Ellagic Acid

Ellagic acid was previously reported to have a high potential for being a promising antimicrobial compound against both Gram-positive and Gram-negative pathogens, including oral pathogens in human (Loo and others 2010; Miguel and others 2010).

The three strains of *Bacillus*; *B. subtilis* (Figure 5.23), *B. cereus* (Figure 5.24) and *B. polymyxa* (Appendix Figure 47) showed similar antimicrobial susceptibility to ellagic acid, at <10 ppm concentrations. Growth of *B. cereus* was inhibited at 24 hours of incubation and it gained resistance afterwards through the 60 hours of incubation period, while *B. polymyxa* showed resistance after 12 hours of incubation.

Ellagic acid was observed to have highly consistent antimicrobial activity when it was tested against LM1, LM2, and LM3. MICs of ellagic acid were determined to be 5 ppm following 60 hours of incubation period for all of the strains. Figure 5.25 shows the average antimicrobial activity of ellagic acid throughout the entire incubation period when tested against *L. monocytogenes* strains.

The two strains of *Clostridium*; *C. perfringens* (Appendix Figure 48) and *C. butyricum* (Appendix Figure 49) were observed to be highly sensitive against ellagic acid, when used at <5 ppm concentrations at 60 hours of incubation. However, *C. perfringens* showed antimicrobial resistance after 36 hours of incubation. When *C. sporogenes* treated with ellagic acid, it was determined to be highly resistant throughout the entire 60 hours of incubation period.

When ellagic acid was tested against *E. coli* strains of EC1, EC2, and EC3, two of them; EC1 (Figure 5.26) and EC2 (Figure 5.27) showed high growth inhibition after 60 hours of incubation. However, EC3 was observed to be highly resistant against ellagic acid, showing no inhibition at any stage of the 60 hour incubation period. The MICs of ellagic acid were determined to be 20 ppm for both EC1 and EC2, following 60 hours. These results confirm the previous research findings where, ellagic acid extracted from pomegranate was found to be highly effective against inhibiting several strains of *E. coli* (Duman and others 2009).

*Salmonella* species, *S. paratyphi* (Appendix Figure 50), *S. cholerasuis subsp.* (Figure 5.28), and *S. enteridis* (Appendix Figure 51) treated with ellagic acid showed varying degrees of antimicrobial susceptibility following the 60 hours of incubation. The MICs of ellagic acid tested against *S. paratyphi*, *S. cholerasuis subsp.* and *S. enteridis* were determined as 20, 10, and 15 ppm, respectively.

### **(-) Epicatechin**

Both Gram-positive and Gram-negative pathogens have been previously reported to have antimicrobial susceptibility when tested against (-) epicatechin (Theivendran and others 2006; Askun and others 2009a).

When treated with (-) epicatechin, *B. subtilis* (Appendix Figure 52), *B. cereus* (Appendix Figure 53) and *B. polymyxa* (Figure 5.29), were observed to have antimicrobial susceptibility when used at 20 ppm concentrations and incubated for 60 hours. These results confirm the previous findings of Askun and others (2009a) where they observed that *Bacillus* species were susceptible when treated with several different levels of (-) epicatechin.

According to Theivendran and others (2006) when (-) epicatechin derived from green tea used on edible film coating, it was observed to be promising as means of controlling the growth and recontamination of *L. monocytogenes* on ready-to-eat meat products. (-) Epicatechin showed close antimicrobial activity for LM2 (Appendix Figure 54) and LM3 (Appendix Figure 55), but at different incubation stages. (-) Epicatechin was effective at inhibiting the growth of both strains with the MIC of 5 ppm. LM2 gained resistance after 36 hours, showing no inhibition afterwards, while LM3 was observed to be resistant after 12 hours of incubation. LM1 was observed to be highly resistant to (-) epicatechin during the entire incubation period.

The two strains of *Clostridium*; *C. perfringens* (Appendix Figure 56) and *C. butyricum* (Figure 5.30) were observed to have similar antimicrobial sensitivity when tested against 20 ppm (-) epicatechin following 60 hours of incubation. *C. perfringens* showed initial antimicrobial susceptibility after 24 hours of incubation. *C. sporogenes* (Figure 5.31) was

determined to have higher antimicrobial sensitivity against (-) epicatechin, when used at 10 ppm concentration following 60 hours of incubation.

(-) Epicatechin was observed to inhibit the growth of EC1 (Figure 5.32), EC2 (Appendix Figure 57) and EC3 (Appendix Figure 58) when used at <20 ppm concentrations. EC2 gained resistance after 12 hours of incubation, showing no antimicrobial susceptibility afterwards. EC1 and EC3 were observed to be consistently susceptible following 60 and 24 hours of incubation time, respectively. The results of this study confirm the findings of previous studies, where *E. coli* species were reported to be inhibited when treated with (-) epicatechin (Taguri and others 2004; Bancirova 2010; Cueva and others 2010) and the susceptibility of *E. coli* being strain dependent (Cueva and others 2010).

In previous studies *Salmonella* species were reported to be highly susceptible to (-) epicatechin (Taguri and others 2004). Confirming those results, *S. paratyphi*, *S. choleraesuis subsp.*, and *S. enteridis* were observed to be consistently sensitive against (-) epicatechin after being incubated for 60 hours. The MIC of (-) epicatechin was determined as 15 ppm for all of those *Salmonella* species and Figure 5.33 shows their average antimicrobial susceptibility against (-) epicatechin.

### **Myricetin**

Myricetin was reported being very effective against inhibiting some species of both Gram-positive and Gram-negative pathogenic bacteria (Tsai and others 2008; Freeman and others 2010).

The two species of *Bacillus*; *B. subtilis* (Appendix Figure 59), and *B. cereus* (Figure 5.34) showed consistently close antimicrobial susceptibility to myricetin. However, *B. poylymxa* showed high resistance during the entire incubation period. The MICs of *B. subtilis*, and *B. cereus* were determined to be 15 ppm, after 48 and 60 hours of incubation period, respectively.

*Listeria* strains; LM1, LM2 and LM3 treated with myricetin were observed to have highly consistent antimicrobial resistance throughout the entire 60 hours of incubation period. Contrarily, according to Yao and others (2011) myricetin extracted from Chinese

bayberry fruit (*Myrica rubra*) has antimicrobial activity against some strains of *L. monocytogenes*.

*C. butyricum* (Figure 5.35), and *C. sporogenes* (Figure 5.36) tested against myricetin were determined to have very similar antimicrobial sensitivity when used at low concentrations (<10 ppm) following 60 hours of incubation. However, *C. perfringens* was observed to be very resistant against myricetin, showing no antimicrobial susceptibility at all.

When *E. coli* species were treated with myricetin, different degrees of antimicrobial activity were observed following 60 hours of incubation. Most of the results were consistent with previous studies of myricetin tested against *E. coli* species (Yao and others 2011). EC1 (Figure 5.37), EC2 (Appendix Figure 60) and EC3 (Appendix Figure 61) were observed to have similar antimicrobial sensitivity against myricetin with the MIC of <20 ppm. However, EC2 gained resistance after 36 hours of incubation and showed no sensitivity afterwards.

*S. paratyphi* (Appendix Figure 62) was observed to have antimicrobial susceptibility against myricetin, when used at 20 ppm concentration at 36 hours of incubation. However, it gained resistance afterwards and showed no inhibition until the end of incubation time. *S. cholerasuis subsp.* (Figure 5.38) and *S. enteridis* (Appendix Figure 63) were also found to be susceptible when treated with lower concentrations (<15 ppm), following 60 hours of incubation. The MICs of myricetin against *S. paratyphi*, *S. cholerasuis subsp.*, and *S. enteridis* were determined as 20, 15, and 5 ppm, respectively. Similarly, Yao and others (2011) also reported myricetin having high *in vitro* antimicrobial activity for *S. paratyphi* and *S. enteridis*.

## **Quercetin**

Quercetin extracted from various plants including, onions, navel oranges and berries was previously studied against pathogenic bacteria, including Gram-positive, Gram-negative, cariogenic and periodontal pathogens. It was found to be highly effective against most of

the strains tested *in vitro* (Tsai et al. 2008; Freeman et al. 2010; Geoghegan et al. 2010; Santas and others 2010; Yao et al. 2011).

When tested against quercetin, *B. cereus* (Appendix Figure 65), and *B. polymyxa* (Figure 5.39) were observed to have similar antimicrobial susceptibility when exposed to <20 ppm concentrations. *B. subtilis* (Appendix Figure 64) also showed inhibition at 36 hours when treated with 15 ppm of quercetin and gained resistance afterwards. *B. cereus* and *B. polymyxa* were consistently susceptible throughout the entire 60 hours of incubation. Similarly, both *B. subtilis* and *B. cereus* (Askun and others 2009b; Santas and others 2010) were previously found to be susceptible when treated with quercetin (Pereira and others 2007b).

*Listeria monocytogenes* strains; LM1, LM2 and LM3 tested with quercetin showed highly consistent antimicrobial resistance, after being incubated for 60 hours. Contrarily, in the previous studies (Santas and others 2010; Yao and others 2011) quercetin was stated being effective against inhibiting the growth of several strains of *L. monocytogenes*.

*C. perfringens* was observed to have no inhibition when treated with quercetin at 60 hours of incubation period. However, *C. butyricum* (Figure 5.40) and *C. sporogenes* (Figure 5.41) were found to have high antimicrobial sensitivity against quercetin following 60 hours of incubation period. MICs of quercetin for both species were determined to be 15 ppm.

Quercetin was observed to have highly consistent antimicrobial activity when it was tested against EC1, EC2, and EC3. MICs of quercetin were determined to be 5 ppm after 24, 60 and 24 hours of incubation period, respectively. The findings of this study confirm the previous studies regarding quercetin, where it was also found to have high antimicrobial activity for *E. coli* strains (Askun and others 2009a; Santas and others 2010; Yao and others 2011). However, according to Pereira and others (2007b) *E. coli* showed antimicrobial resistance against quercetin, which was extracted from walnuts. Figure 5.42 exhibits the average antimicrobial activity of EC1, EC2, and EC3.

According to Yao and others (2011) *S. paratyphi* and *S. enteridis* showed high antimicrobial susceptibility *in vitro* when treated with quercetin. In this study, *S. paratyphi* (Appendix Figure 66), *S. choleraesuis subsp.* (Figure 5.43) and *S. enteridis* (Appendix Figure 67) were observed to be consistently sensitive against quercetin after being incubated for 60 hours. The MICs of quercetin were determined as <20 ppm for all of those strains.

## **Rutin**

Rutin was previously reported as having high antimicrobial activity for Gram-positive and mostly for fastidious Gram-negative pathogenic bacteria (Pereira and others 2007a; Askun and others 2009b; Maddox and others 2010). Especially, *E. coli* and *Bacilli* species were found to be the most susceptible pathogens (Pereira, Ferreira and others 2007; Askun, Tumen and others 2009).

*B. subtilis* (Figure 5.43), *B. cereus* (Figure 5.44), and *B. polymyxa* (Figure 5.45) showed varying degrees of antimicrobial susceptibility when tested against rutin. The MICs of rutin for *B. subtilis*, *B. cereus* and *B. polymyxa* were determined as <15 ppm. Rutin showed a consistent antimicrobial activity for *B. subtilis* and *B. cereus* following 60 hours of incubation. However, *B. polymyxa* was observed to be susceptible after 36 hours of incubation. Lee and Lee (2010) previously reported that rutin extracted from olives does not have any antimicrobial affect against *B. cereus*. However, according to Singh and others (2008) rutin, extracted from Brake Fern (*Pteris vittata*) exhibited potent activity for *B. cereus* with the MIC value of 0.03 mg/ml. *B. subtilis* was also previously found to show antimicrobial susceptibility when tested with rutin *in vitro* (Pereira and others 2007a; Askun and others 2009b).

Rutin showed similar antimicrobial activity against *Listeria monocytogenes* strains tested at 60 hours of incubation period. LM1 (Figure 5.46), LM2 (Figure 5.47) and LM3 (Appendix Figure 68) were observed to be sensitive against rutin, with MIC of <15 ppm, following 60 hours.

The two strains of *Clostridium*; *C. butyricum*, and *C. sporogenes* were observed to have high antimicrobial resistance when tested against rutin, at 60 hours of incubation period. However, *C. perfringens* (Appendix Figure 69) showed antimicrobial susceptibility to rutin. MIC of rutin for *C. perfringens* was determined as 10 ppm following 60 hours of incubation.

Rutin was observed to inhibit the growth of tested *E. coli* strains showing varying degrees of antimicrobial activity. EC1 (Appendix Figure 70), EC2 (Appendix Figure 71) and EC3 (Appendix Figure 72) were observed to be sensitive when incubated for 60 hours. MICs of rutin against EC1, EC2 and EC3 were determined as <20 ppm. Confirming the findings of this study, previous studies also reported *E. coli* O157:H7 and other *E. coli* strains being highly susceptible to rutin *in vitro* (Pereira and others 2007a; Askun and others 2009b). However, according to Lee and Lee (2010) some *E. coli* strains were not inhibited when treated and incubated with rutin *in vitro*.

Previous studies reported that rutin was highly effective against inhibiting the growth of some *Salmonella* species *in vitro* (Askun and others 2009a). *S. cholerasuis subsp.* (Appendix Figure 74) and *S. enteridis* (Appendix Figure 75) were observed to be consistently sensitive against rutin following 60 hours of incubation. The MICs of rutin were determined as 15 ppm for both of those species. Moreover, rutin also showed antimicrobial activity against *S. paratyphi* (Appendix Figure 73) but when used at 20 ppm concentration following 60 hours of incubation (MIC of 20 ppm). However, Lee and Lee (2010) previously reported *S. enteridis* being not susceptible to rutin, which was extracted from olives.

### **Tannic Acid**

When tested against tannic acid, only *B. subtilis* (Appendix Figure 76) was observed to have antimicrobial susceptibility until the incubation period of 36 hours. Afterwards it gained resistance and showed no susceptibility. *B. polymyxa* and *B. cereus* were found to be highly resistant to tannic acid even when used at higher concentrations. MIC of *B. subtilis* was determined as 20 ppm after being incubated for 36 hours.

Tannic acid showed varying degrees of antimicrobial activity against LM1 (Appendix Figure 77) and LM2 (Appendix Figure 78) and LM3 (Appendix Figure 79) when used at different concentrations. LM1 was observed to be the most sensitive when used at concentration as low as 10 ppm. LM2 gained resistance after 36 hours when treated with 15 ppm concentration of tannic acid, while LM1 and LM3 were consistently sensitive throughout the entire incubation period. MICs of tannic acid for LM1, LM2, and LM3 were determined to be 10, 15 and 20 ppm, respectively.

*C. perfringens* (Figure 5.48), *C. butyricum* (Figure 5.49) and *C. sporogenes* (Appendix Figure 80) were observed to have varying degrees of antimicrobial sensitivity when tested against tannic acid at 60 hours of incubation. MIC of tannic acid for *C. perfringens*, *C. butyricum* and *C. sporogenes* were determined to be <20 ppm.

Previous studies regarding tannic acid showed that several strains of *E. coli* were observed to have high antimicrobial susceptibility for tannic acid (Taguri and others 2004; Bancirova 2010). The present study confirms the previous findings, where tannic acid was observed to inhibit the growth of EC1 (Appendix Figure 81) and E3 (Figure 5.50) when used at 5 ppm and 20 ppm concentrations, respectively. However EC2 was observed to be highly resistant against tannic acid during the entire incubation period. MICs of tannic acid were determined as 5 and 20 against EC1 and EC3, respectively.

Tannic acid was observed to have high antimicrobial activity against *S. paratyphi* (Appendix Figure 82), and *S. cholerasuis subsp.* (Appendix Figure 83) and *S. enteridis* (Appendix Figure 84) when used at 15, 10 and 15 ppm concentrations, respectively. The previous studies regarding tannic acid (Taguri and others 2004) confirmed the findings of the present study, where *Salmonella* species were also found to have antimicrobial susceptibility.



### 5.3 Phenolic Compounds having Antimicrobial Activity only against *Bacillus* spp.

Some of the natural phenolic compounds did not exhibit any antimicrobial activity for any kind of pathogens but *Bacillus* spp. Caffeic acid, naringenin, *p*-coumaric acid and syringic acid (Kosina and others 2010) were previously reported to have antimicrobial activity towards Gram-positive, *Listeria innocua* (Mandalari and others 2007), *Bacillus* spp. (Mandalari and others 2007; Askun and others 2009a), *Salmonella* spp. (Mandalari and others 2007; Tsai and others 2008; Askun and others 2009a) and Gram-negative bacteria including *E. coli* (Mandalari and others 2007; Askun and others 2009a), *Salmonella* spp., and *E. aerogenes* (Askun and others 2009b). The results of this study confirm these studies having similar antimicrobial activities (Table 5.6).

Caffeic acid was observed to have antimicrobial activity on *B. subtilis* following the 24 hour incubation period, when used at 15 ppm concentrations. According to Askun and others (2009a) *B. cereus* was reported being sensitive to caffeic acid between the MIC range of 1280-10240 µg/ml. However, in this study the results revealed *B. cereus* being highly resistant to caffeic acid. Naringenin was determined to have high antimicrobial effects against *B. subtilis*, *B. cereus*, *B. polymyxa* with the MICs of <10 ppm following 60, 12 and 12 hours of incubation time.

*B. subtilis* and *B. polymyxa* were observed to have high antimicrobial susceptibility against syringic acid, with the MICs of 5 ppm following 60 and 12 hours of incubation time, respectively. Previous studies show that syringic acid, extracted from olive leaves was highly resistant against *B. cereus* (Korukluoglu and others 2010), however Yang and others (2010) reported *B. subtilis* being highly susceptible when treated with syringic acid extracted from *Canthium horridum* stems.

When treated with *p*-coumaric acid, *B. subtilis* and *B. cereus* were found to be highly susceptible, with MICs of 5 ppm following 36 and 12 hours of incubation time.

Confirming these results Acar and others (2010) reported that *p*-coumaric acid extracted from *Crocus baytopiorum* has high antimicrobial activity against both *B. subtilis* and *B. cereus*.

## 5.4 Conclusion

The results revealed that different bacterial species exhibit varying antimicrobial sensitivities towards the tested phenolic compounds. Generally, the Gram-negative bacteria (*E. coli* and *Salmonella*) were observed to have more antimicrobial susceptibility than Gram-positives (*Bacilli*, *Listeria*, and *Clostridium*) following 60 hours of incubation.

In addition, different strains of the same bacterial species showed differences in antimicrobial sensitivity, with varying concentrations of phenolic compounds. Therefore, each phenolic compound had different MIC for each bacterial strain. Figure 5.7 and Figure 5.8 depict the summary of MICs of both Gram-positive and Gram-negative pathogens, respectively. However, chlorogenic acid and ellagic acid had the same MICs (<10 ppm) for all strains of *Listeria monocytogenes*, where capsaicin had MIC of 5 ppm for all species of *Clostridium* and MIC of 20 ppm for *Salmonella* species. Moreover, all three strains of *E. coli* showed consistent antimicrobial susceptibility against quercetin, with the MICs of 5 ppm, where chlorogenic acid had MICs of 20 ppm for all strains of *Salmonella*. (-) Epicatechin was also consistently effective inhibiting the growth of all *Salmonella* species (MIC 15 ppm) and *Bacillus* species (MIC 20 ppm). Interestingly, quercetin and myricetin obtained from berries were determined to have no antibacterial activity for *Listeria monocytogenes* species.

Overall, ellagic acid, (-) epicatechin, capsaicin and rutin determined to be the most effective natural phenolic compounds against both Gram-positive and Gram-negative pathogens. The investigated strength of antimicrobial activity was followed by decanol, tannic acid, coumarin, chlorogenic acid, quercetin and myricetin, respectively. Each vegetable exhibits peculiar biochemical and nutritional characteristics, influenced by the species of belonging and, within the same species, by the variety, stage of ripening and technique of breeding (Andarwulan and Shetty 1999). Thus, the entire interaction among different factors becomes essential, influencing the content and composition of antibacterial and antioxidant biomolecules (Nazzaro and others 2009).

For the first time in literature, current study reported the antimicrobial activity of phenolics through 60 hours of incubation. The MICs were determined for both 24 hours

and 60 hours of incubation where the change in growth characteristics of foodborne pathogens was revealed. These findings provide evidence for the impact of natural phenolic compounds in food safety where the food is exposed to long term storage. Table 5.9 and Table 5.10 depict the MICs of phenolics for both Gram-positive and Gram-negative pathogens after 24 hours of incubation.

The results of this study suggest that the sensitivity of the pathogenic bacteria to natural phenolic compounds depends on bacterial species, source and polyphenol structure of the phenolics. Incubation period is another factor affecting the bacterial growth, where some of the sensitive pathogens may recover and become resistant after 24 hours of incubation. The usage of plant based antimicrobials can be alternatives for chemicals used in food preservation. In fact, phenolic and non-phenolic compounds in the plant extracts have the potential inhibitory activity against pathogenic bacteria (Cowan 1999). Furthermore, the genetic diversity, agronomical practices, and environmental conditions, the composition of critical compounds in herb and plant extracts exhibit differences in their efficacy against foodborne pathogens (Theivendran and others 2006).

**Table 5.1. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *Bacillus* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco S.	<i>B. polymyxa</i> ATCC 842	<i>Bacillus</i> species
Capsaicin	5.4	ETOH	< 15.0	< 20.0	< 20.0	< 20.0
Chlorogenic a.	5.62	ETOH	NA	< 10.0	< 10.0*	< 10.0
Coumarin	6.1	ETOH	< 20.0	< 5.0	< 15.0	< 20.0
Decanol	4.77	Prop. g.	< 5.0	< 10.0*	< 5.0*	< 10.0
Ellagic a.	5.44	Prop. g.	< 10.0	< 10.0	< 5.0*	< 10.0
(-) Epicatechin	5.6	ETOH	< 20.0	< 20.0	< 20.0	< 20.0
Myricetin	6.46	ETOH	< 15.0	< 15.0	NA	< 15.0
Quercetin	7.18	ETOH	< 15.0	< 20.0	< 10.0	< 20.0
Rutin	5.65	ETOH	< 15.0	< 5.0	< 10.0	< 15.0
Tannic a.	5.4	ETOH	< 20.0	NA	NA	< 20.0

NA: Not active

\*: Incubation period before 24 hours

**Table 5.2. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *Listeria monocytogenes* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	LM spp.
Capsaicin	5.4	ETOH	NA	< 20.0	< 10.0	< 20.0
Chlorogenic a.	5.62	ETOH	< 10.0	< 10.0	< 10.0	< 10.0
Coumarin	6.1	ETOH	< 5.0*	< 5.0	< 15.0	< 15.0
Decanol	4.77	Prop. g.	NA	< 20.0	< 20.0	< 20.0
Ellagic a.	5.44	Prop. g.	< 5.0	< 5.0	< 5.0	< 5.0
(-) Epicatechin	5.6	ETOH	NA	< 5.0	< 5.0*	< 5.0
Myricetin	6.46	ETOH	NA	NA	NA	NA
Quercetin	7.18	ETOH	NA	NA	NA	NA
Rutin	5.65	ETOH	< 5.0	< 10.0	< 15.0	< 15.0
Tannic a.	5.4	ETOH	< 10.0	< 15.0	< 20.0	< 20.0

NA: Not active

\*: Incubation period before 24 hours

**Table 5.3. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *Clostridium* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	C1 RNR	C2 ATCC 8260	C3 ATCC 7955	<i>Clostridium</i> species
Capsaicin	5.4	ETOH	< 5.0	< 5.0	< 5.0	< 5.0
Chlorogenic a.	5.62	ETOH	NA	< 20.0	< 5.0	< 20.0
Coumarin	6.1	ETOH	NA	< 10.0	< 5.0	< 10.0
Decanol	4.77	Prop. g.	< 5.0	< 5.0	NA	< 5.0
Ellagic a.	5.44	Prop. g.	< 5.0	< 15.0	NA	< 15.0
(-) Epicatechin	5.6	ETOH	< 20.0	< 20.0	< 10.0	< 20.0
Myricetin	6.46	ETOH	NA	< 5.0	< 10.0	< 10.0
Quercetin	7.18	ETOH	NA	< 15.0	< 15.0	< 15.0
Rutin	5.65	ETOH	< 10.0	NA	NA	< 10.0
Tannic a.	5.4	ETOH	< 20.0	< 20.0	< 10.0	< 20.0

NA: Not active

**Table 5.4. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *E.coli* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	EC1 FTJ	EC2 ATCC 43895	EC3 ATCC 35150	<i>E. coli</i> species
Capsaicin	5.4	ETOH	< 20.0	< 20.0	NA	< 20.0
Chlorogenic a.	5.62	ETOH	< 15.0	NA	< 10.0	< 15.0
Coumarin	6.1	ETOH	< 20.0	NA	NA	< 20.0
Decanol	4.77	Prop. g.	< 10.0	< 5.0	< 5.0	< 10.0
Ellagic a.	5.44	Prop. g.	< 20.0	< 20.0	NA	< 20.0
(-) Epicatechin	5.6	ETOH	< 20.0	< 5.0*	< 10.0	< 20.0
Myricetin	6.46	ETOH	< 20.0	< 10.0	< 15.0	< 20.0
Quercetin	7.18	ETOH	< 5.0	< 5.0	< 5.0	< 5.0
Rutin	5.65	ETOH	< 20.0	< 5.0	< 15.0	< 20.0
Tannic a.	5.4	ETOH	< 5.0	NA	< 20.0	< 20.0

NA: Not active

\*: Incubation period before 24 hours

**Table 5.5. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *Salmonella* spp.**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	S1 UKM 29A	S2 ATCC 10708	S3 (-) H <sub>2</sub> S	<i>Salmonella</i> species
Capsaicin	5.4	ETOH	< 20.0	< 20.0	< 20.0	< 20.0
Chlorogenic a.	5.62	ETOH	< 10.0	< 10.0	< 10.0	< 10.0
Coumarin	6.1	ETOH	< 15.0	< 15.0	< 5.0	< 15.0
Decanol	4.77	Prop. g.	< 15.0	< 15.0*	< 10.0	< 15.0
Ellagic a.	5.44	Prop. g.	< 20.0	< 10.0	< 15.0	< 20.0
(-) Epicatechin	5.6	ETOH	< 15.0	< 15.0	< 15.0	< 15.0
Myricetin	6.46	ETOH	< 20.0	< 15.0	< 5.0	< 20.0
Quercetin	7.18	ETOH	< 20.0	< 15.0	< 10.0	< 20.0
Rutin	5.65	ETOH	< 20.0	< 15.0	< 15.0	< 20.0
Tannic a.	5.4	ETOH	< 15.0	< 10.0	< 15.0	< 15.0

NA: Not active

\*: Incubation period before 24 hours

**Table 5.6. Minimum Inhibitory Concentration of Phenolic Compounds Derived from Vegetables and Fruits against *Bacillus* spp. (2)**

Minimum Inhibitory Concentrations (ppm)						
Compound	pH	Solvent	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco S.	<i>B. polymyxa</i> ATCC 842	<i>Bacillus</i> spp.
Caffeic a.	6.1	H <sub>2</sub> O	< 15.0	NA	NA	< 15.0
Naringenin	6.04	ETOH	< 5.0	< 5.0*	NA	< 5.0
<i>p</i> -Coumaric a.	6.15	H <sub>2</sub> O	< 5.0	< 5.0*	NA	< 5.0
Syringic a.	6.08	ETOH	< 5.0	NA	< 5.0*	< 5.0

NA: Not active

\*: Incubation period before 24 hours

**Table 5.7. MICs (ppm) of Phenolic Compounds Derived from Vegetables and Fruits for Gram-positive bacteria.**

Gram-positive bacteria										
Compound	pH	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco Spores	<i>B. polymyxa</i> ATCC 842	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	C1 RNR	C2 ATCC 8260	C3 ATCC 7955
Capsaicin	5.4	< 15.0	< 20.0	< 20.0	NA	< 20.0	< 10.0	< 5.0	< 5.0	< 5.0
Chlorogenic a.	5.62	NA	< 10.0	< 10.0	< 10.0	< 10.0	< 10.0	NA	< 20.0	< 5.0
Coumarin	6.1	< 20.0	< 5.0	< 15.0	< 5.0*	< 5.0	< 15.0	NA	< 10.0	< 5.0
Decanol	4.77	< 5.0	< 10.0*	< 5.0*	NA	< 20.0	< 20.0	< 5.0	< 5.0	NA
Ellagic a.	5.44	< 10.0	< 10.0	< 5.0*	< 5.0	< 5.0	< 5.0	< 5.0	< 15.0	NA
(-) Epicatechin	5.6	< 20.0	< 20.0	< 20.0	NA	< 5.0	< 5.0*	< 20.0	< 20.0	< 10.0
Myricetin	6.46	< 15.0	< 15.0	NA	NA	NA	NA	NA	< 5.0	< 10.0
Quercetin	7.18	< 15.0	< 20.0	< 10.0	NA	NA	NA	NA	< 15.0	< 15.0
Rutin	5.65	< 15.0	< 5.0	< 10.0	< 5.0	< 10.0	< 15.0	< 10.0	NA	NA
Tannic a.	5.4	< 20.0	NA	NA	< 10.0	< 15.0	< 20.0	< 20.0	< 20.0	< 10.0

NA: Not active, \*: Incubation period before 24 hours

**Table 5.8. MICs (ppm) of Phenolic Compounds Derived from Vegetables and Fruits for Gram-negative bacteria.**

Gram-negative bacteria							
Compound	pH	<i>E. coli</i> FTJ	<i>E. coli</i> 0157:H7 ATCC 43895	<i>E. coli</i> 0157:H7 ATCC 35150	<i>S. paratyphi</i> UK Micro 29A	<i>S. cholerae</i> subsp. ATCC 10708	<i>S. enteridis</i> (-) H <sub>2</sub> S
Capsaicin	5.4	< 20.0	< 20.0	NA	< 20.0	< 20.0	< 20.0
Chlorogenic a.	5.62	< 15.0	NA	< 10.0	< 10.0	< 10.0	< 10.0
Coumarin	6.1	< 20.0	NA	NA	< 15.0	< 15.0	< 5.0
Decanol	4.77	< 10.0	< 5.0	< 5.0	< 15.0	< 15.0*	< 10.0
Ellagic a.	5.44	< 20.0	< 20.0	NA	< 20.0	< 10.0	< 15.0
(-) Epicatechin	5.6	< 20.0	< 5.0*	< 10.0	< 15.0	< 15.0	< 15.0
Myricetin	6.46	< 20.0	< 10.0	< 15.0	< 20.0	< 15.0	< 5.0
Quercetin	7.18	< 5.0	< 5.0	< 5.0	< 20.0	< 15.0	< 10.0
Rutin	5.65	< 20.0	< 5.0	< 15.0	< 20.0	< 15.0	< 15.0
Tannic a.	5.4	< 5.0	NA	< 20.0	< 15.0	< 10.0	< 15.0

NA: Not active, \*: Incubation period before 24 hour

**Table 5.9. MICs (ppm) of Phenolic Compounds Derived from Vegetables and Fruits for Gram-positive bacteria (after 24 hours).**

Gram-positive bacteria										
Compound	pH	<i>B. subtilis</i> ATCC 6051	<i>B. cereus</i> Difco Spores	<i>B. polymyxa</i> ATCC 842	LM1 ATCC 7644	LM2 UK ADL	LM3 ATCC 49594	C1 RNR	C2 ATCC 8260	C3 ATCC 7955
Capsaicin	5.4	NA	< 20.0	< 20.0	NA	< 20.0	< 10.0	< 5.0	< 10.0	< 10.0
Chlorogenic a.	5.62	NA	< 20.0	NA	NA	< 20.0	< 15.0	NA	< 5.0	< 5.0
Coumarin	6.1	< 20.0	NA	NA	NA	< 10.0	NA	NA	NA	< 20.0
Decanol	4.77	NA	NA	NA	< 20.0	< 20.0	< 20.0	< 15.0	< 20.0	NA
Ellagic a.	5.44	< 20.0	< 20.0	NA	< 5.0	< 5.0	< 5.0	< 5.0	< 15.0	NA
(-) Epicatechin	5.6	NA	< 10.0	NA	NA	NA	NA	NA	NA	< 5.0
Myricetin	6.46	< 15.0	< 15.0	NA	NA	NA	NA	NA	NA	< 5.0
Quercetin	7.18	< 10.0	< 10.0	< 10.0	NA	NA	NA	NA	NA	< 15.0
Rutin	5.65	NA	< 20.0	< 20.0	NA	NA	NA	< 20.0	NA	NA
Tannic a.	5.4	< 20.0	NA	NA	< 5.0	< 10.0	< 15.0	< 5.0	< 5.0	< 10.0

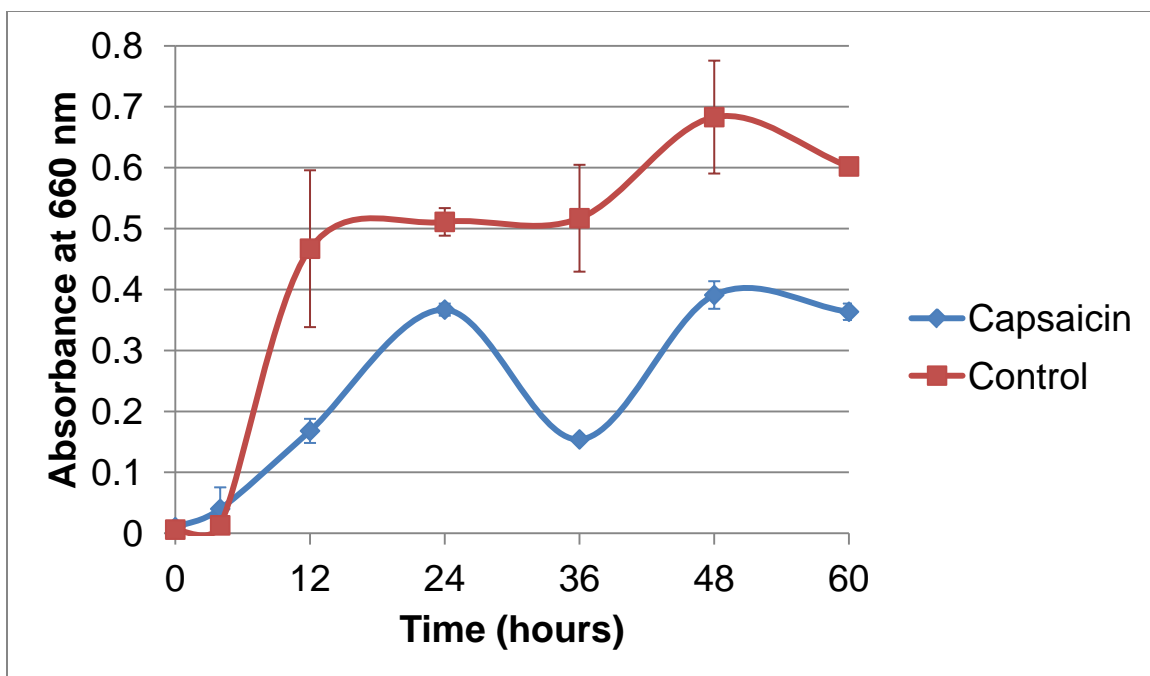
NA: Not active

**Table 5.10. MICs (ppm) of Phenolic Compounds Derived from Vegetables and Fruits for Gram-negative bacteria (after 24 hours).**

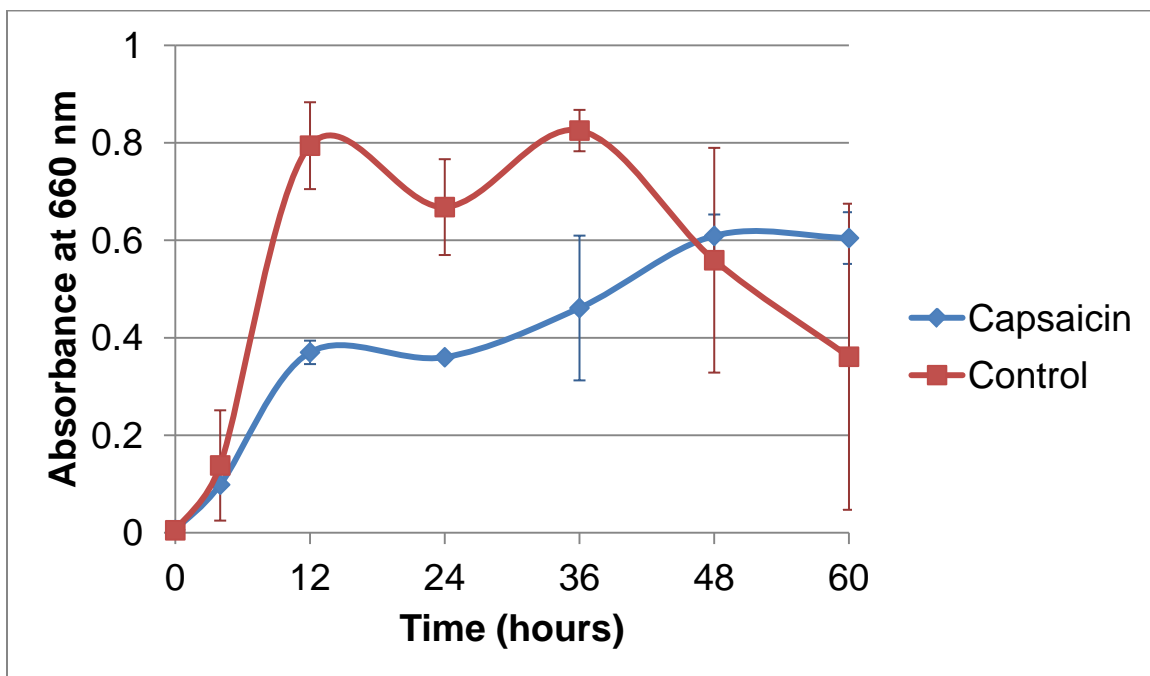
Gram-negative bacteria							
Compound	pH	<i>E. coli</i> FTJ	<i>E. coli</i> 0157:H7 ATCC 43895	<i>E. coli</i> 0157:H7 ATCC 35150	<i>S. paratyphi</i> UK Micro 29A	<i>S. cholerae</i> subsp. ATCC 10708	<i>S. enteridis</i> (-) H <sub>2</sub> S
Capsaicin	5.4	NA	NA	NA	< 10.0	< 20.0	< 15.0
Chlorogenic a.	5.62	< 10.0	NA	< 15.0	< 5.0	< 15.0	NA
Coumarin	6.1	< 20.0	NA	NA	< 20.0	< 15.0	< 20.0
Decanol	4.77	< 20.0	< 20.0	< 20.0	< 15.0	< 5.0	< 10.0
Ellagic a.	5.44	< 10.0	< 5.0	NA	NA	< 15.0	< 15.0
(-) Epicatechin	5.6	< 20.0	NA	< 15.0	< 10.0	NA	NA
Myricetin	6.46	< 15.0	< 10.0	< 10.0	< 15.0	NA	< 10.0
Quercetin	7.18	NA	NA	< 5.0	< 20.0	< 15.0	NA
Rutin	5.65	< 20.0	NA	< 15.0	NA	NA	NA
Tannic a.	5.4	< 20.0	NA	< 10.0	< 15.0	< 20.0	< 10.0

NA: Not active

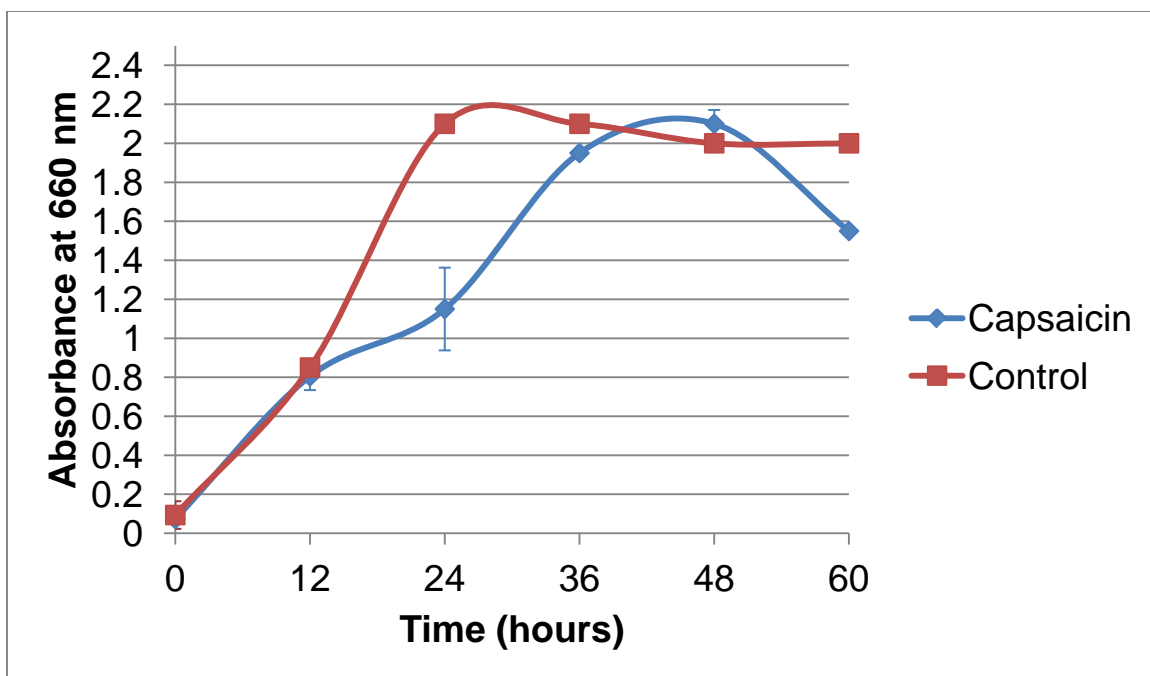




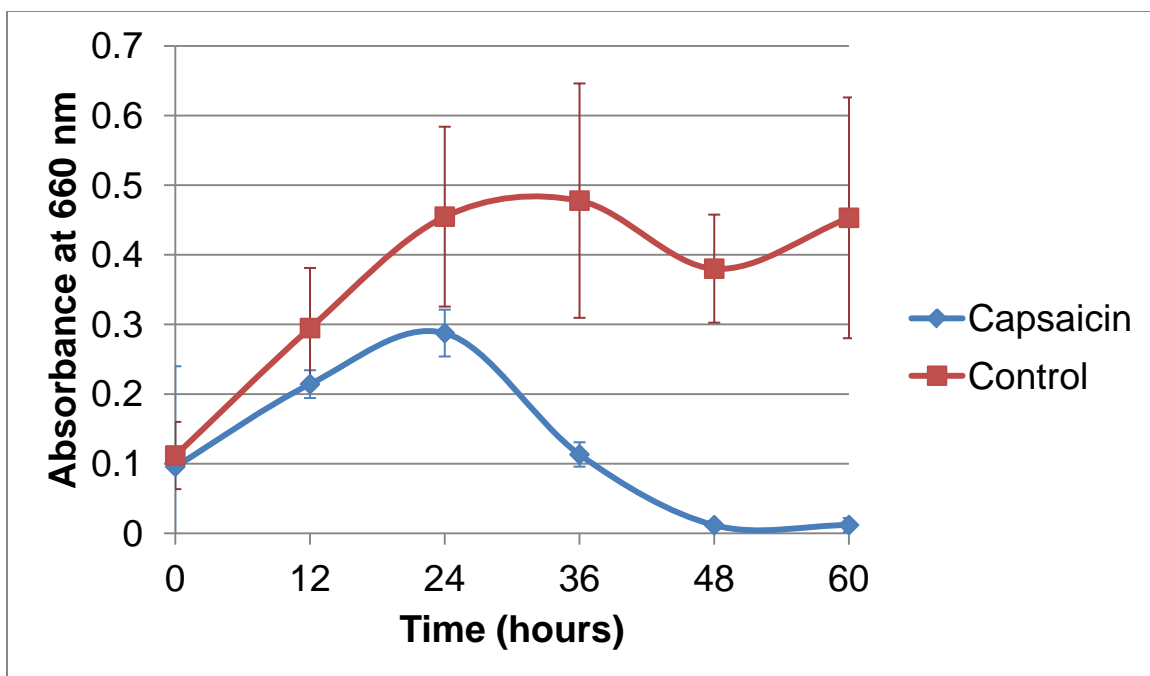
**Figure 5.1.** The antimicrobial activity of capsaicin at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



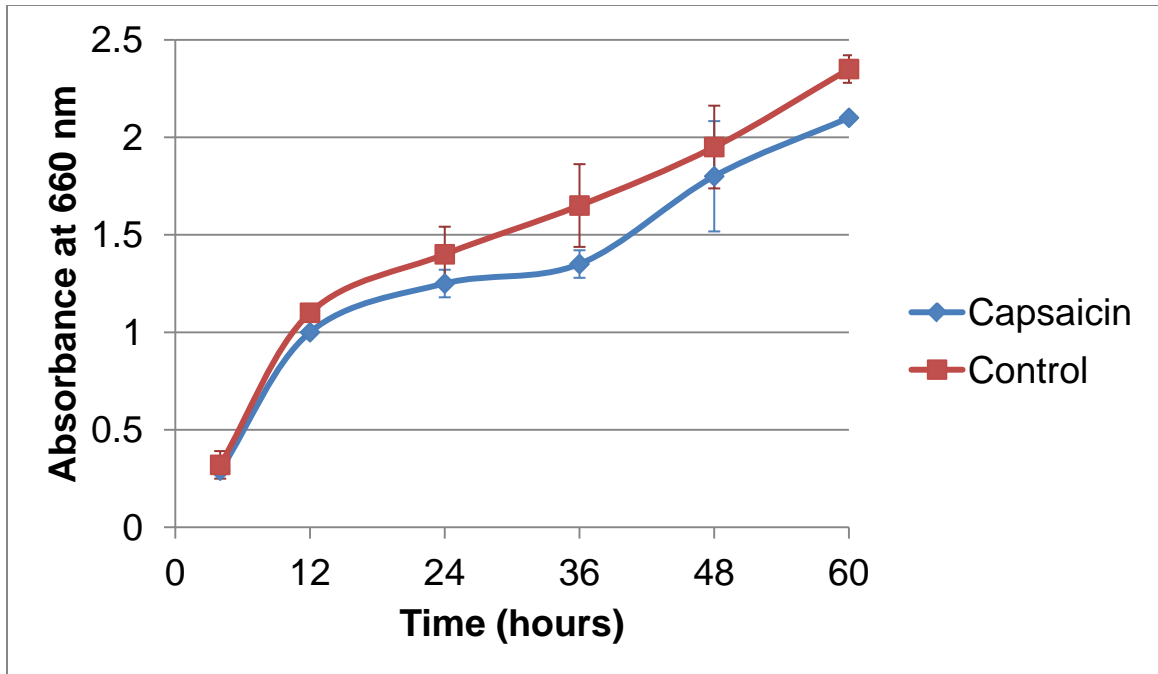
**Figure 5.2.** The antimicrobial activity of capsaicin at MIC of 20 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



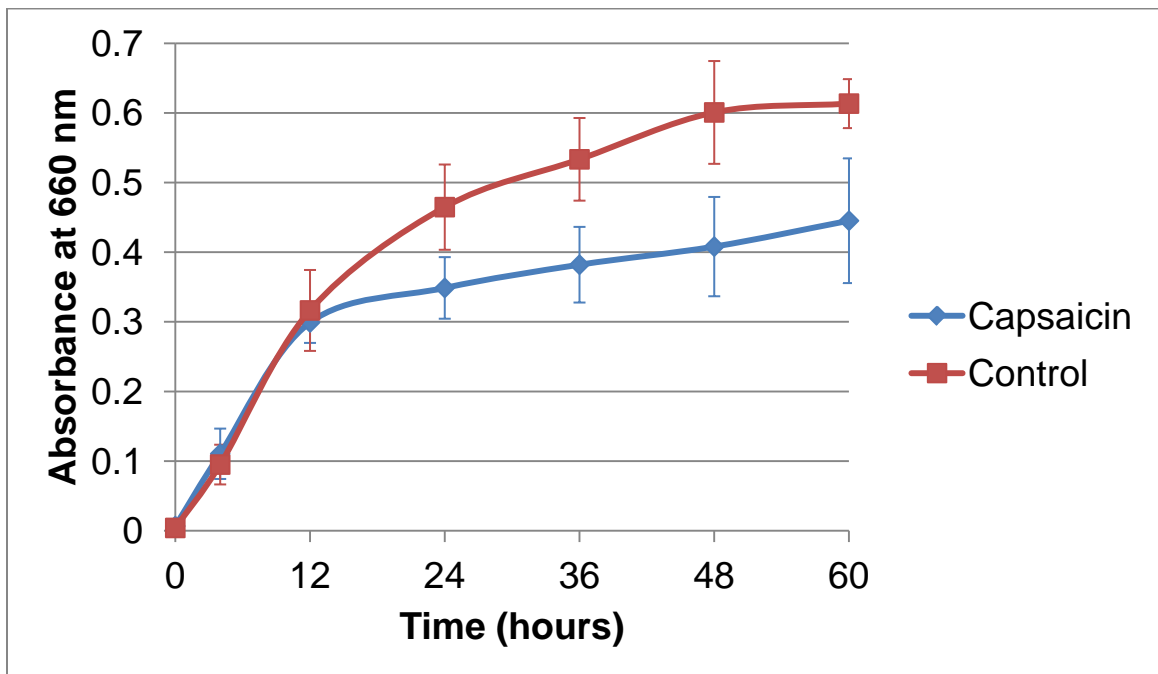
**Figure 5.3.** The antimicrobial activity of capsaicin at MIC of 10 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



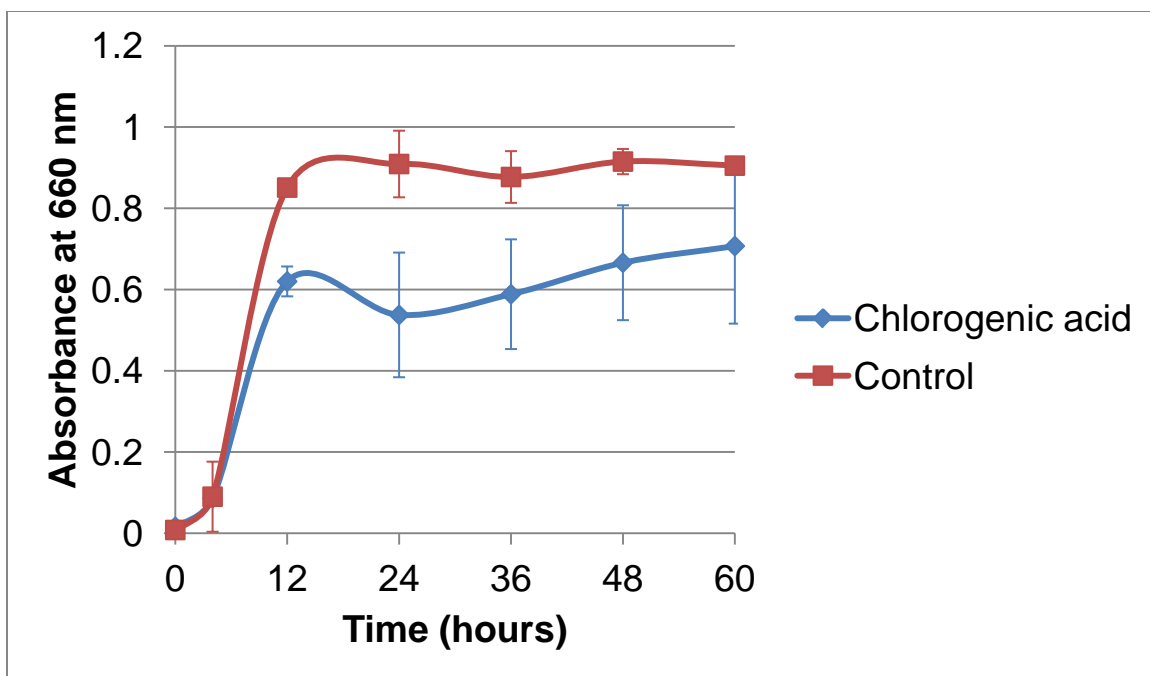
**Figure 5.4.** The average antimicrobial activity of capsaicin at MIC of 5 ppm against *C. perfringens*, RNR, *C. butyricum*, ATCC 8260 and *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



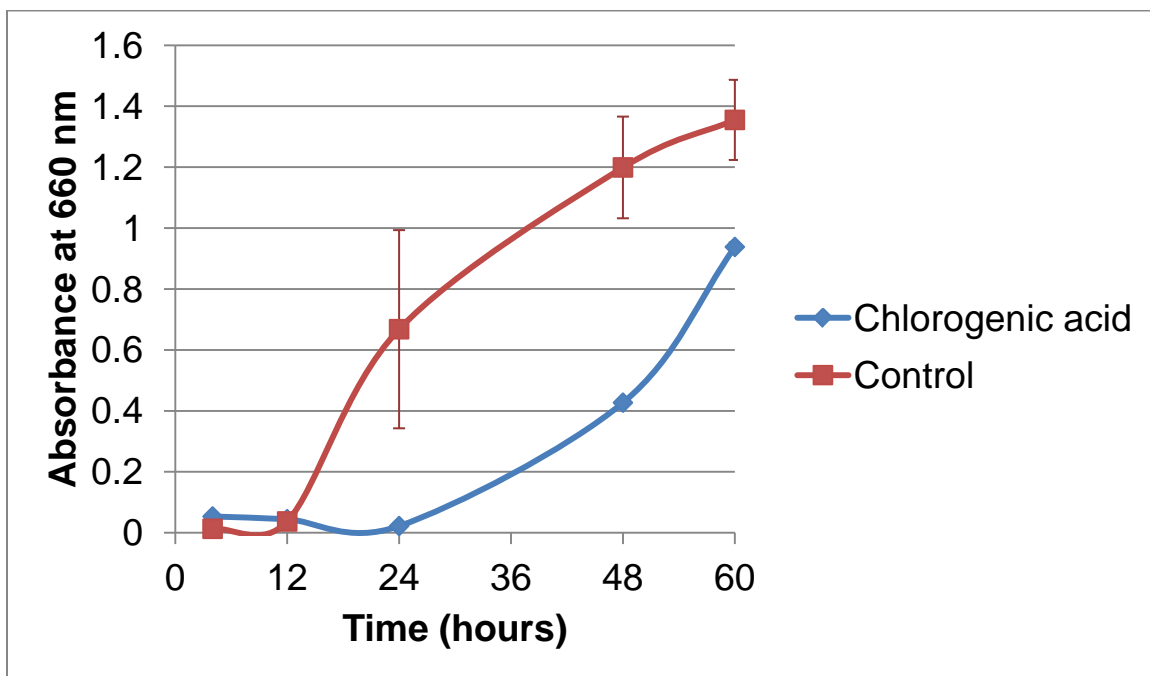
**Figure 5.5.** The antimicrobial activity of capsaicin at MIC of 20 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



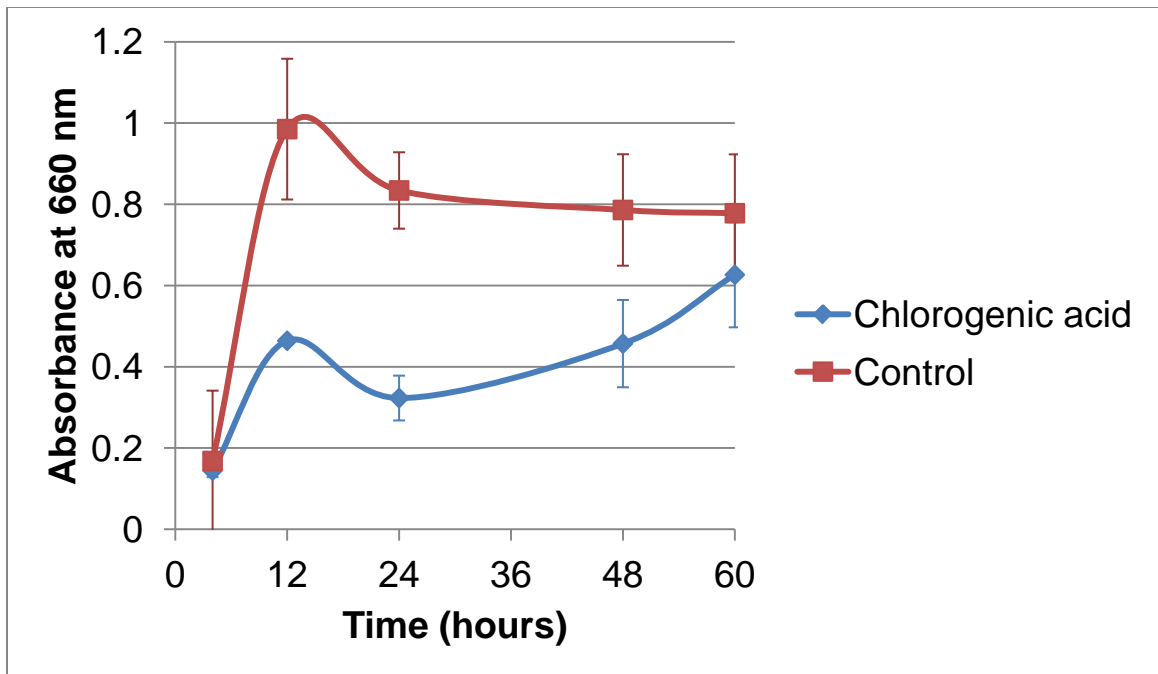
**Figure 5.6.** The average antimicrobial activity of capsaicin at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A, *S. choleraesuis* subsps., ATCC 10708 and *S. enteritidis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean,  $P < 0.05$ .



