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Natural phenolic extracts from cardamom (*Elettaria cardamomum*), sumac (*Rhus coriaria*) and pomegranate (*Punica granatum*)- Potential application to control pathogenic bacteria in foods

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Natural phenolic extracts from cardamom (*Elettaria cardamomum*), sumac (*Rhus coriaria*) and pomegranate (*Punica granatum*)- Potential application to control pathogenic bacteria in foods

by

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**Natural phenolic extracts from cardamom, (*Elettaria cardamomum*),
sumac (*Rhus coriaria*) and pomegranate (*Punica granatum*) - Potential
application to control pathogenic bacteria in foods**

Abstract

Food-borne disease is still a concern for both consumer and food industry in spite of the use of various preservation techniques. Plant extracts are becoming more popular as naturally occurring antibacterial agents. Antibacterial activity of cardamom, sumac, pomegranate, star anise and cranberry extracts and their minimum inhibitory concentration (MIC) against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Clostridium perfringens* were determined using agar well diffusion and broth dilution methods. Sumac and pomegranate alcoholic extracts and cardamom oleoresin showed strong antibacterial activity, and their MIC range were: for sumac (3-10 mgml⁻¹), for pomegranate (6-12 mgml⁻¹) and cardamom (3-25 mgml⁻¹). Sumac alcoholic extract and cardamom oleoresin had higher phenolic content than other extracts. Positive correlations between the antibacterial activities of cardamom, sumac and pomegranate extracts and the level of phenolics were found.

The mechanisms of action of the effective extracts were studied by scanning electron microscopy (SEM) observations of morphological alterations of the cell and the measurement of bacterial cell content release and intra and extracellular ATP. A significant ($P < 0.05$) higher release of cell constituents was observed, on bacterial cells treated with effective extracts at the MIC when compared to controls. Both pomegranate and cardamom extracts dramatically decreased the intracellular ATP concentration, and increased the extracellular ATP concentration. All measurements suggested that selected extracts affected the cell membrane integrity, resulting in the loss of cell homeostasis and compromise of the cytoplasmic membranes.

Experiments to understand combined effects of cardamom and pomegranate extracts and a range of environmental conditions of (temperature (25-35°C), pH (5-7) and NaCl (0-5%)) were performed. Growth kinetic parameters of selected bacteria were computed using Baranyi and Robert's model and a response surface analysis model (RSA). Precise and reliable data obtained from both models, predicted parameters for all tested bacteria showed a high correlation coefficient $R^2 > 0.081$. Both extracts considerably reduced the growth rate and prolonged the lag time of all tested strains compared with control samples. Highest reduction on the growth rate was obtained when cardamom and pomegranate incorporated with 5% NaCl and pH 5. In conclusion, this study showed that selected extracts when used in conjunction with other hurdles can help to control food-borne bacteria.

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List of Abbreviation

Abbreviation	Glossary of terms used
(GRAS)	Generally recognized as safe
%	Percentage
(DIZ)	Diameter of the inhibition zone
(Eos)	Essential oils
(MIC)	Minimum inhibitory concentration
°C	Degree centigrade
µl	Microliter
µm	Micrometre
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHI	Brain heart infusion
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
<i>B. cereus</i>	<i>Bacillus cereus</i>
G ^{+ve}	Gram positive
G ^{-ve}	Gram negative
<i>L. innocua</i>	<i>Listeria innocua</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
Log	Logarithm
M	Molar
Mg	Milligram
Min	Minute
ml	Millilitre
Mm	Millimetre
Mol	Mole
MTC	Maximum tolerance concentration
NaCl	Sodium chloride
nm	Nanometre
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBS	Phosphate buffer saline
pmol	Picomol

R ²	Coefficient of determination
Rate h ⁻¹	Maximum growth rate
RMSE	Root mean square error
RSA	Response surface analysis model
<i>Staph. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. typhi</i>	<i>Salmonella typhi</i>
S. Typhimurium	Salmonella Typhimurium
<i>S. enteritidis</i>	<i>Salmonella enteritidis</i>
SE	Standard error
SEM	Scanning electron microscope
TPC	Total phenolic content
UV	Ultra violet
v/v	Volume to volume
yEnd	Maximum microbial load

Dedication

To my family with my love

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Author's Declaration

At no time during the registration for the degree of Master of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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1 Chapter 1: General introduction, aim and objective and hypothesis

1.1 Introduction

Food borne diseases are a serious and global problem, mainly observed in infants, young children, the elderly and the immunocompromised. Annually around 2.2 million of people worldwide die as a result of foodborne and waterborne diarrhoeal diseases (WHO 2012). The enormous burden of disease happens everywhere in the world including the UK, which imposes a significant burden not only on infected individuals but also on the economy. Although the majority of cases are mild, occasionally cases can lead to serious or long-term conditions or even death (Food Standards Agency 2011a; 2014).