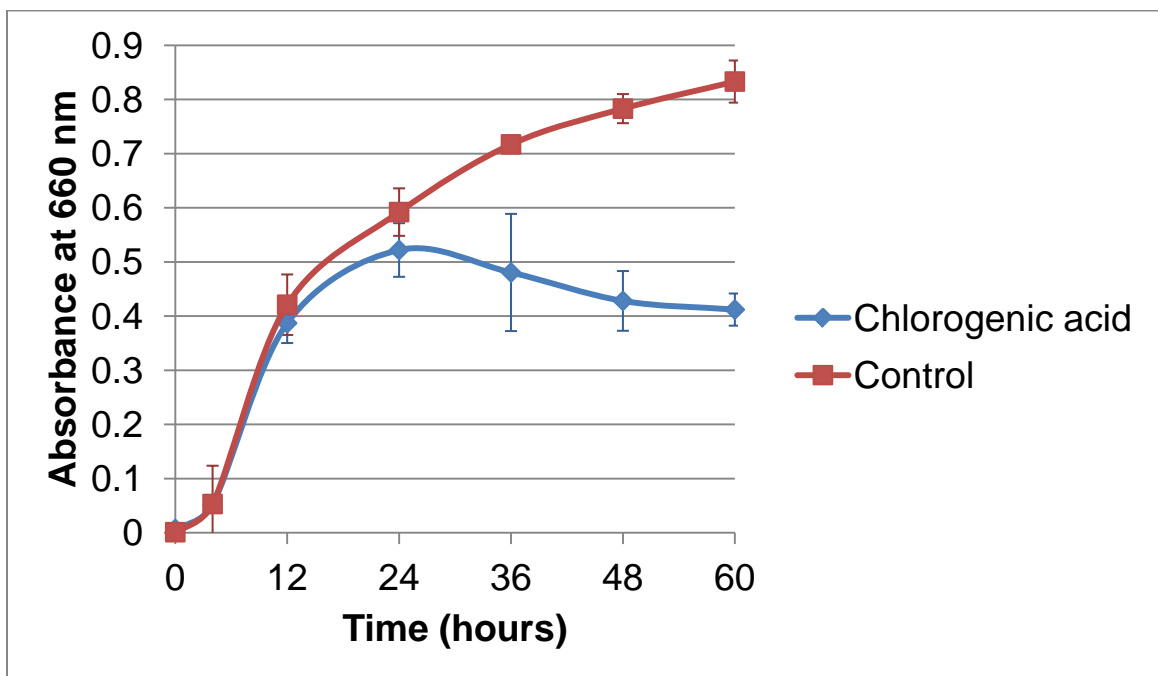
**Figure 5.7.** The antimicrobial activity of chlorogenic acid at MIC of 10 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



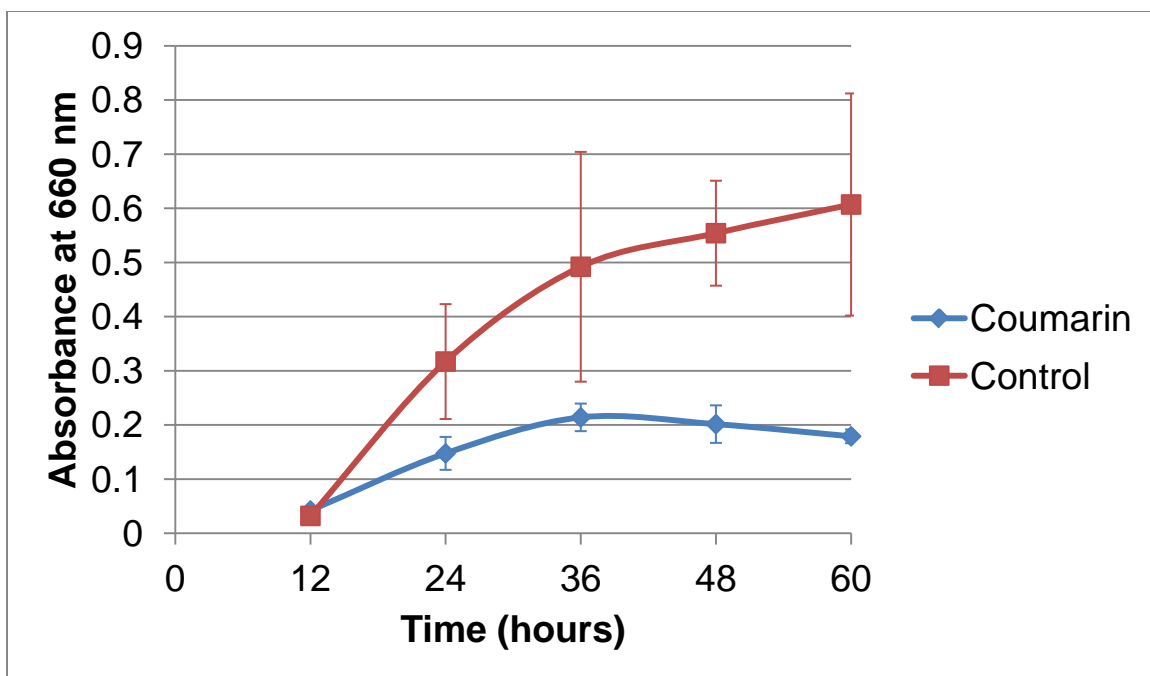
**Figure 5.8.** The antimicrobial activity of chlorogenic acid at MIC of 20 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



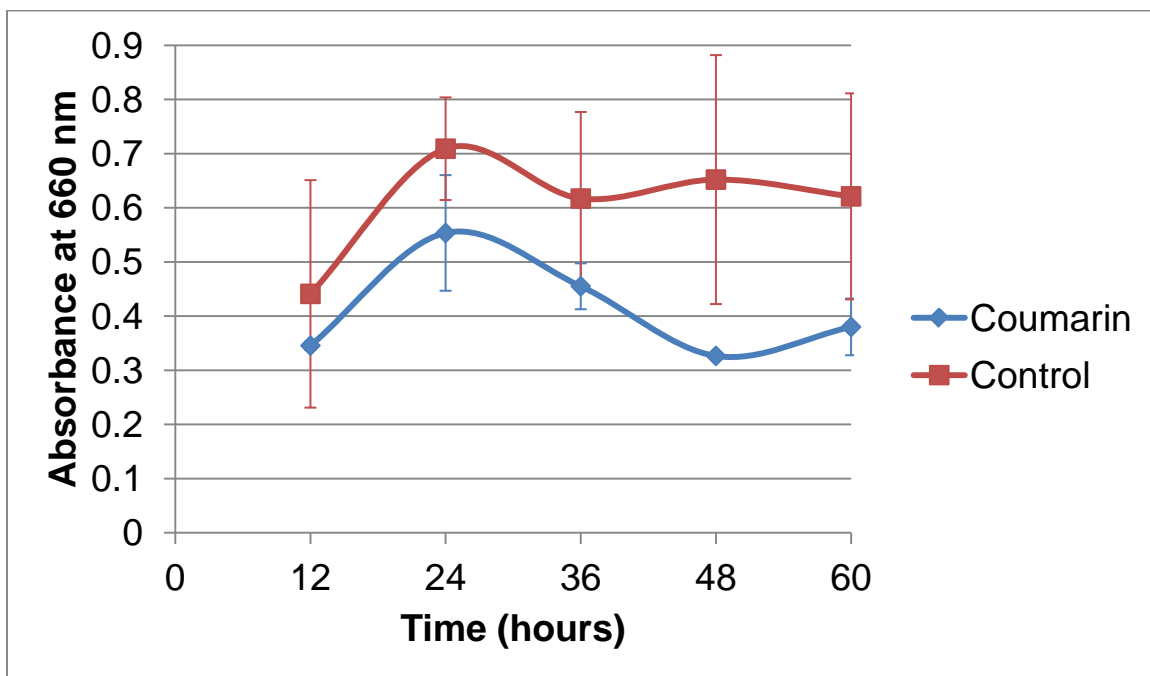
**Figure 5.9.** The antimicrobial activity of chlorogenic acid at MIC of 5 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



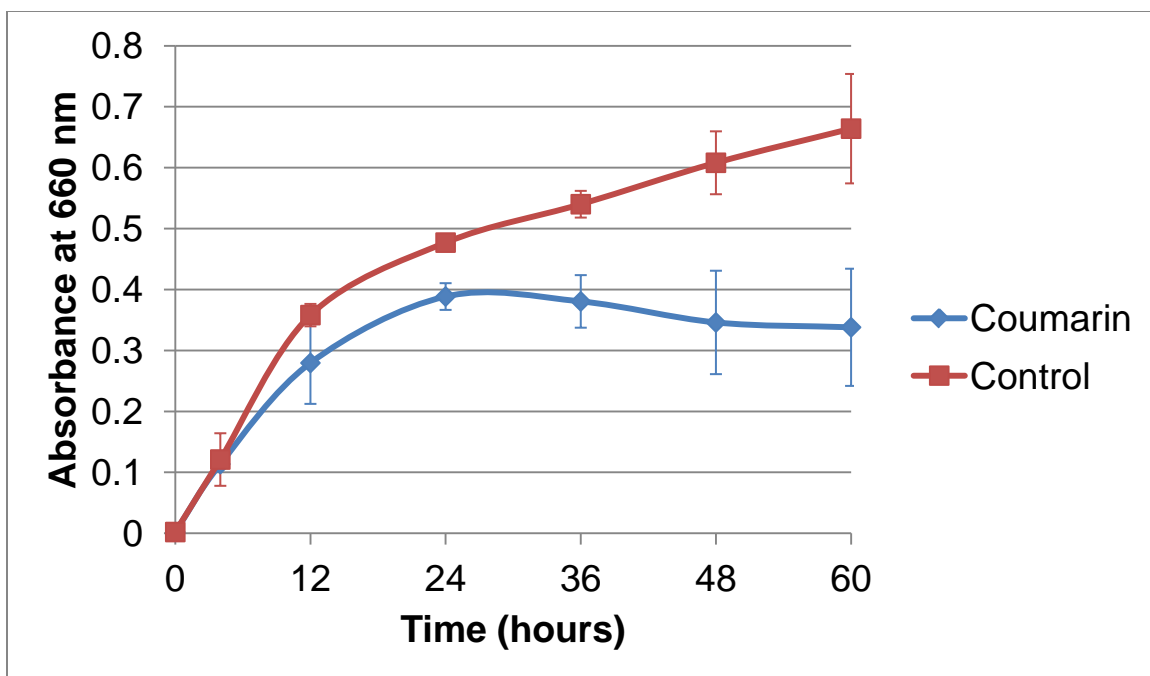
**Figure 5.10.** The antimicrobial activity of chlorogenic acid at MIC of 10 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



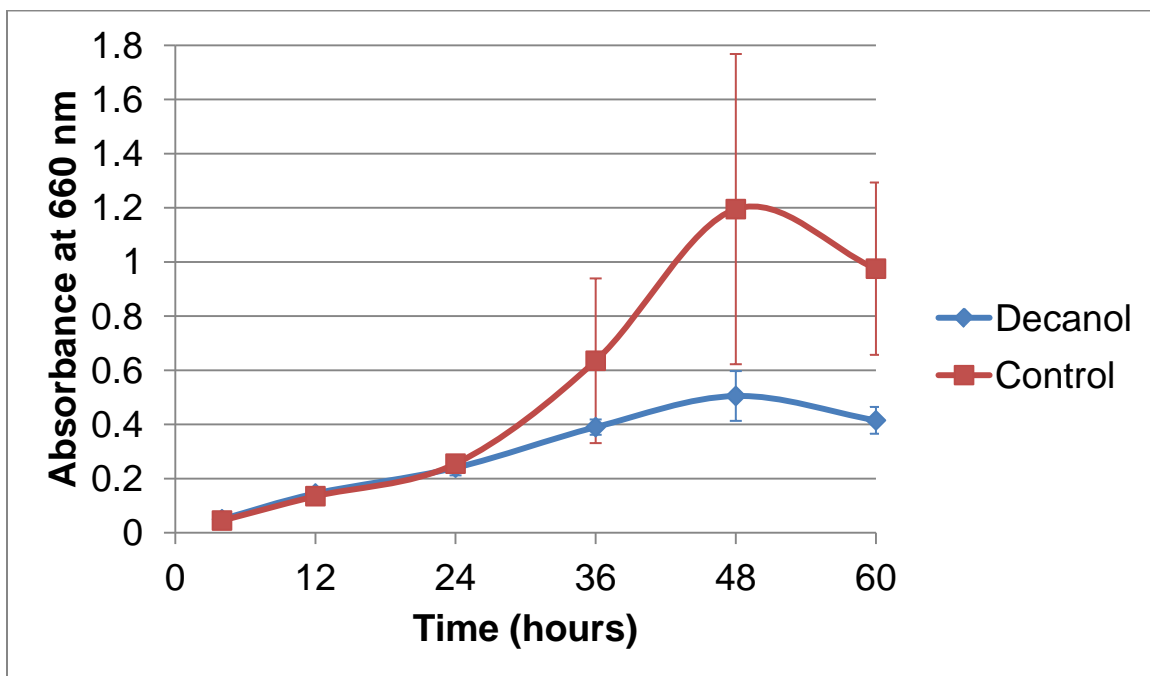
**Figure 5.11.** The antimicrobial activity of coumarin at MIC of 10 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



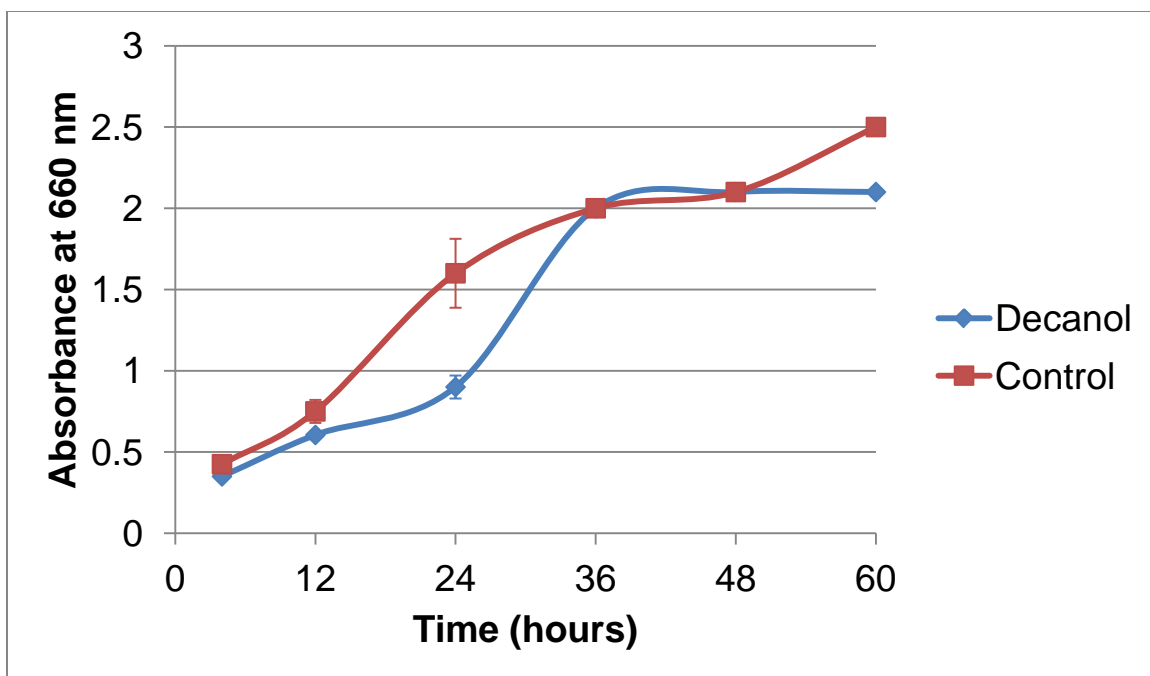
**Figure 5.12.** The antimicrobial activity of coumarin at MIC of 5 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



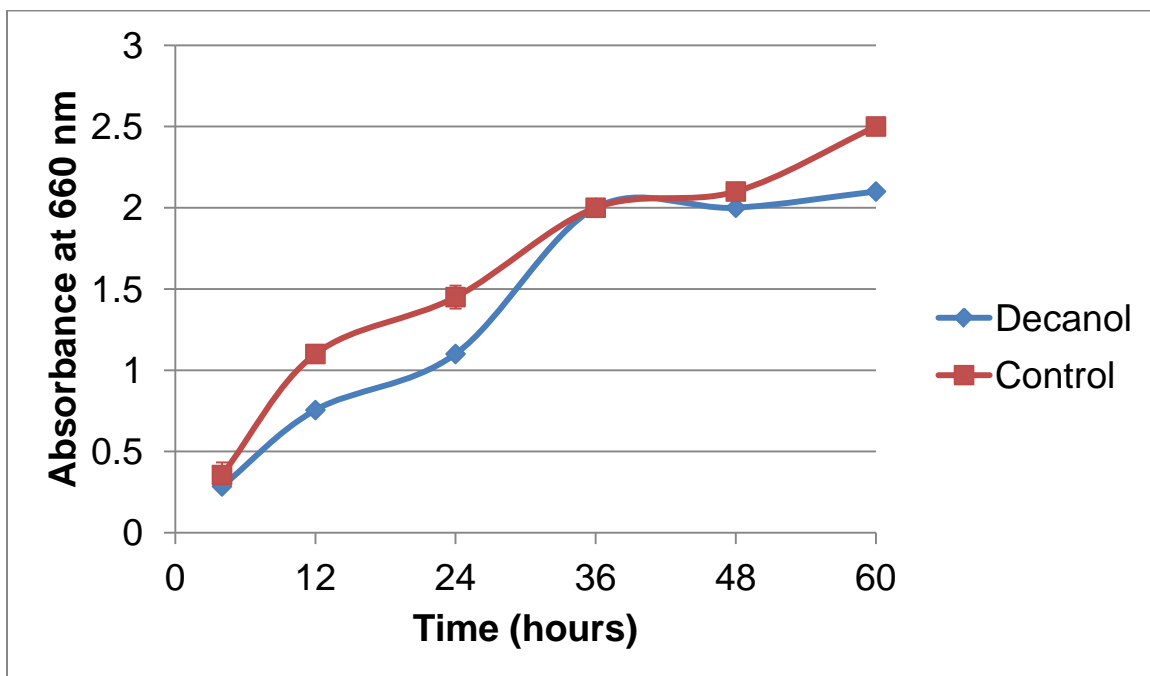
**Figure 5.13.** The antimicrobial activity of coumarin at MIC of 5 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



**Figure 5.14.** The antimicrobial activity of decanol at MIC of 5 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.

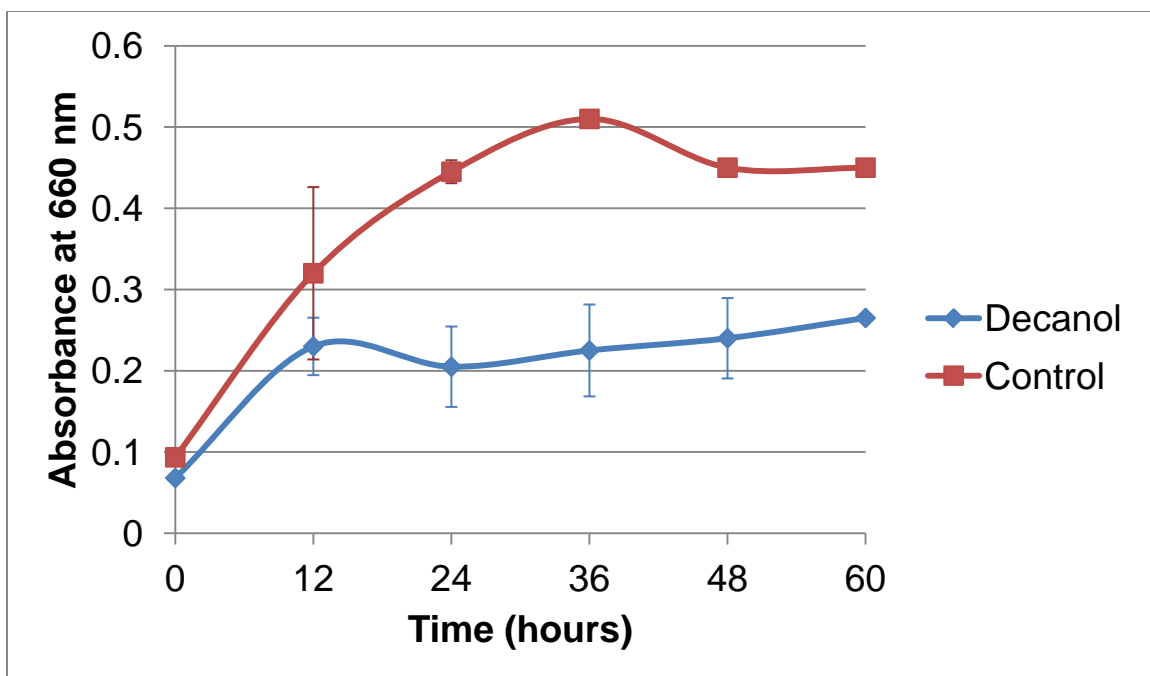


**Figure 5.15.** The antimicrobial activity of decanol at MIC of 10 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .

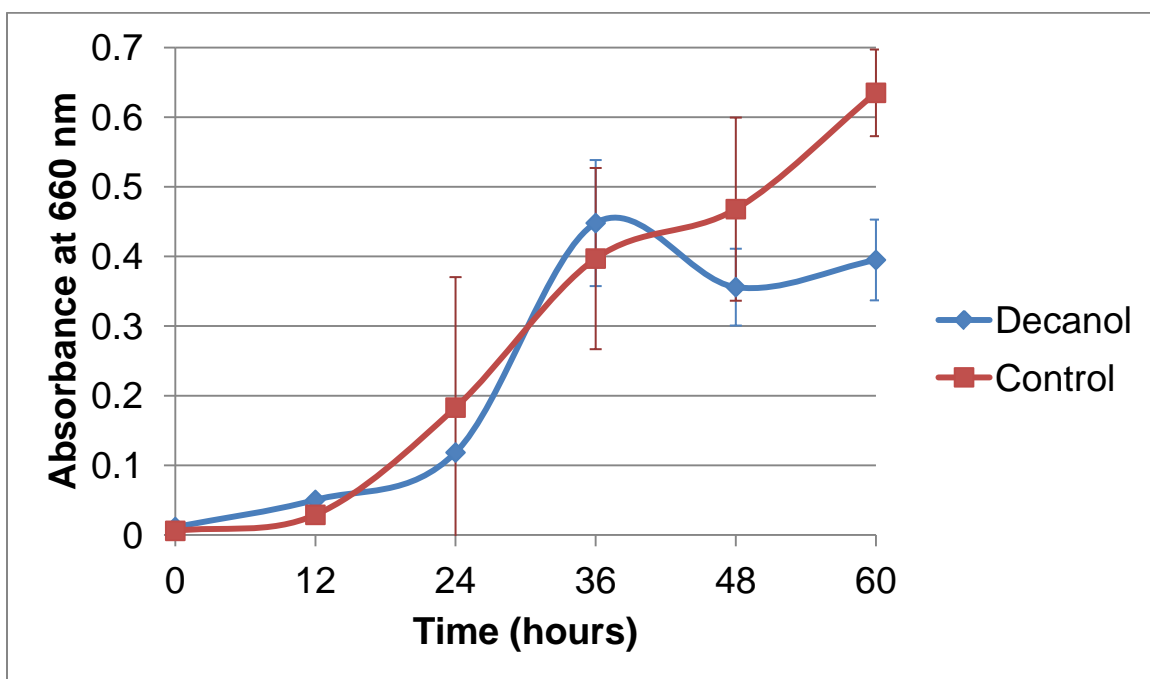


**Figure 5.16.** The antimicrobial activity of decanol at MIC of 5 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .

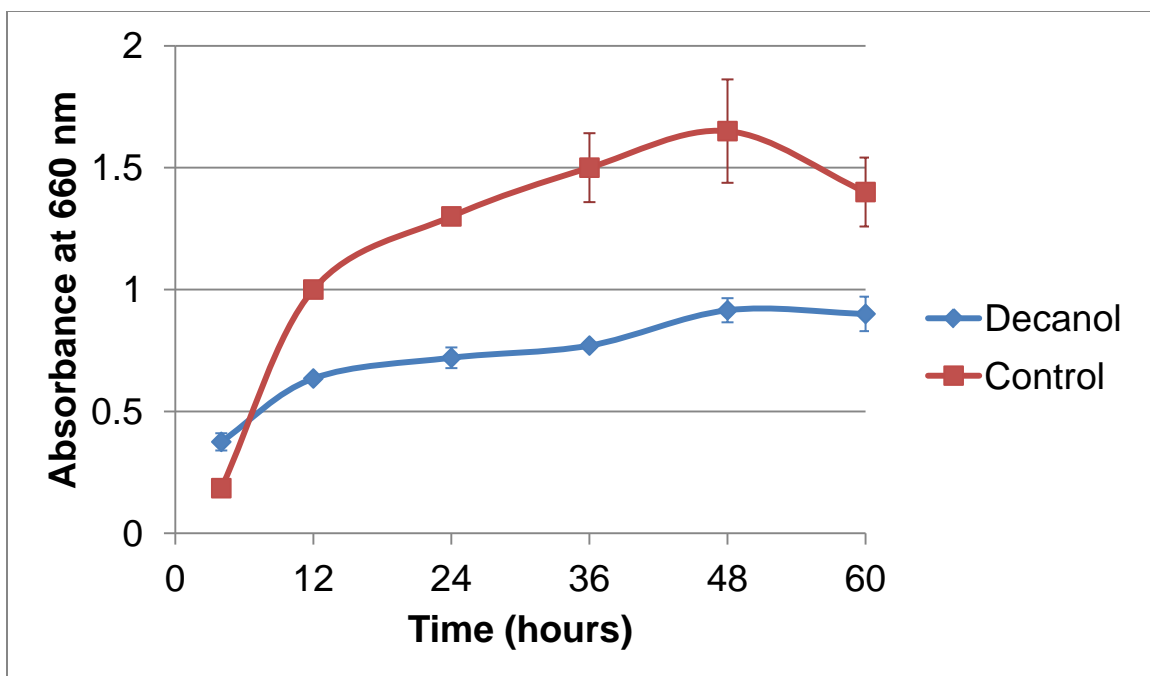




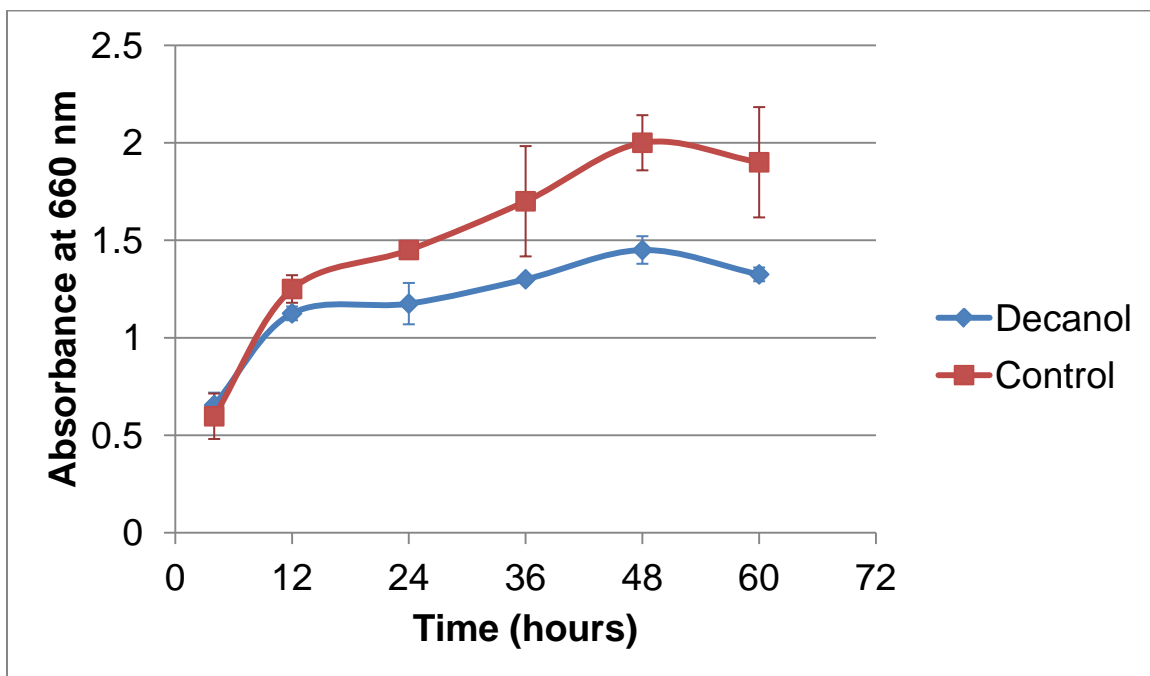
**Figure 5.17.** The antimicrobial activity of decanol at MIC of 20 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



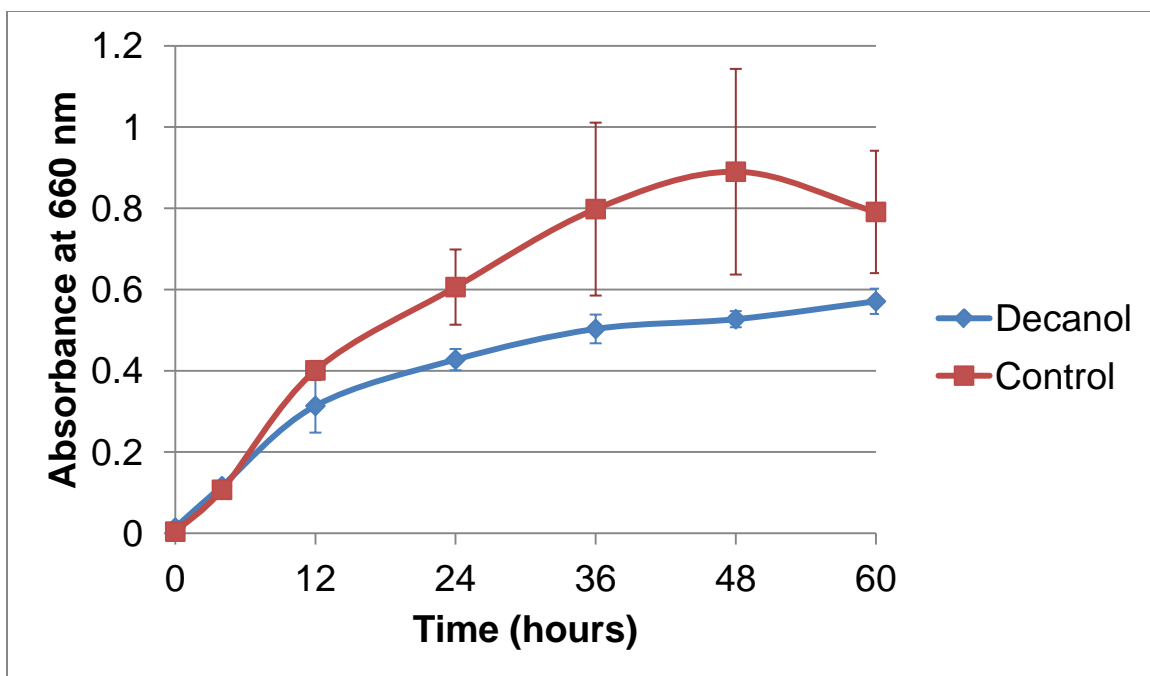
**Figure 5.18.** The antimicrobial activity of decanol at MIC of 5 ppm against *C. butyricum* ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



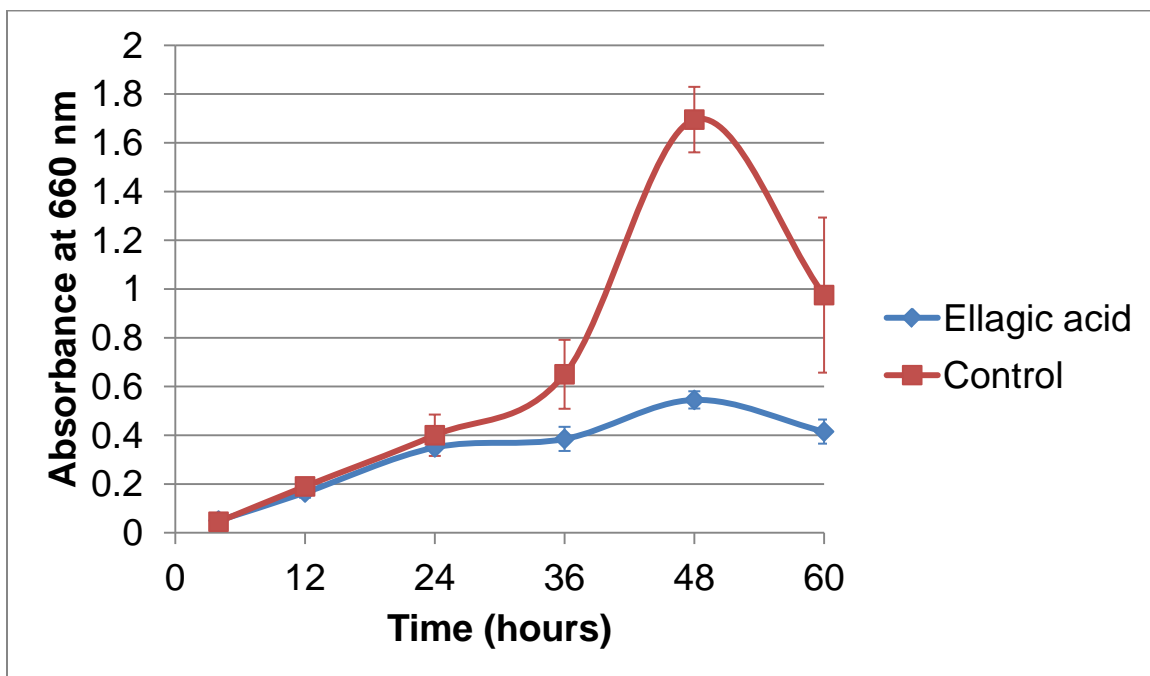
**Figure 5.19.** The antimicrobial activity of decanol at MIC of 10 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



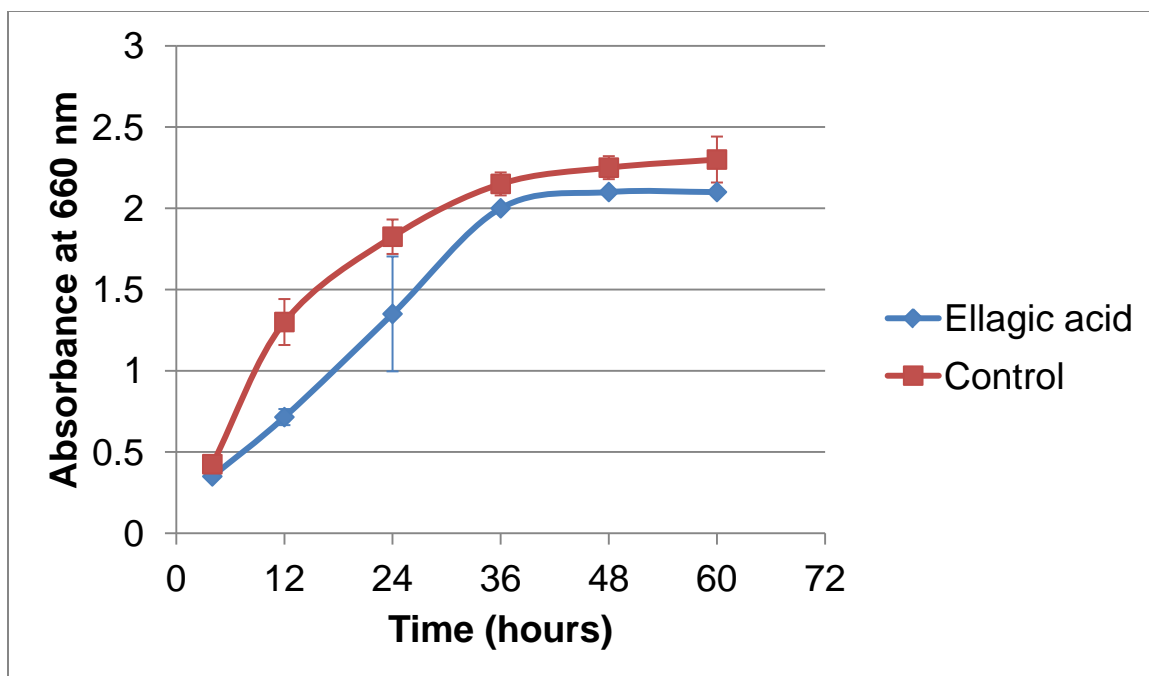
**Figure 5.20.** The average antimicrobial activity of decanol at MIC of 5 ppm against *E. coli* O157:H7, ATCC 43895 and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



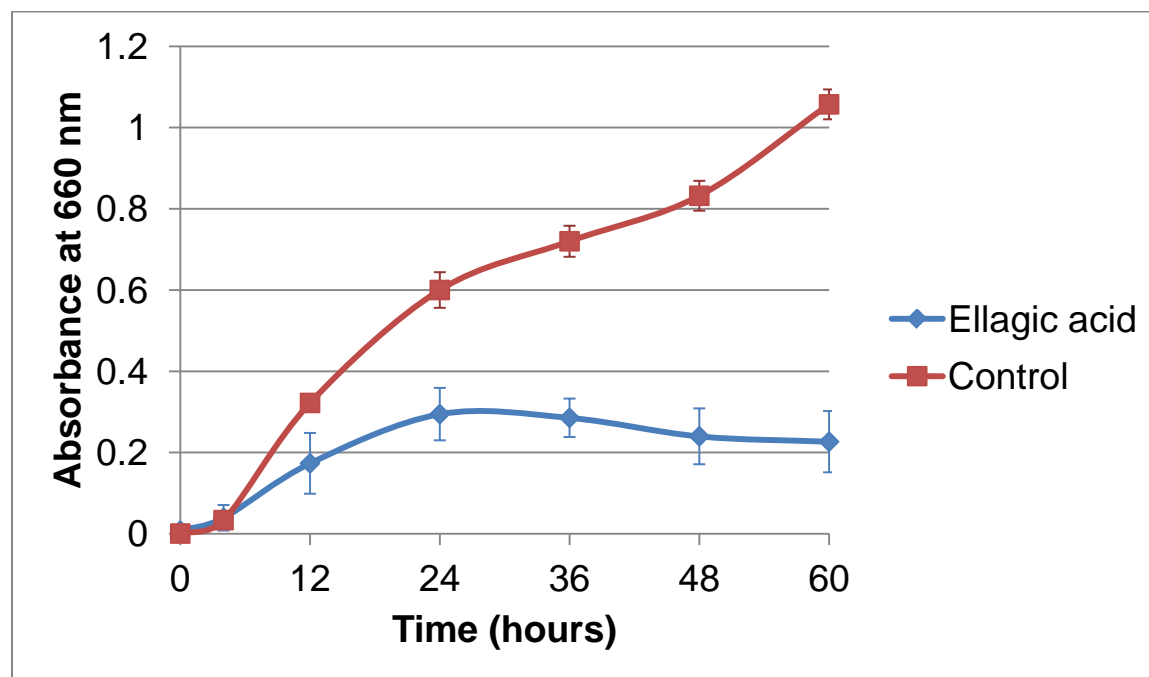
**Figure 5.21.** The antimicrobial activity of decanol at MIC of 10 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



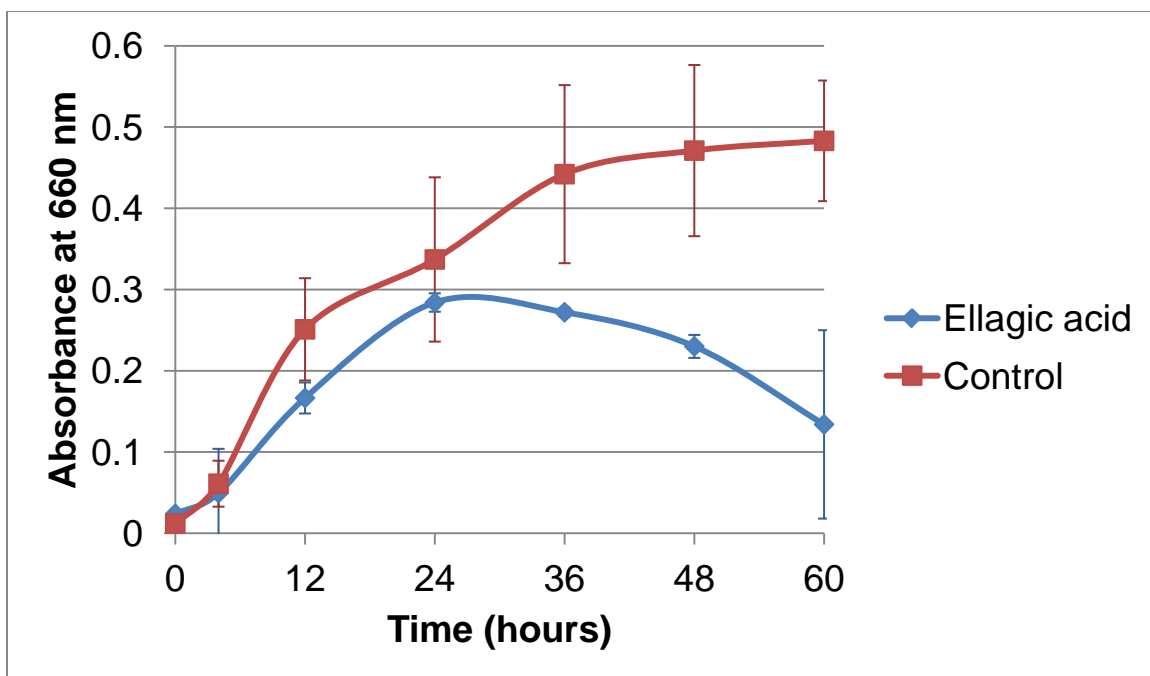
**Figure 5.22.** The antimicrobial activity of ellagic acid at MIC of 10 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.



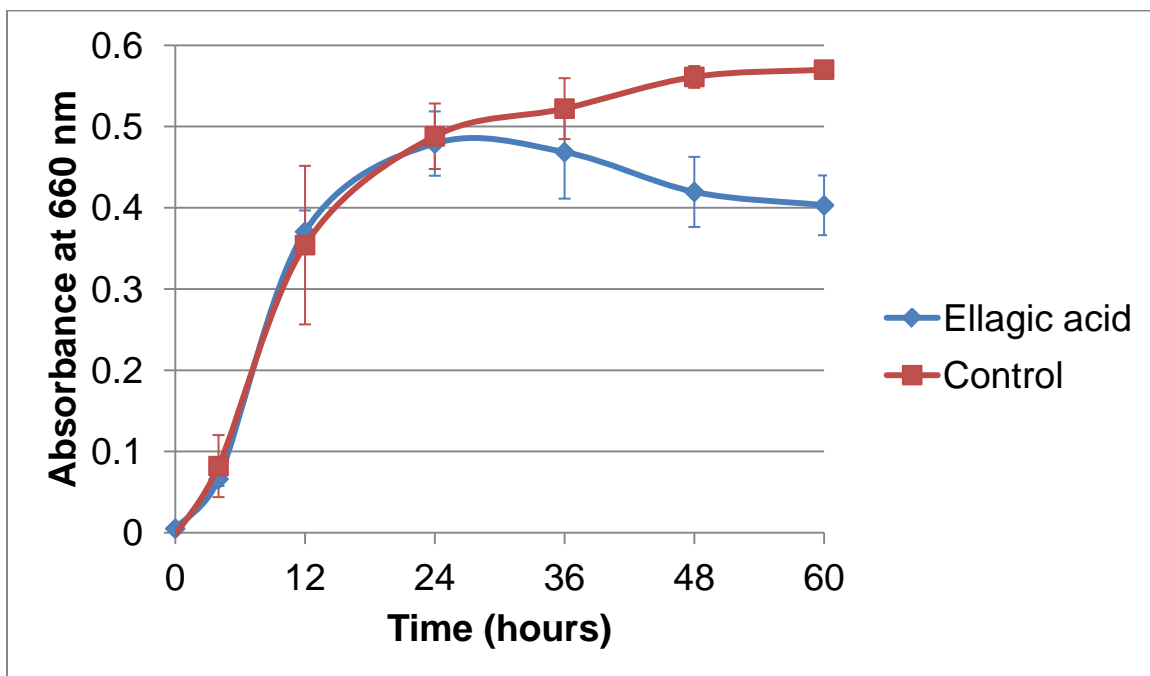
**Figure 5.23.** The antimicrobial activity of ellagic acid at MIC of 10 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



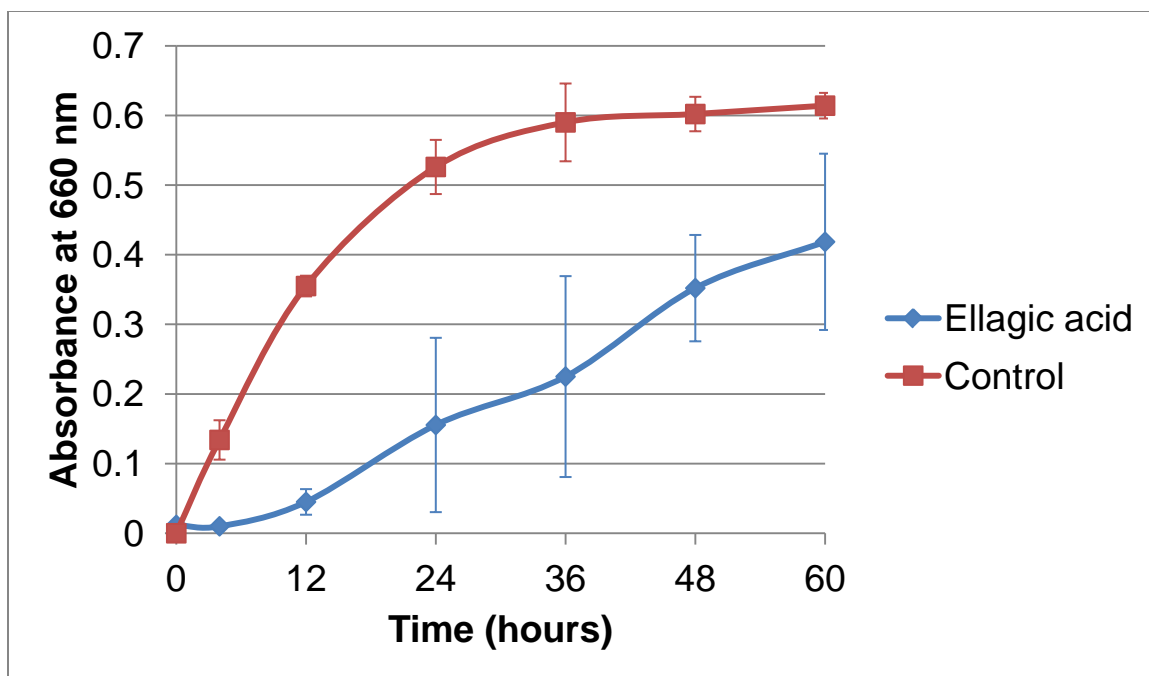
**Figure 5.24.** The average antimicrobial activity of ellagic acid at MIC of 5 ppm against *L. monocytogenes*, ATCC 7644, *L. monocytogenes*, UK ADL. and *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .



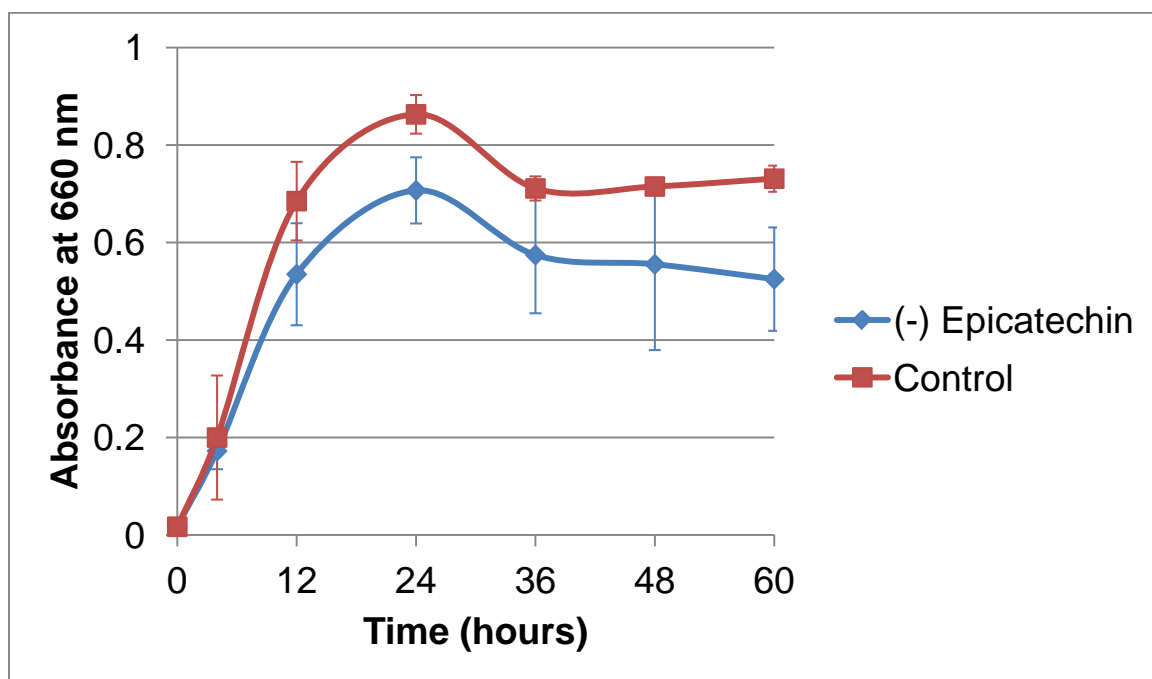
**Figure 5.25.** The antimicrobial activity of ellagic acid at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



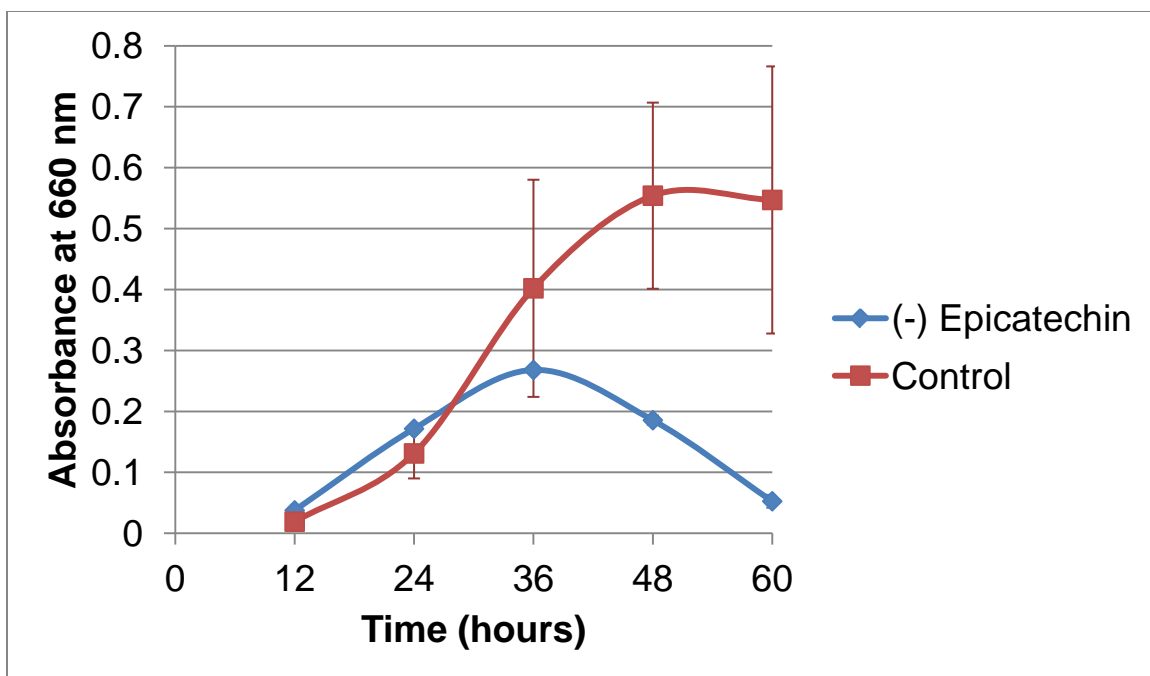
**Figure 5.26.** The antimicrobial activity of ellagic acid at MIC of 20 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



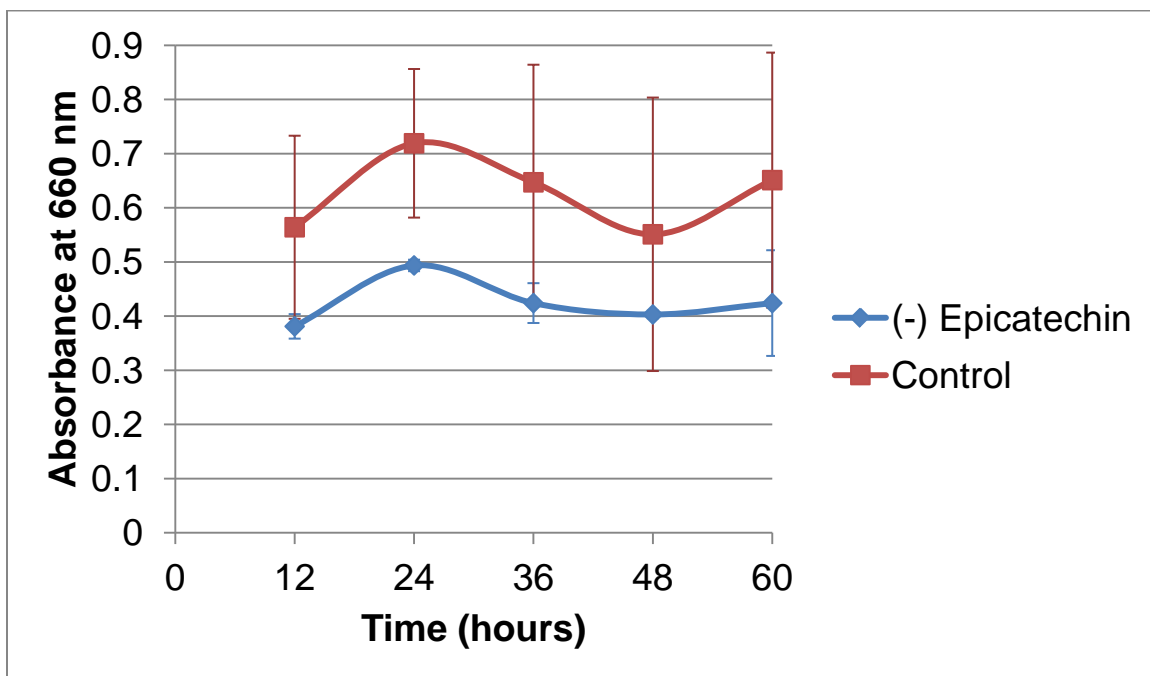
**Figure 5.27.** The antimicrobial activity of ellagic acid at MIC of 10 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



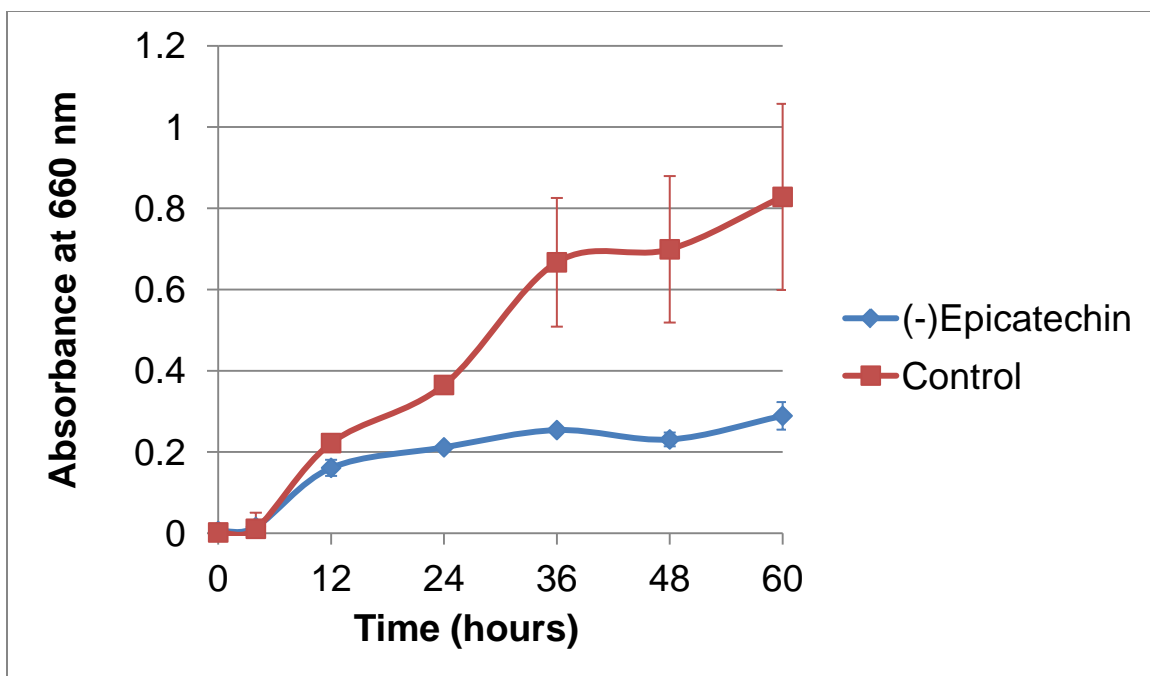
**Figure 5.28.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



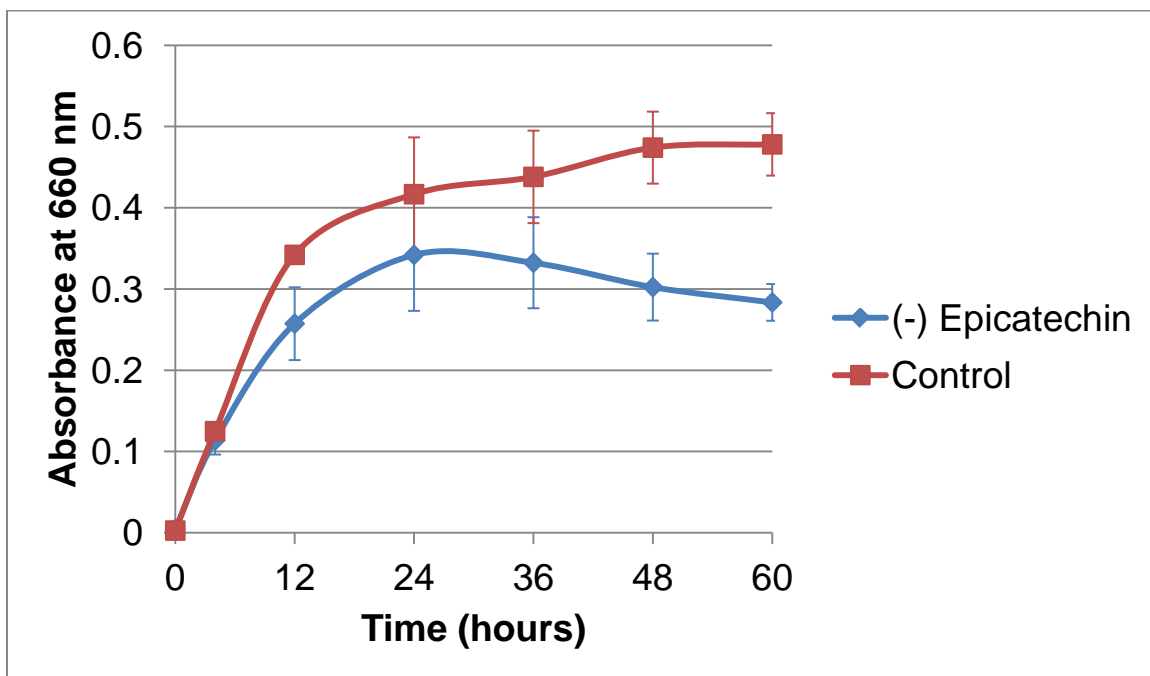
**Figure 5.29.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 5.30.** The antimicrobial activity of (-) epicatechin at MIC of 10 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .

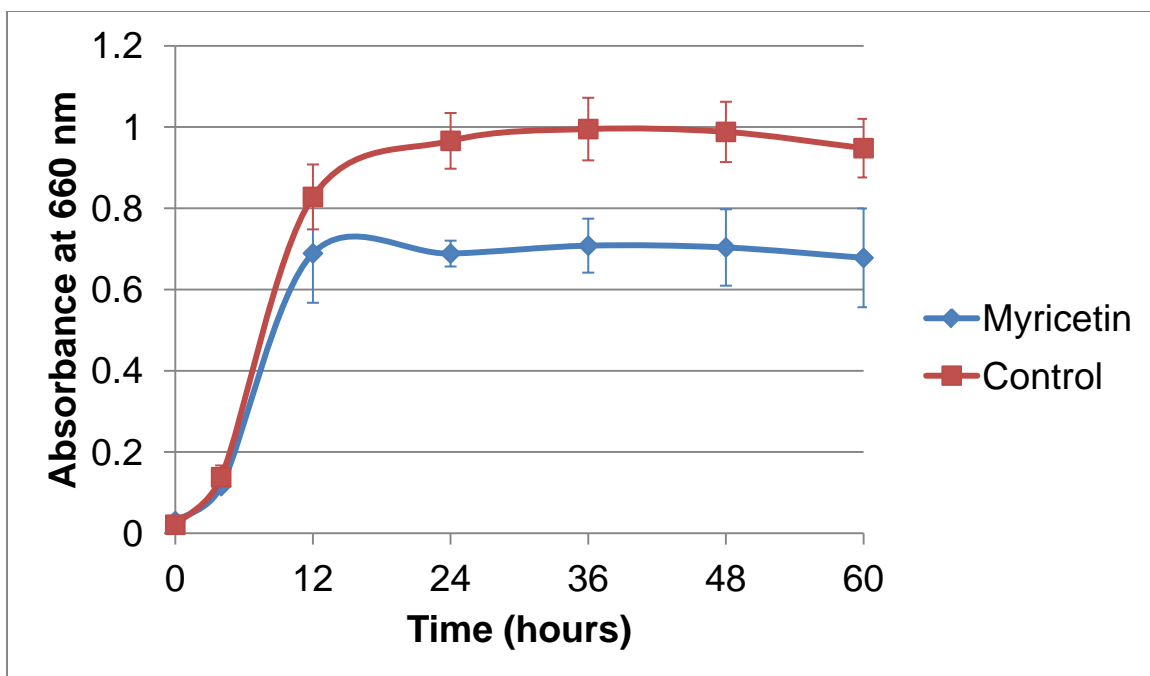


**Figure 5.31.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .

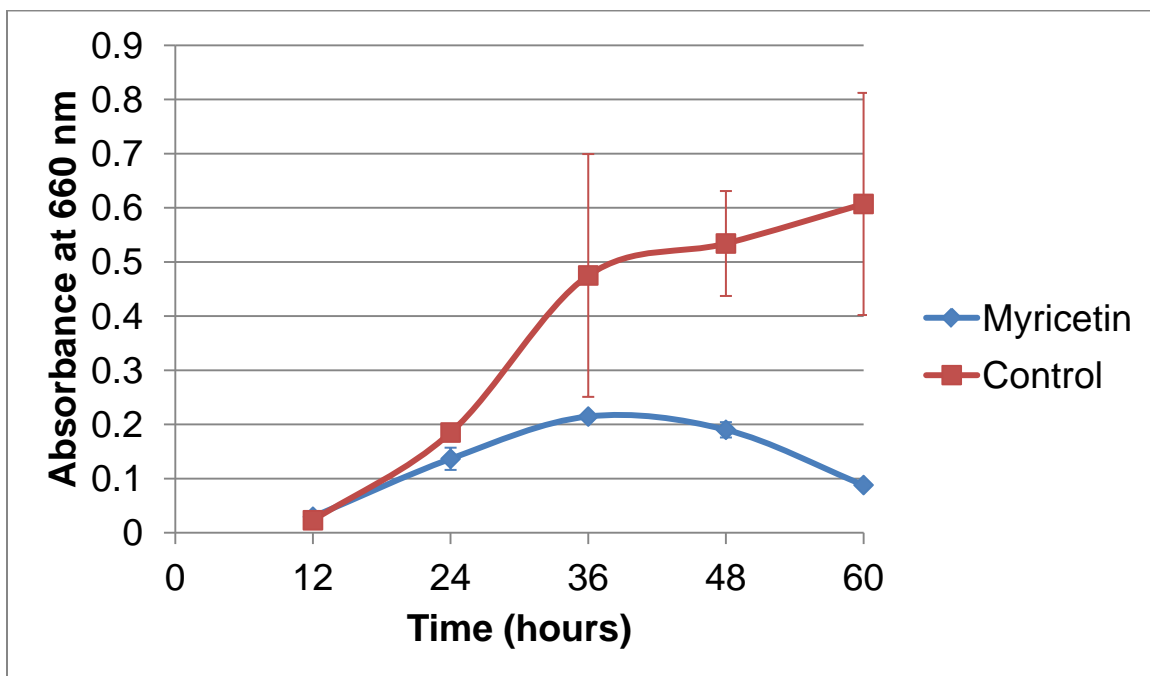


**Figure 5.32.** The average antimicrobial activity of (-) epicatechin at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A, *S. cholerasuis* subsps., ATCC 10708 and *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean,  $P < 0.05$ .

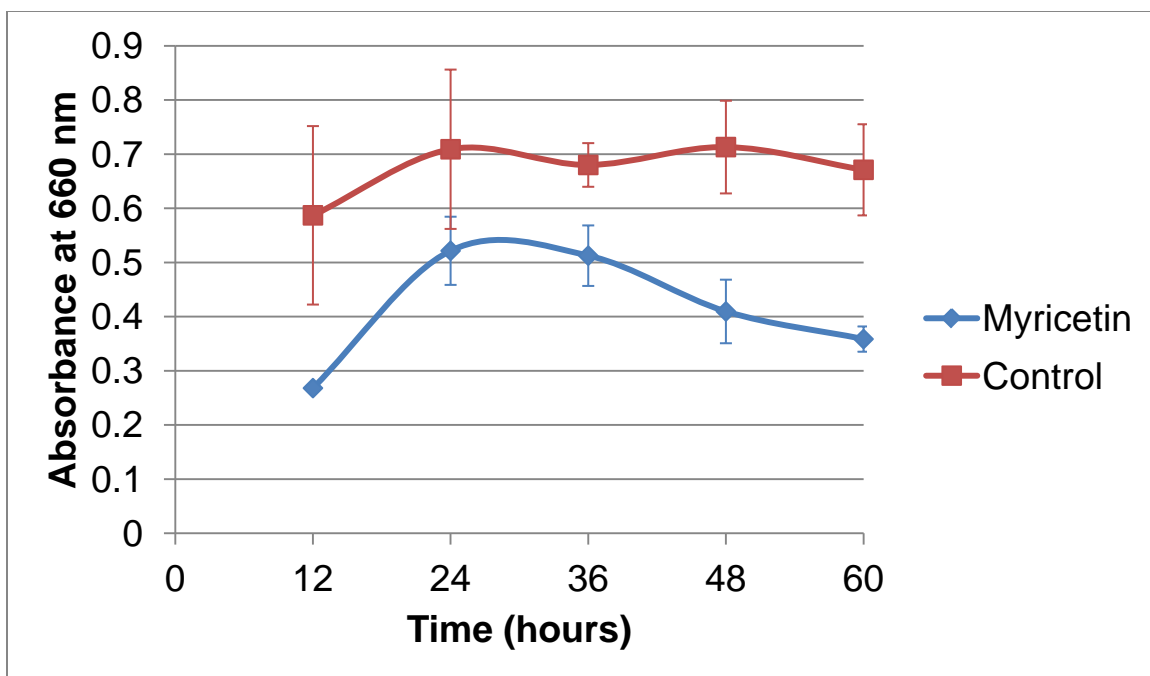




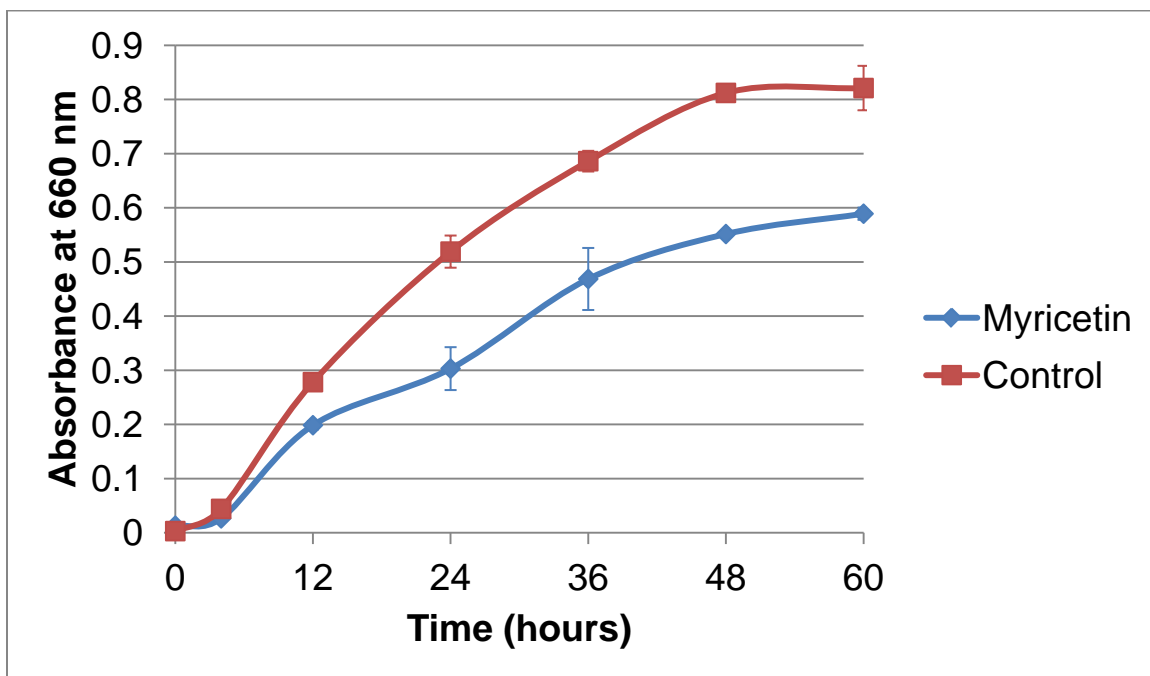
**Figure 5.33.** The antimicrobial activity of myricetin at MIC of 15 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



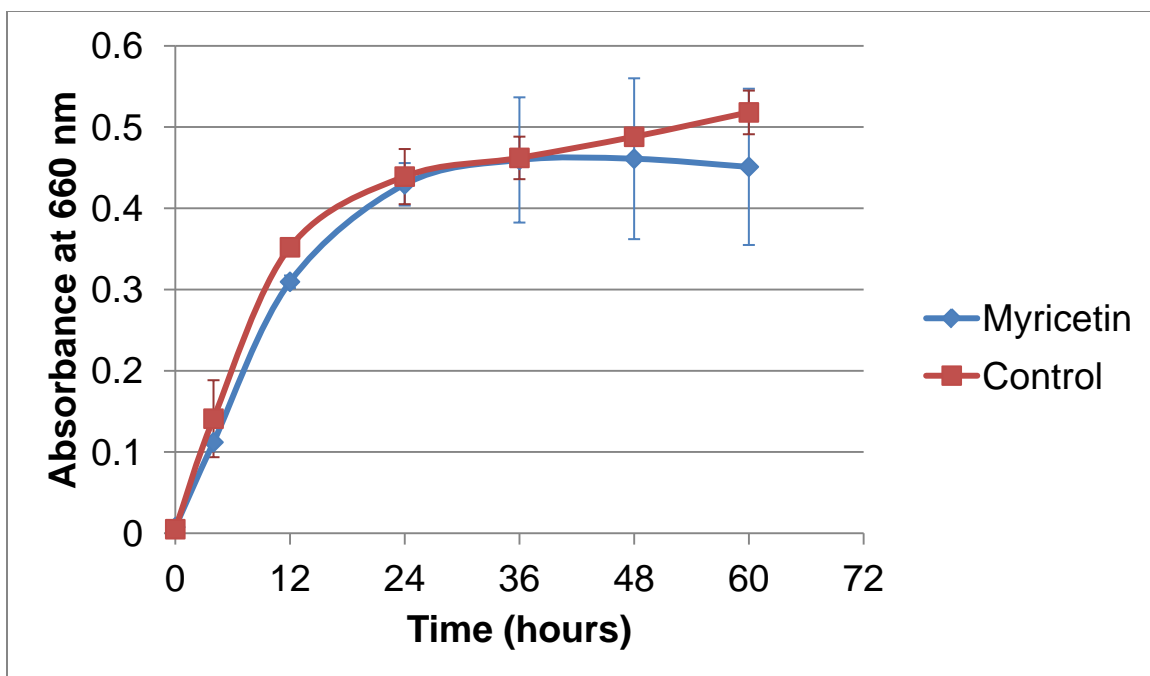
**Figure 5.34.** The antimicrobial activity of myricetin at MIC of 5 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



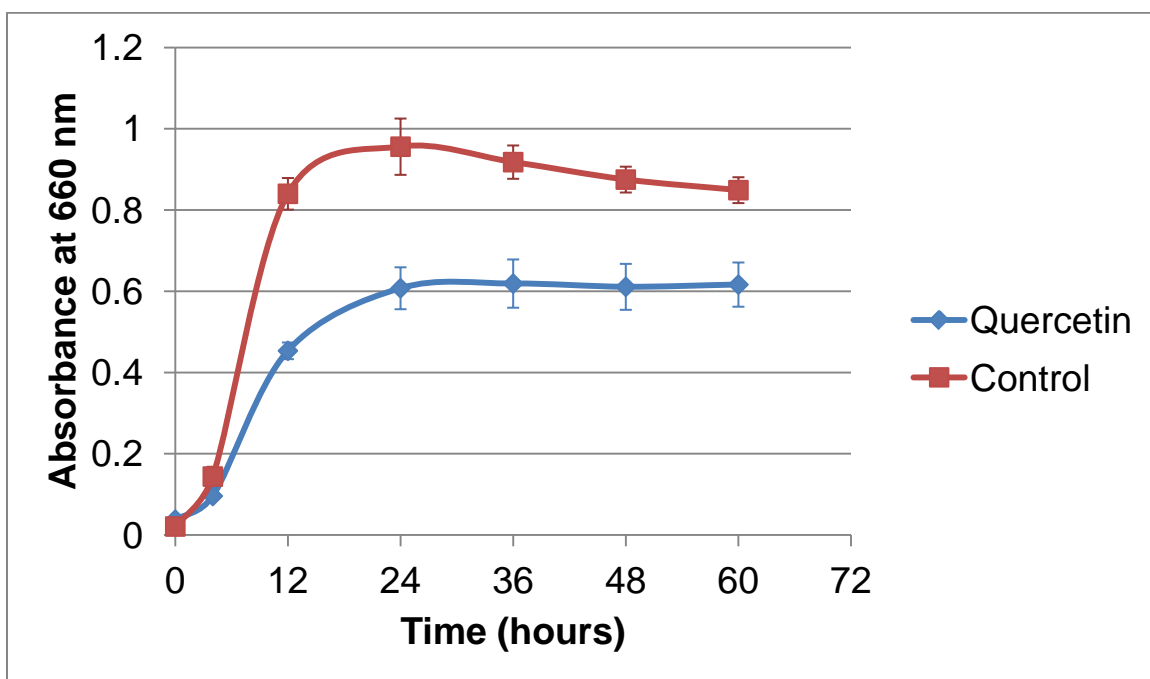
**Figure 5.35.** The antimicrobial activity of myricetin at MIC of 10 ppm against *C. sporogenes*, ATCC 8955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



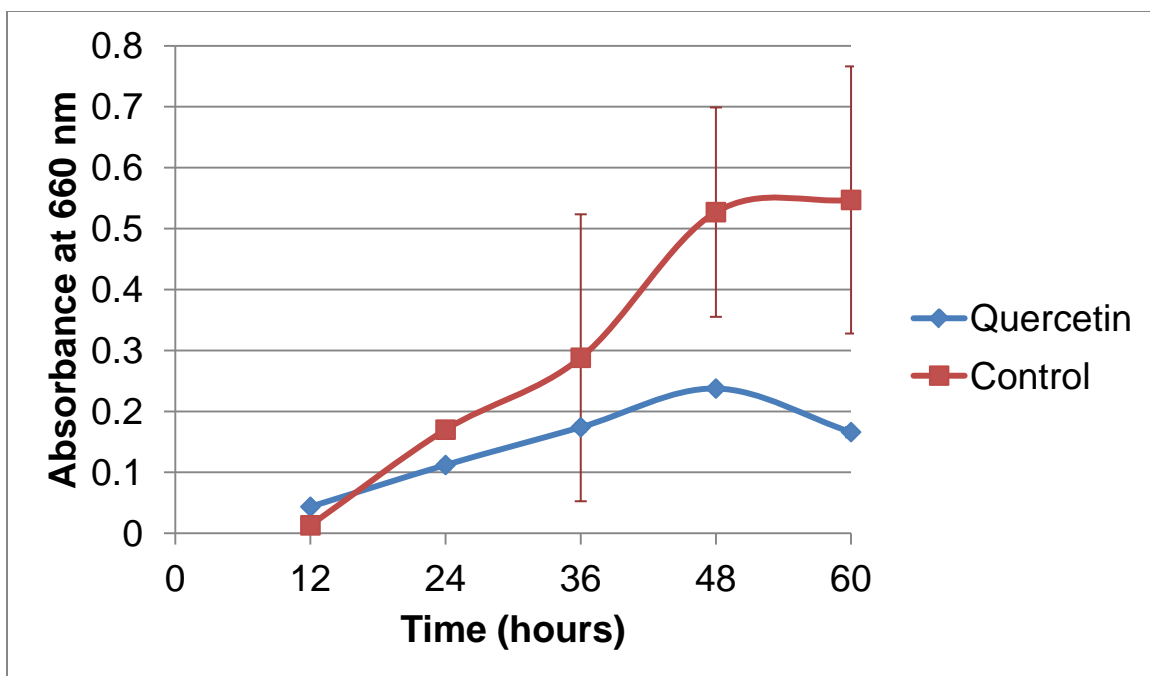
**Figure 5.36.** The antimicrobial activity of myricetin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



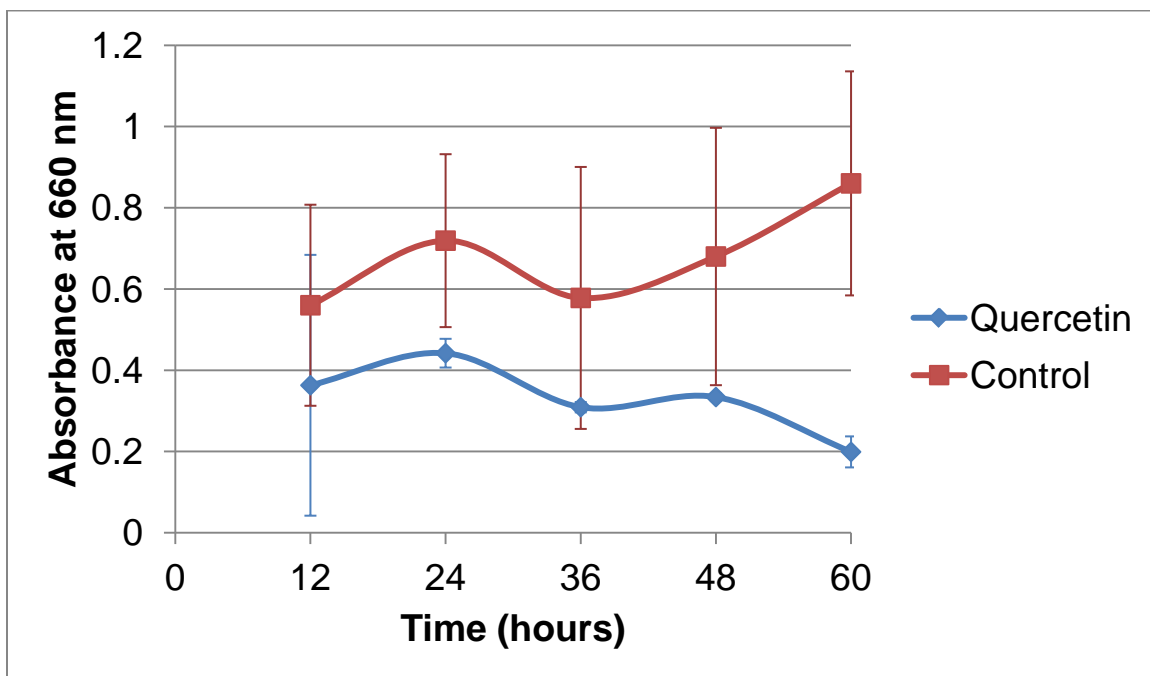
**Figure 5.37.** The antimicrobial activity of myricetin at MIC of 15 ppm against *S. cholerae subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



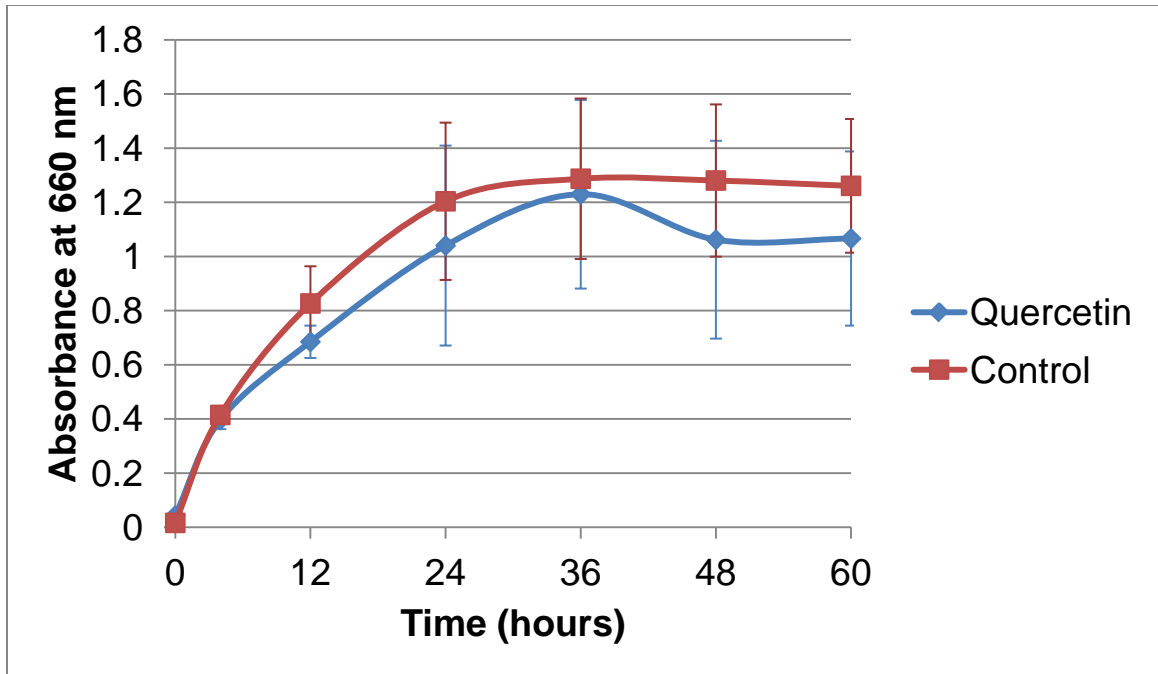
**Figure 5.38.** The antimicrobial activity of quercetin at MIC of 10 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



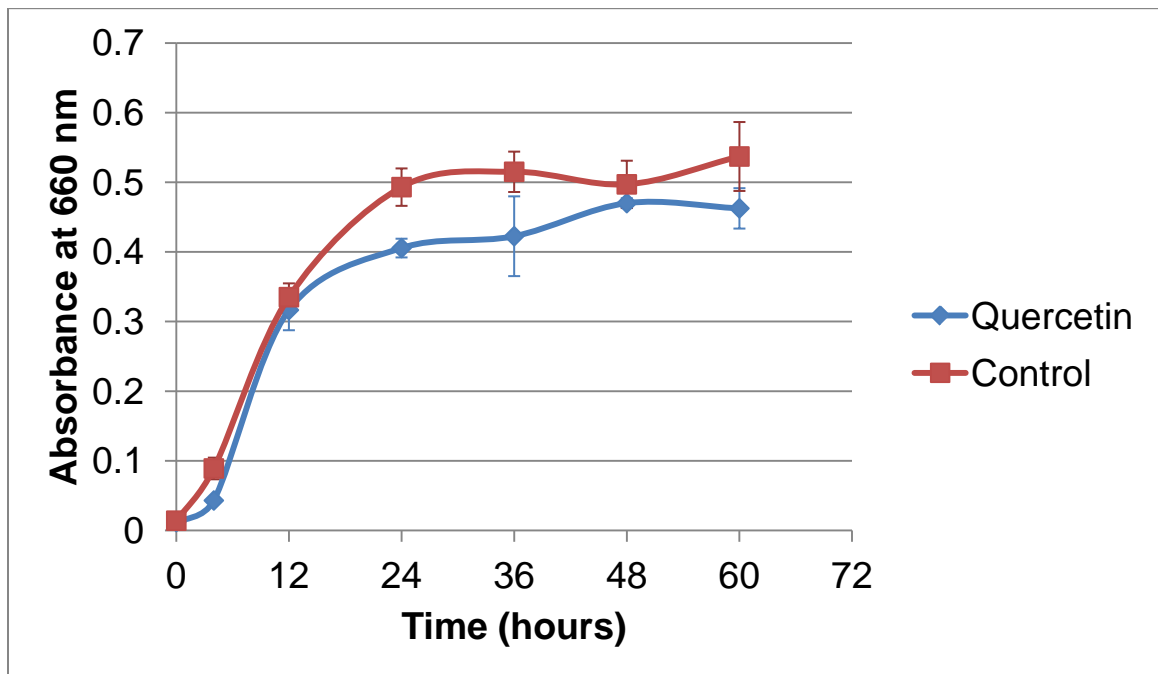
**Figure 5.39.** The antimicrobial activity of quercetin at MIC of 15 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P<0.05$ .



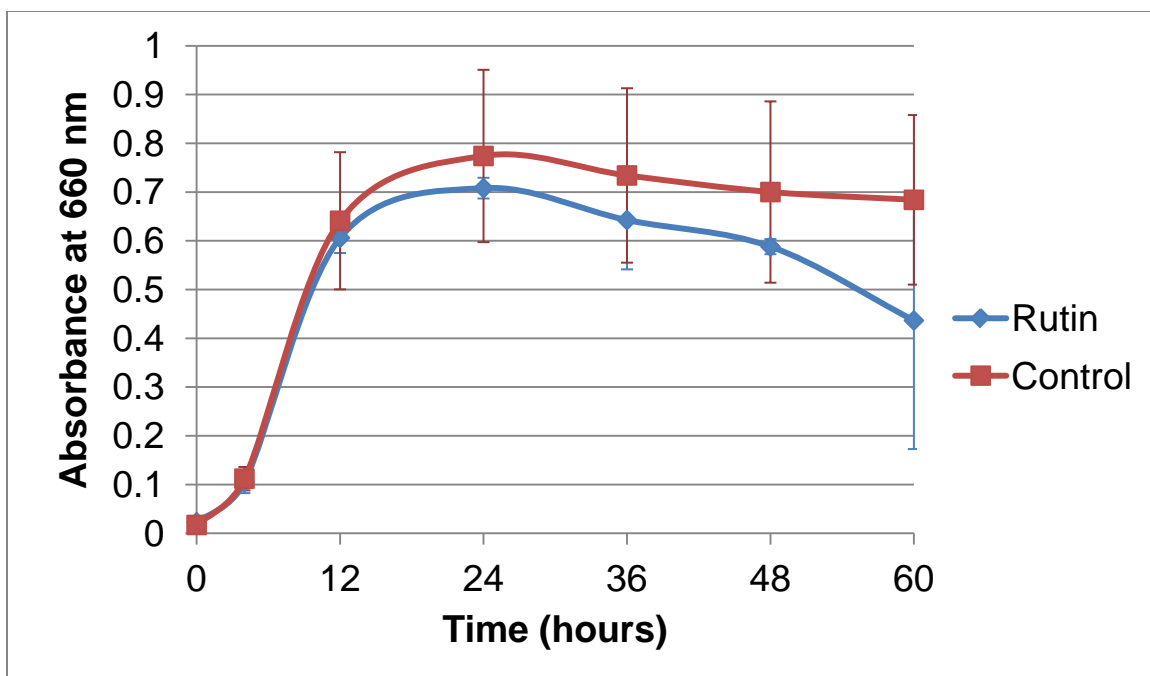
**Figure 5.40.** The antimicrobial activity of quercetin at MIC of 15 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P<0.05$ .



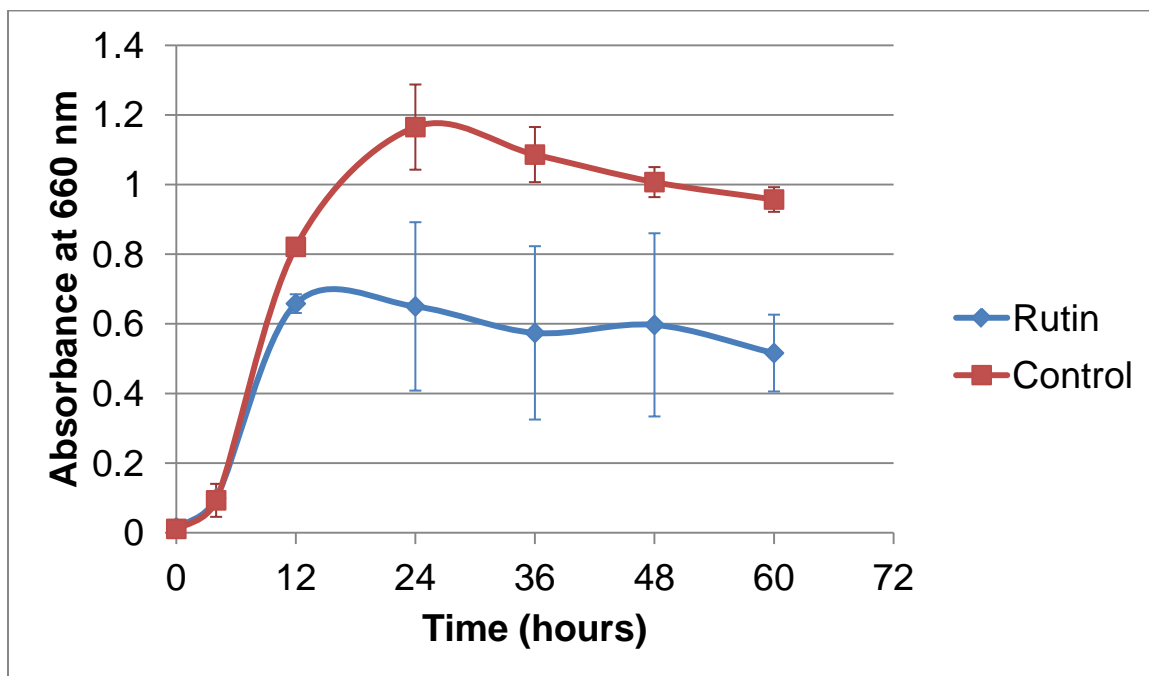
**Figure 5.41.** The average antimicrobial activity of quercetin at MIC of 5 ppm against *E. coli*, FTJ, *E. coli* O157:H7, ATCC 43895, and *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



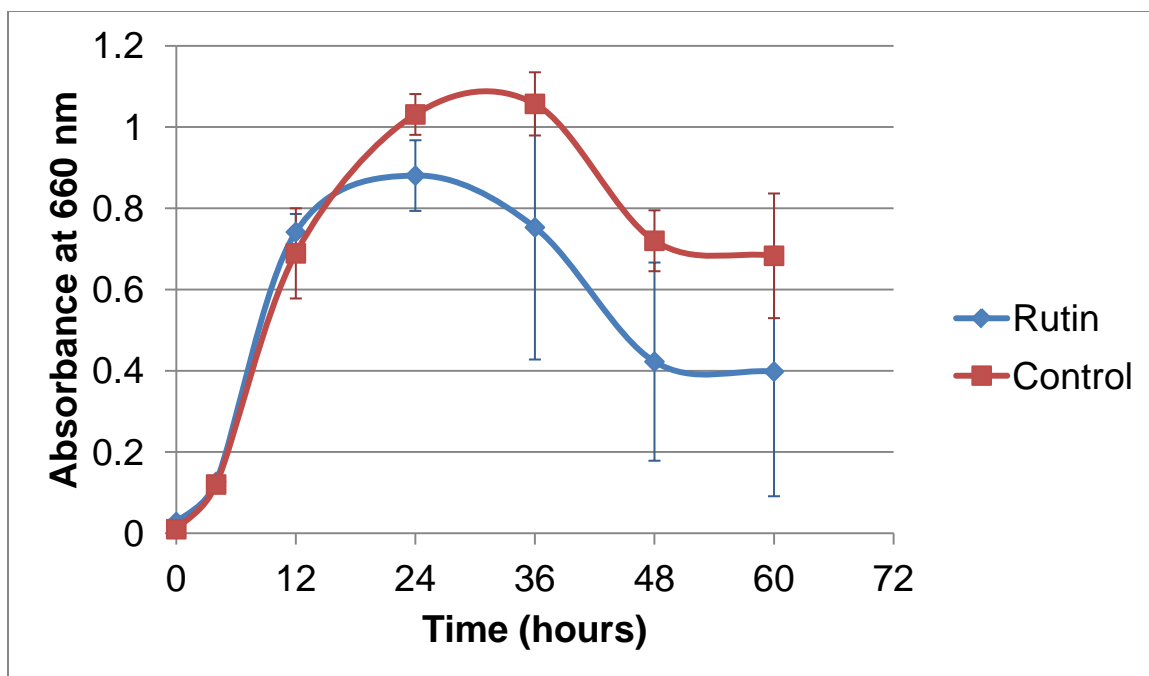
**Figure 5.42.** The antimicrobial activity of quercetin at MIC of 15 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



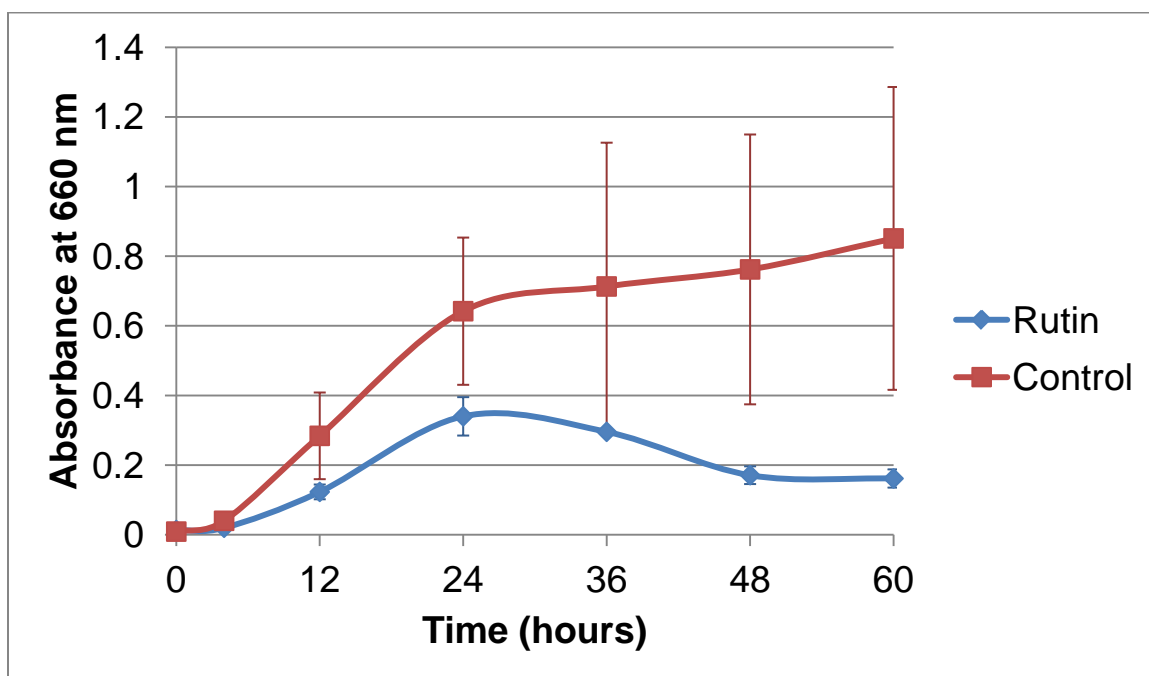
**Figure 5.43.** The antimicrobial activity of rutin at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



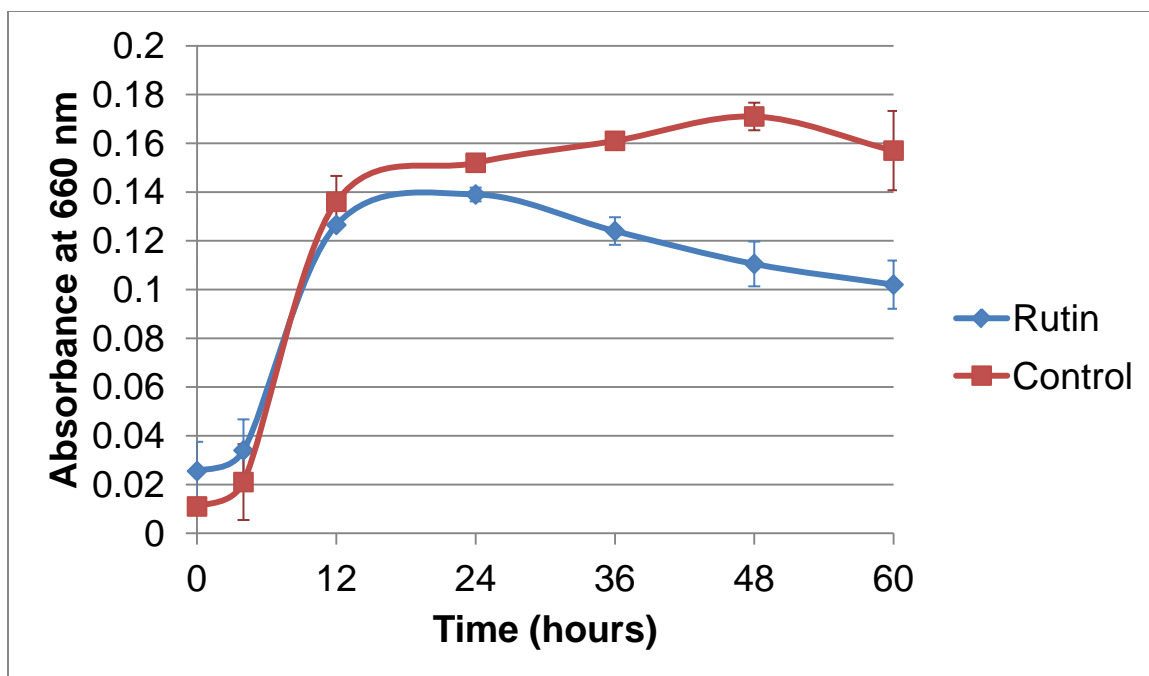
**Figure 5.44.** The antimicrobial activity of rutin at MIC of 5 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



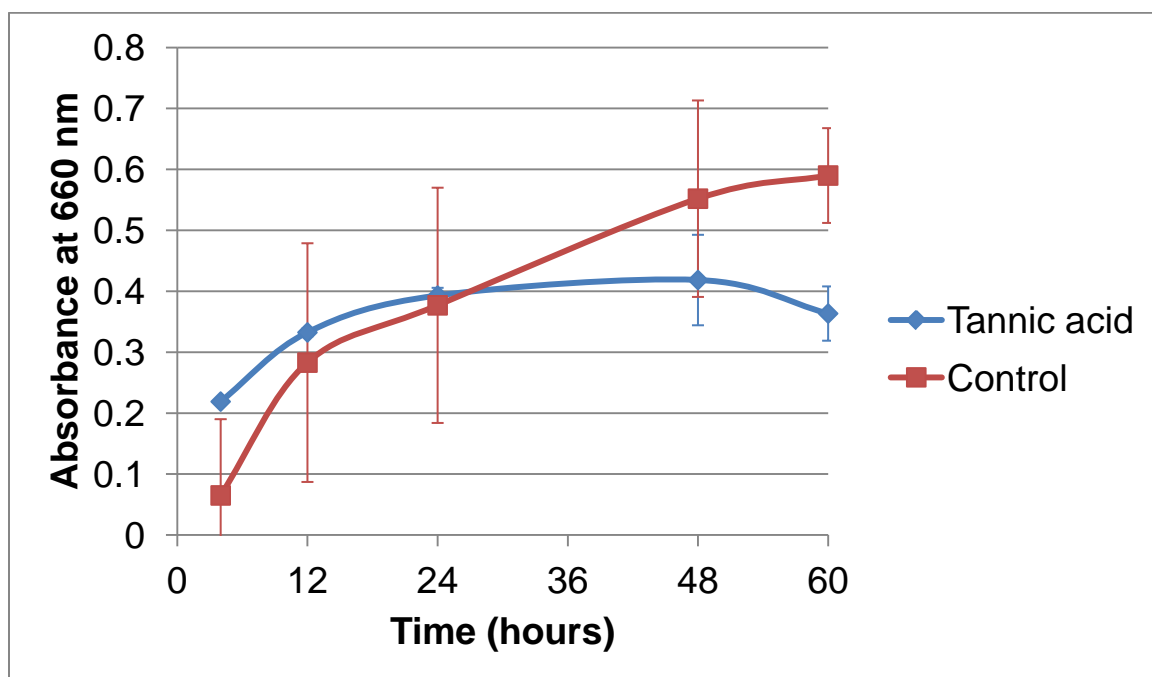
**Figure 5.45.** The antimicrobial activity of rutin at MIC of 10 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 5.46.** The antimicrobial activity of rutin at MIC of 5 ppm against *L. monocytogenes*, ATCC 7644. Error bars represent standard deviation from the mean,  $P < 0.05$ .

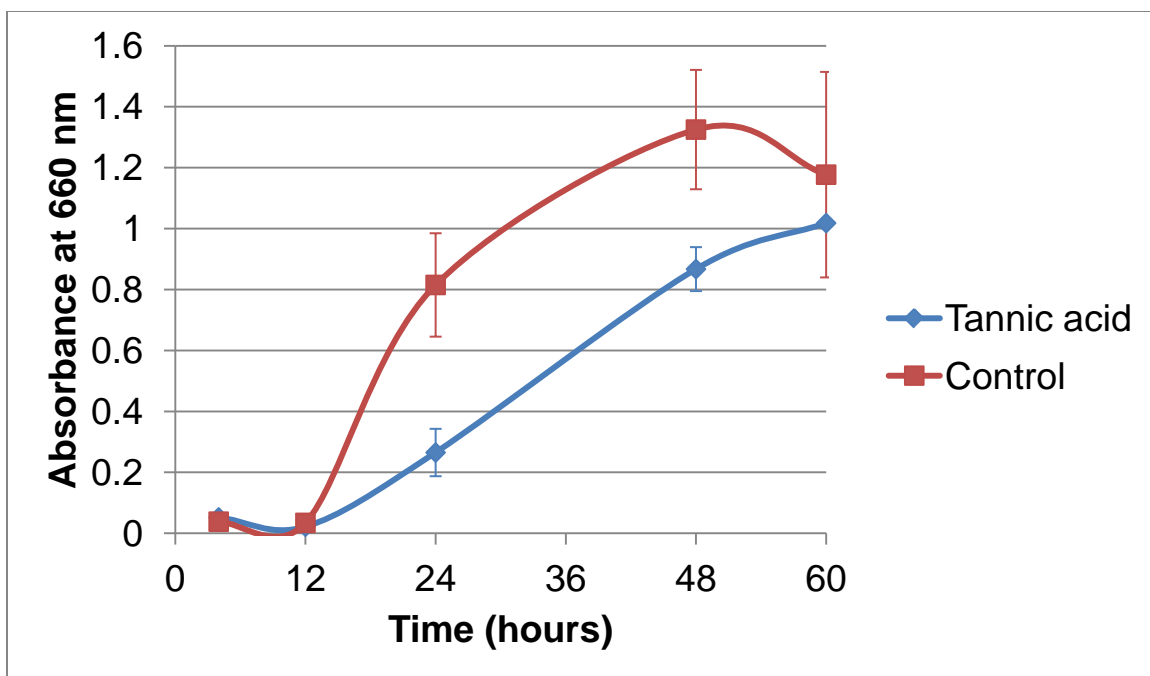


**Figure 5.47.** The antimicrobial activity of rutin at MIC of 10 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .

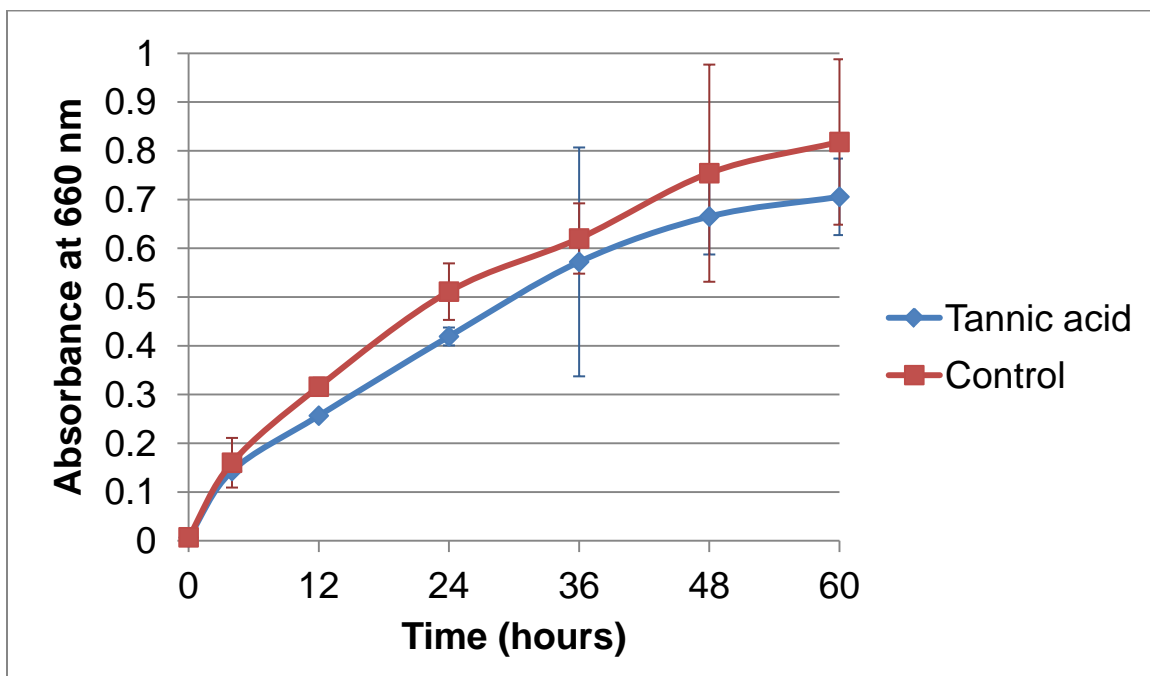


**Figure 5.48.** The antimicrobial activity of tannic acid at MIC of 20 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .





**Figure 5.49.** The antimicrobial activity of tannic acid at MIC of 20 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 5.50.** The antimicrobial activity of tannic acid at MIC of 20 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .

## CHAPTER 6

### STRUCTURAL CHANGES OF ANTIMICROBIAL TREATED AND NON-TREATED PATHOGENIC BACTERIA

#### 6.1 Introduction

Bacteria are present everywhere: in the soil, deep in the rocks, in all bodies of water, in the atmosphere, including the clouds, and also on and inside other living organisms. Their effects on higher life forms are known for only a limited number of bacterial species. Some are harmful - pathogenic – causing diseases in plants or in animals including humans, and some are useful to humans either as “probiotic” bacteria protecting health (Hassan and others 2003), or industrially by participating in the production of various commodities. The effects of most bacteria are not known. A new trend is in progress (Sachs 2008) not to exterminate harmful bacteria in human and animal environments but to replace them with beneficial ones. Bacteria which are genetically programmed to die after their mission has been accomplished in humans or animals are part of the new trend. These minute microorganisms have far reaching macroscopic consequences. They are of interest to agriculture because they may harm or improve the production of foods, either animal or plant, and thus affect human civilization.

Electron microscopy is one of a limited number of techniques suitable to show bacteria in great detail in their natural environment. There are two basic modes, scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and each of them uses specially designed microscopes. SEM is relatively easy to perform and the results are easy to interpret, because the specimens are shown as three-dimensional objects. TEM produces different kinds of images based on the fact that the electron beam passes through the specimen and forms a shadow-like image on a fluorescent screen which is photographed (Kalab and others 2008). The term Scanning Electron Microscope (SEM) is used to describe a microscope used to create high magnification, black and white images of devices with dimensions from a few tenths of a micron to several millimeters in diameter. SEM is also used to describe the photographs taken by the Scanning Electron

Microscope. Hence, SEM refers both to the microscope, and the photograph created by the microscope.

Images created with traditional optical microscopes have only a small area that is in focus. They can create quality images of structures that are flat, but can not create good images of three dimensional structures because of the focus issues. Scanning Electron Microscopes, on the other hand, have a very large depth of field. They can image complex three dimensional structures, with all parts of the device being imaged being in focus. This large depth of field results in stunning, sharp photographs of very small structures.

SEM also consists of different techniques:

1. Conventional SEM: It is used to visualize bacteria which had been fixed, dehydrated, and dried.
2. Environmental SEM (ESEM): It makes it possible to examine hydrated specimens by not exposing them to high vacuum.

A sophisticated technical design keeps the specimen at a temperature several degrees above the freezing point of water in a small area inside the microscope where a low partial pressure of water vapor provides ions at a concentration sufficient to neutralize electrons and thus to prevent charging artifacts (Kaláb and others 1995).

A scanning electron microscope operates by using a beam of electrons to image extremely small structures. The electron beam is created by a cathode typically made of tungsten. An electric field is used to accelerate the electron beam towards the cathode, where the structure to be imaged is located. Coils or deflector plates are used to scan the beam across the sample to be imaged. The resolution that can be achieved depends on the spot size of the electron beam. The smaller the beam, the better the resolution can be achieved. In order to achieve proper creation, control, and detection of the electron beam, the imaging must be done in a vacuum (Bacteria world 2011). In addition, in order to get good contrast and resolution, the surface of the device to be imaged must be very conductive.

In principle, the preparation for SEM consists of isolating the bacteria or trimming the specimen where they are present, fixing them, dehydrating in ethanol, critical-point drying, mounting on an SEM stub, sputter-coating with conductive material (gold, carbon and etc.), and recording images at an appropriate accelerating voltage. Dehydration and critical-point drying may disturb bacterial flagella. Sputter coating the fixed and dried bacteria with a 20 nm thick layer of conductive material obscures fine structures such as pili and fimbriae. However, such structures may be visualized by TEM using negative staining or shadowing with platinum (Kalab and others 2008). Glutaraldehyde is a commonly used preservative for biological tissues, and it is used in preparation for a wide variety of imaging procedures. Glutaraldehyde is an alkylating agent, killing organisms and producing a much tougher cell that will resist lysis for extended time periods. It can also be used as a buffer solution to maintain pH and osmotic conditions (Lohnes and Demirel 1978). For conventional SEM imaging of a non-conductive surface, a metallic coating is required to prevent uncontrolled charging and sample damage (Goldstein and others 1992). After being treated with phenolic compounds, pathogenic bacteria were observed to have morphological changes including deformity in cell walls, cytoplasmic membrane and loss of integrity in cells (Filipowicz and others 2003; Packiyasothy and Kyle 2002; de Billerbeck and others 2001).

The purpose of this study was to confirm the antimicrobial activity of natural phenolic compounds against pathogenic bacteria including both Gram-positives (*B. subtilis*, *B. cereus*, *B. polymyxa*, *L. monocytogenes* spp., *C. perfringens*, *C. sporogenes*, and *C. butyricum*) and Gram-negatives (non-pathogenic *E. coli*, *E. coli* O157:H7, *S. paratyphi*, *S. choleraesuis subsp.*, and *S. enteridis*). Scanning electron microscopy (SEM) was conducted to get the images of morphological damages in selected pathogenic bacteria.

## **6.2 Results and Discussion**

Treated samples of pathogenic bacteria using relevant MICs for each, were incubated for 24 hours at appropriate incubation temperature and conditions. They were then, observed

by SEM to investigate the morphological changes in the appearance of the cells. SEM observations confirmed the physical damage and considerable morphological alteration to all tested Gram-positive and Gram-negative pathogenic bacteria treated with the natural phenolic compounds.

These images directly illustrate the destructive effects of the phenolics on the tested bacteria. Non-treated cells (control) were intact and showed a smooth surface (Figure 6.1; A1, B1 and Figure 6.2; C1, D1) while bacterial cells treated with the phenolics underwent considerable damage (Figure 6.1; A2-A6, B2-B6 and Figure 6.2; C2-C6, D2-D6). Although the samples were not prepared quantitatively, it was clearly observed that the number of the damaged cells was significantly greater in the treatments than in the control.

There are many possible explanations for the observations. The literature suggests that the active components of the plant extracts might bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes (Shan and others 2007a). The effects might include the inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. Uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides might follow (Farag and others 1989; Kim and others 1995a).

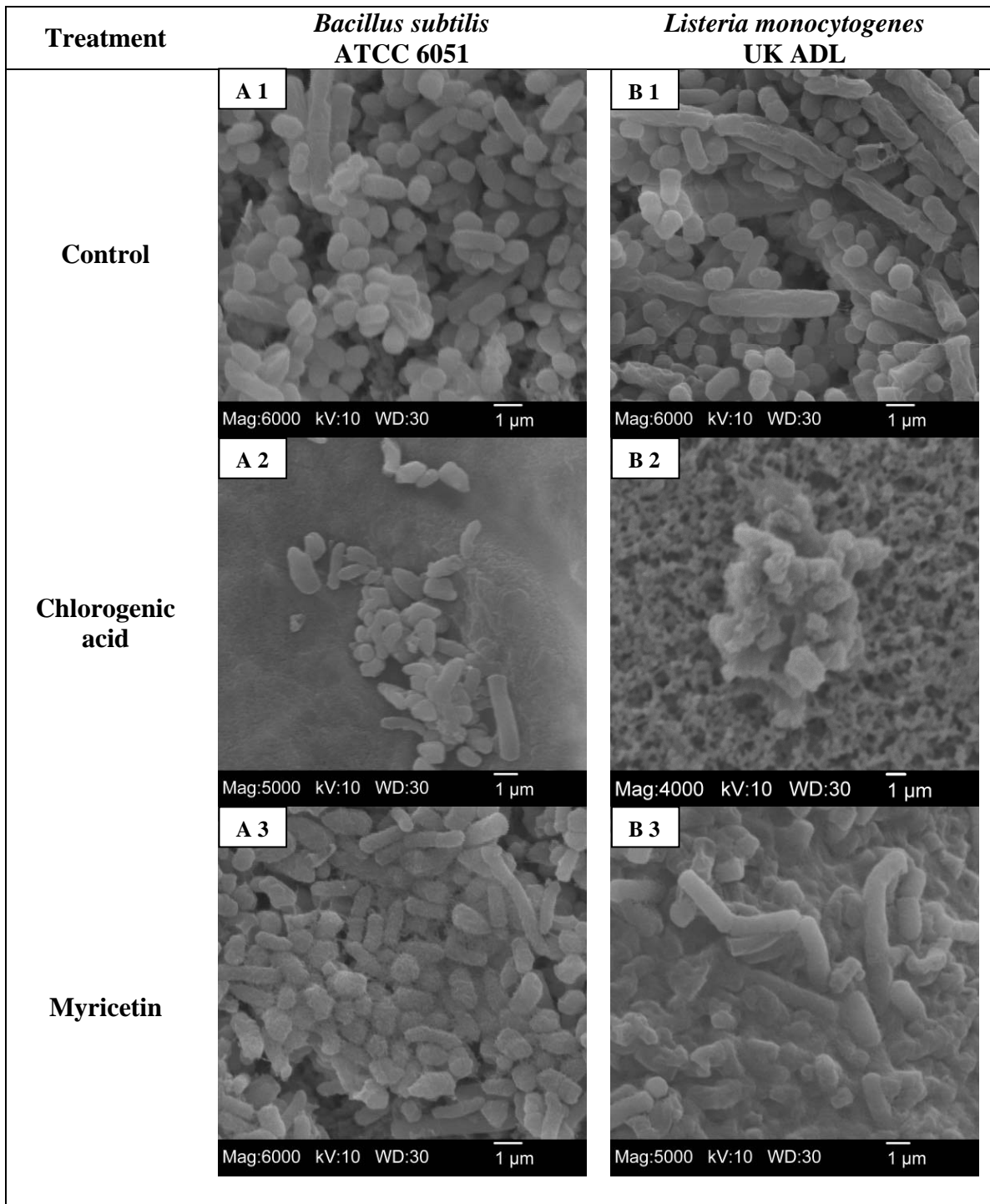
The SEM images show that some cells present damage as pores or deformity in the cell walls (Figure 6.1; A6, B2, B4 and Figure 6.2; C4 and D3). Some authors have suggested that the damage to the cell wall and cytoplasmic membrane was the loss of structural integrity and the ability of the membrane to act as a permeability barrier (de Billerbeck and others 2001; Packiyasothy and Kyle 2002; Filipowicz and others 2003). Most of the cells were observed to get clustered and stick to each other (Figure 6.1; A2, A3, A4, A5, A6, B2, B4, B5, and Figure 6.2; C2, C3, C4, C5, C6 and D2, D3, D5, D6). The distortion of the cell physical structure would cause the expansion and destabilization of the membrane and would increase membrane fluidity, which in turn would increase passive

permeability (Ultee and others 2002) and manifest itself as a leakage of various vital intracellular constituents, such as ions, ATP, nucleic acids, and amino acids (Kim and others 1995b; Cox and others 1998; Helander and others 1998). Most of the Gram-negative cells (Figure 6.2; C2, C3, C4, C5, C6, D2, D5, D6) and some of the Gram-positive cells (Figure 6.1; A2, A5, A6, B2, B3, B4) appeared to be shrunk and even some were empty, and the remains were flaccid. Furthermore, most of the Gram-negative cells (Figure 6.2; C2, C5, C6, D3, D5, D6) and two of the *L. monocytogenes* cells (Figure 6.1; B4 and B5) appeared to be stuck together and melted. These images confirm the loss of shape and integrity which was followed by the cell death. Cell death may have been the result of the extensive loss of cell contents, the exit of critical molecules and ions, or the initiation of autolytic processes (Denyer 1990).

### **6.3 Conclusion**

From all the SEM observations, it seemed that the phenolic compounds caused severe damage to the bacteria. In addition, the modes of action of bacterial agents depend on the type of microorganisms and are mainly related to their cell wall structure and to the outer membrane arrangement. This study and many previous studies (Smith and others 1998; Ceylan and others 2004; Lopez and others 2005; Shan and others 2005) indicated that the most bioactive compounds of plant extracts were more active against Gram-positive bacteria than Gram-negative bacteria. This is likely due to the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space which is not found in Gram-positive bacteria (Duffy and Power 2001). The resistance of Gram-negative bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules, and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (Gao and others 1999; Shan and others 2007). Gram-positive bacteria do not have such an outer membrane and cell wall structure.

Consequently, antibacterial substances can penetrate the bacterial cells and easily destroy the bacterial cell wall and cytoplasmic membrane and result in a leakage of the cytoplasm and its coagulation (Shan and others 2007). Eventually, these disruptions may cause the loss of cell integrity and death.



**Figure 6.1.** Scanning electron microscope observations of two selected Gram-positive pathogenic bacteria (**A1-A6**, *B. subtilis* **B1-B6**, *L. monocytogenes*) treated with the natural phenolic compounds (**1**, control; **2**, chlorogenic acid; **3**, myricetin; **4**, quercetin; **5**, thymoquinine; **6**, xanthohumol).



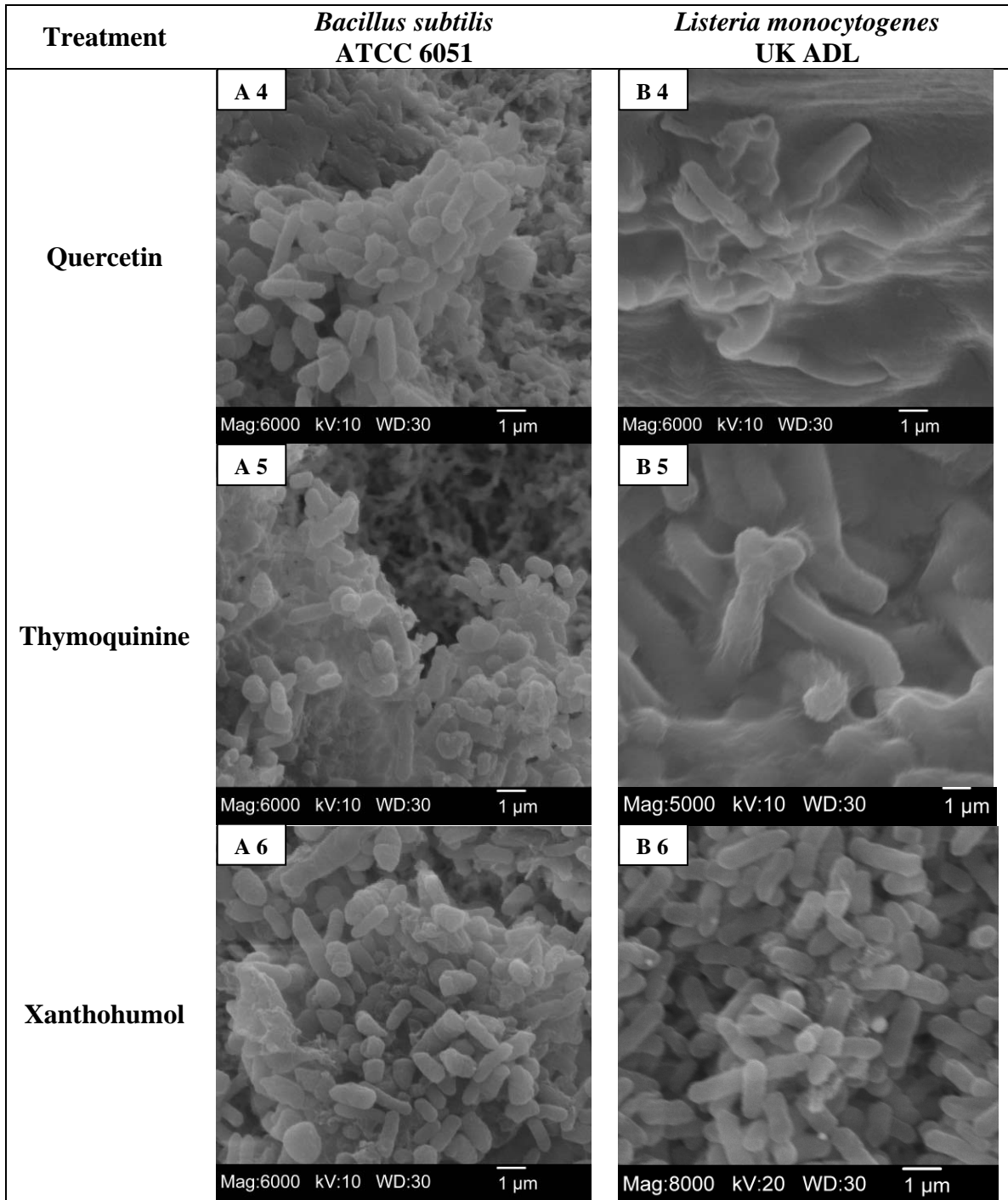
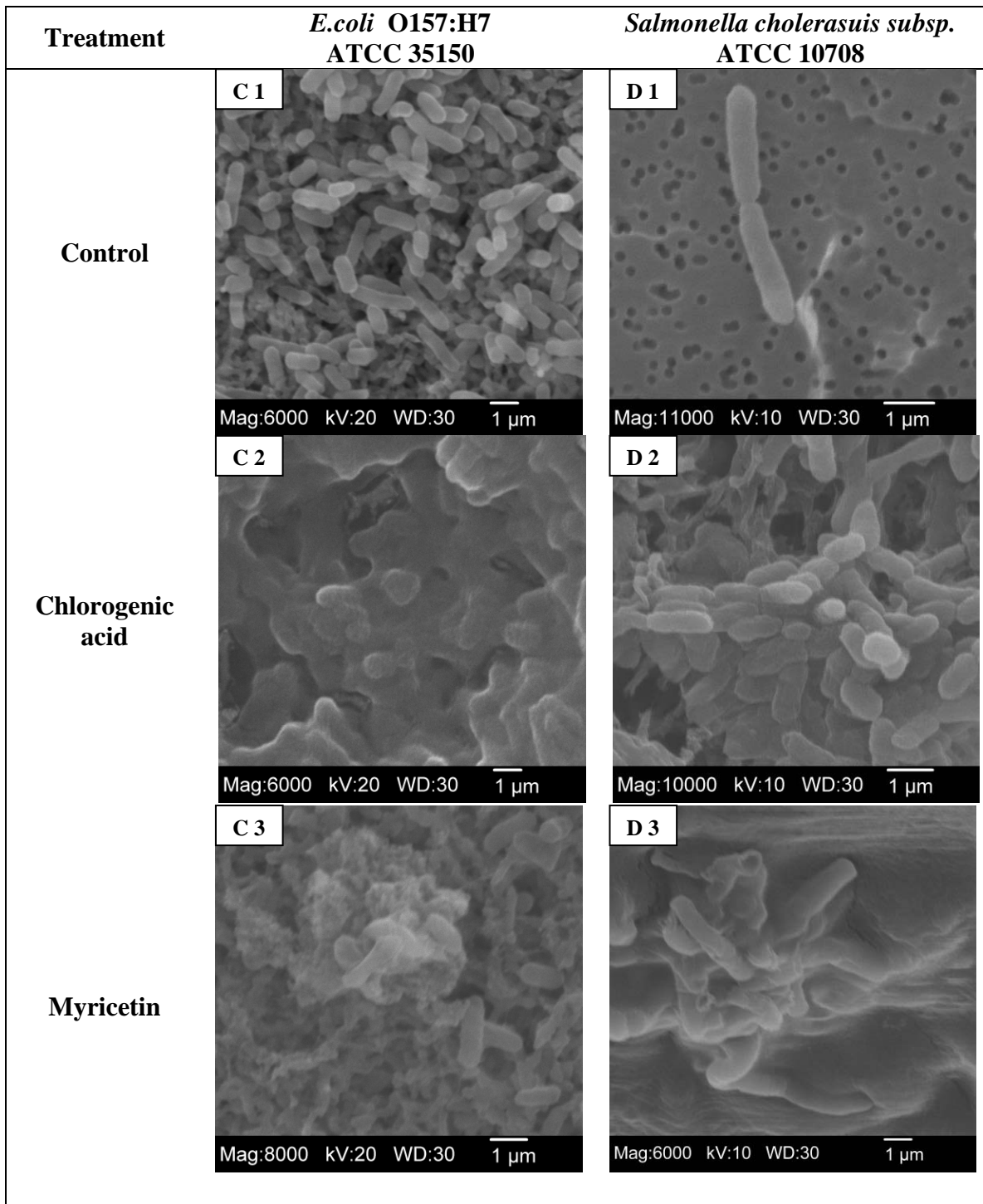


Figure 6.1. continues



**Figure 6.2.** Scanning electron microscope observations of two selected Gram-negative pathogenic bacteria (**C1-C6**, *E. coli* O 157: H7 **D1-D6**, *S. cholerasuis* subsp.) treated with the natural phenolic compounds (**1**, control; **2**, chlorogenic acid; **3**, myricetin; **4**, quercetin; **5**, thymoquinine; **6**, xanthohumol).

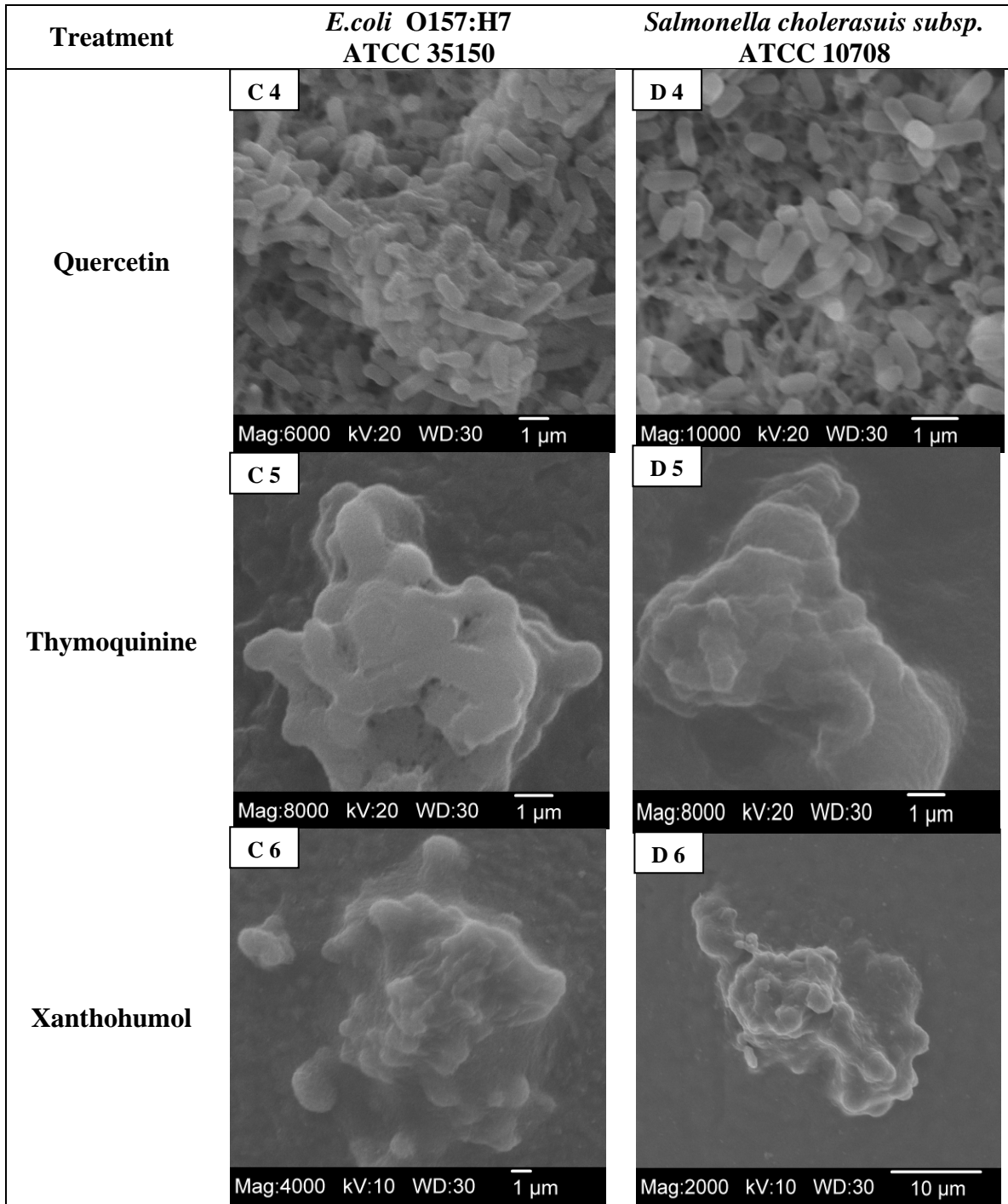


Figure 6.2. continues

## CHAPTER 7

### OVERALL CONCLUSIONS

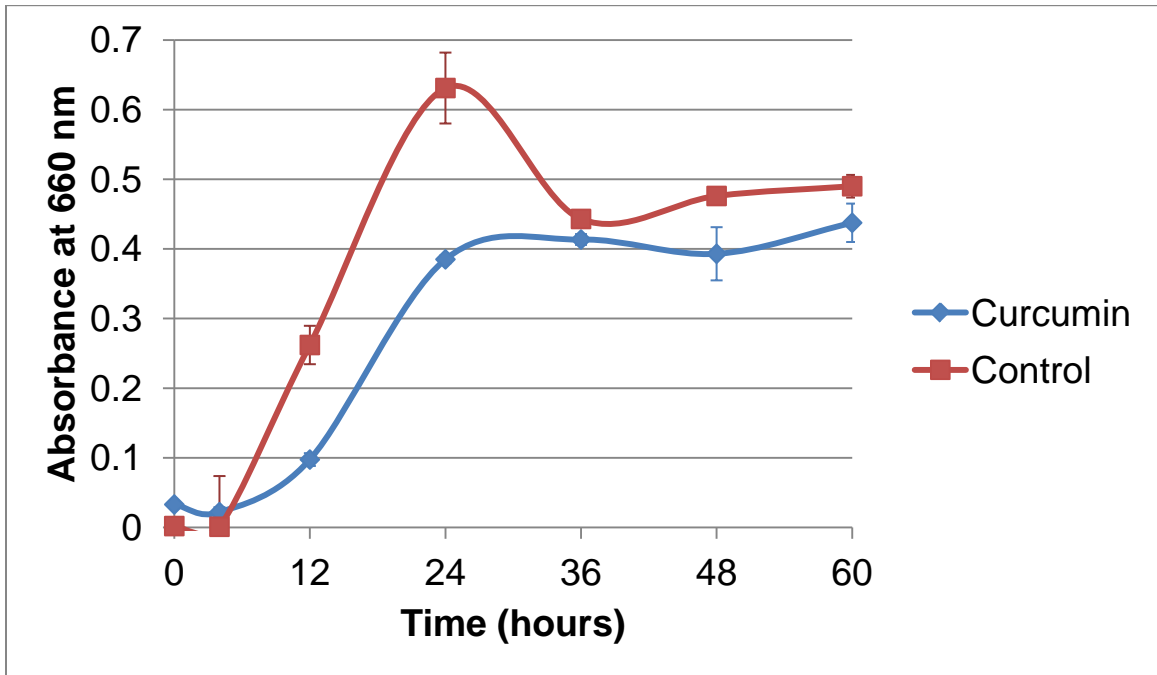
The results of these studies demonstrate that different natural antimicrobials of plant origin can effectively reduce or inhibit pathogenic and spoilage microorganisms, thus providing a good alternative to the use of traditional antimicrobials. This project also shows that chemical structure plays an important role in pathogenic bacteria's cell growth inhibition.

The natural phenolic compounds extracted from vegetables, fruits, herbs and spices have high potential to reduce and/or inhibit the growth of most common pathogenic bacteria. Generally, Gram-negative bacteria (*E. coli* and *Salmonella*) and Gram-positives (*Bacilli*, *Listeria monocytogenes*, and *Clostridium*) were observed to have different degrees of antimicrobial susceptibility. These variations may stem from the differences in cell surface structures between Gram-negative and Gram-positive bacteria. In particular, the outer membrane of Gram-negative bacteria functions as a preventive barrier against hydrophobic compounds.

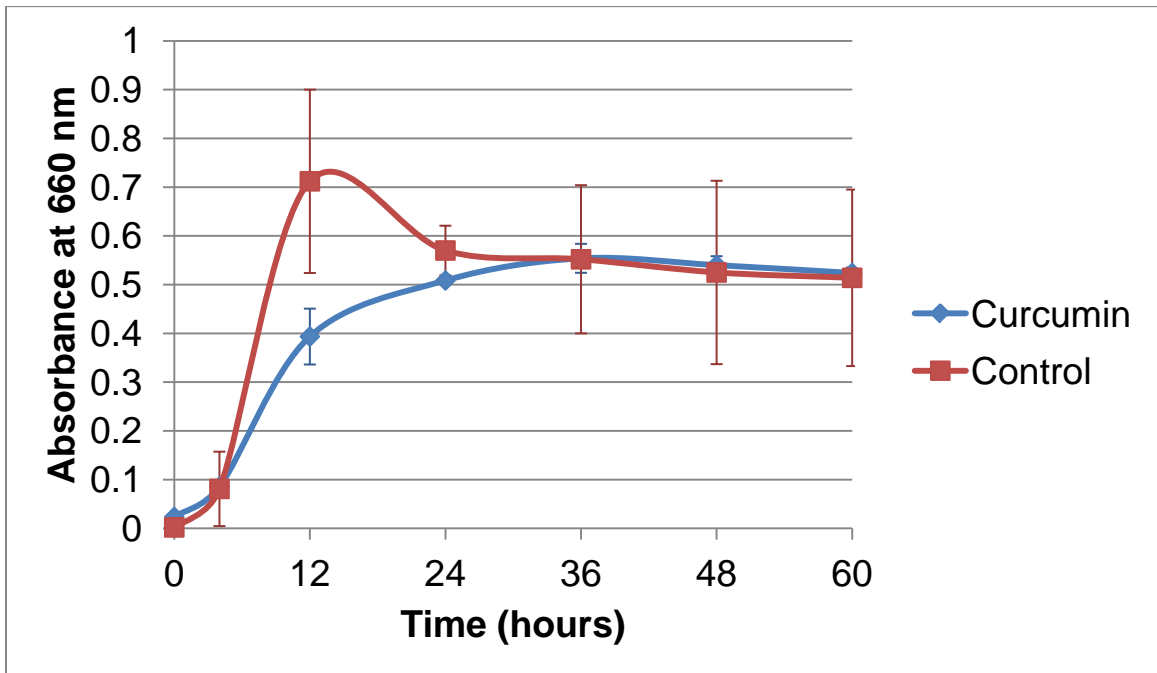
Furthermore, different strains of the same family showed varying degrees of antimicrobial susceptibility, with different concentrations of phenolic compounds. Therefore, each phenolic compound had different MIC for each bacterial strain. The results of this study suggest that the sensitivity of the pathogenic bacteria to phenolic compounds depends on bacterial species and polyphenol structure of the phenolics. The usage of plant based antimicrobials can be alternatives for chemicals used in food preservation. In fact, phenolic compounds in plant extracts have potential inhibitory activity against pathogenic bacteria. Moreover, the genetic diversity, agronomical practices, environmental conditions, and the composition of critical compounds in herb and plant extracts exhibit differences in their efficacy against foodborne pathogens. It should be kept in mind that the addition of antimicrobials to the food products without adversely affecting the sensory characteristics is still a challenge for researchers, since the concentrations that are necessary to ensure safety of food and food products are several times higher than those accepted by consumers from sensory point of view. Therefore, new studies combining the use of antimicrobials with other methodologies of

food preservation are necessary to reduce the impact of these compounds on sensory properties. Further research is also needed to better understand the impact of natural phenolic compounds on pathogens, their organoleptic properties on food and their relevant use in food applications.

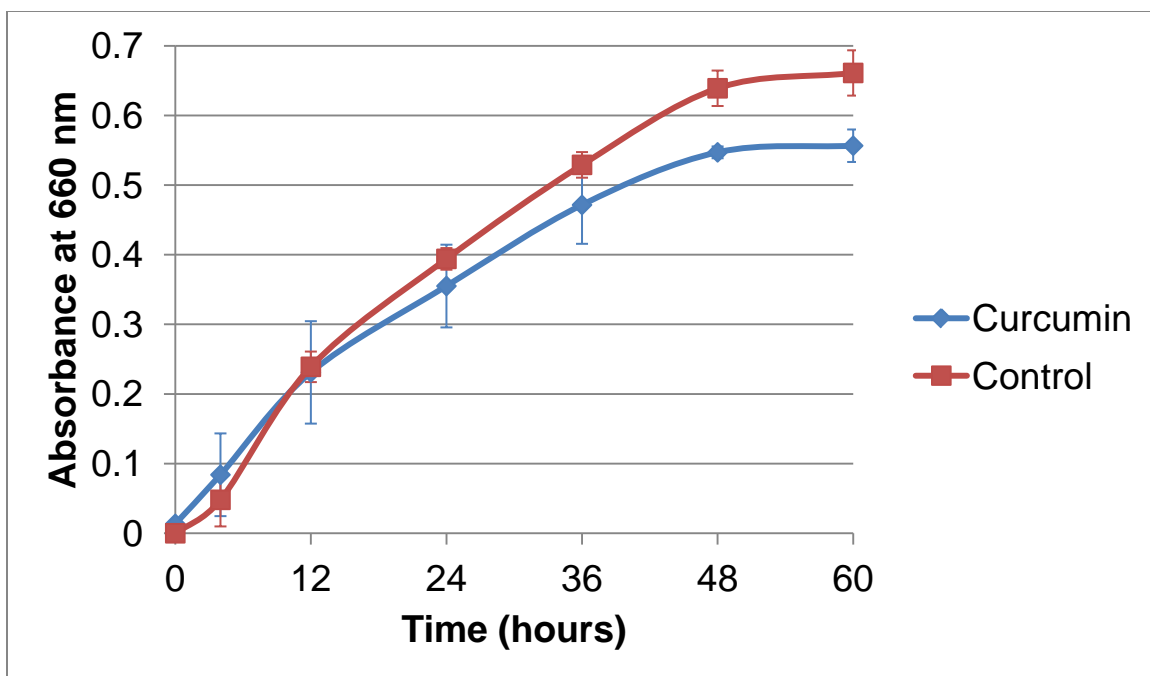
## APPENDICES



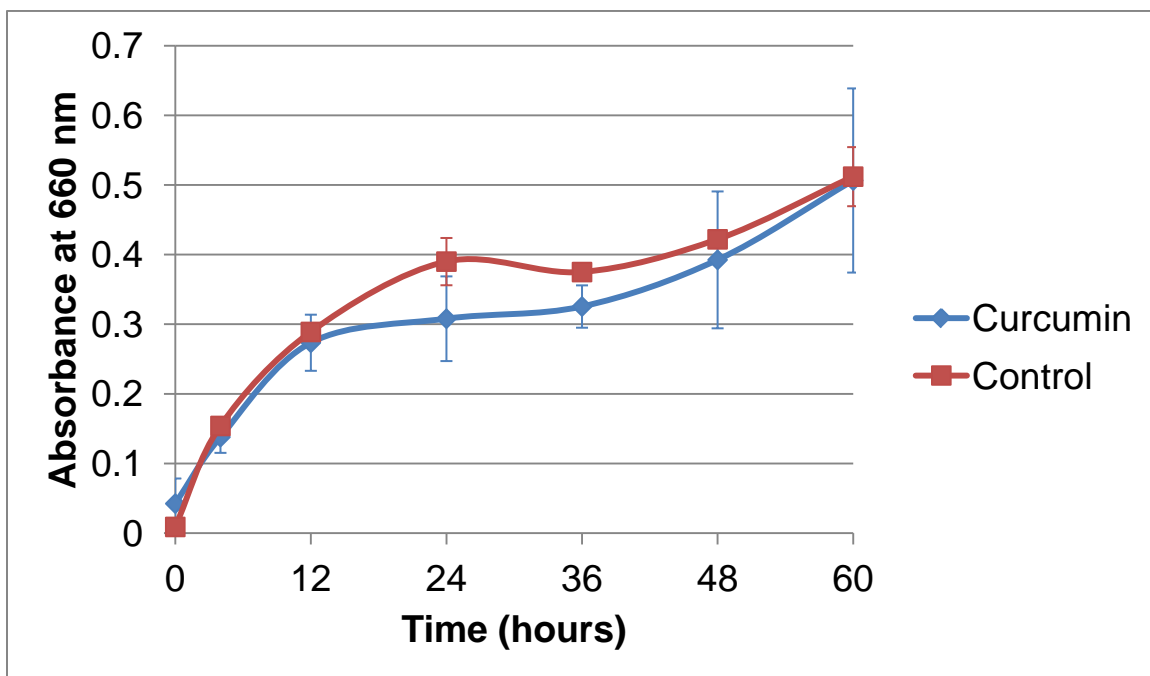
**Figure 1.** The antimicrobial activity of curcumin at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



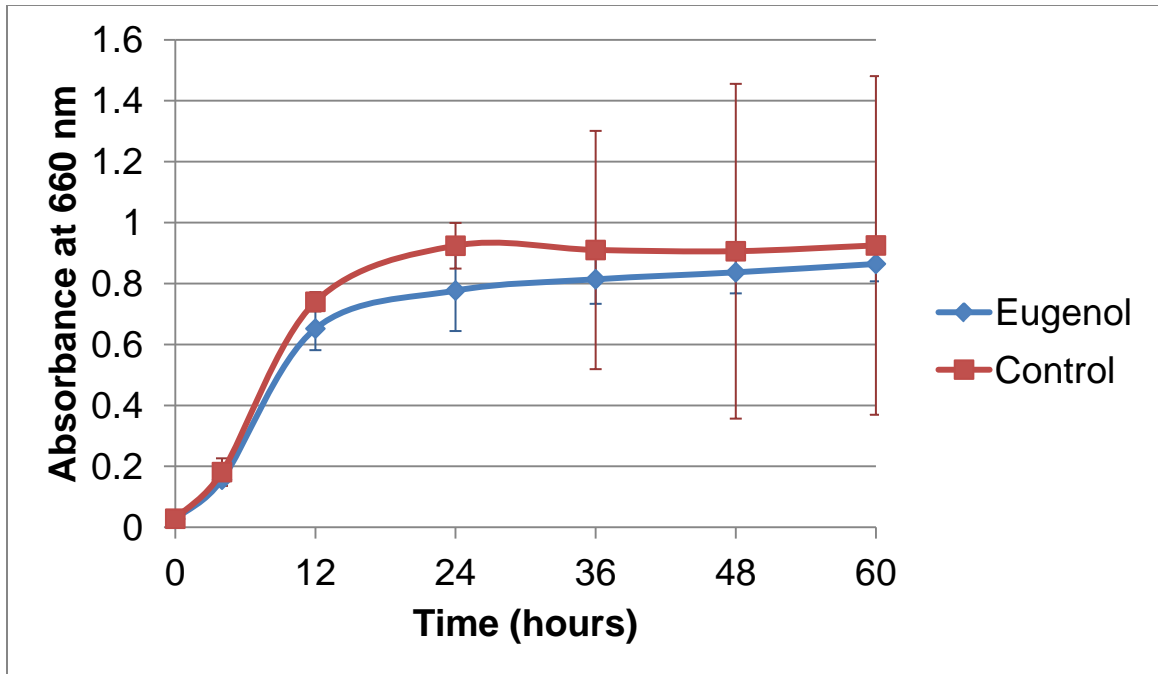
**Figure 2.** The antimicrobial activity of curcumin at MIC of 5 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



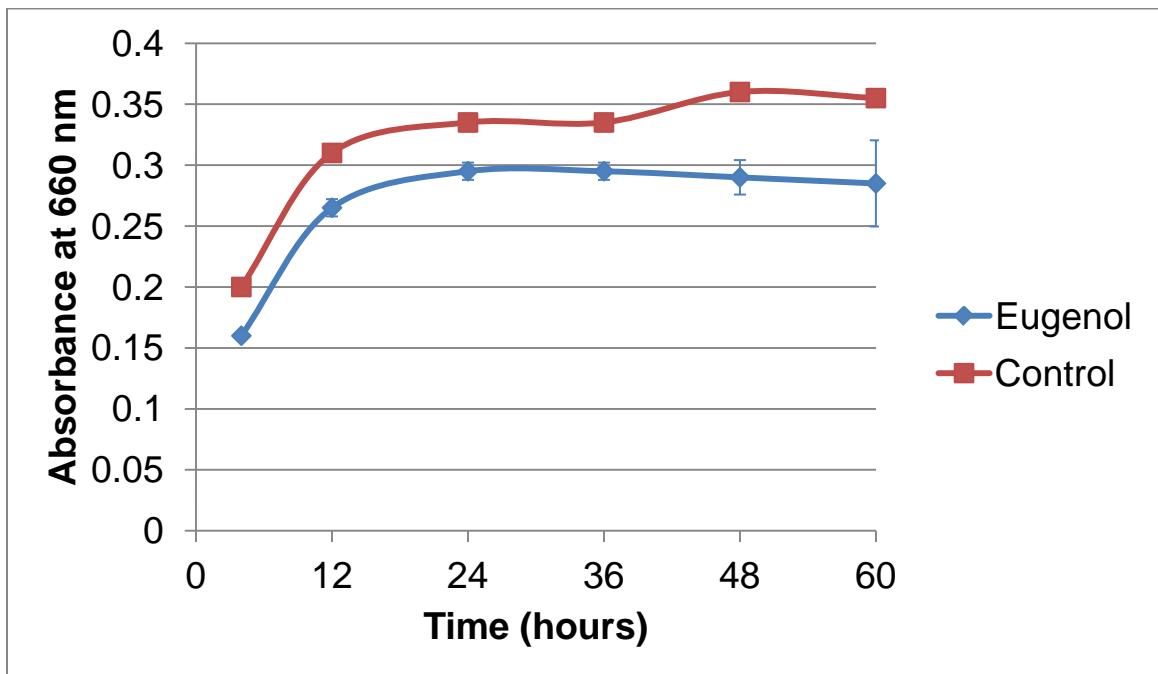
**Figure 3.** The antimicrobial activity of curcumin at MIC of 5 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 4.** The antimicrobial activity of curcumin at MIC of 20 ppm against *S. enteridis*, (-)  $H_2S$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .

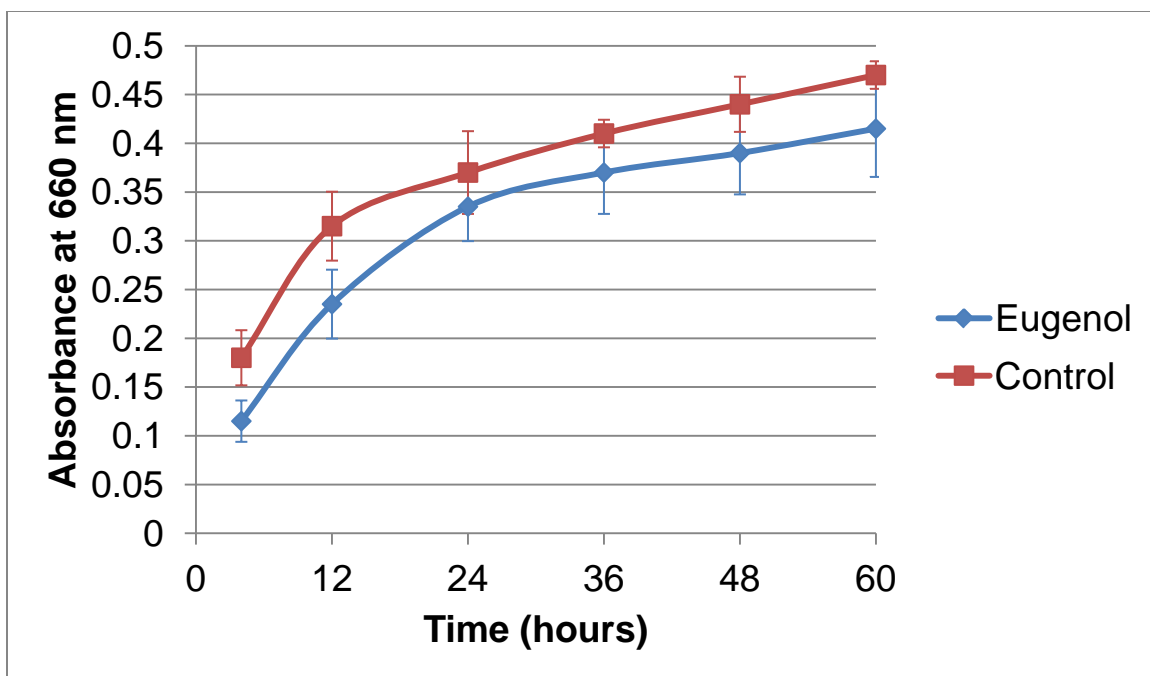


**Figure 5.** The antimicrobial activity of Eugenol at MIC of 20 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .

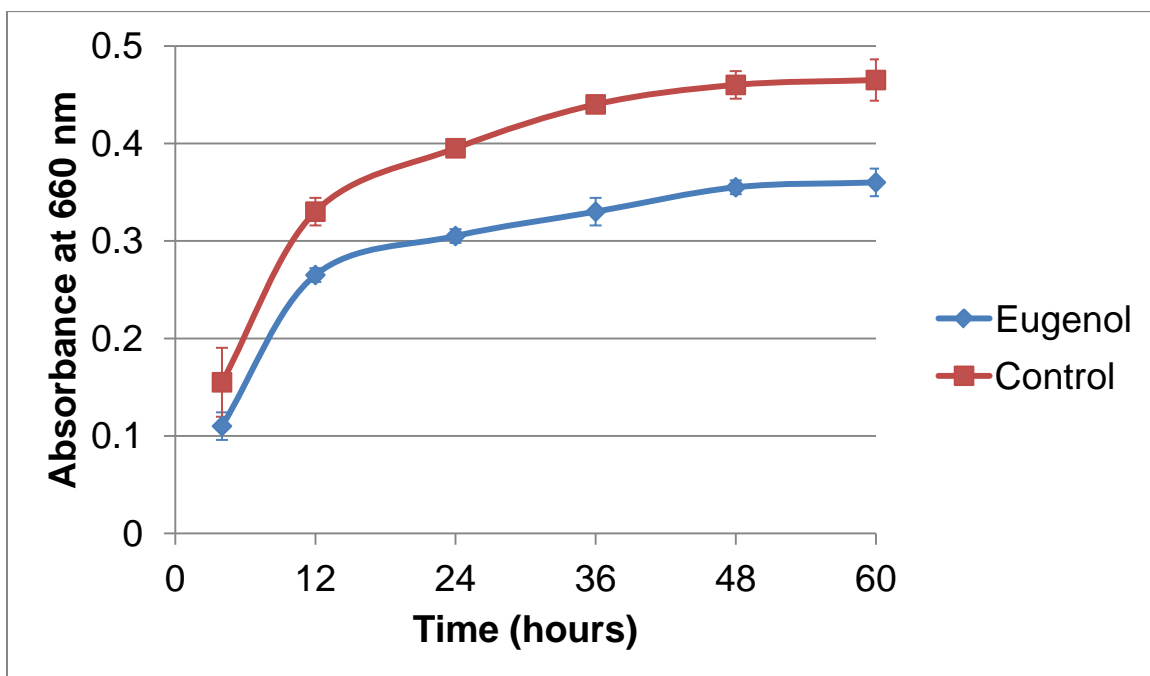


**Figure 6.** The antimicrobial activity of eugenol at MIC of 5 ppm against *L. monocytogenes*, ATCC 7644. Error bars represent standard deviation from the mean,  $P < 0.05$ .

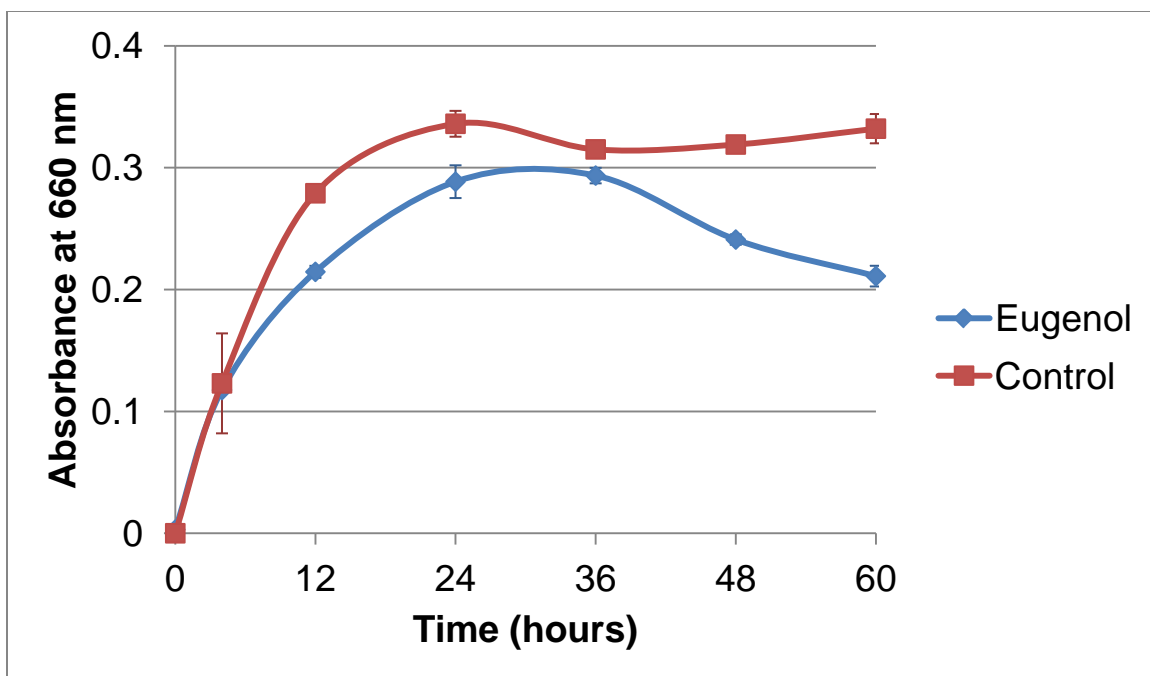




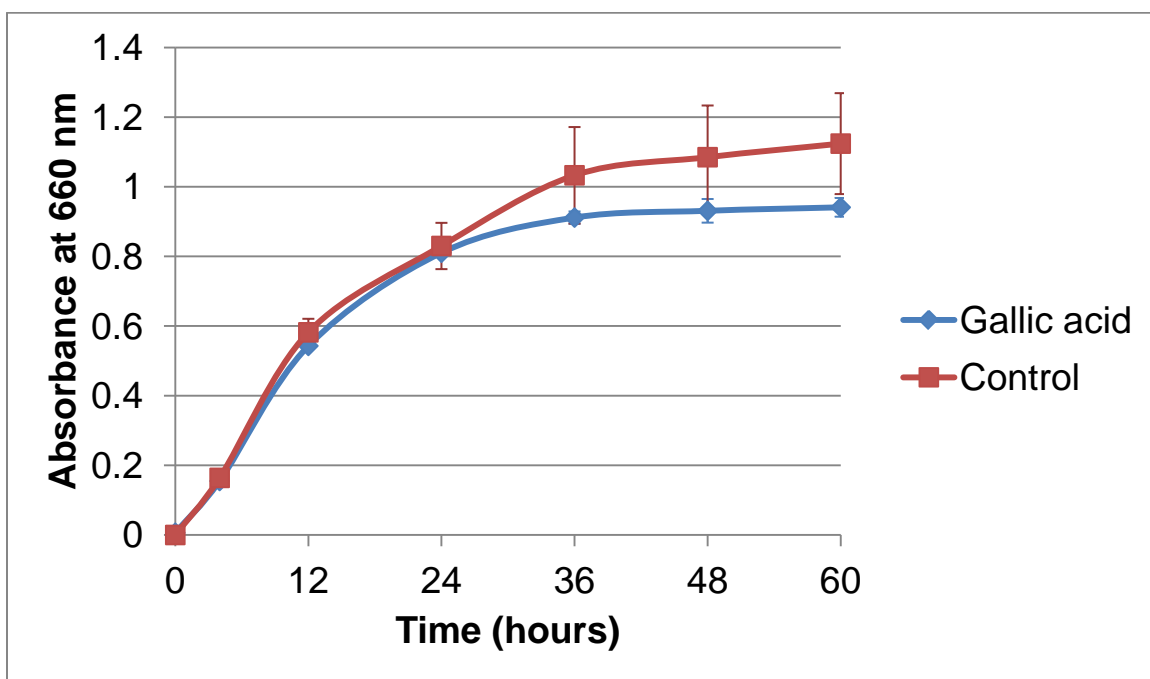
**Figure 7.** The antimicrobial activity of eugenol at MIC of 10 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



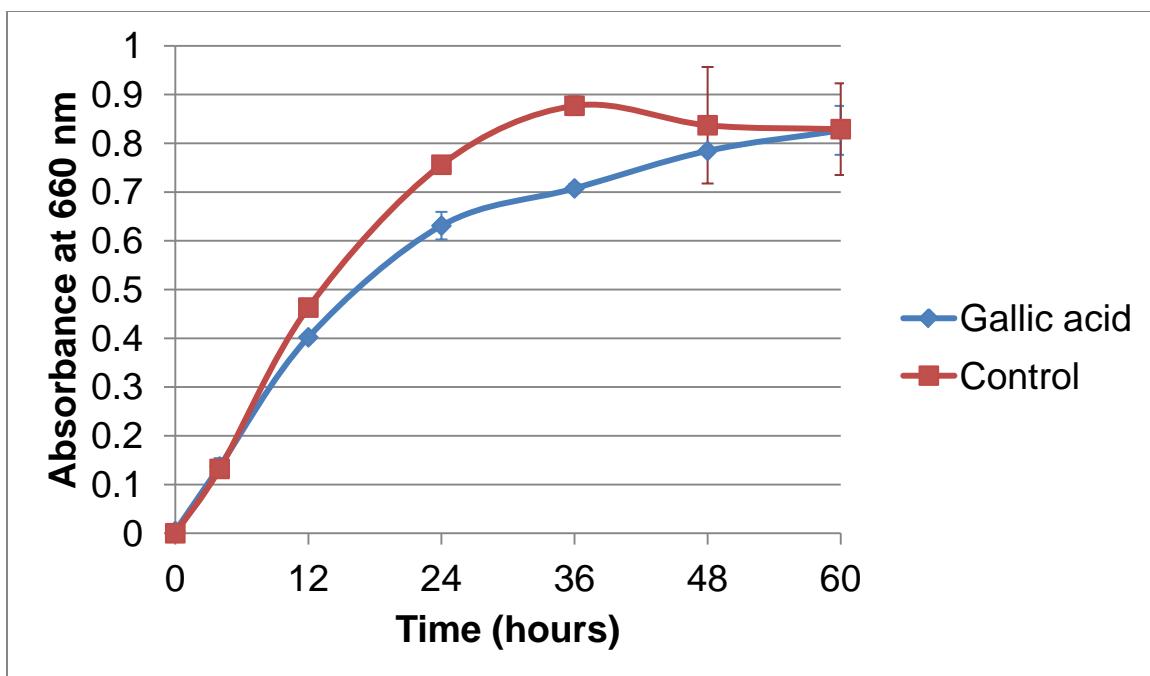
**Figure 8.** The antimicrobial activity of eugenol at MIC of 5 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



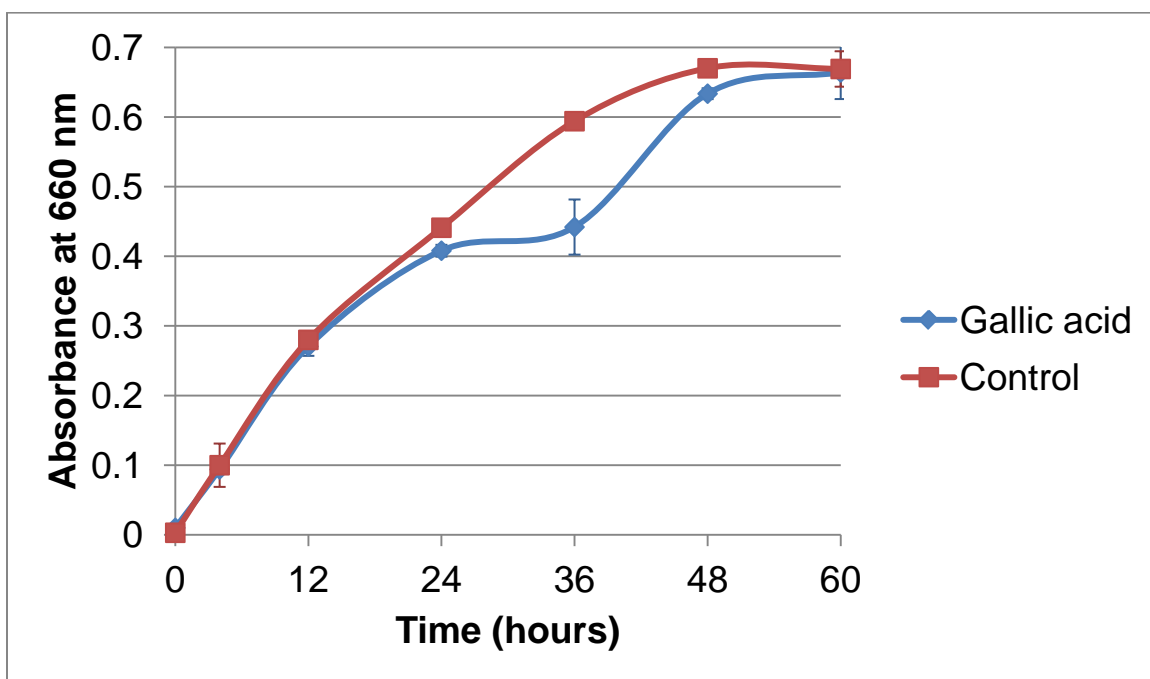
**Figure 9.** The antimicrobial activity of eugenol at MIC of 10 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



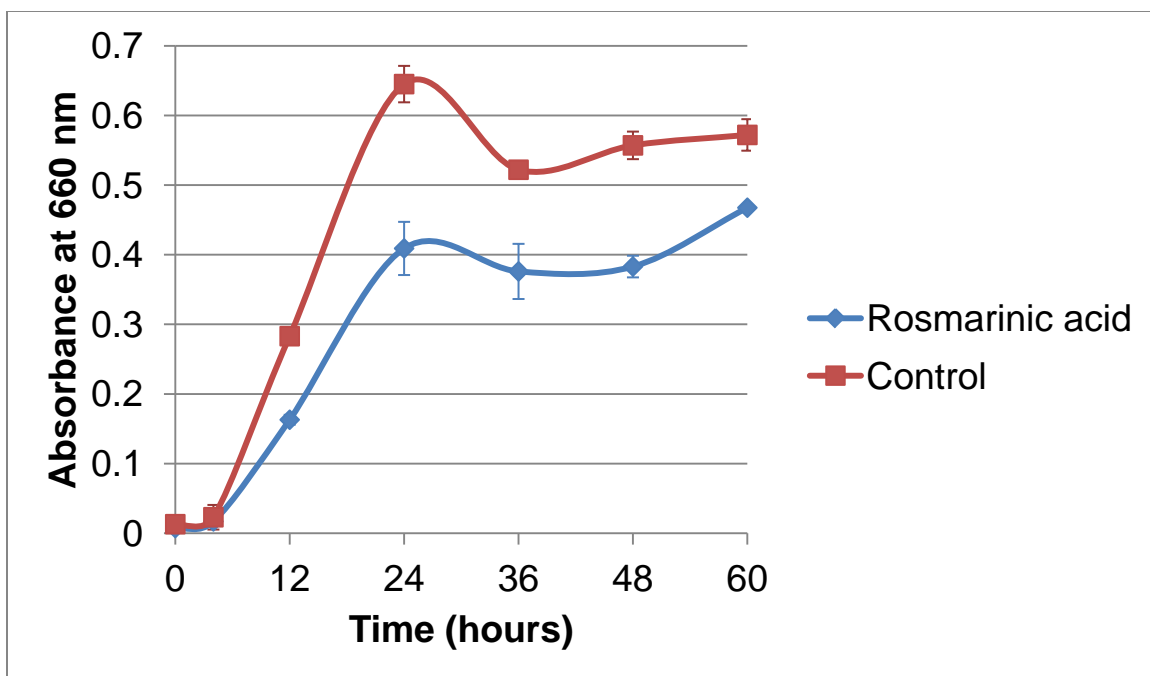
**Figure 10.** The antimicrobial activity of gallic acid at MIC of 5 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



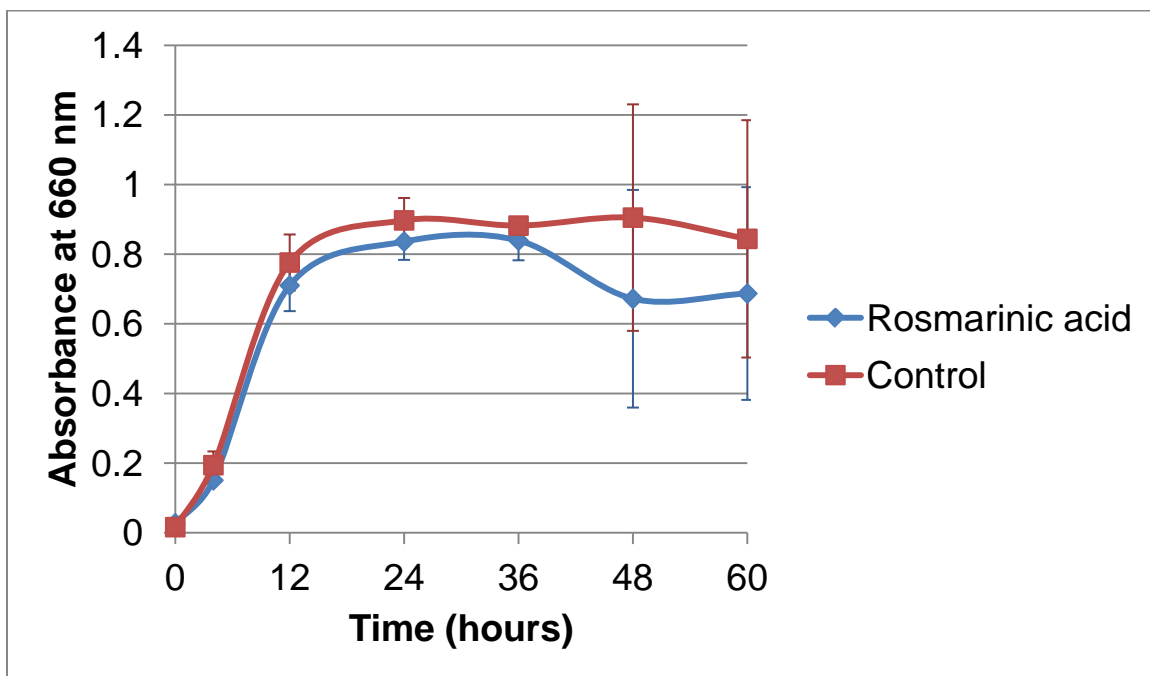
**Figure 11.** The antimicrobial activity of gallic acid at MIC of 10 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



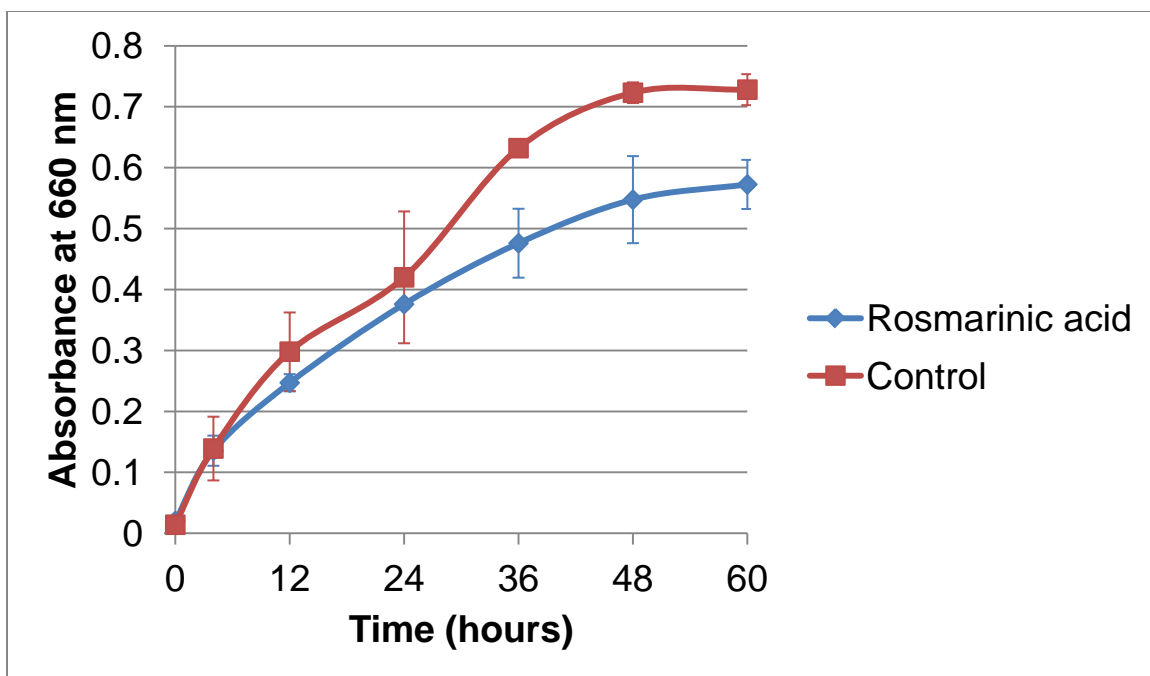
**Figure 12.** The antimicrobial activity of gallic acid at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



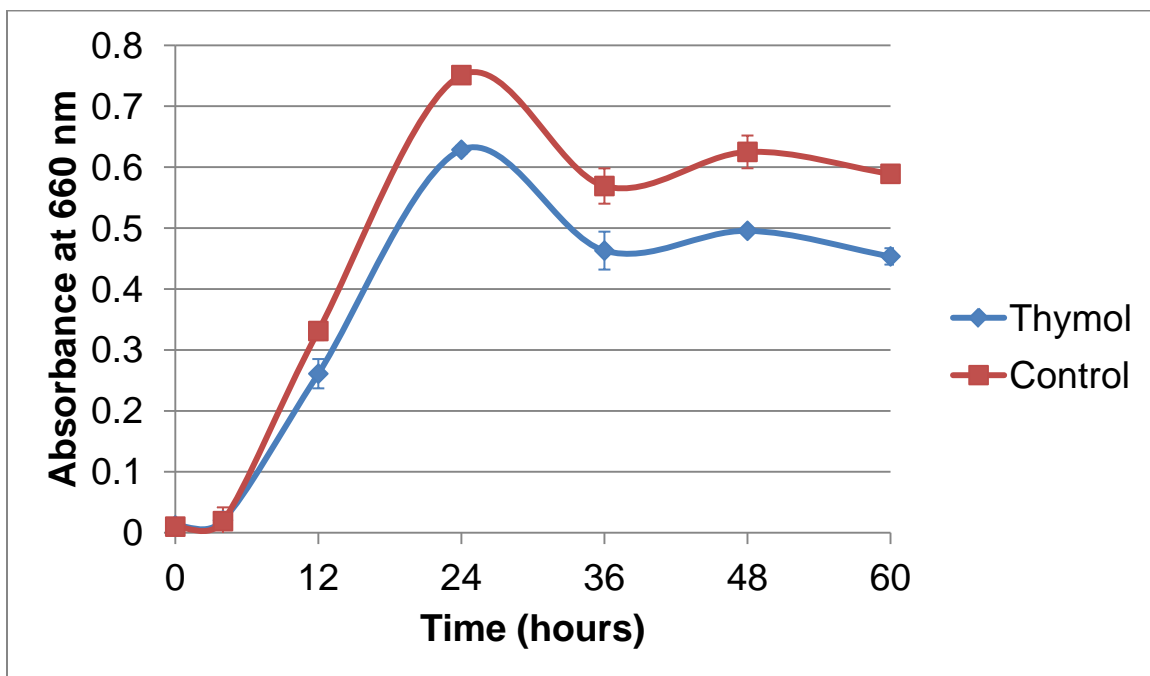
**Figure 13.** The antimicrobial activity of rosmarinic acid at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



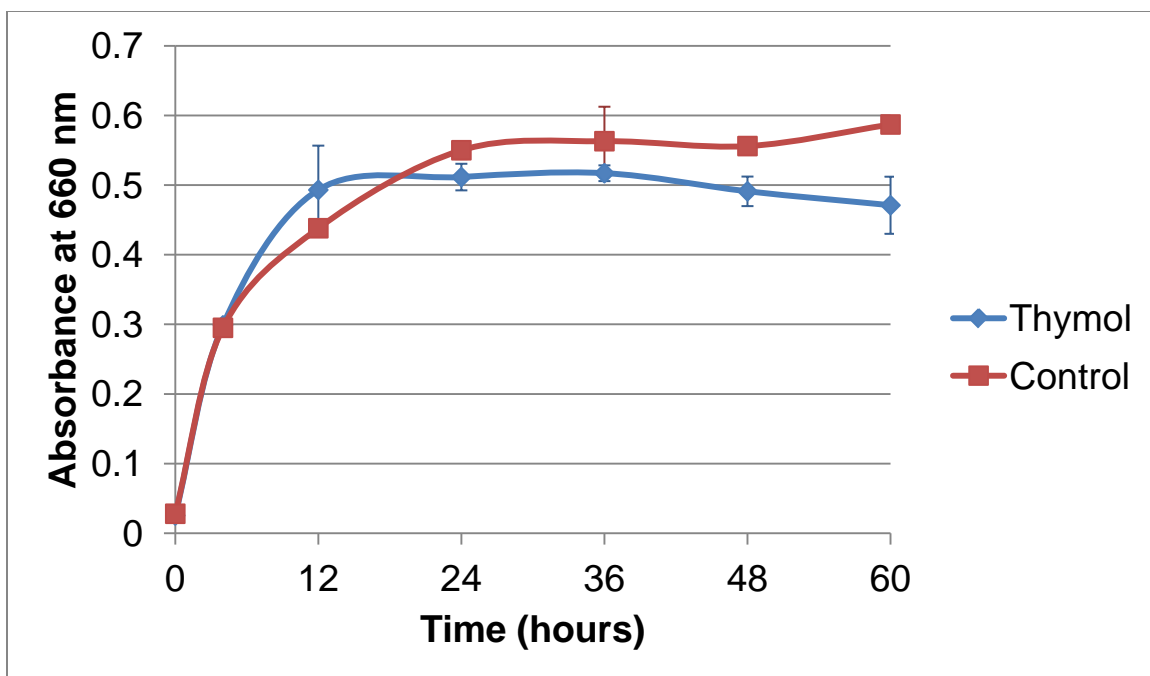
**Figure 14.** The antimicrobial activity of rosmarinic acid at MIC of 15 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



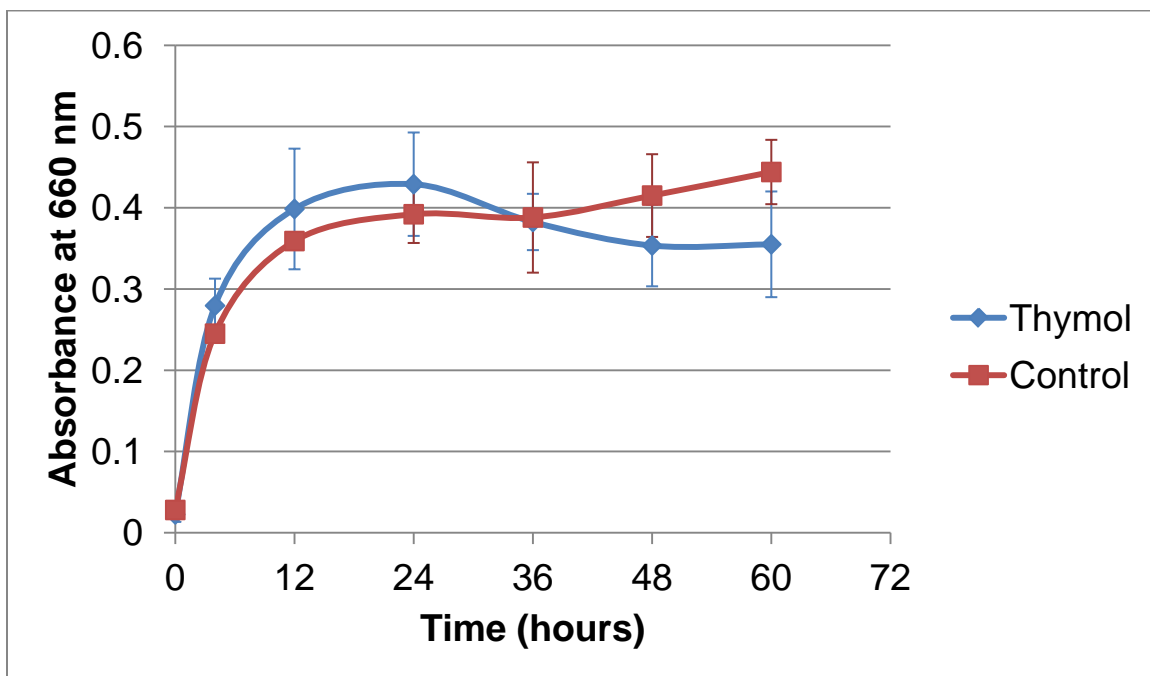
**Figure 15.** The antimicrobial activity of rosmarinic acid at MIC of 15 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



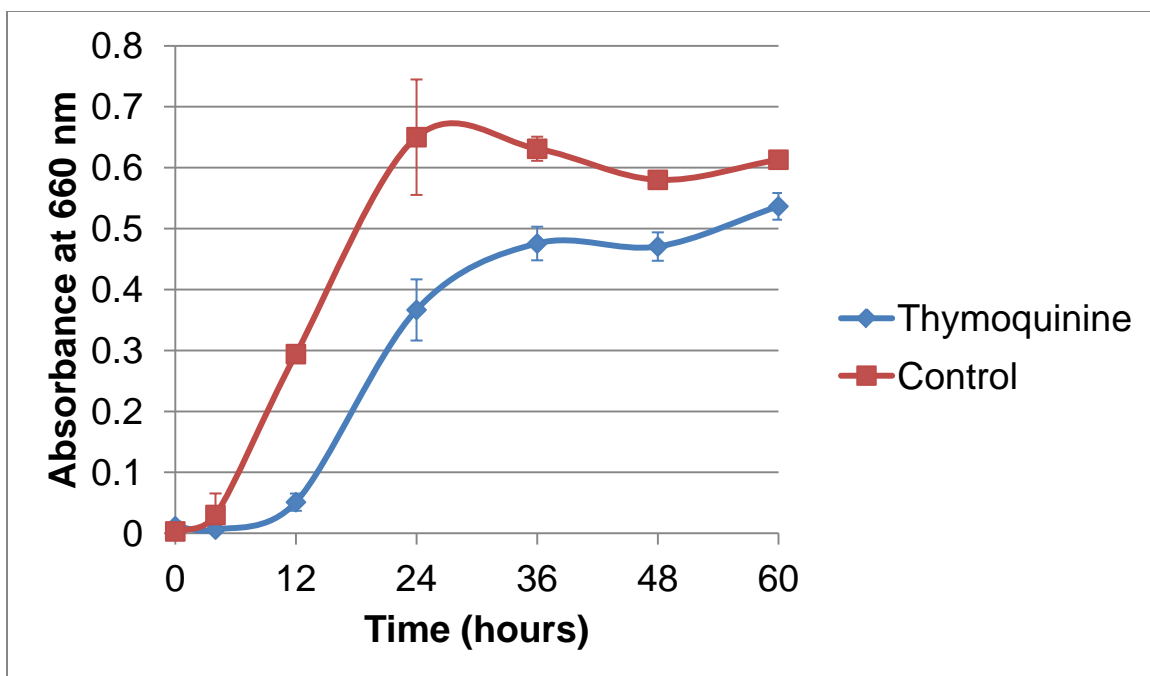
**Figure 16.** The antimicrobial activity of thymol at MIC of 20 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.



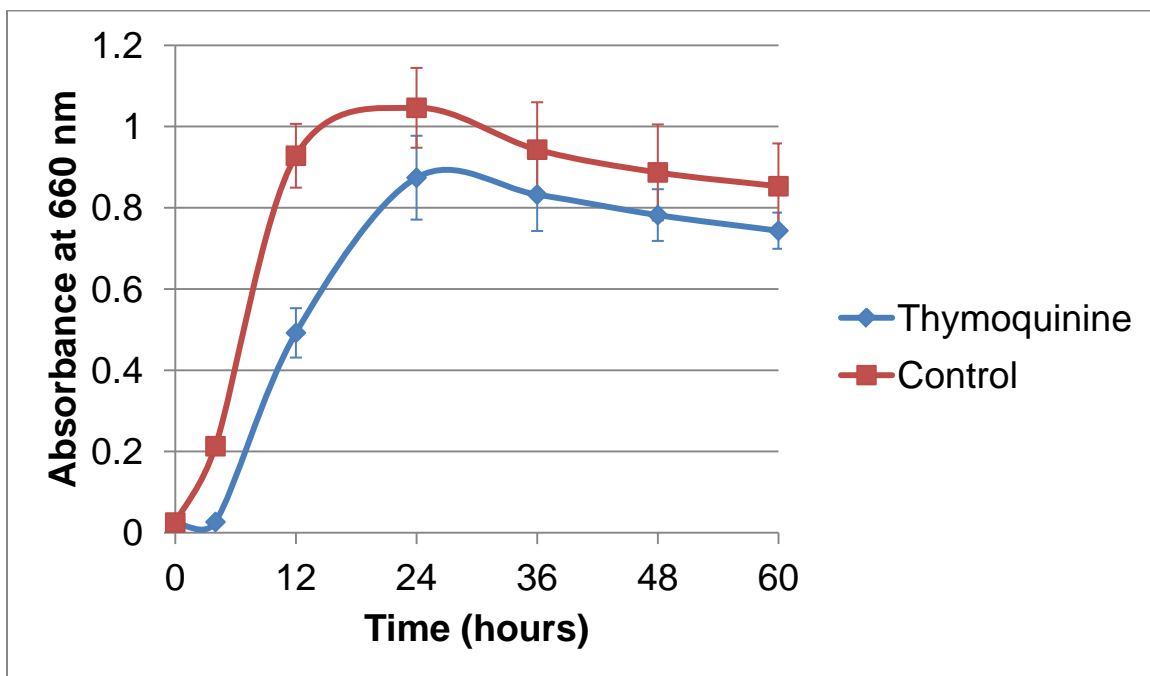
**Figure 17.** The antimicrobial activity of thymol at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



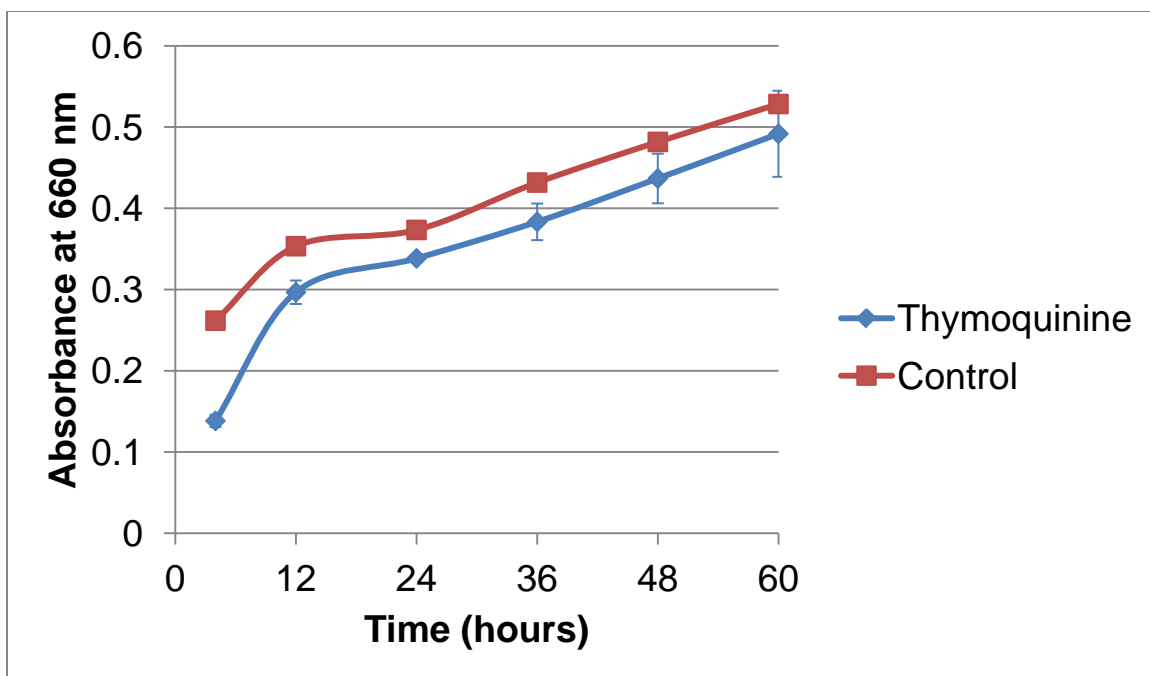
**Figure 18.** The antimicrobial activity of thymol at MIC of 15 ppm against *S. cholerasuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



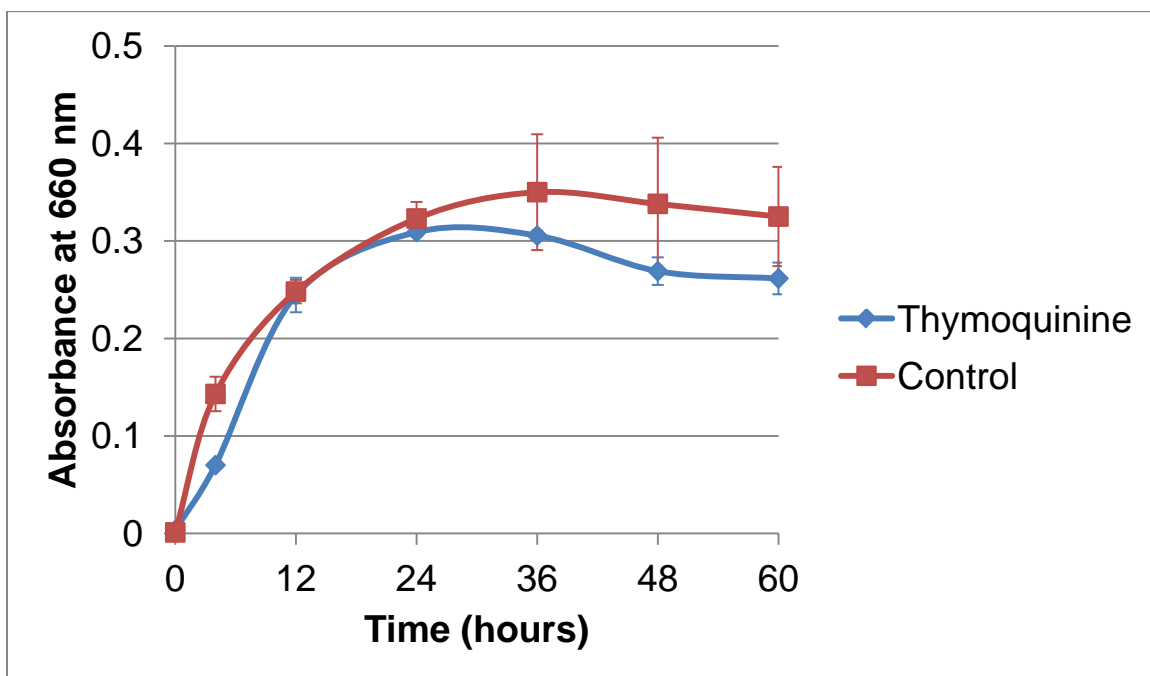
**Figure 19.** The antimicrobial activity of thymoquinine at MIC of 5 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 20.** The antimicrobial activity of thymoquinine at MIC of 10 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .

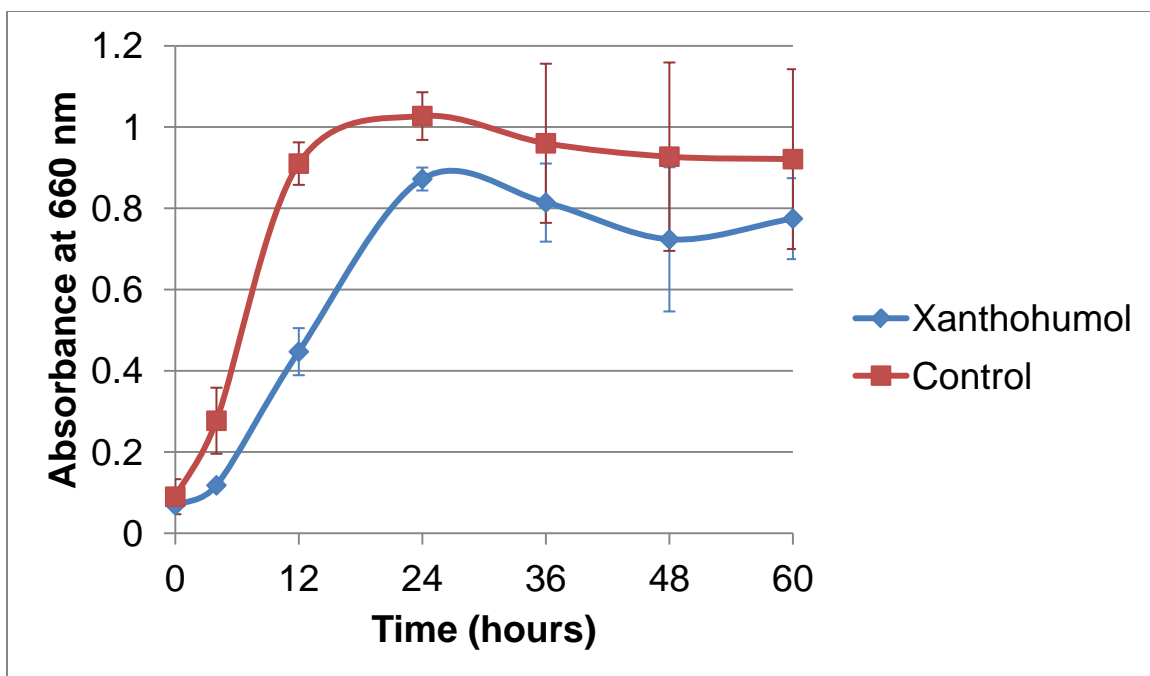


**Figure 21.** The average antimicrobial activity of thymoquinine at MIC of 10 ppm against *L. monocytogenes*, ATCC 7644, *L. monocytogenes*, UK ADL and *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .

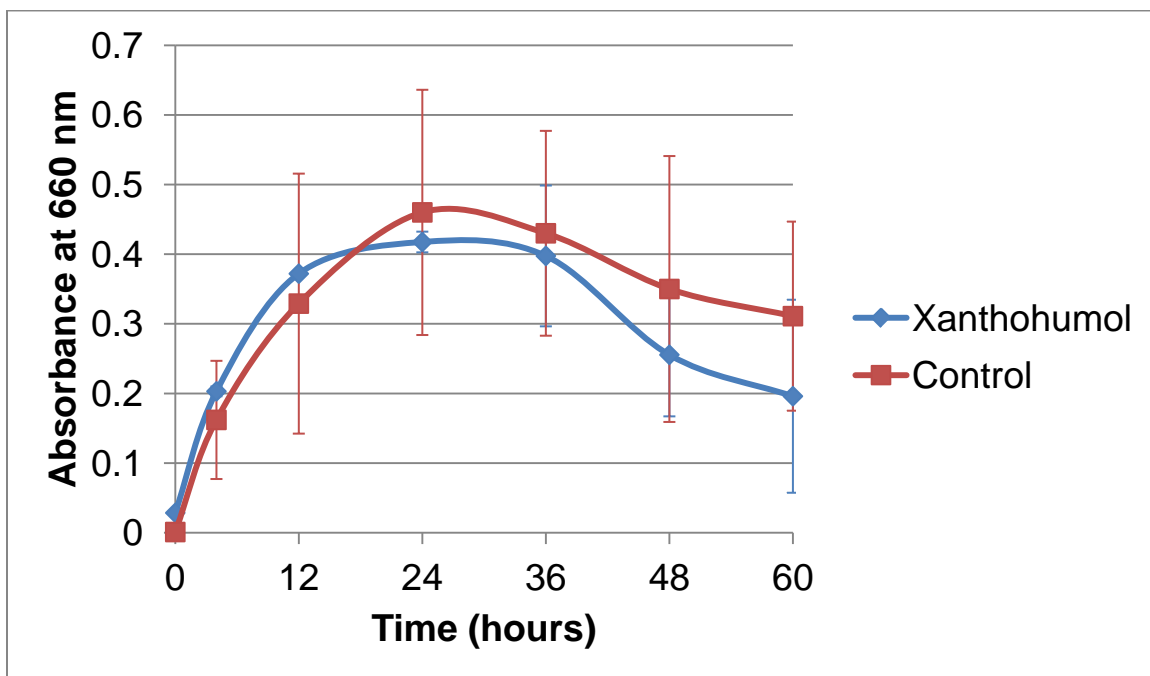


**Figure 22.** The antimicrobial activity of thymoquinine at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .

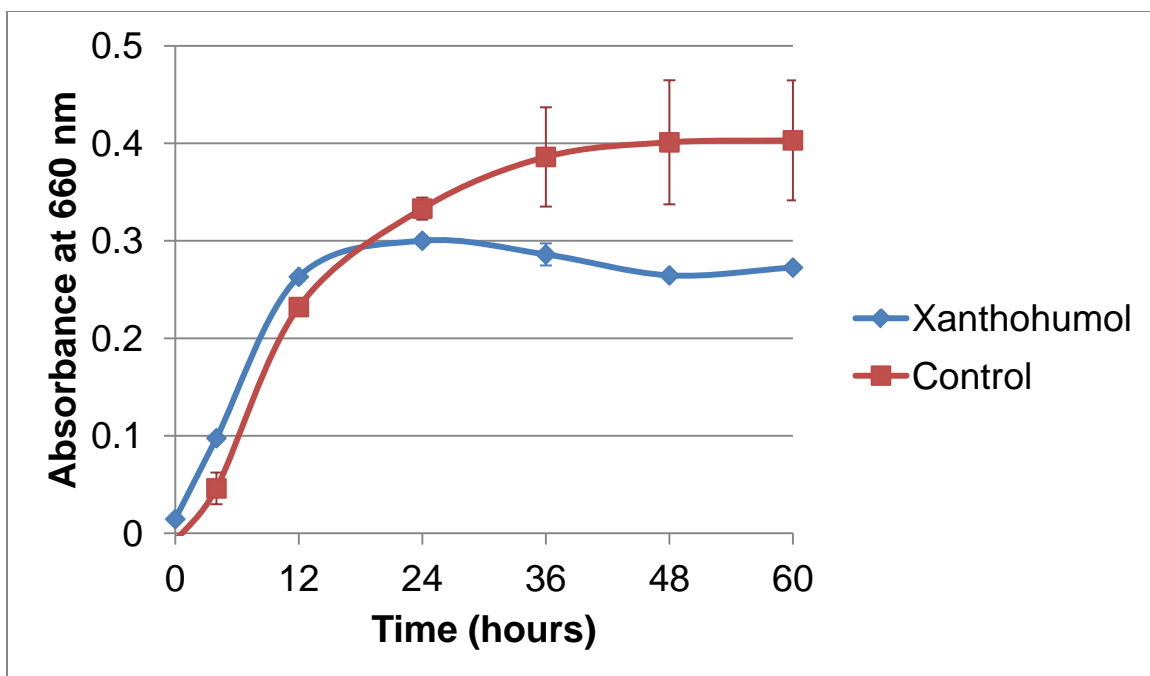




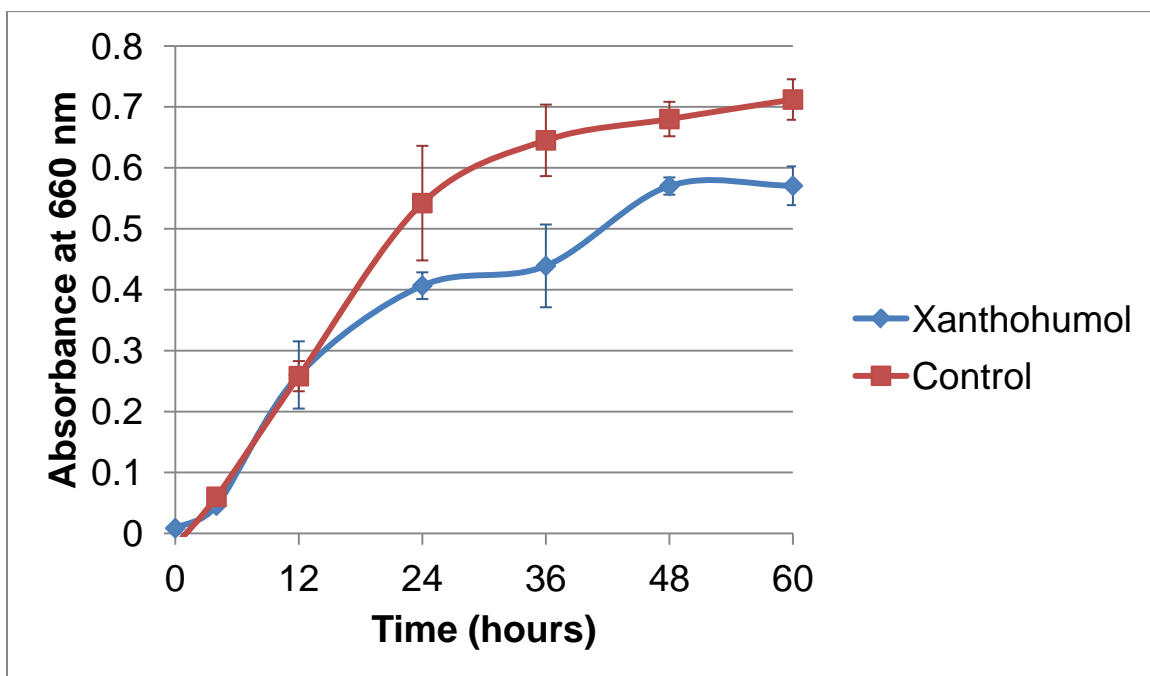
**Figure 23.** The antimicrobial activity of xanthohumol at MIC of 5 ppm against *B. polymyxa*, ATCC 840. Error bars represent standard deviation from the mean,  $P < 0.05$ .



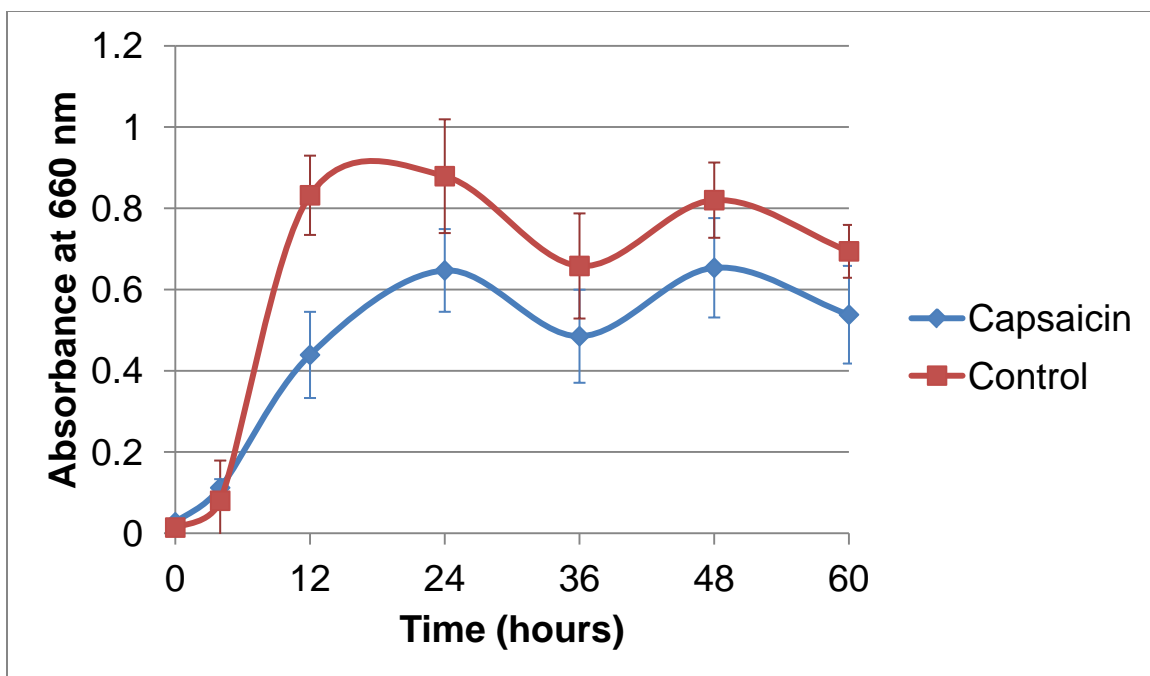
**Figure 24.** The antimicrobial activity of xanthohumol at MIC of 15 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



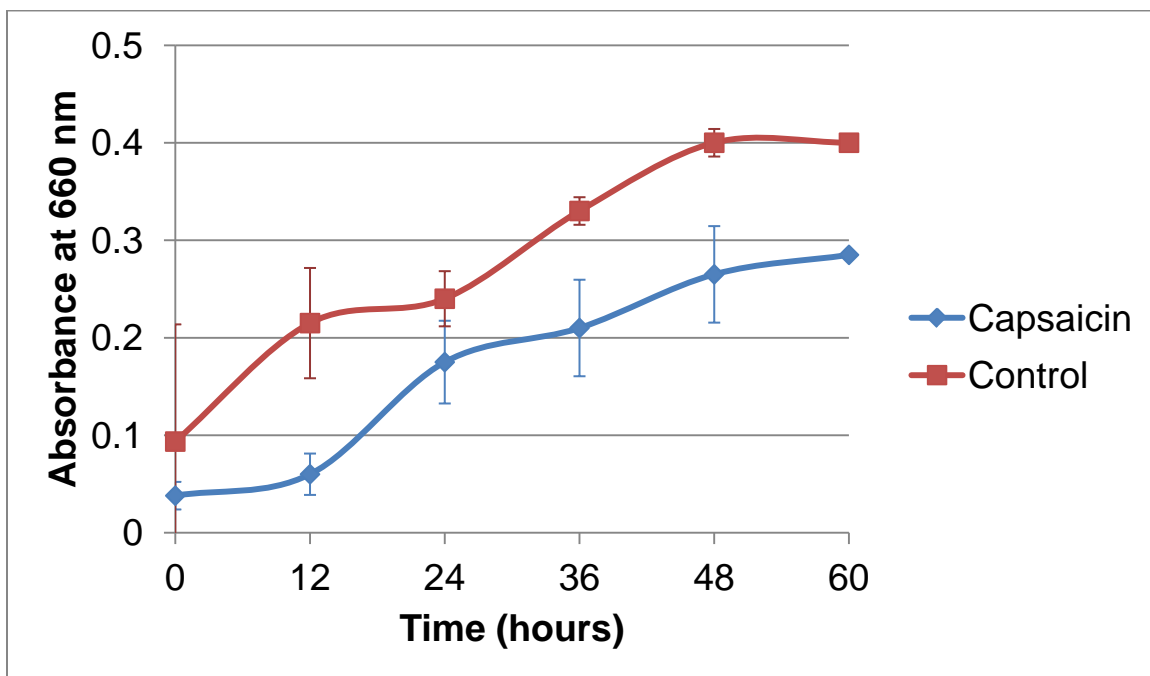
**Figure 25.** The antimicrobial activity of xanthohumol at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



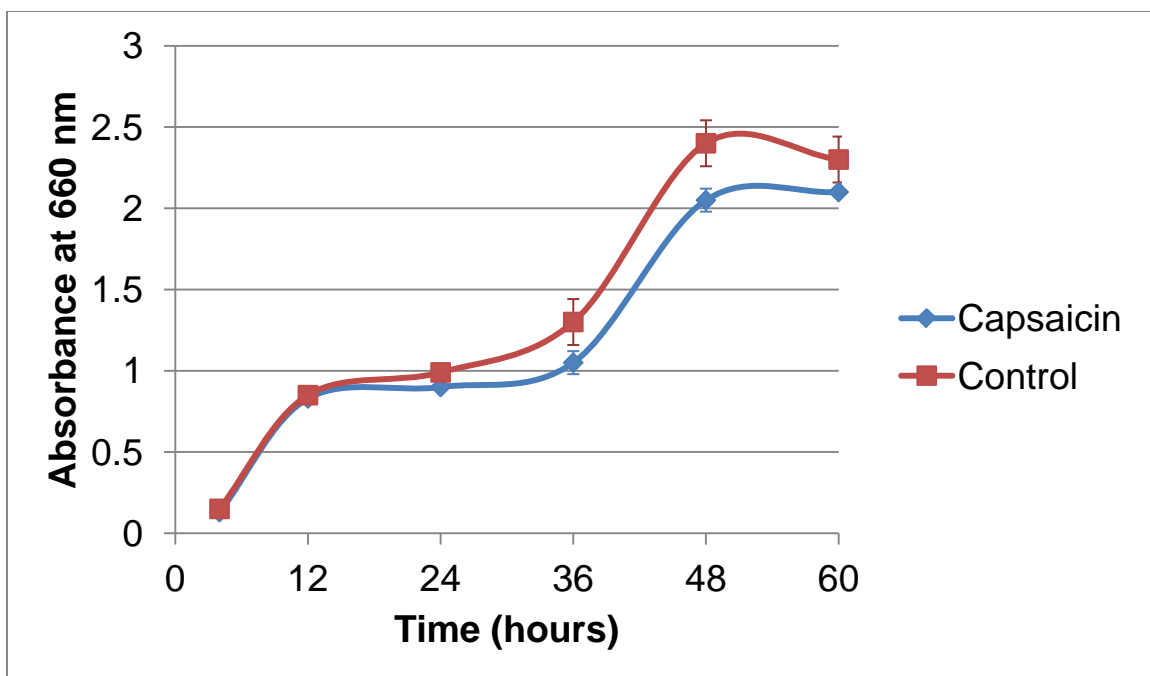
**Figure 26.** The antimicrobial activity of xanthohumol at MIC of 15 ppm against *S. choleraesuis subsps.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



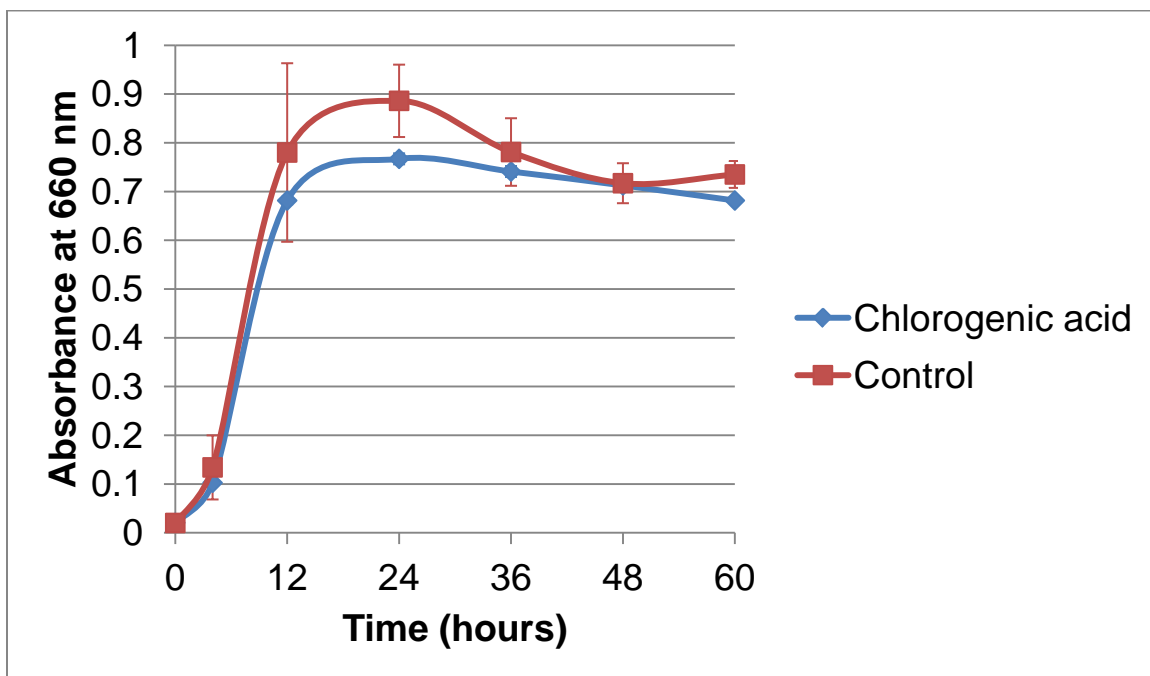
**Figure 27.** The antimicrobial activity of capsaicin at MIC of 20 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



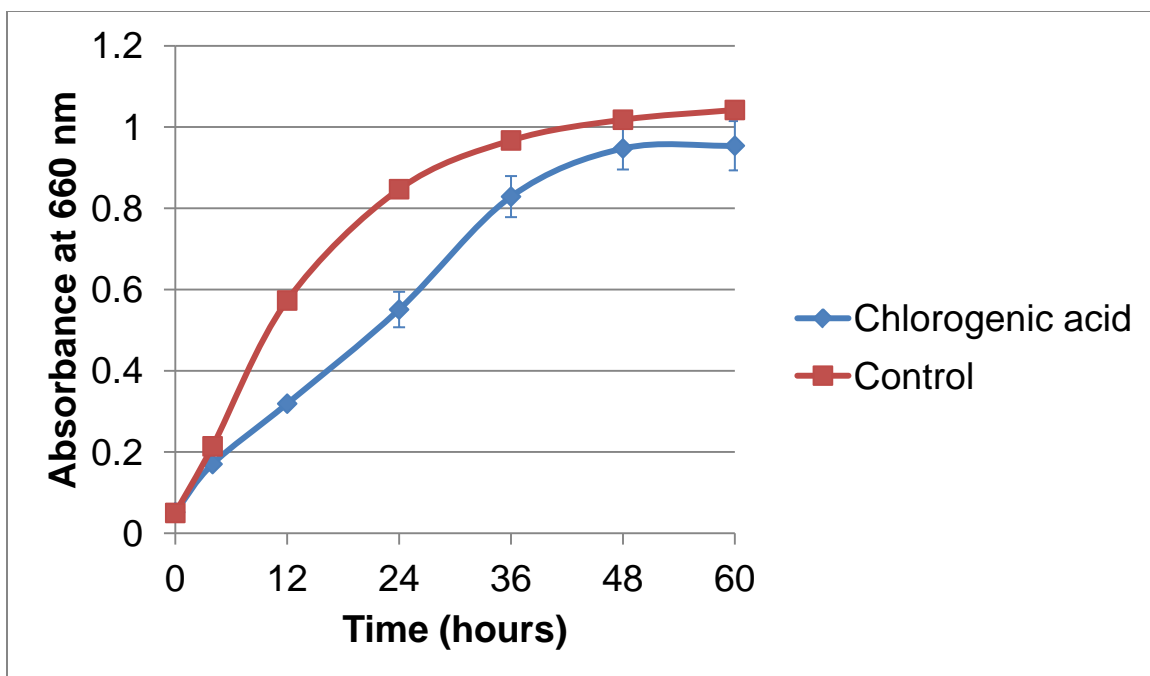
**Figure 28.** The antimicrobial activity of capsaicin at MIC of 20 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



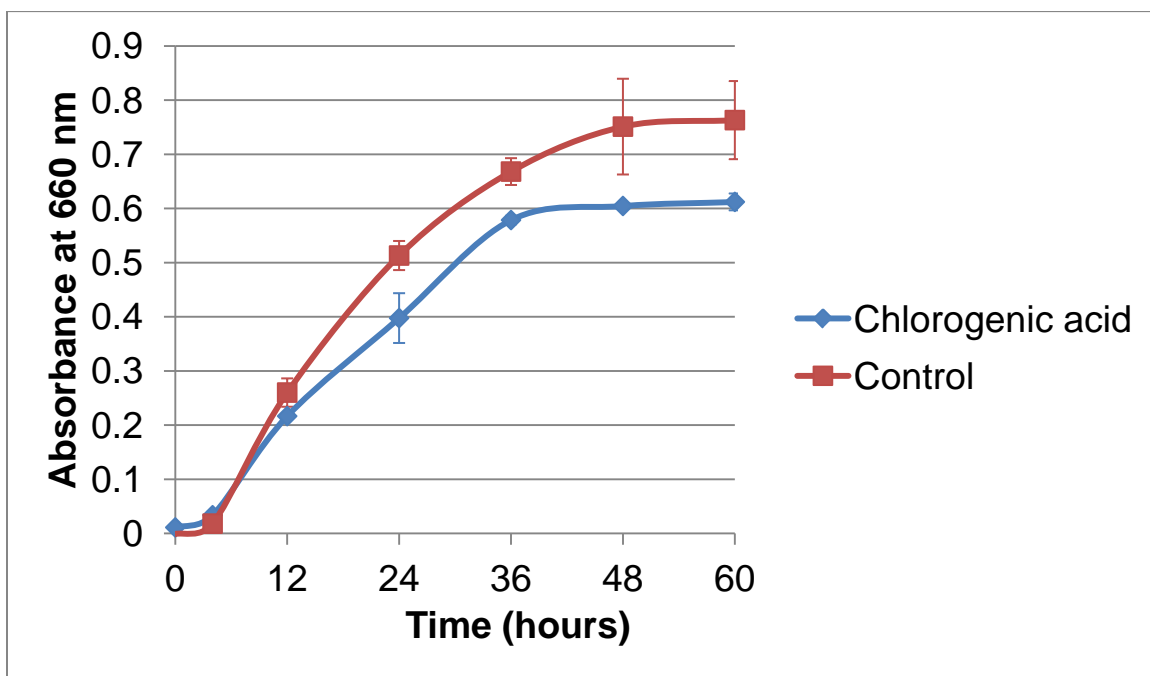
**Figure 29.** The antimicrobial activity of capsaicin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



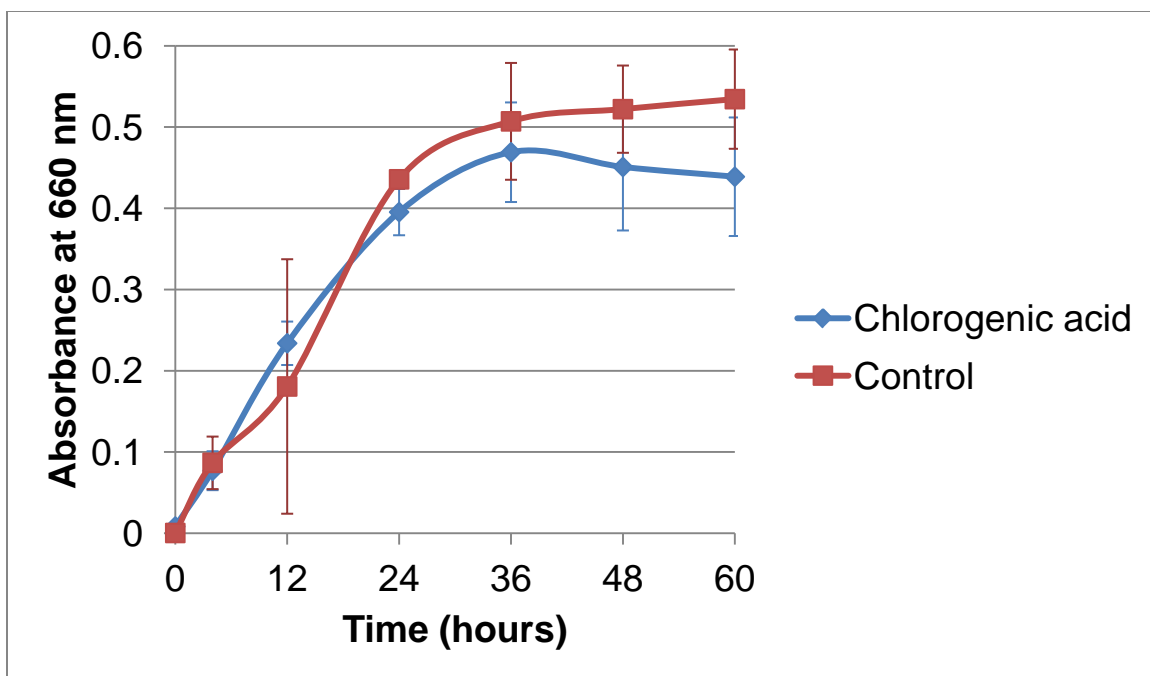
**Figure 30.** The antimicrobial activity of chlorogenic acid at MIC of 10 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



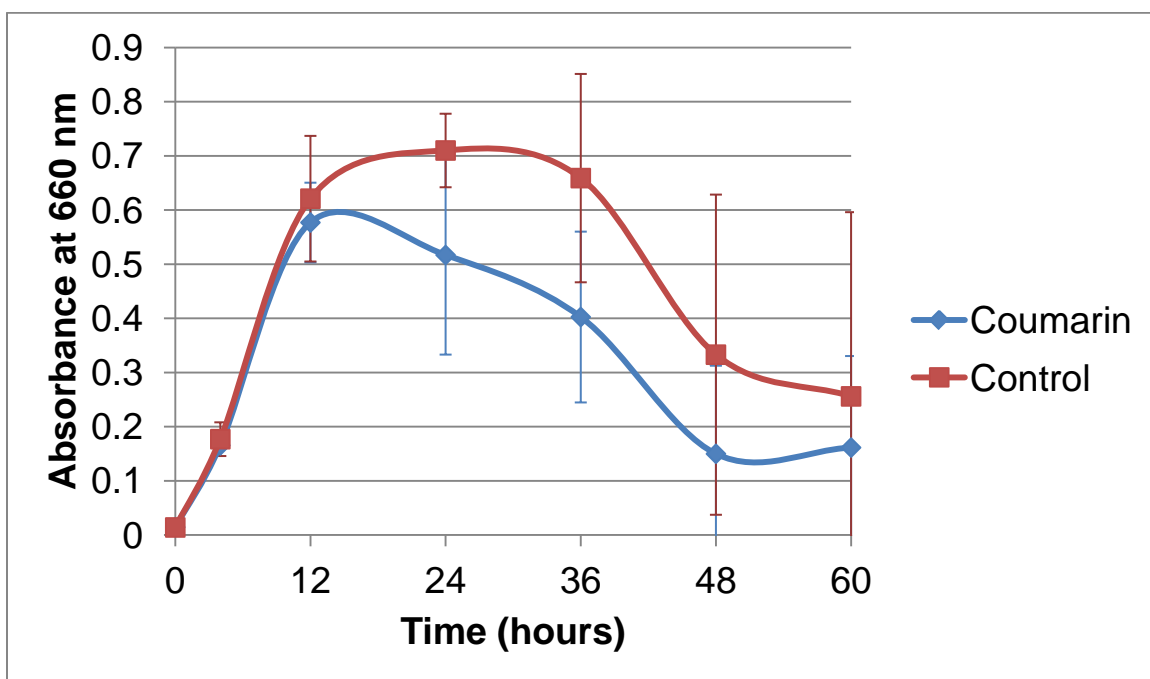
**Figure 31.** The average antimicrobial activity of chlorogenic acid at MIC of 10 ppm against *L. monocytogenes*, ATCC 7644, *L. monocytogenes*, UK ADL and *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .



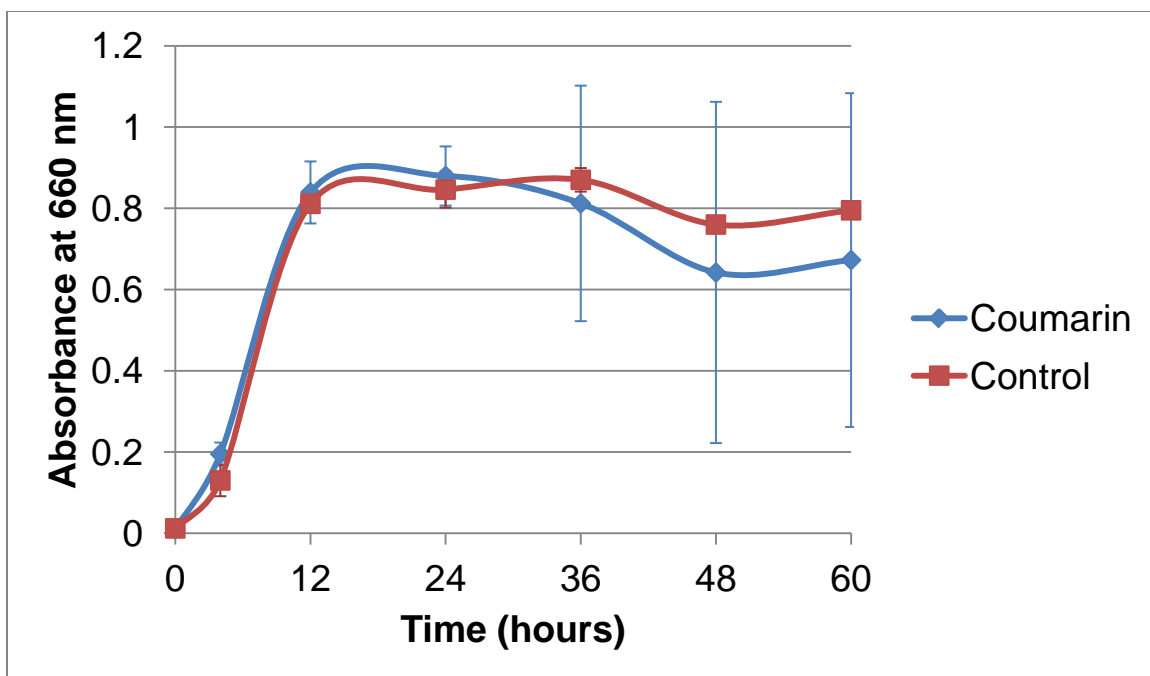
**Figure 32.** The antimicrobial activity of chlorogenic acid at MIC of 15 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



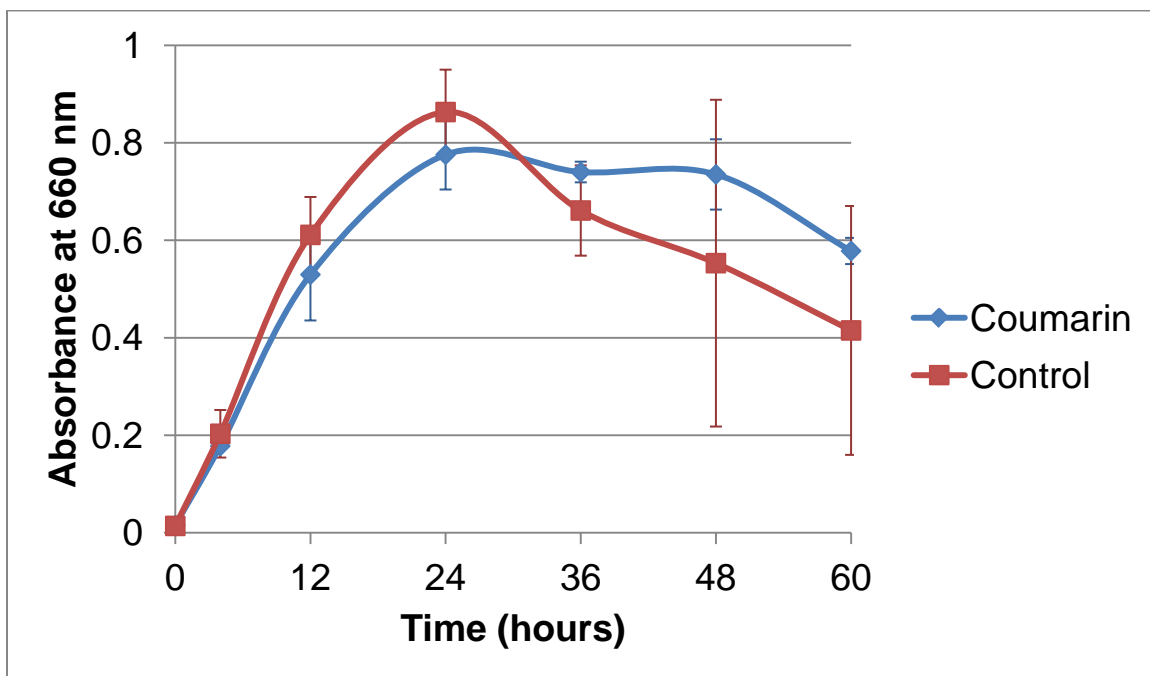
**Figure 33.** The average antimicrobial activity of chlorogenic acid at MIC of 10 ppm against *S. paratyphi*, UK Micro 29A, *S. cholerasuis subsp.* ATCC 10708 and *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



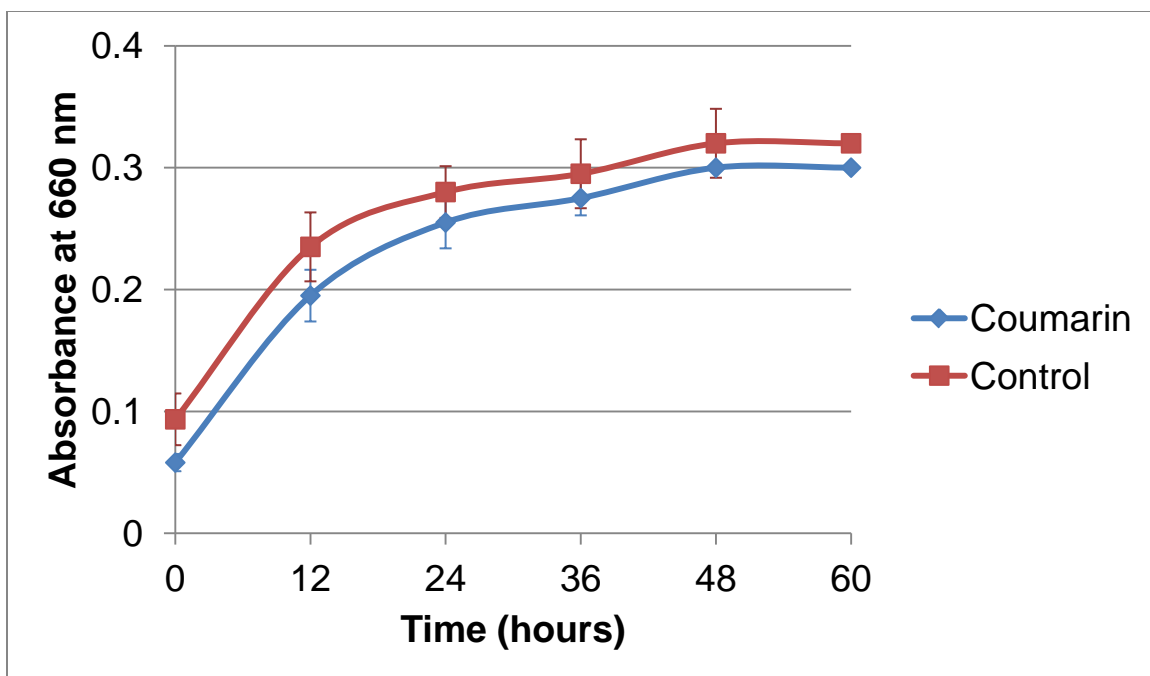
**Figure 34.** The antimicrobial activity of coumarin at MIC of 20 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.



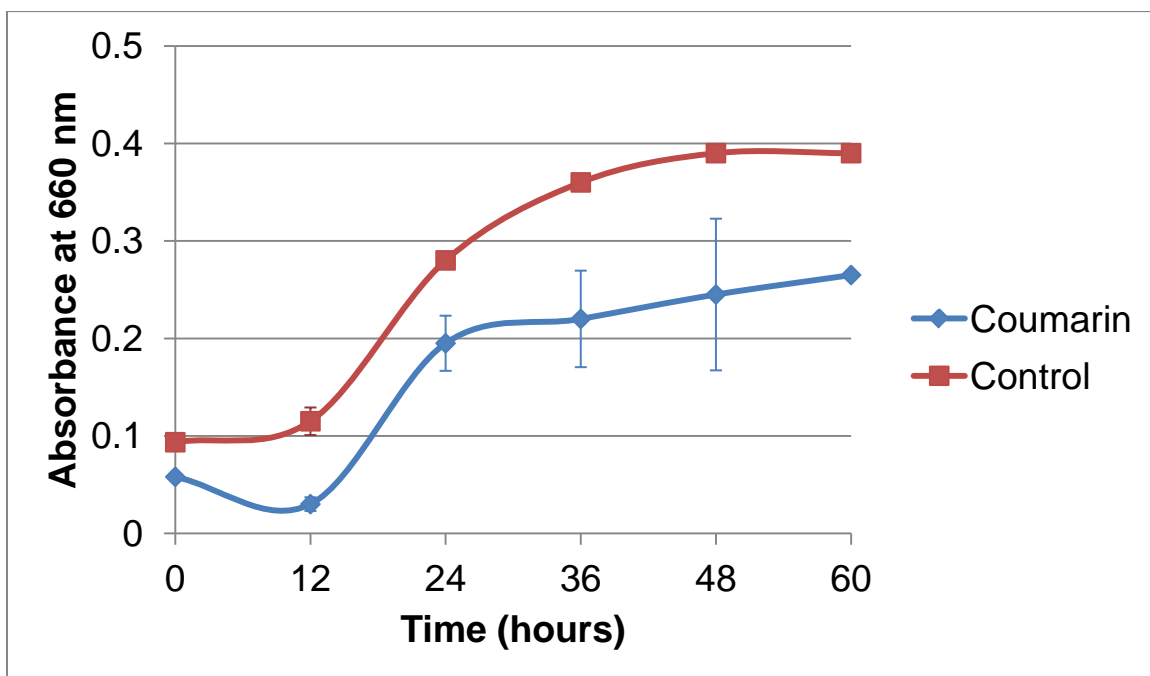
**Figure 35.** The antimicrobial activity of coumarin at MIC of 5 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 36.** The antimicrobial activity of coumarin at MIC of 15 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .

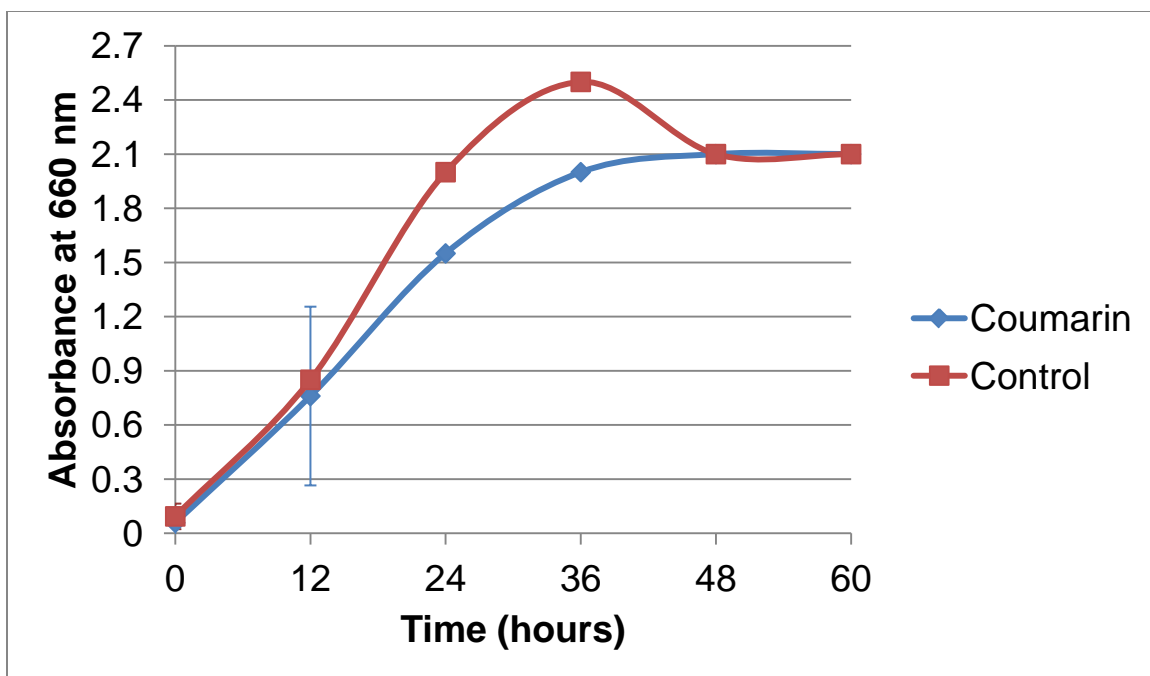


**Figure 37.** The antimicrobial activity of coumarin at MIC of 5 ppm against *L. monocytogenes*, ATCC 7644. Error bars represent standard deviation from the mean,  $P < 0.05$ .

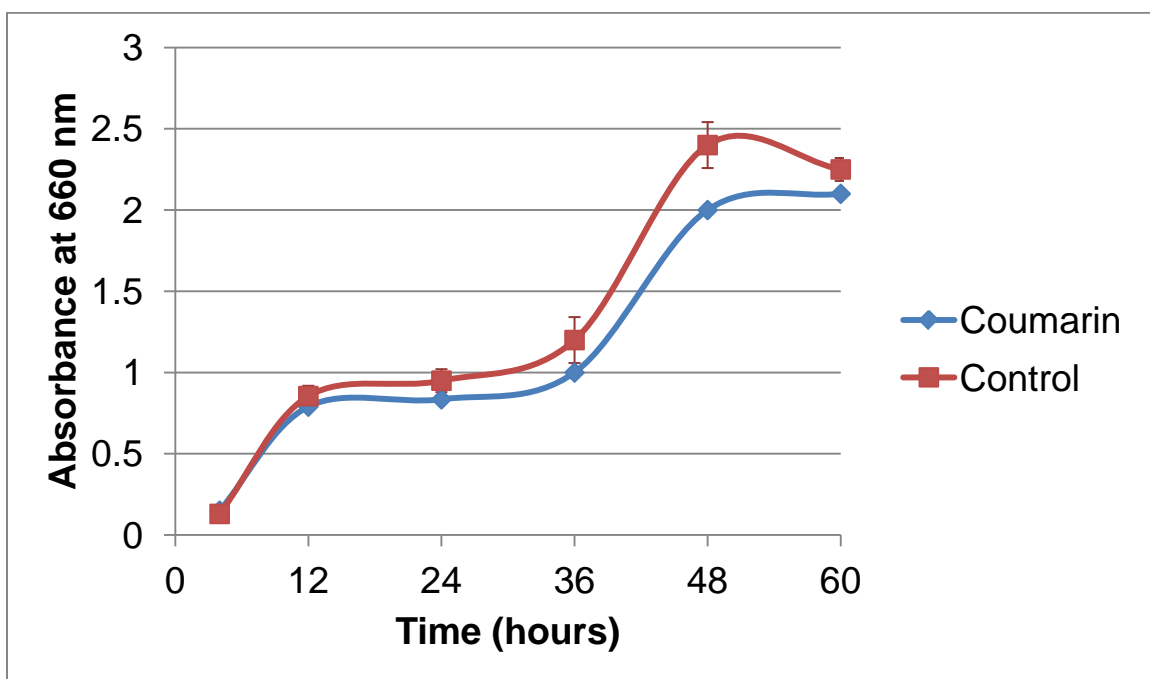


**Figure 38.** The antimicrobial activity of coumarin at MIC of 5 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .

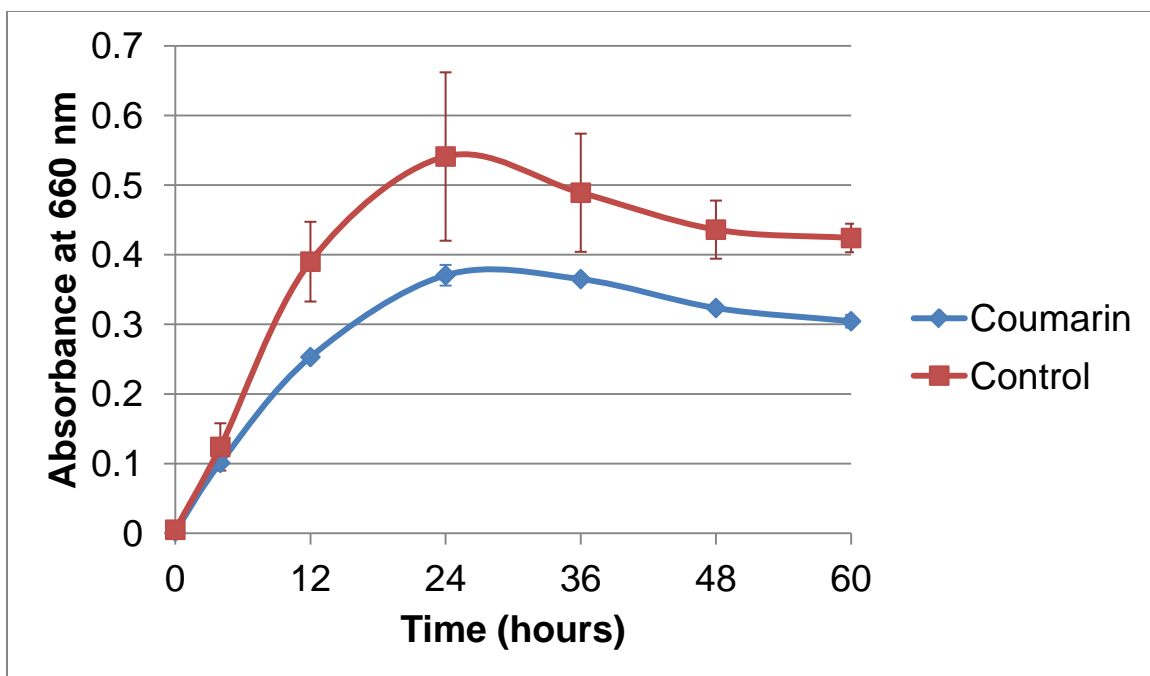




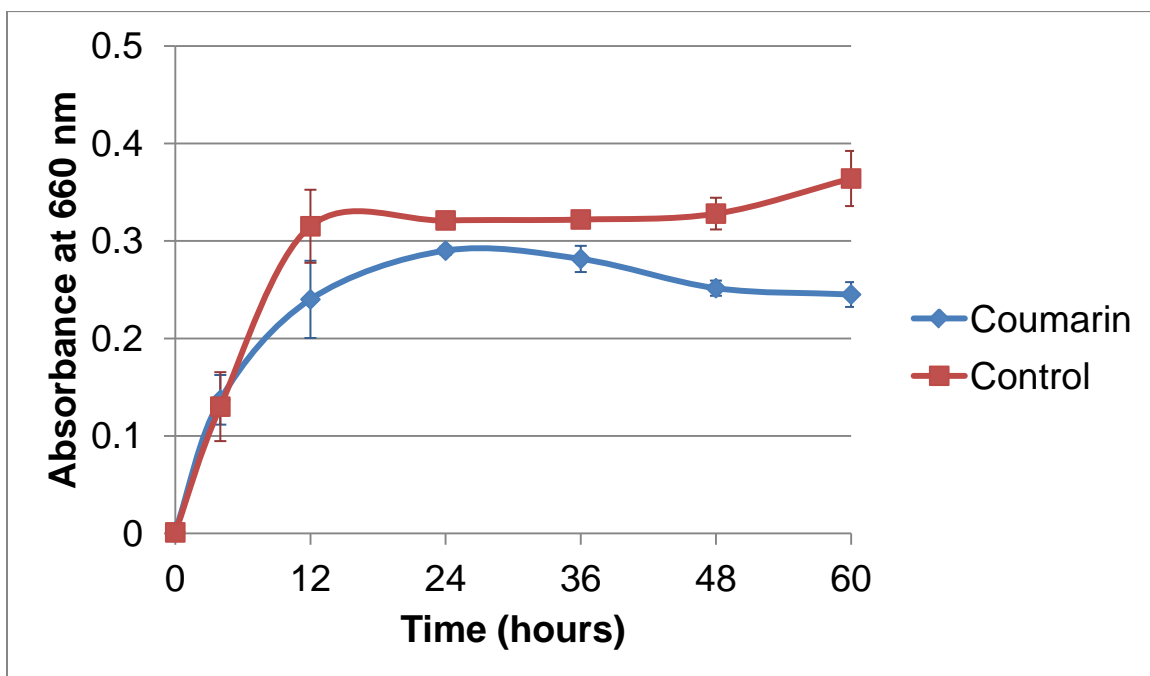
**Figure 39.** The antimicrobial activity of coumarin at MIC of 15 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



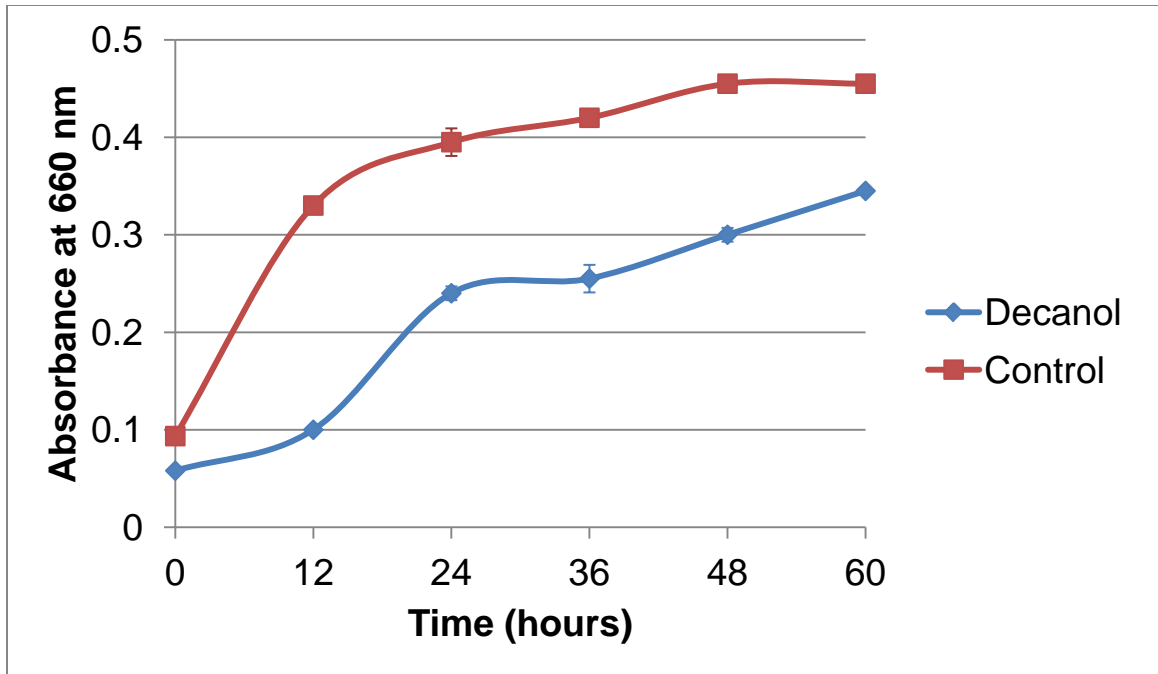
**Figure 40.** The antimicrobial activity of coumarin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



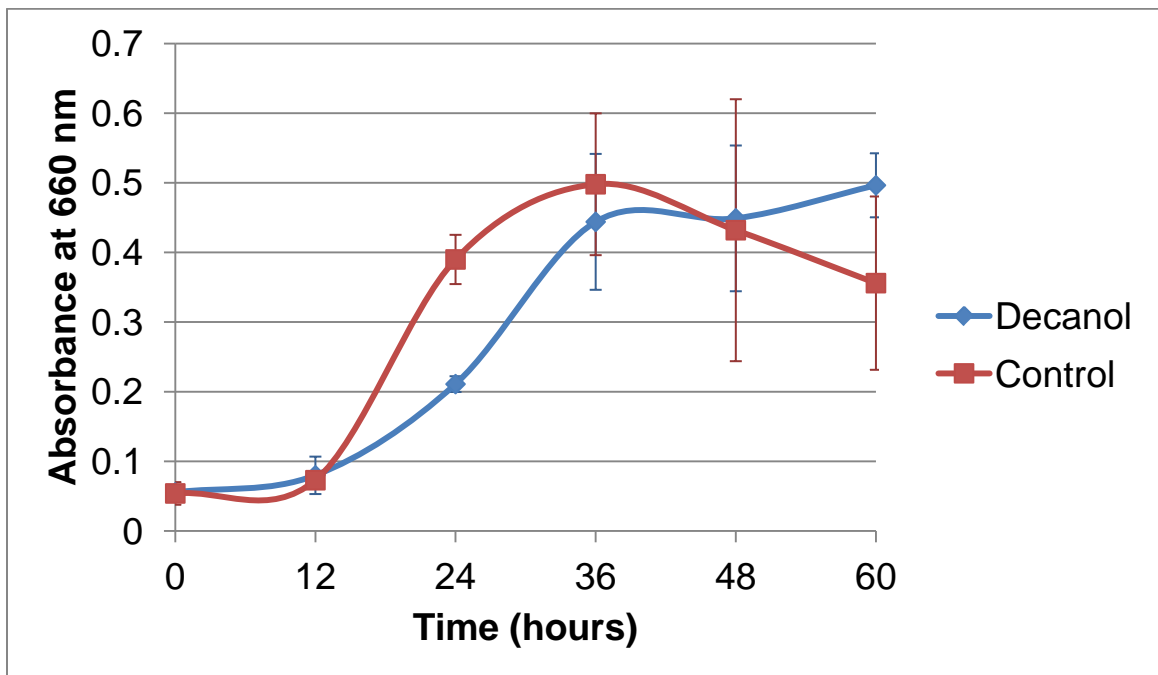
**Figure 41.** The antimicrobial activity of coumarin at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



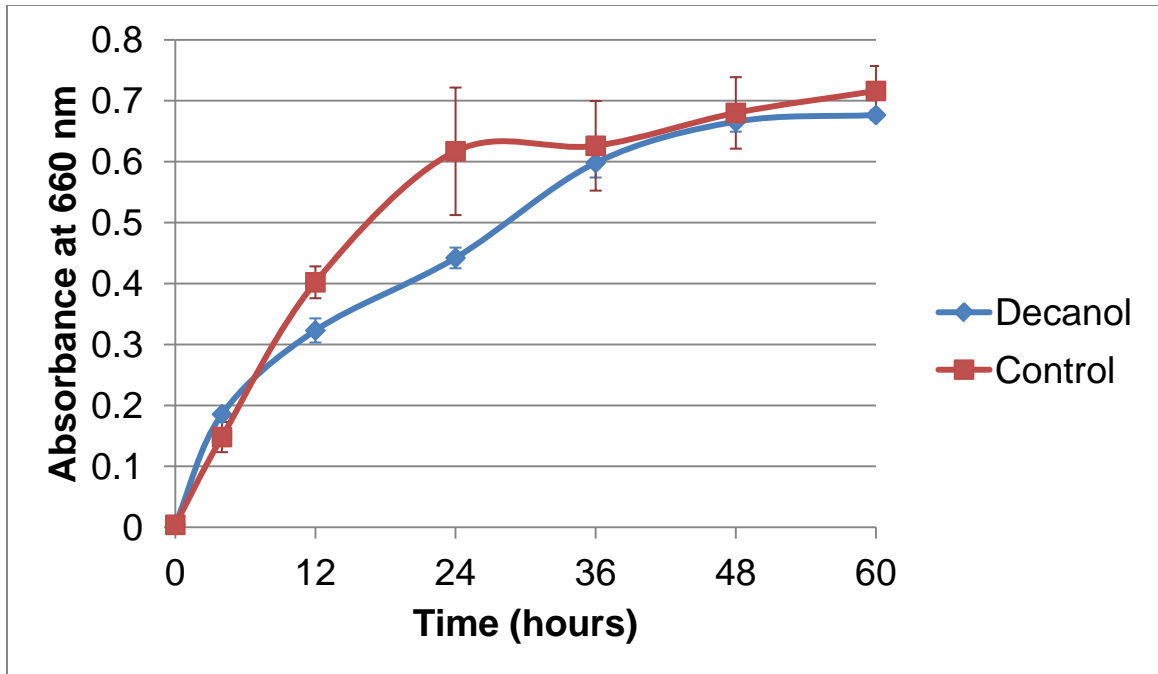
**Figure 42.** The antimicrobial activity of coumarin at MIC of 15 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



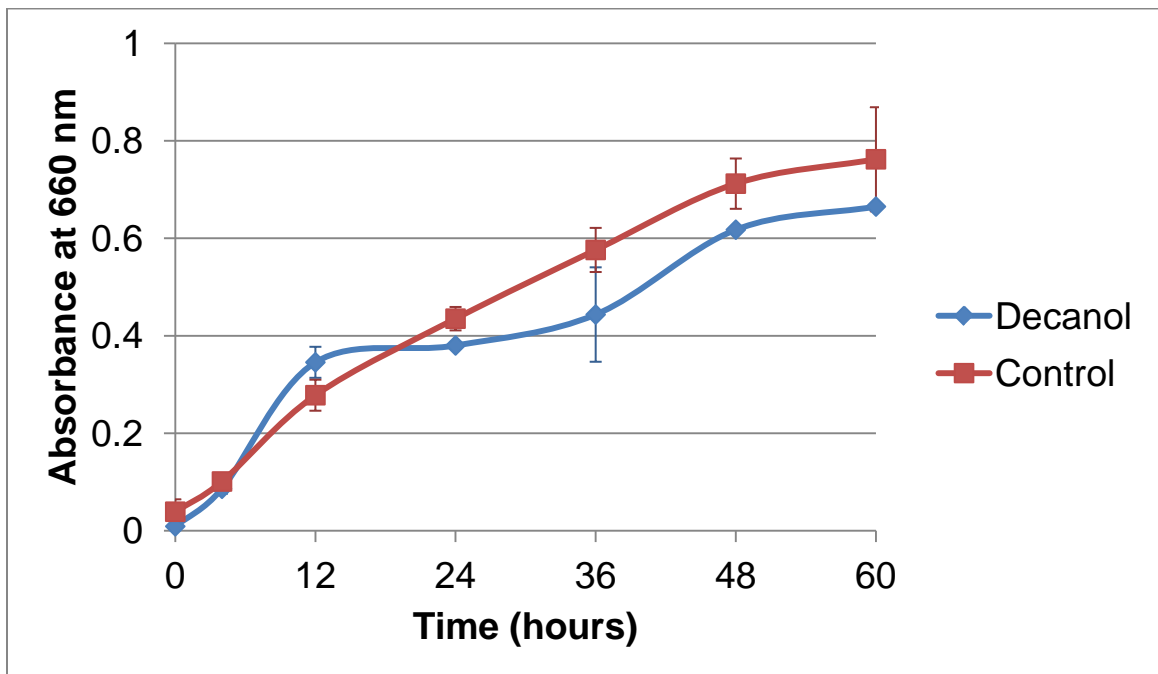
**Figure 43.** The antimicrobial activity of decanol at MIC of 20 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



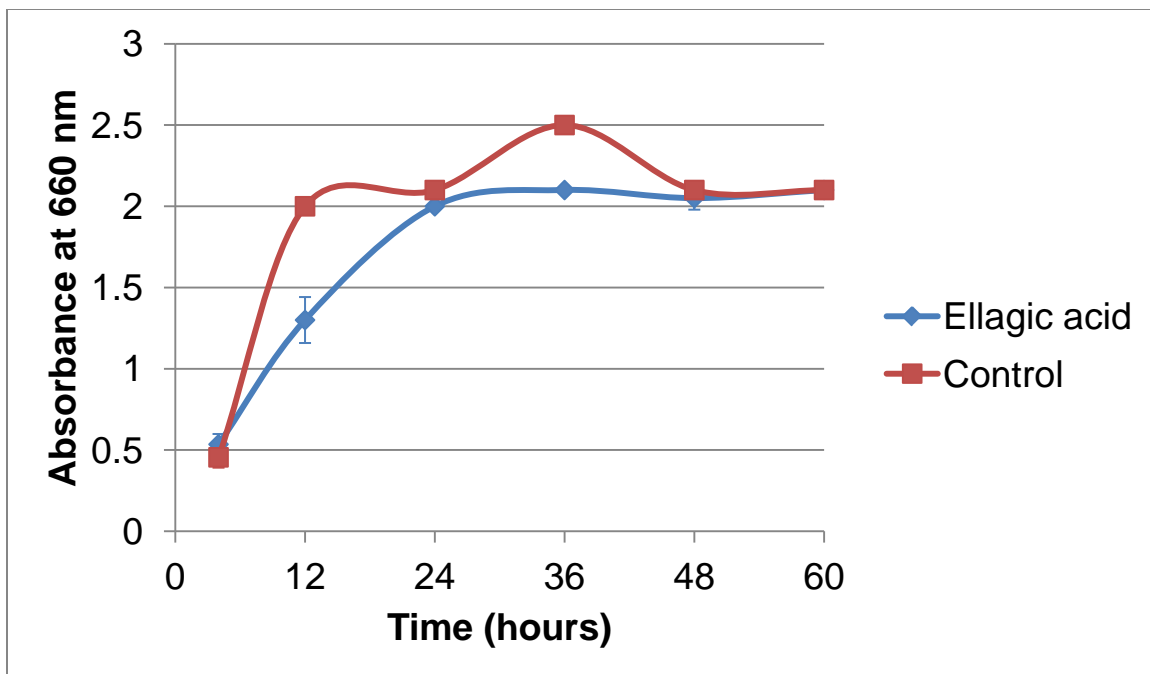
**Figure 44.** The antimicrobial activity of decanol at MIC of 5 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .



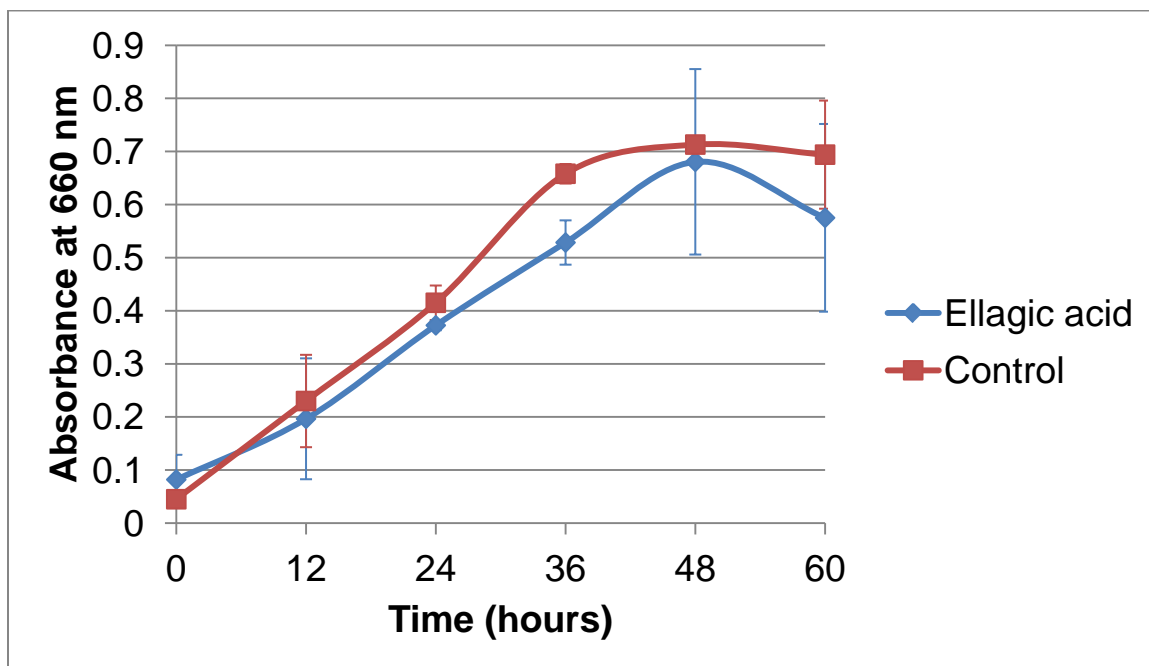
**Figure 45.** The antimicrobial activity of decanol at MIC of 15 ppm against *S. paratyphi*, UK Micro Lab. Error bars represent standard deviation from the mean,  $P < 0.05$ .



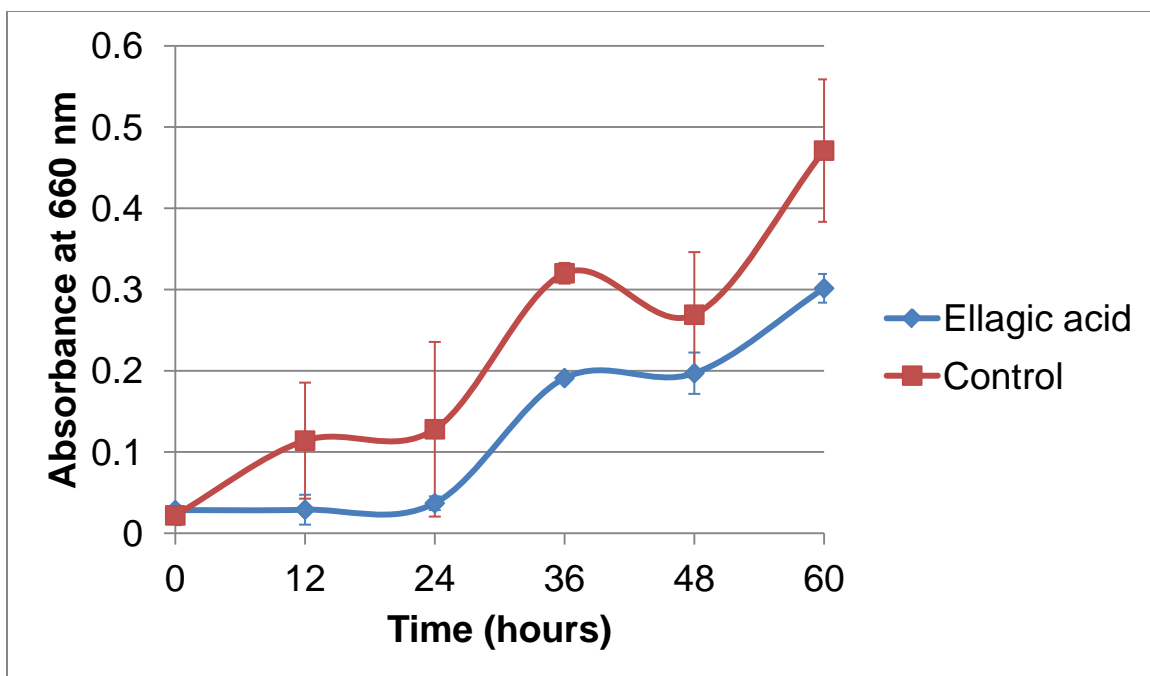
**Figure 46.** The antimicrobial activity of decanol at MIC of 15 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



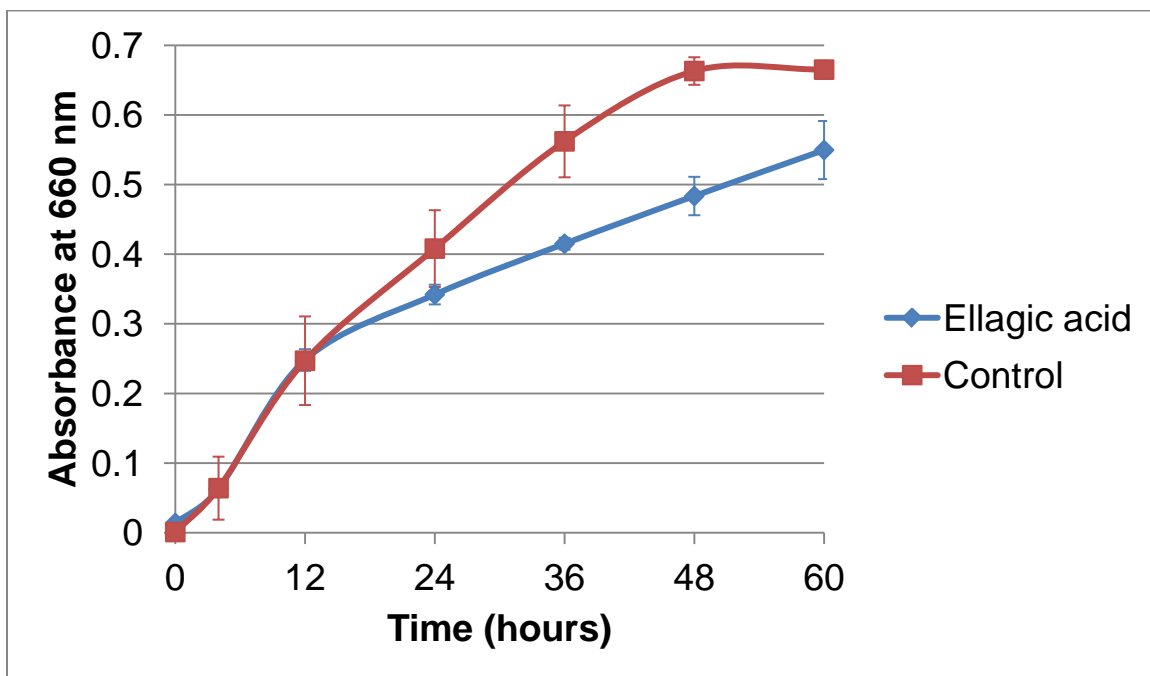
**Figure 47.** The antimicrobial activity of ellagic acid at MIC of 5 ppm against *B. polymyxa*, ATCC 842. Error bars represent standard deviation from the mean,  $P < 0.05$ .



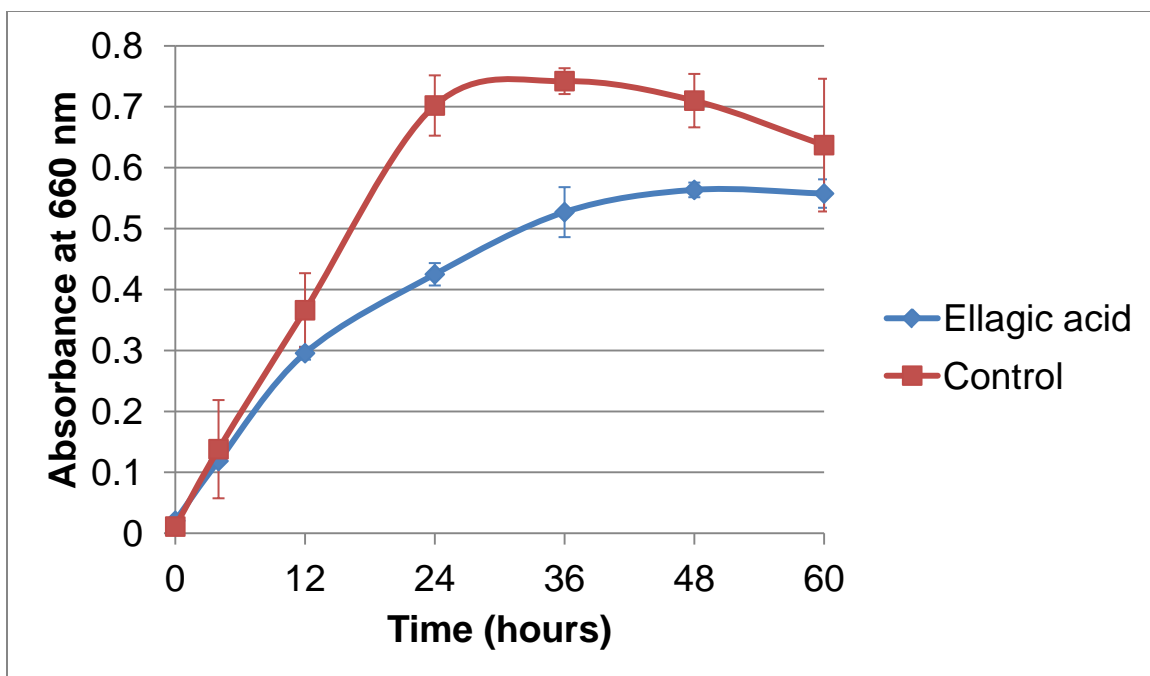
**Figure 48.** The antimicrobial activity of ellagic acid at MIC of 5 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .



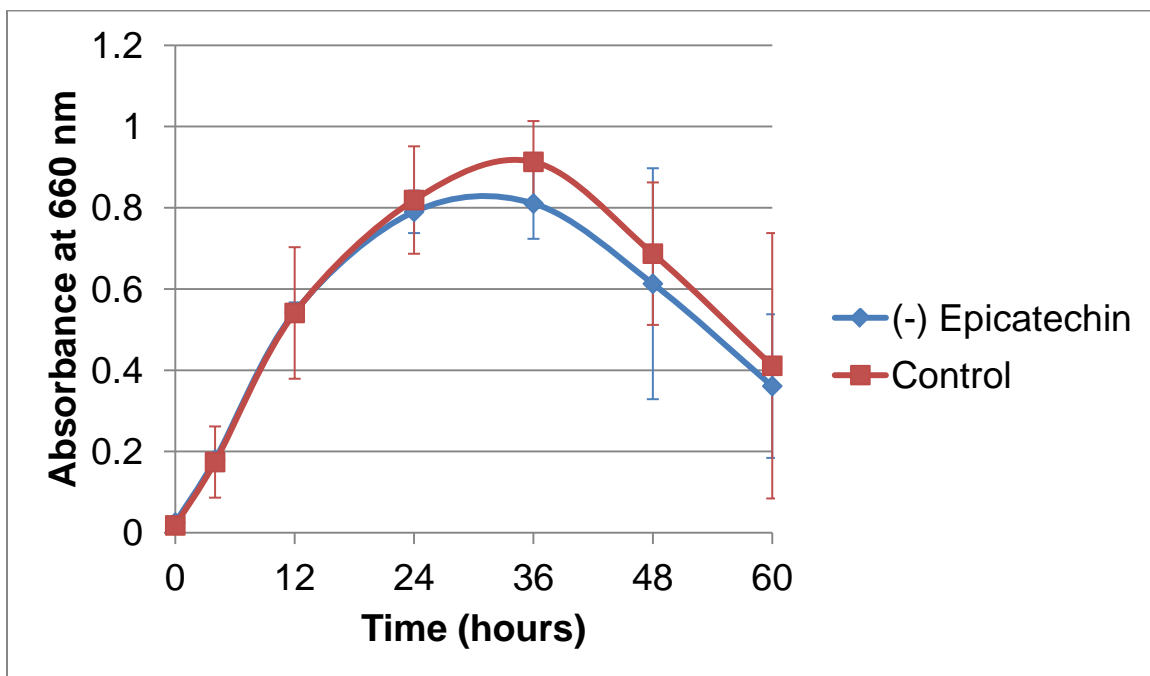
**Figure 49.** The antimicrobial activity of ellagic acid at MIC of 15 ppm against *C. butyricum*, ATCC 8260. Error bars represent standard deviation from the mean,  $P < 0.05$ .



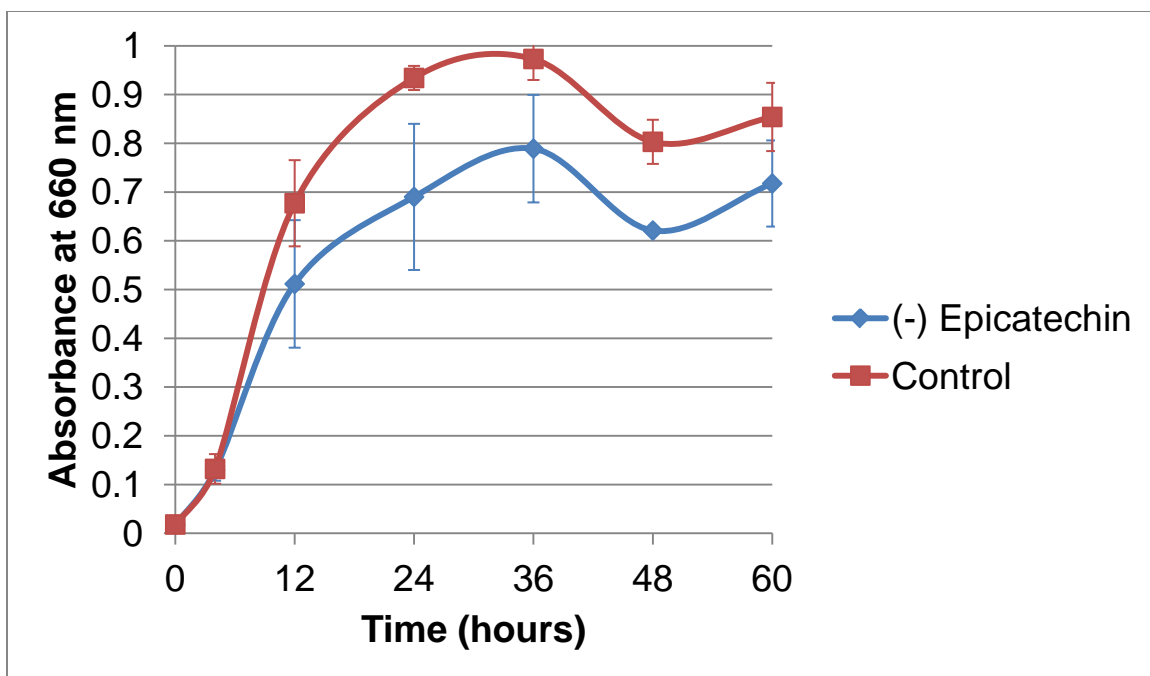
**Figure 50.** The antimicrobial activity of ellagic acid at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



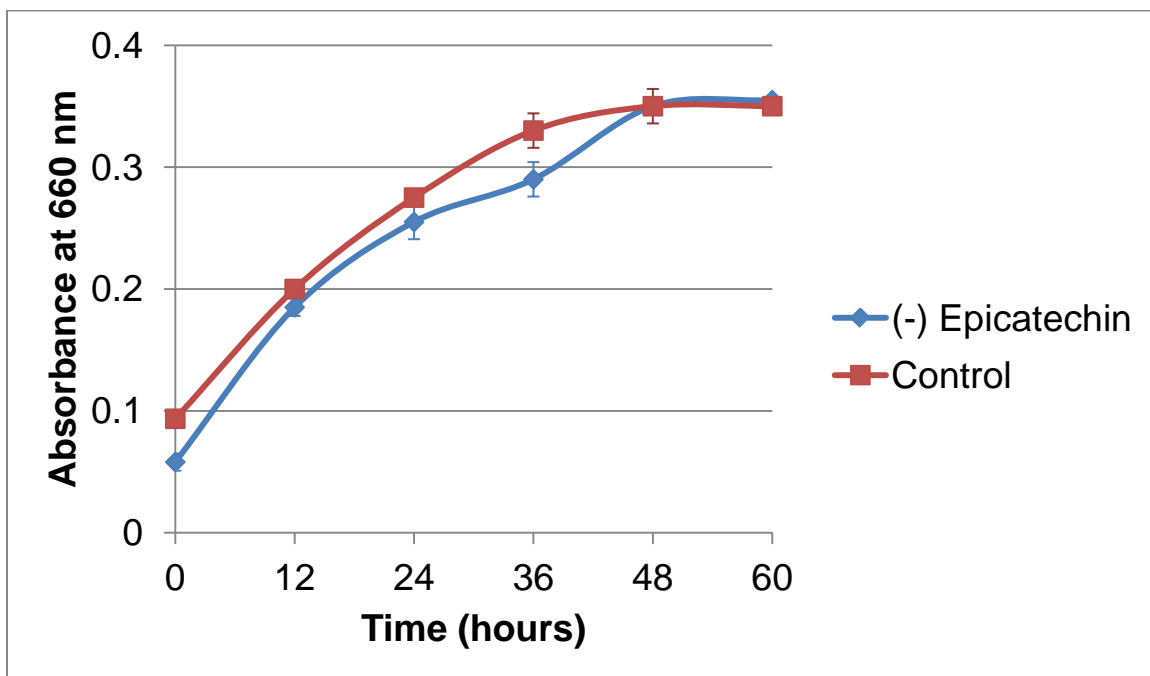
**Figure 51.** The antimicrobial activity of ellagic acid at MIC of 15 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



**Figure 52.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.

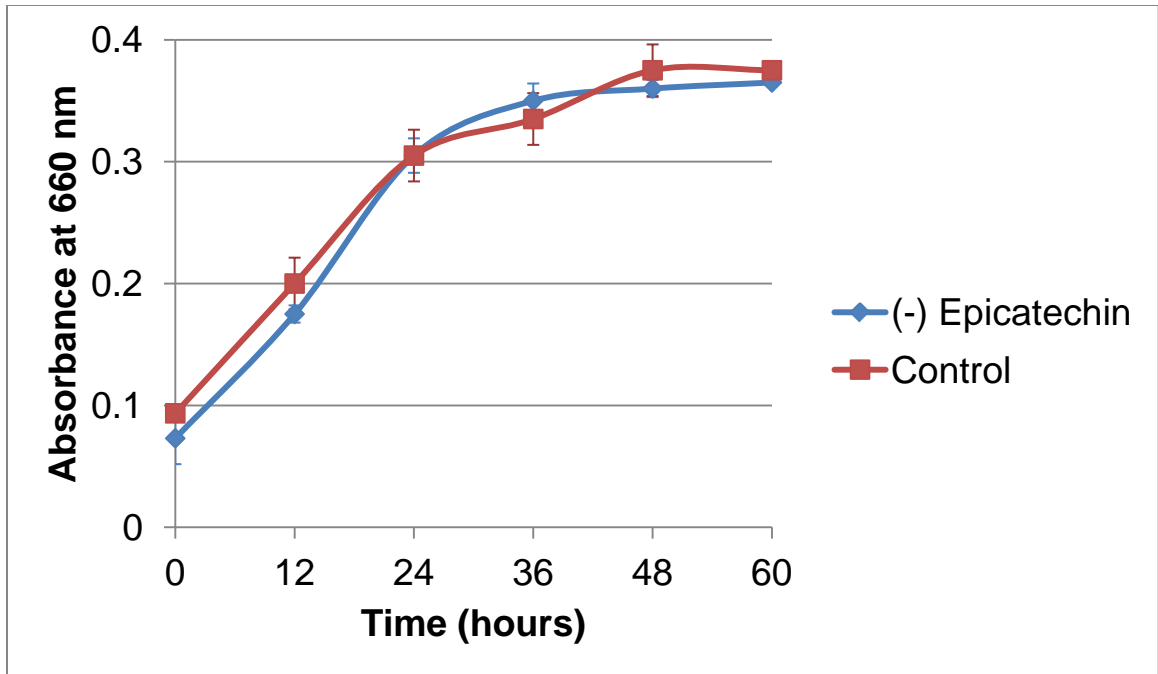


**Figure 53.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .

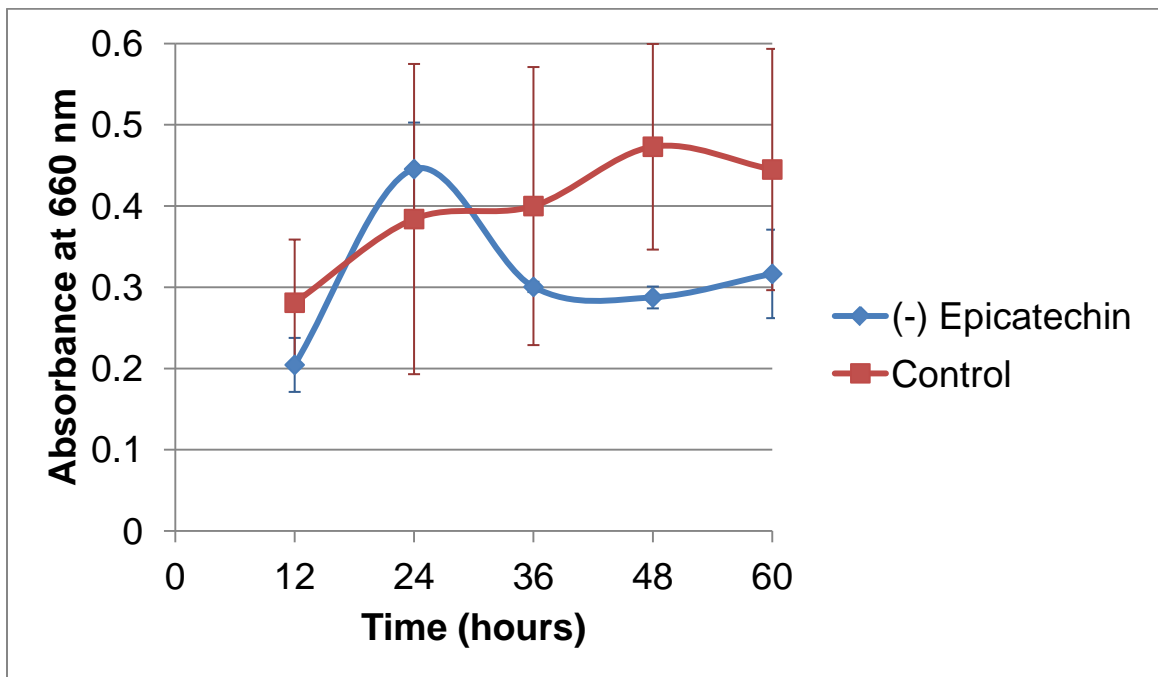


**Figure 54.** The antimicrobial activity of (-) epicatechin at MIC of 5 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .

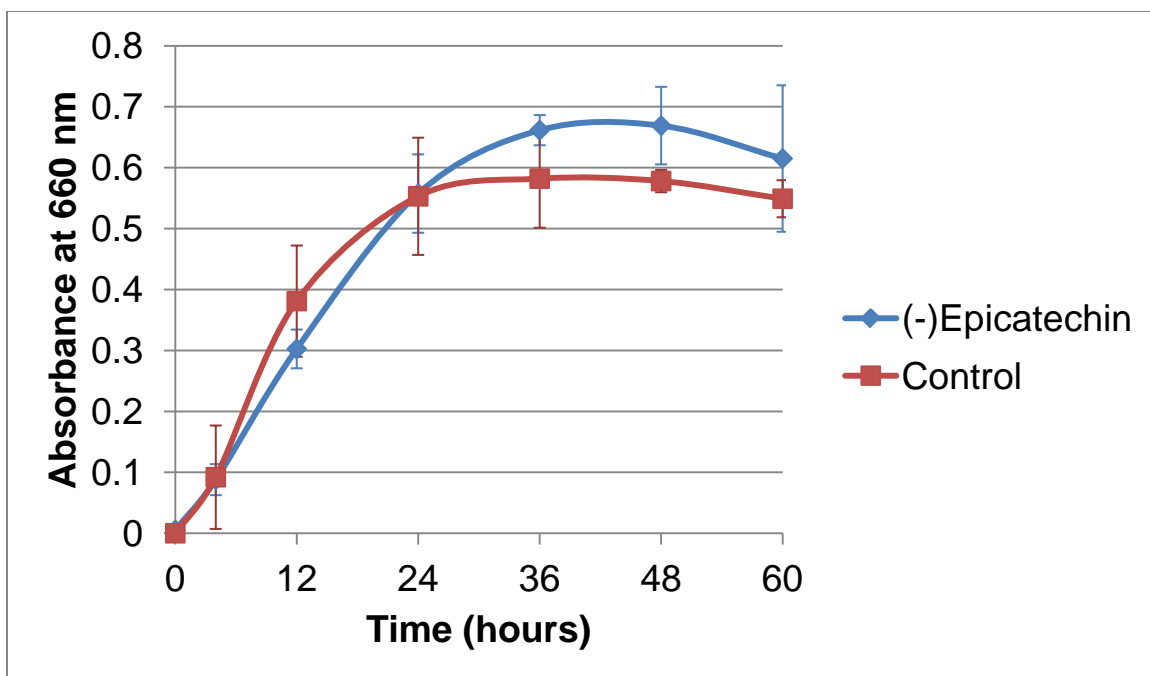




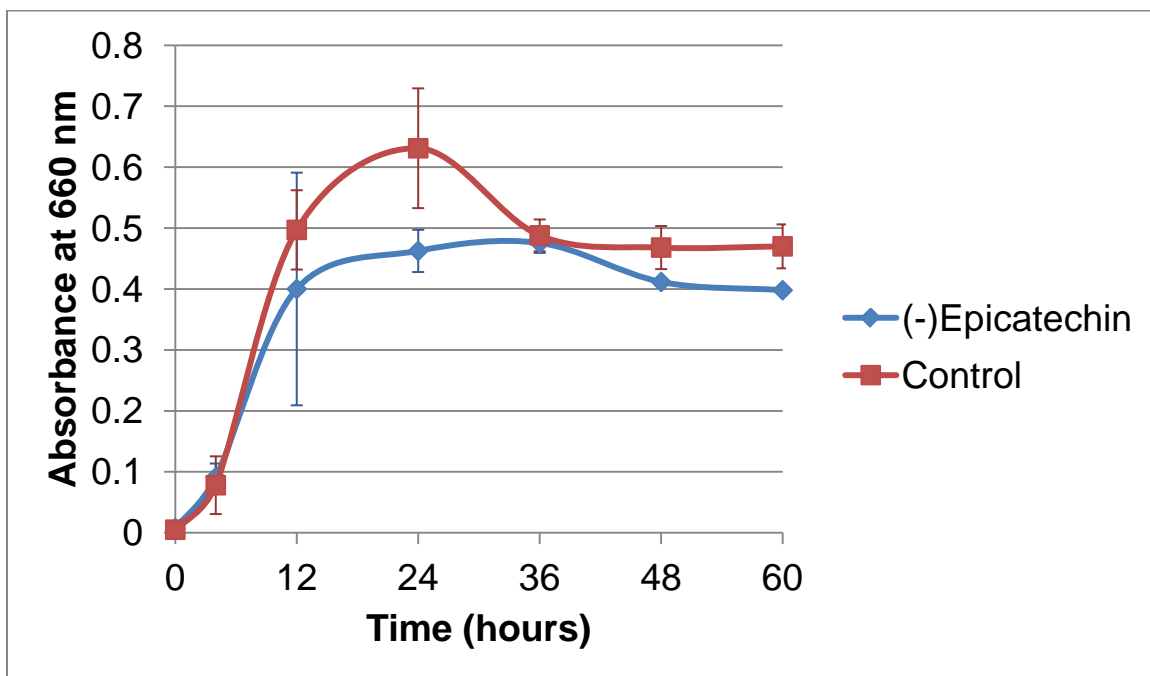
**Figure 55.** The antimicrobial activity of (-) epicatechin at MIC of 5 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



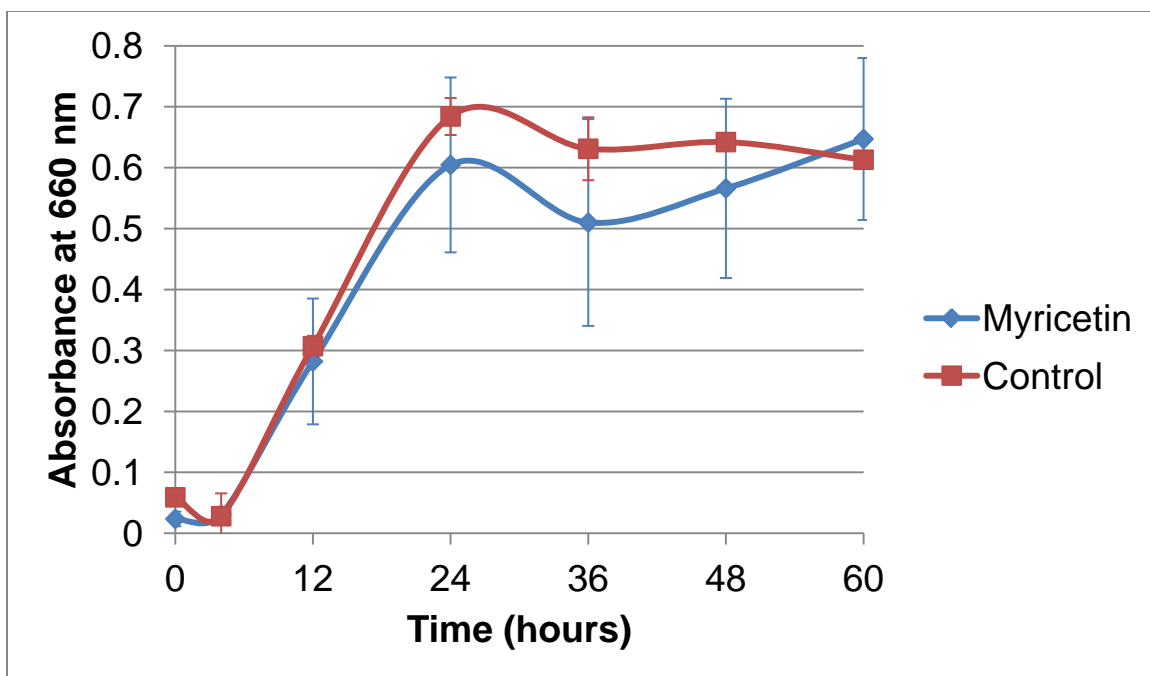
**Figure 56.** The antimicrobial activity of (-) epicatechin at MIC of 20 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .



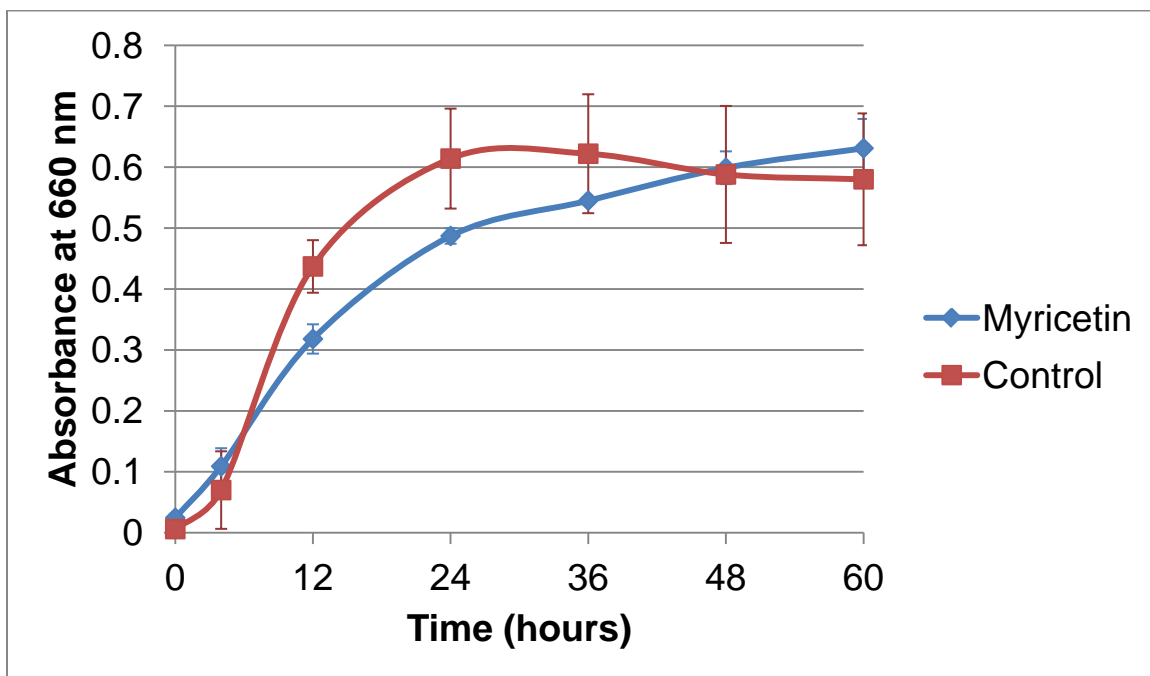
**Figure 57.** The antimicrobial activity of (-) epicatechin at MIC of 5 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



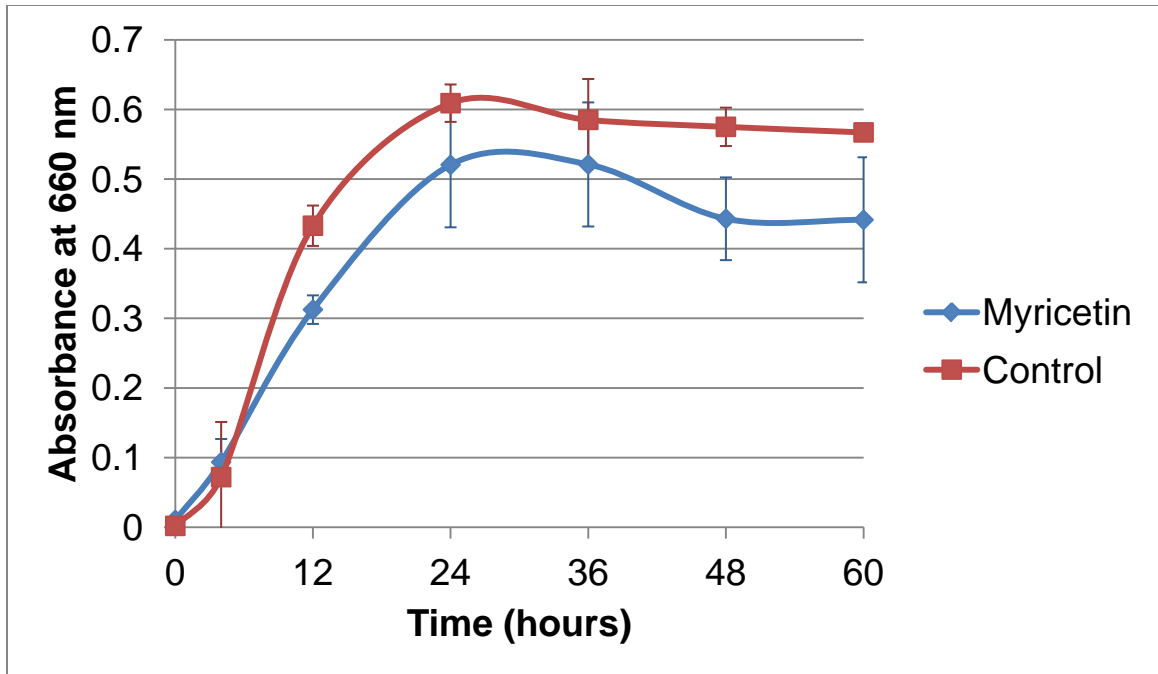
**Figure 58.** The antimicrobial activity of (-) epicatechin at MIC of 10 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



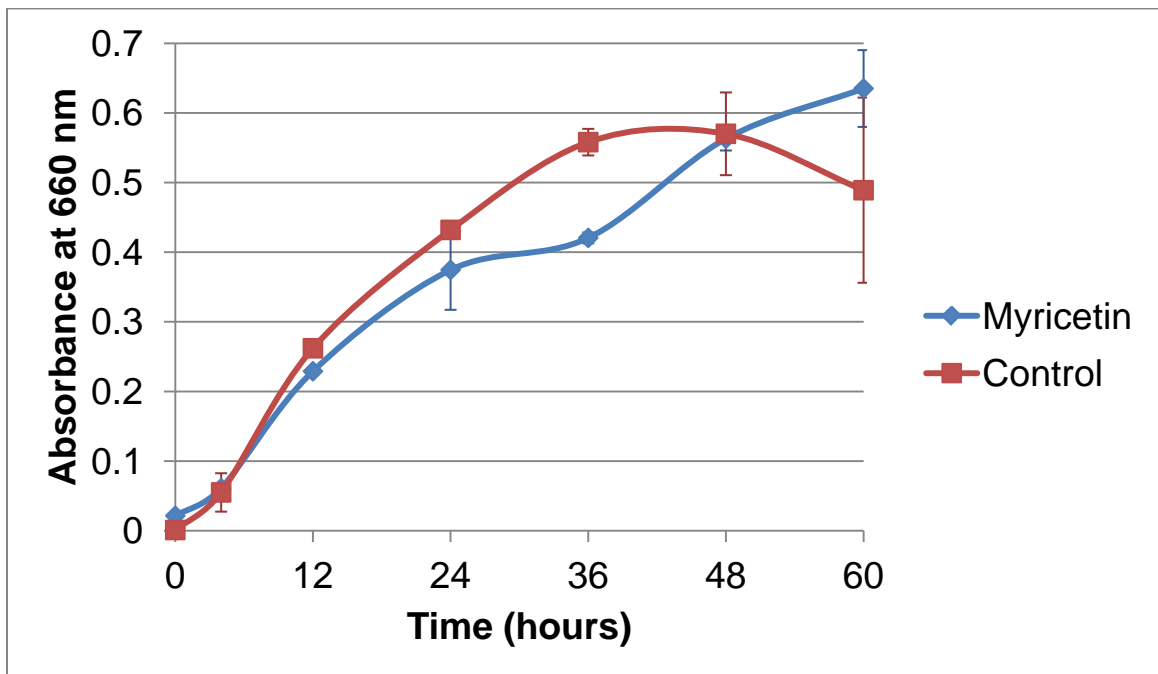
**Figure 59.** The antimicrobial activity of myricetin at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean,  $P < 0.05$ .



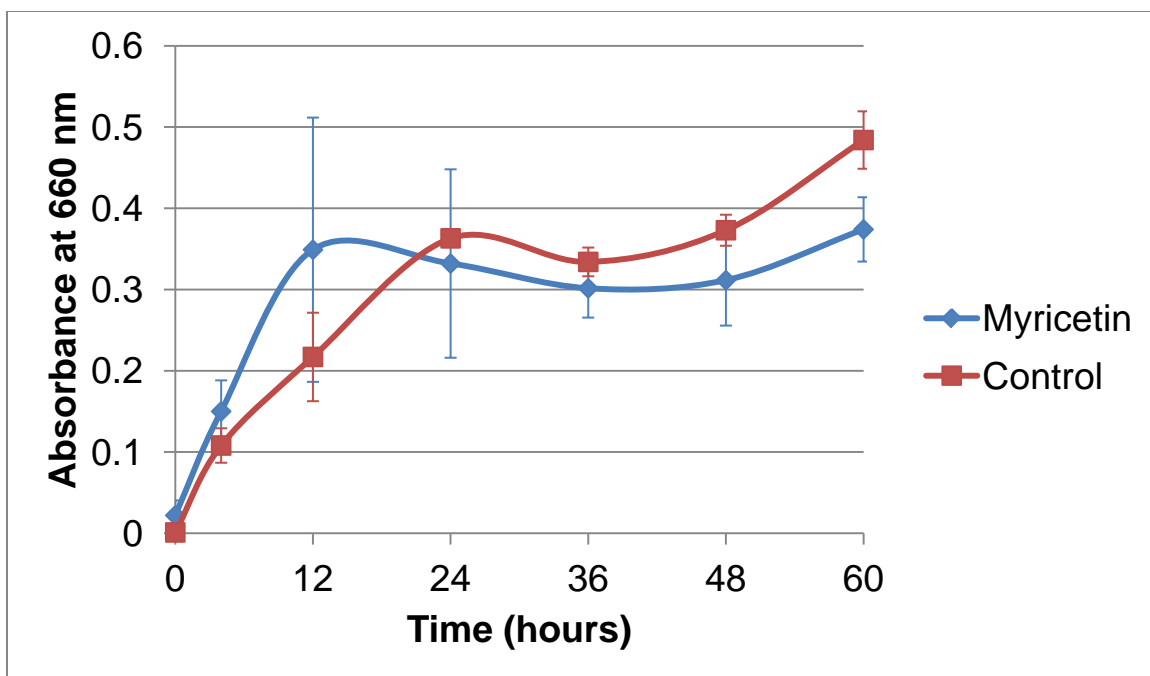
**Figure 60.** The antimicrobial activity of myricetin at MIC of 10 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



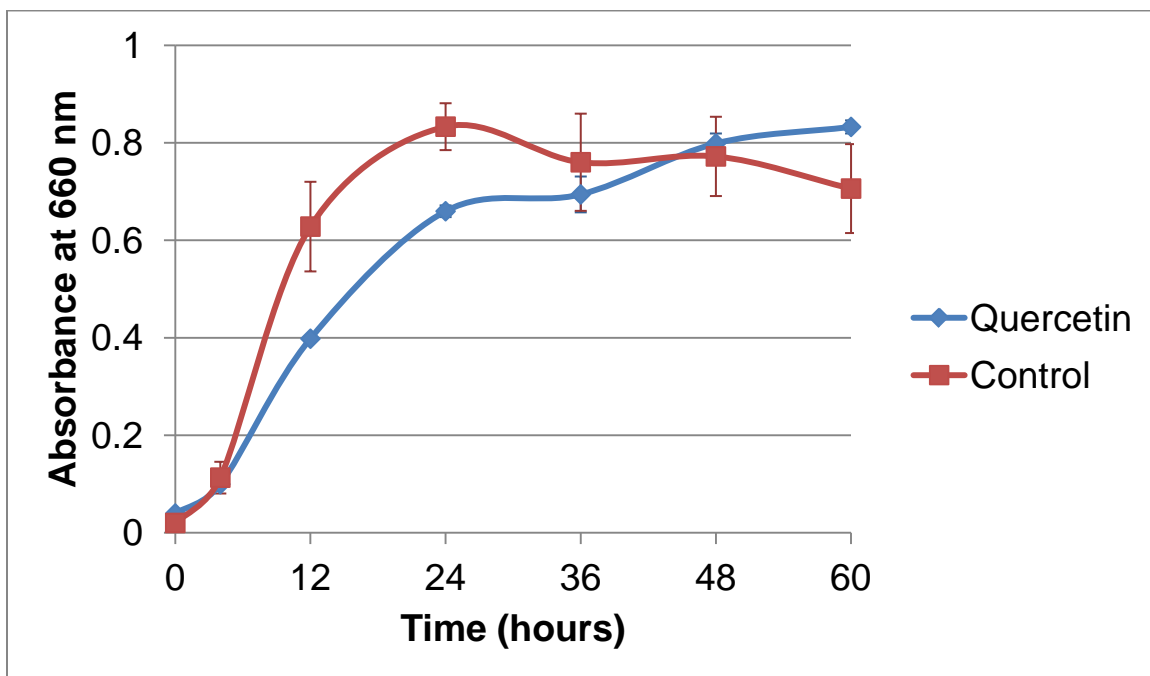
**Figure 61.** The antimicrobial activity of myricetin at MIC of 15 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



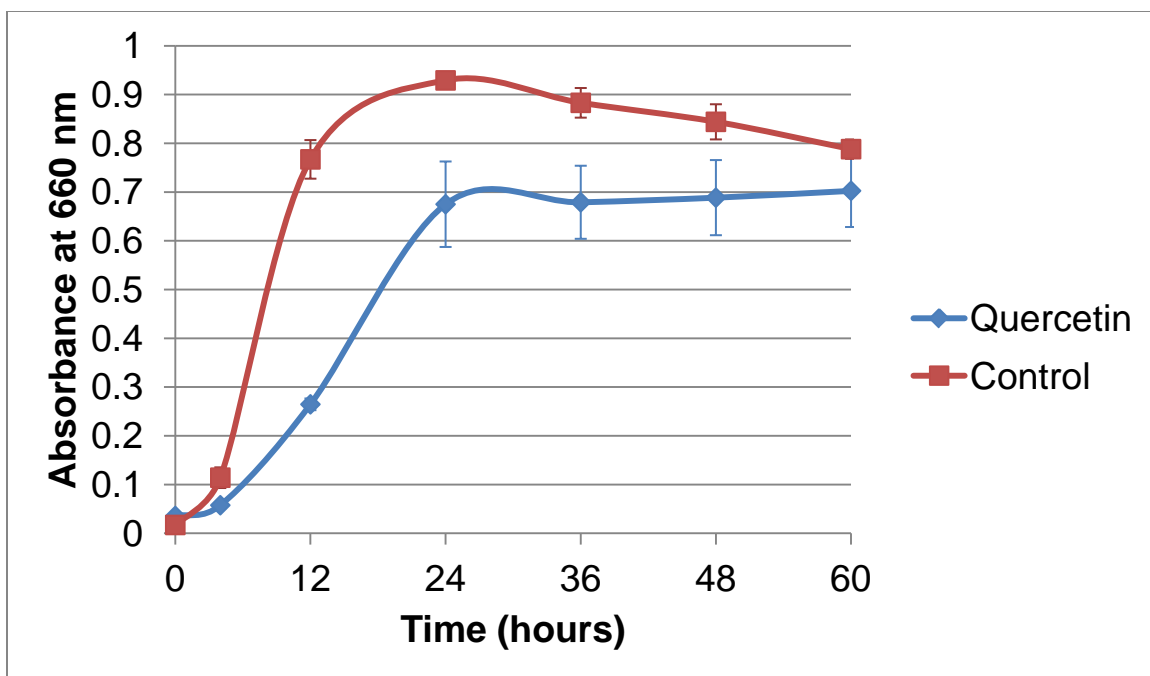
**Figure 62.** The antimicrobial activity of myricetin at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



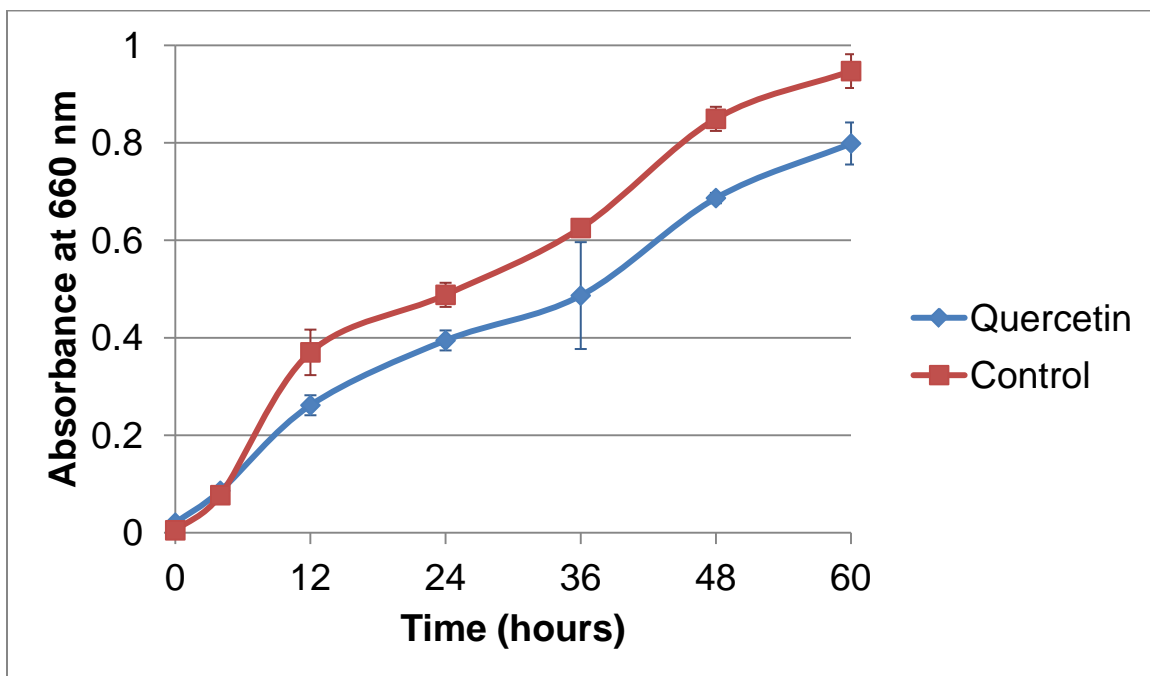
**Figure 63.** The antimicrobial activity of Myricetin at MIC of 5 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



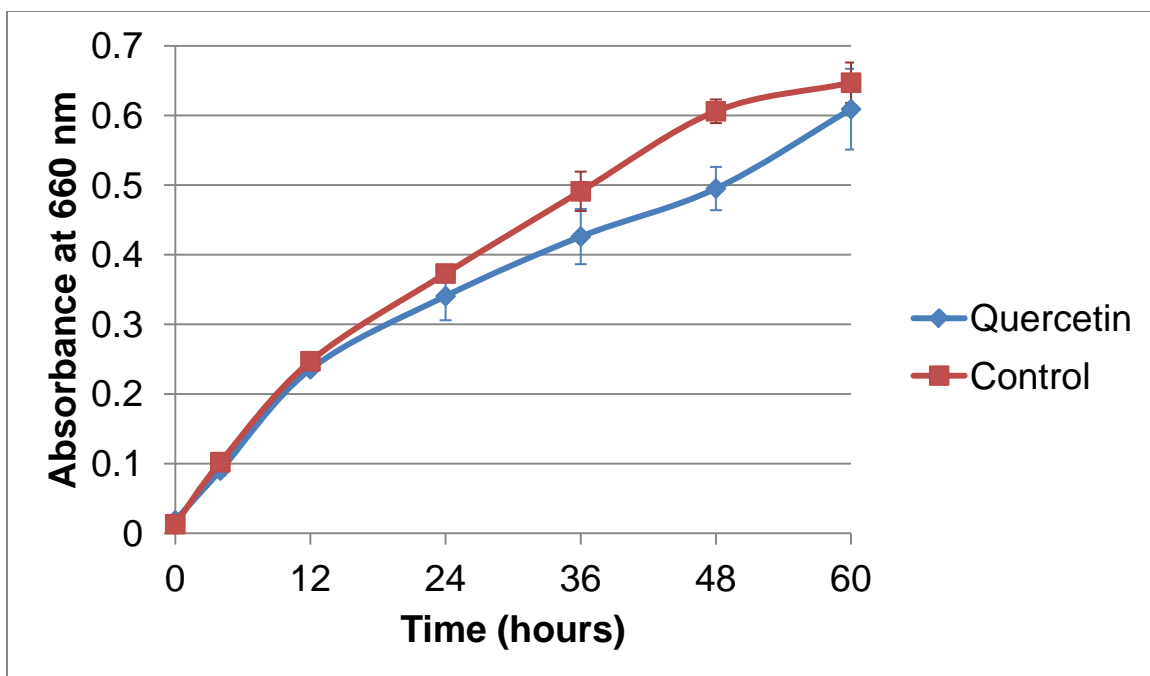
**Figure 64.** The antimicrobial activity of quercetin at MIC of 15 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.



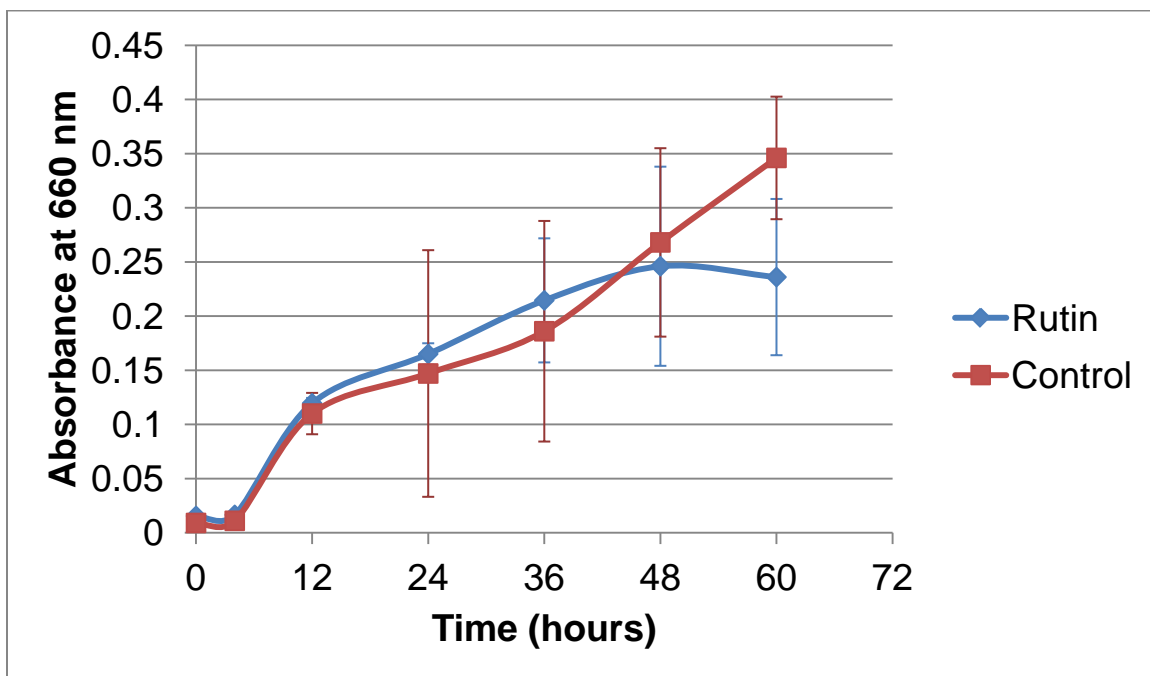
**Figure 65.** The antimicrobial activity of quercetin at MIC of 20 ppm against *B. cereus*, Difco Spores. Error bars represent standard deviation from the mean,  $P < 0.05$ .



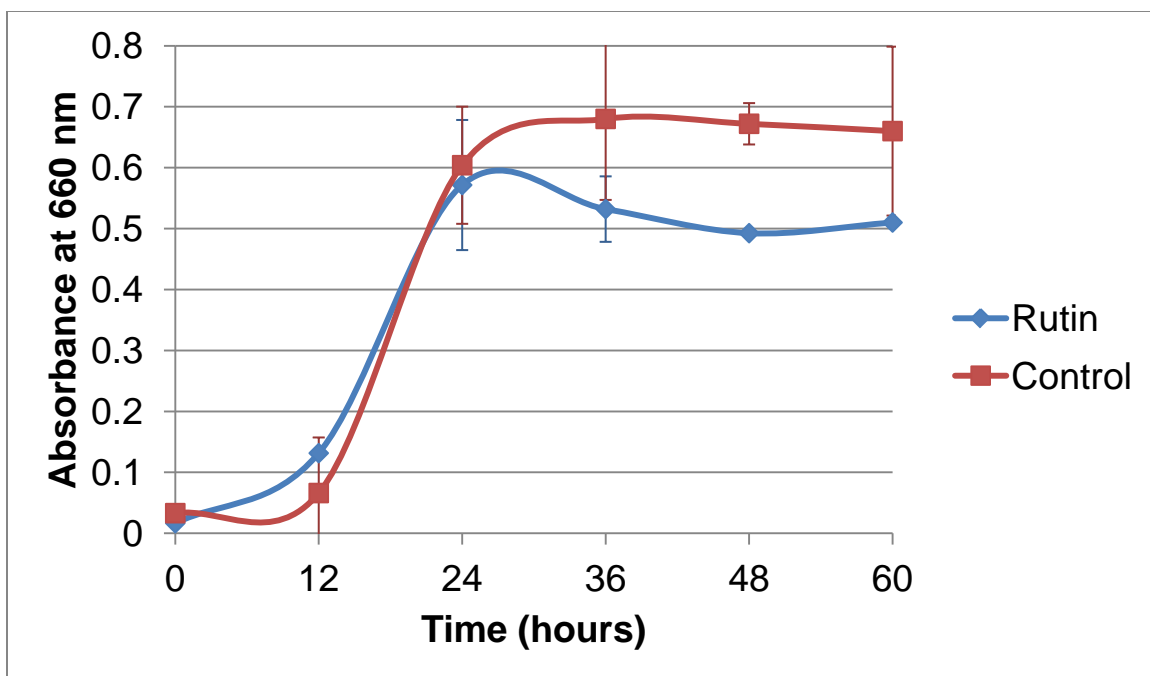
**Figure 66.** The antimicrobial activity of quercetin at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



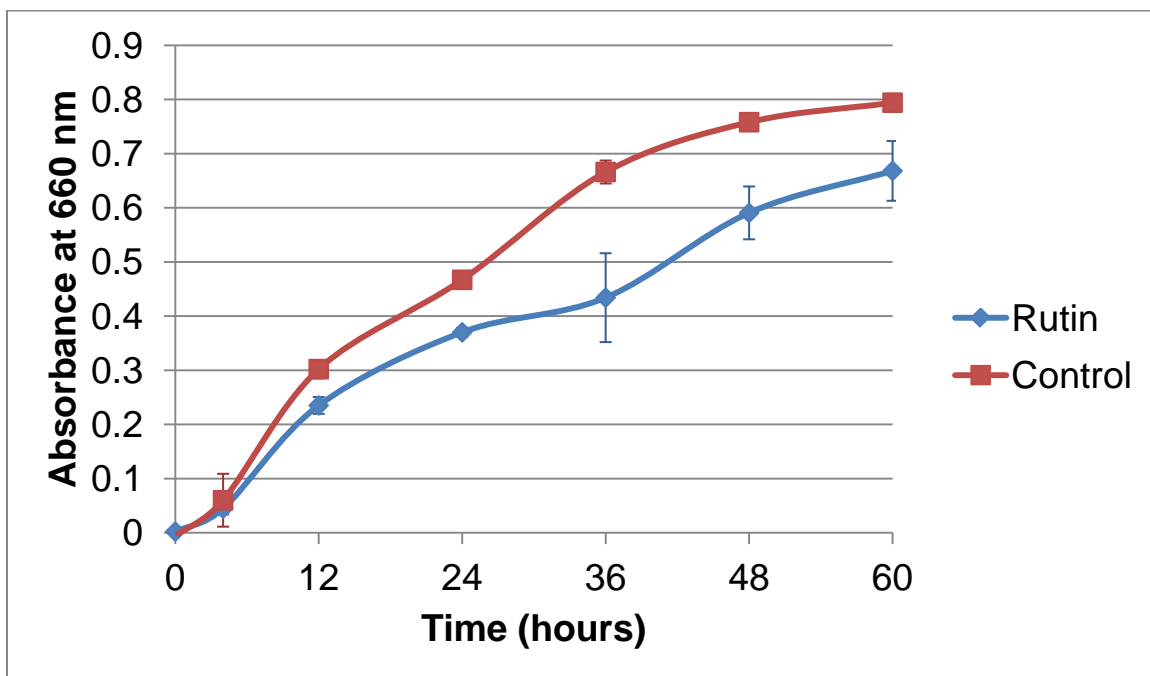
**Figure 67.** The antimicrobial activity of quercetin at MIC of 10 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



**Figure 68.** The antimicrobial activity of rutin at MIC of 15 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean, P<0.05.

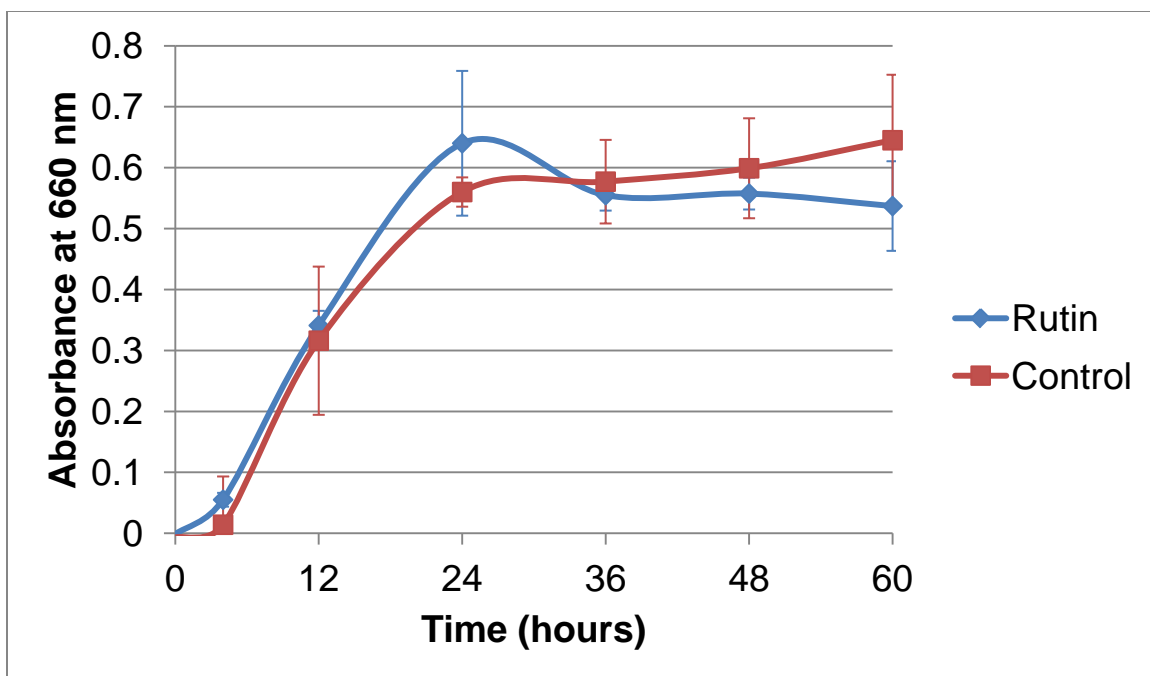


**Figure 69.** The antimicrobial activity of rutin at MIC of 10 ppm against *C. perfringens*, RNR. Error bars represent standard deviation from the mean,  $P < 0.05$ .

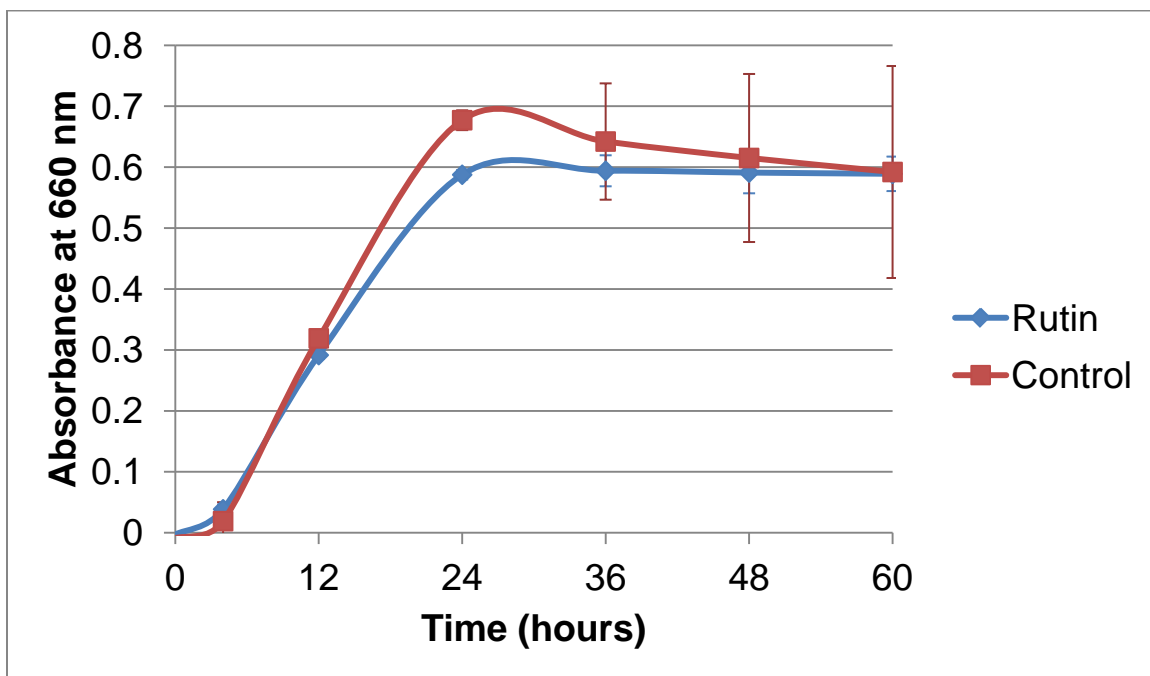


**Figure 70.** The antimicrobial activity of rutin at MIC of 20 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .

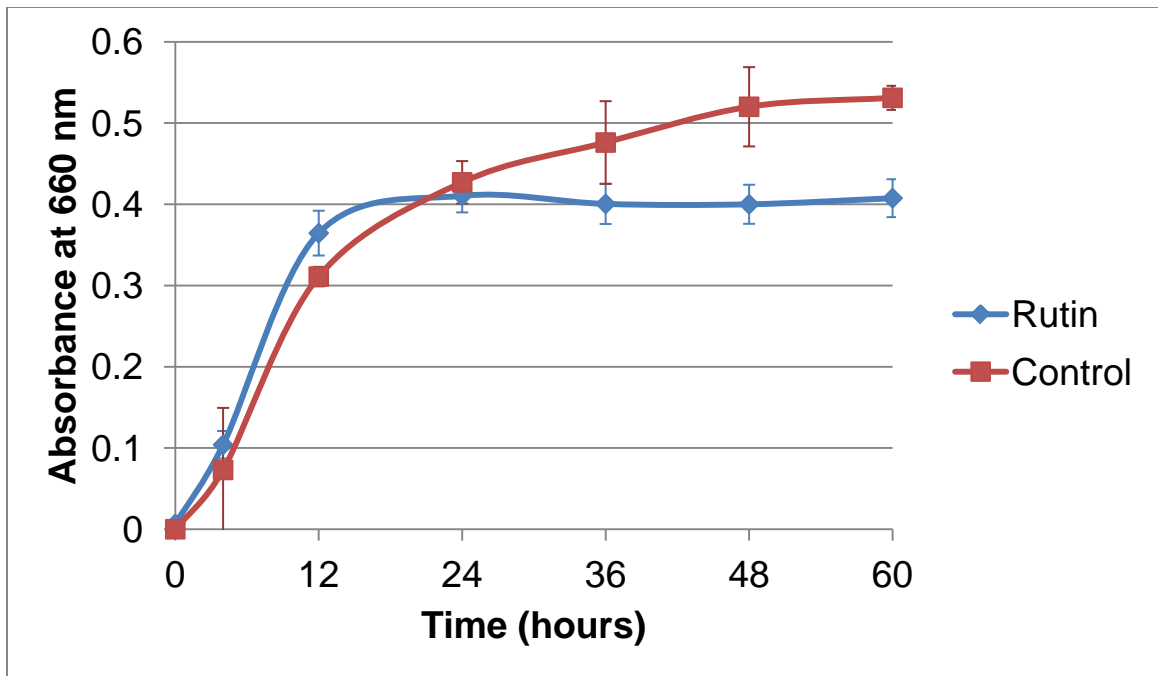




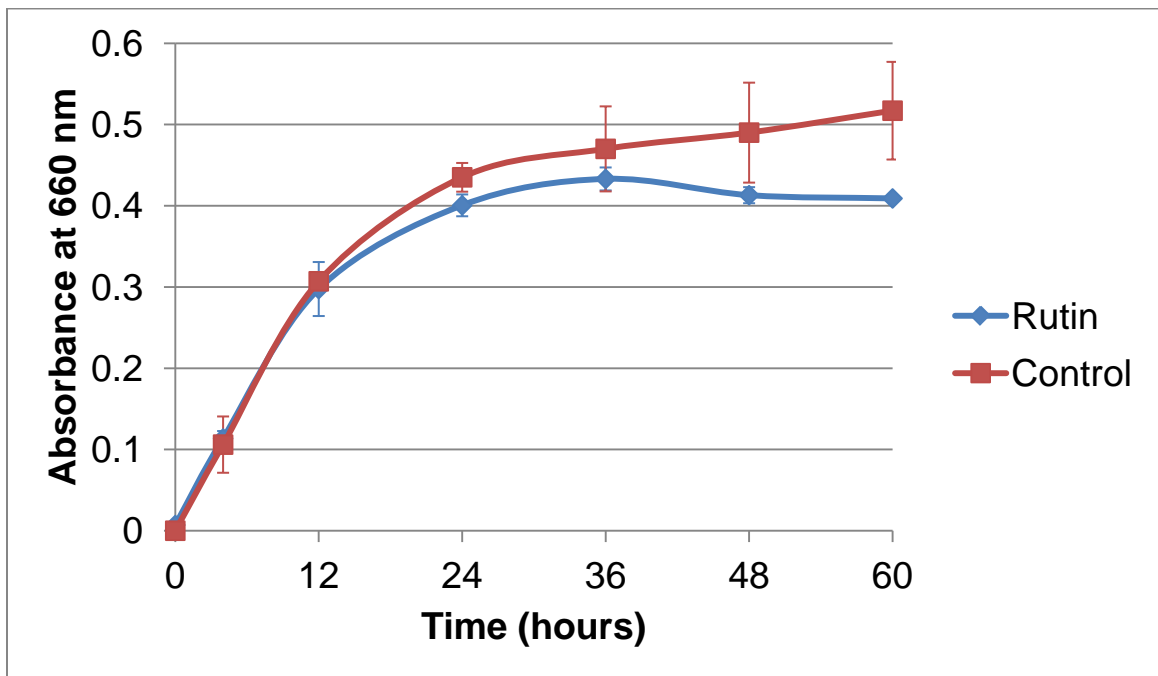
**Figure 71.** The antimicrobial activity of rutin at MIC of 5 ppm against *E. coli* O157:H7, ATCC 43895. Error bars represent standard deviation from the mean,  $P < 0.05$ .



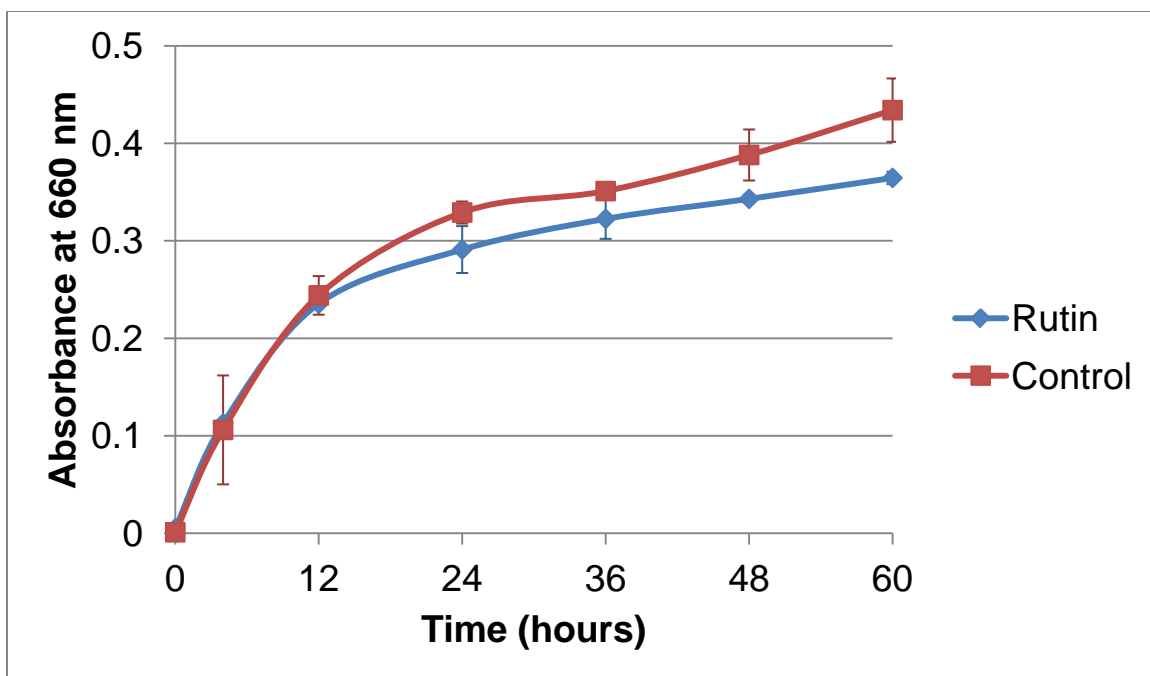
**Figure 5.72.** The antimicrobial activity of rutin at MIC of 15 ppm against *E. coli* O157:H7, ATCC 35150. Error bars represent standard deviation from the mean,  $P < 0.05$ .



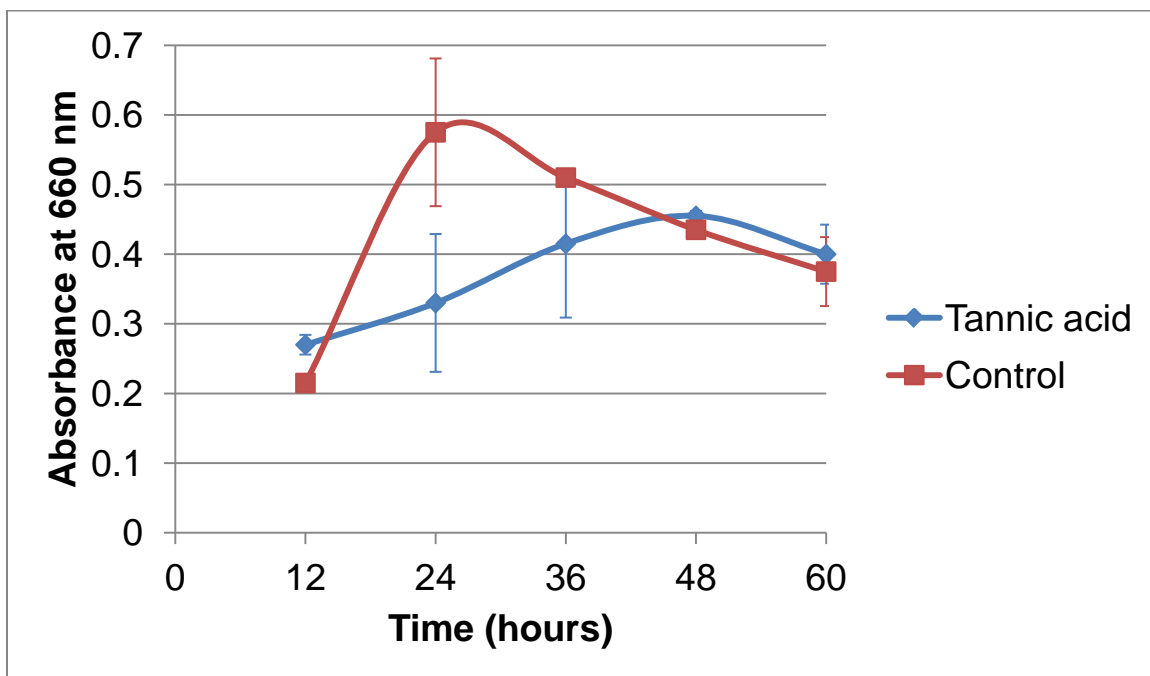
**Figure 73.** The antimicrobial activity of rutin at MIC of 20 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



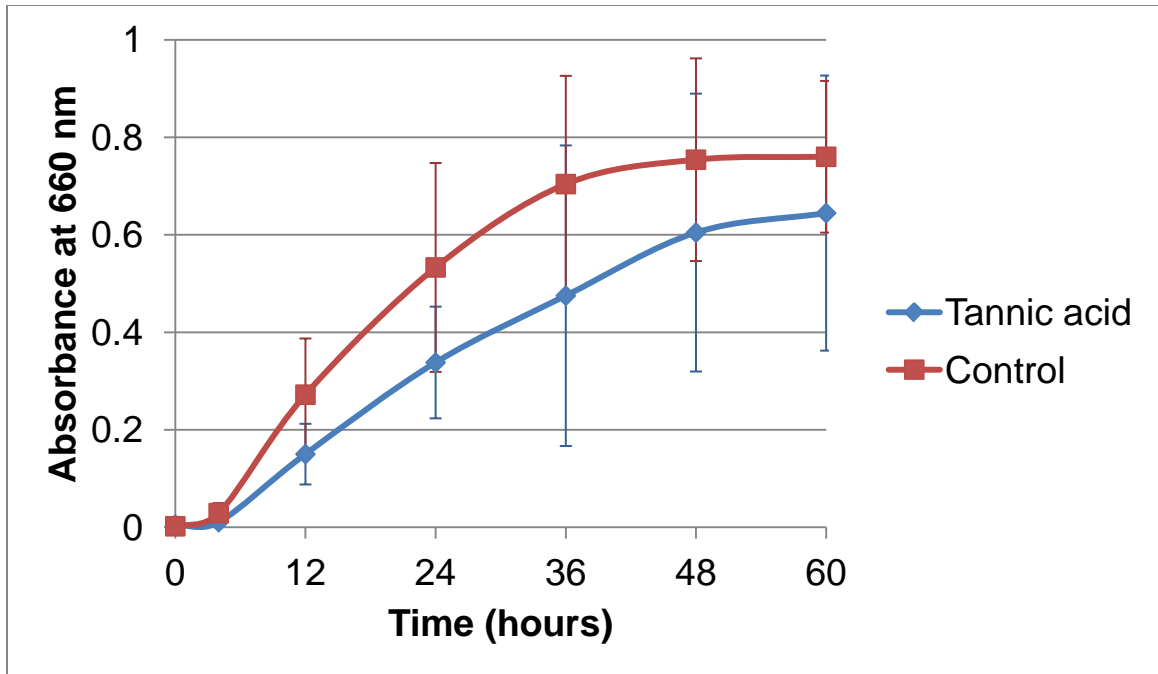
**Figure 74.** The antimicrobial activity of rutin at MIC of 15 ppm against *S. choleraesuis* subsp., ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



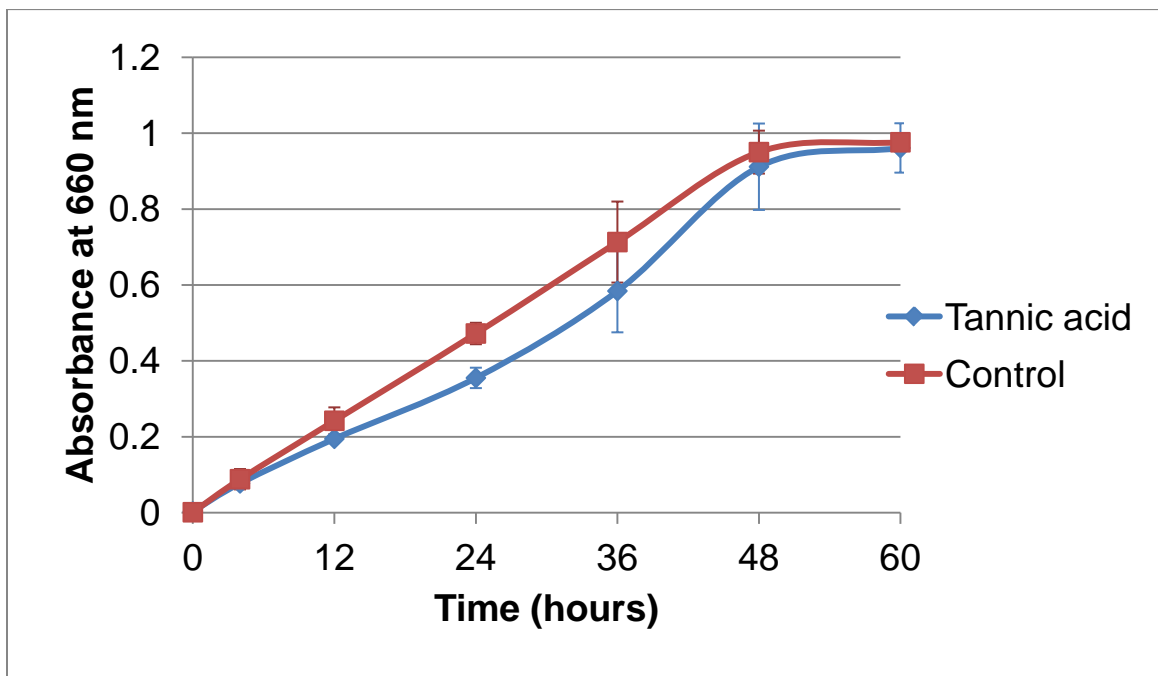
**Figure 75.** The antimicrobial activity of rutin at MIC of 15 ppm against *S. enteridis*, (-) H<sub>2</sub>S. Error bars represent standard deviation from the mean, P<0.05.



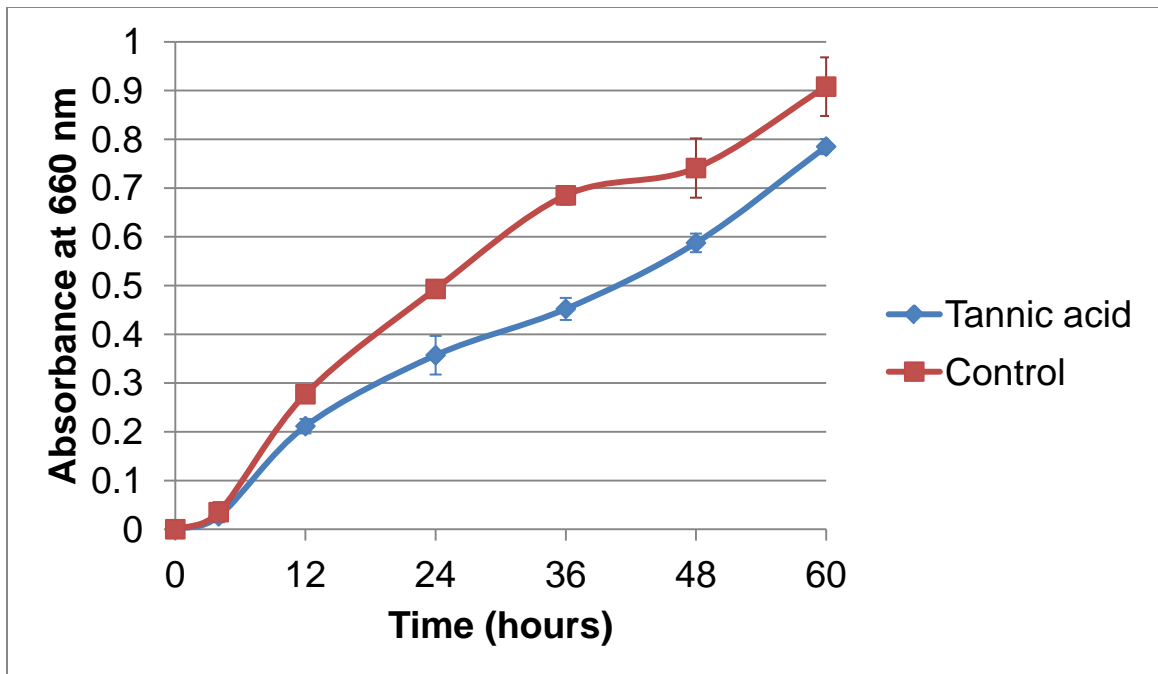
**Figure 76.** The antimicrobial activity of tannic acid at MIC of 20 ppm against *B. subtilis*, ATCC 6051. Error bars represent standard deviation from the mean, P<0.05.



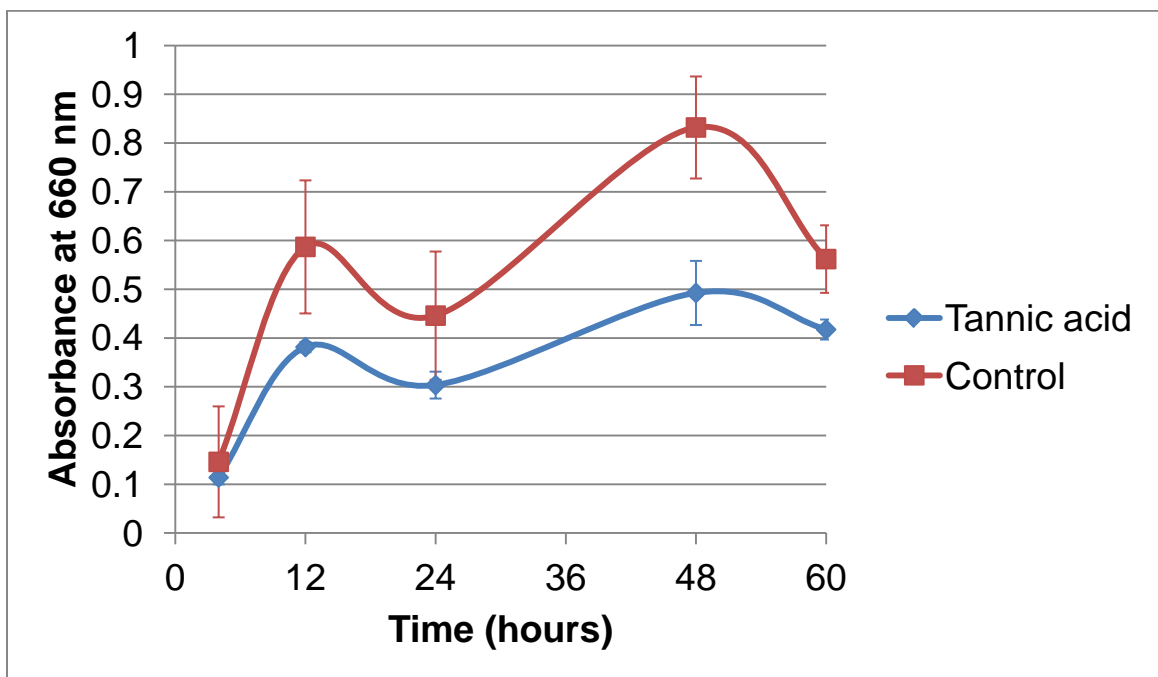
**Figure 77.** The antimicrobial activity of tannic acid at MIC of 10 ppm against *L. monocytogenes*, ATCC 7644. Error bars represent standard deviation from the mean,  $P < 0.05$ .



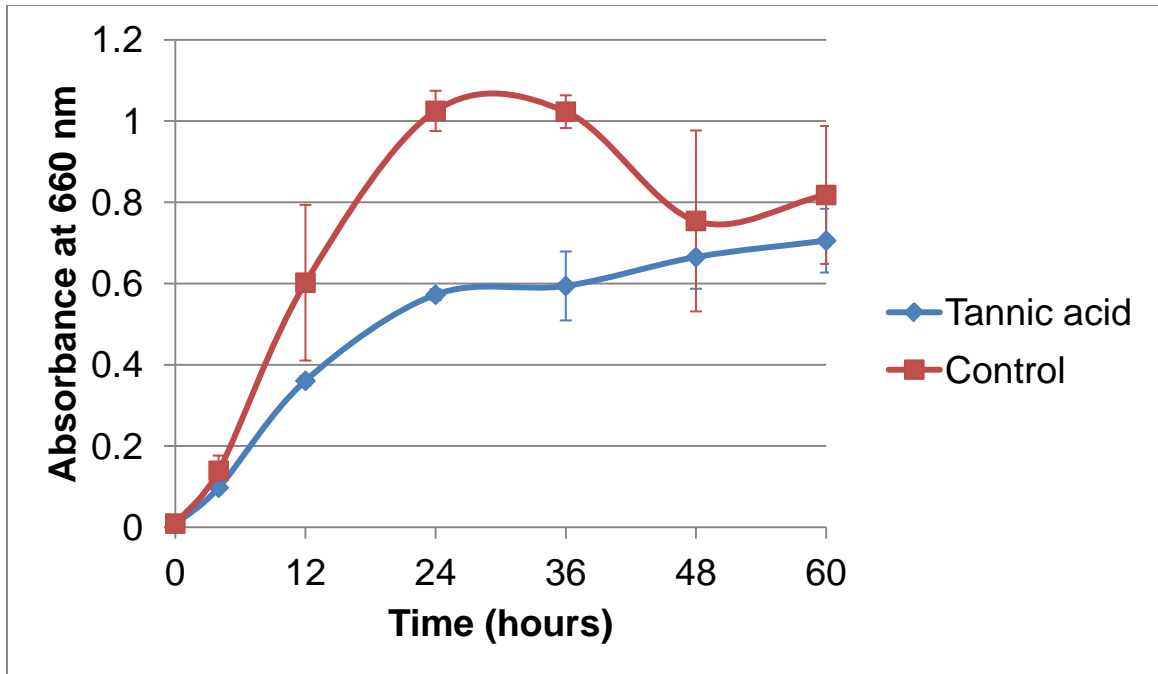
**Figure 78.** The antimicrobial activity of tannic acid at MIC of 15 ppm against *L. monocytogenes*, UK ADL. Error bars represent standard deviation from the mean,  $P < 0.05$ .



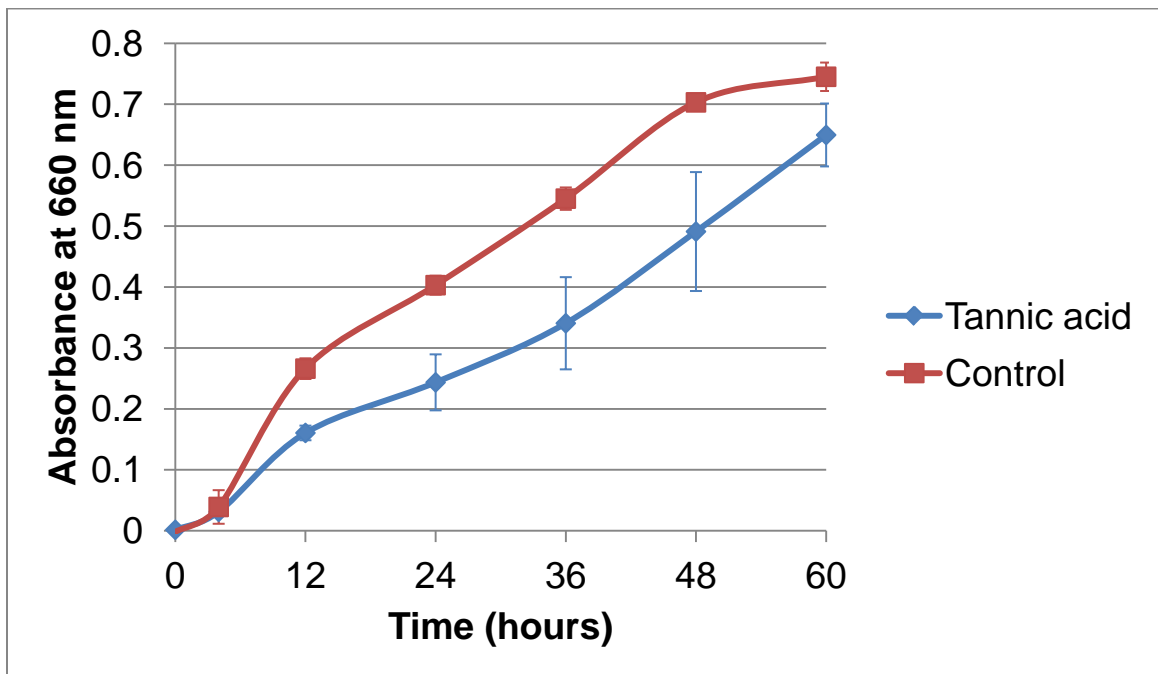
**Figure 79.** The antimicrobial activity of tannic acid at MIC of 20 ppm against *L. monocytogenes*, ATCC 49594. Error bars represent standard deviation from the mean,  $P < 0.05$ .



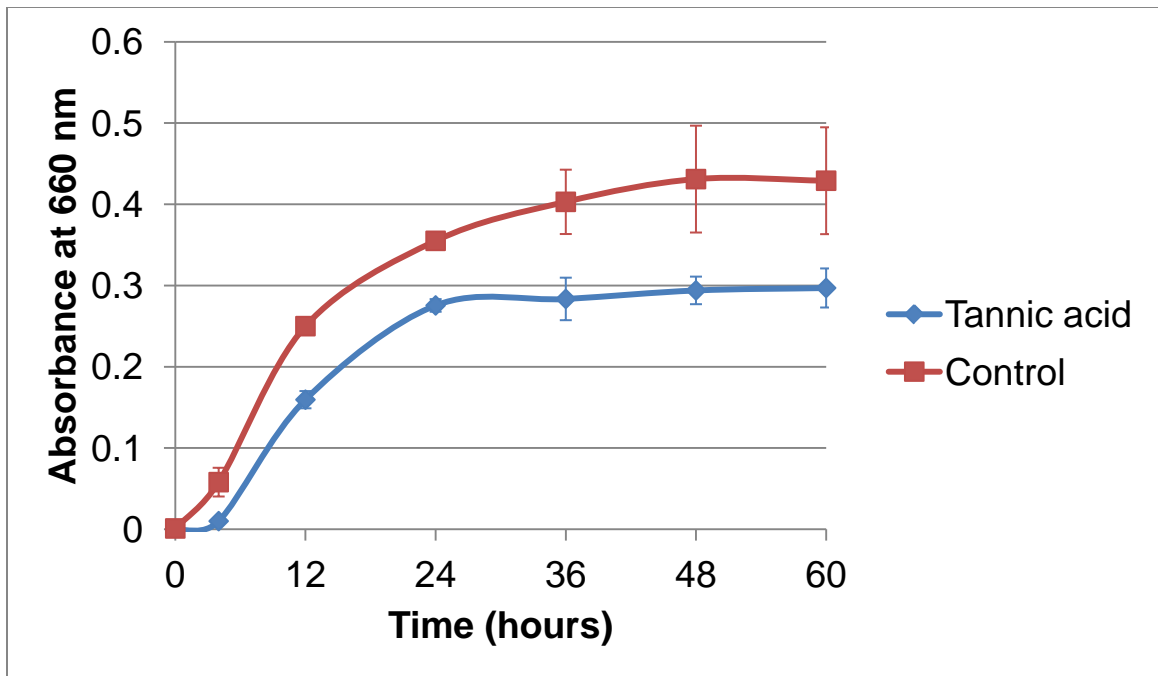
**Figure 80.** The antimicrobial activity of tannic acid at MIC of 10 ppm against *C. sporogenes*, ATCC 7955. Error bars represent standard deviation from the mean,  $P < 0.05$ .



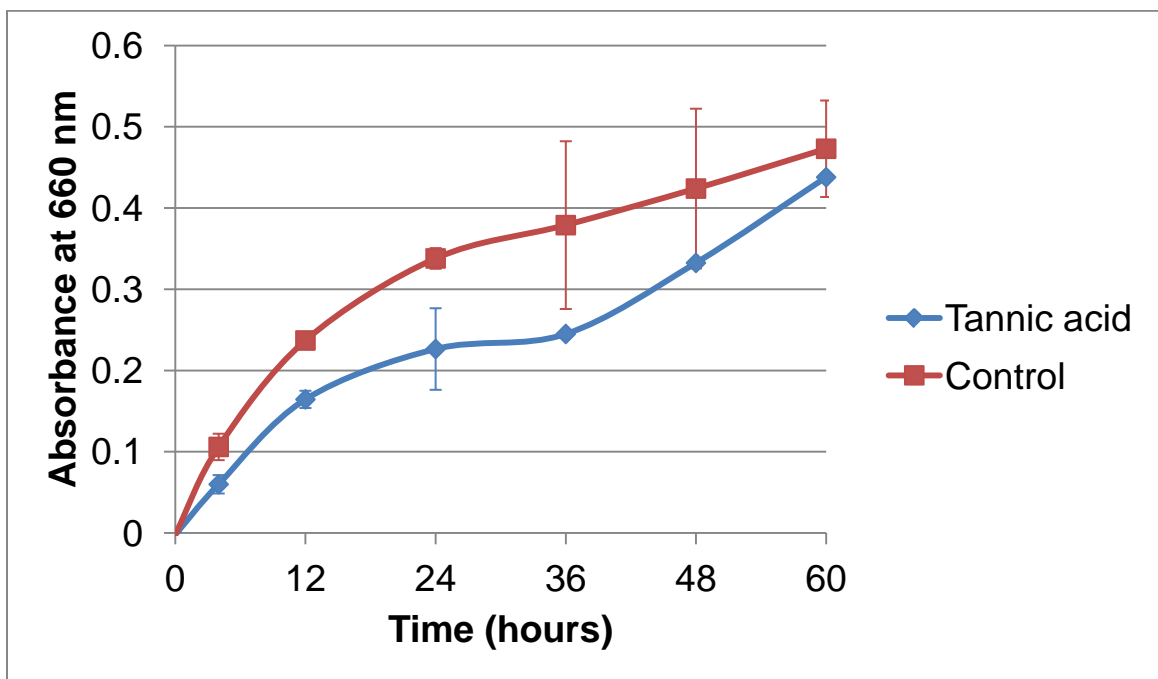
**Figure 81.** The antimicrobial activity of tannic acid at MIC of 5 ppm against *E. coli*, FTJ. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 82.** The antimicrobial activity of tannic acid at MIC of 15 ppm against *S. paratyphi*, UK Micro 29A. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 83.** The antimicrobial activity of tannic acid at MIC of 10 ppm against *S. choleraesuis subsp.*, ATCC 10708. Error bars represent standard deviation from the mean,  $P < 0.05$ .



**Figure 84.** The antimicrobial activity of tannic acid at MIC of 15 ppm against *S. enteridis*, (-)  $H_2S$ . Error bars represent standard deviation from the mean,  $P < 0.05$ .

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## VITA

Hayriye Cetin-Karaca, daughter of Hikmet and Husniye Cetin, elder sister of Ayse Cetin and wife of Haluk Karaca was born in Bourgas, Bulgaria in 1983.

The author graduated from Sehit Osman Altinkuyu Anatolian High School, Yalova, Turkiye, in 2002. The following fall, she started attending the Celal Bayar University and in May of 2006 graduated with honors from, Department of Food Engineering. Following graduation, Hayriye was employed as a food process manager in industry. In spring 2009 she enrolled in University of Kentucky to pursue a Master of Science degree in Food Science.

Hayriye Cetin-Karaca  
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