Food contaminated with pathogenic microorganisms is the causative agent of food borne disease (Celiktas et al. 2007; Jacob et al. 2010). Foods such as meat, poultry and fish are most frequently associated with food borne illness. Therefore, the development of new effective techniques to reduce the risk of contamination is required to maintain food safety.

Several preservation techniques such as temperature (high or low), packaging, preservatives, drying pulsed light, and high pressure have been applied worldwide to control pathogenic and spoilage bacteria in foods. Cases of food-borne outbreaks continue to rise in many developed countries (Gaysinsky and Weiss 2007; Abou-Taleb and Kawai 2008).

Strong consumer awareness for safe and good quality food, free from synthetic additives has been increased recently. As well as, concerns about the greater frequencies of antibiotic resistance among bacteria isolated from food animals have been detected (Palaniappan and Holley 2010). In this regards, knowledge

concerning the application of natural antimicrobials in foods is important to improve the microbiological safety of perishable food, and to control the antibiotic resistant pathogens particularly in meat products, fresh vegetables and fruits.

Plant oil extracts are secondary metabolic aromatic and volatile compounds, naturally formed in specialised cells, found in flowers, buds, seeds, fruits, roots and leaves (Aidi Wannes et al. 2010). Some plant extracts showed antibacterial potential against several species of bacteria such as, *Listeria monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli*, *Shigella dysenteriae*, *Bacillus cereus* and *Staphylococcus aureus* (Patrignani et al. 2008; Raybaudi-Massilia et al. 2008). It has been observed that sumac, cardamom, pomegranate, clove, coriander, cinnamon, garlic, rosemary sage and vanillin showed strong antimicrobial activity against some groups of G^{+ve} and G^{-ve} pathogens (Angioni et al. 2004; Kosar et al. 2007; Arqués et al. 2008; Kossah et al. 2011). However, factors such as plant origin, composition of the extract and extraction methods, species and concentration of microorganisms, processing and storage conditions can affect the antimicrobial activity of the extracts (Pandit and Shelef 1994; Marino et al. 2001; Özcan and Erkmen 2001). It has been found that higher concentrations of extracts should be applied in food to attain equivalent effect than in *in vitro* (Lv et al. 2011). Thus, combination of natural plant extracts with other preservatives and evaluating their mechanism of action against pathogenic bacteria could reduce the risk associated with foodborne diseases.

1.2 Aim and objectives

Risk assessment programmes assure safety on key points of the food chain, and indicate that some control is required at the retail and preparation points to reduce the risk for foodborne disease. This project aims to establish the factors influencing the survival and growth of pathogenic and spoilage bacteria, by applying novel methods of pathogen inactivation, including plant natural extracts to achieve disease risk reduction. Natural extracts are rich of phenolic compound and other secondary metabolite and some have antimicrobial, antioxidant, anti-inflammatory and anti-cancer activity (Ismail et al. 2012; Wu et al. 2013), thus investigating for new sources of antimicrobial agents from plant part that can be use as alternative to synthetic preservatives. There is a lack of information regarding the antibacterial action and phenolic contain of sumac, cardamom and pomegranate against most common food-borne pathogens and also there is no information about using of these extracts in predicted microbiological model to determine the growth survival and /or inactivation of these pathogens in a food system. Therefore, this study focuses on investigation their antimicrobial effect, by preparing specific bacterial cell samples treated with extracts and then measuring the bacterial cell constituents release and the ATP concentration and finally tested under an electron microscope. Moreover, application of the mathematical models and investigating factors that can interact with these extract such as pH, temperature and NaCl. The schematic outline of the thesis is summarized in Figure 1.2.

1.3 The main hypothesis

- Plant natural extracts would be effective against pathogenic bacteria and their antimicrobial efficacy would be phenolic and concentration dependent.
- The mechanism of action of natural plant extracts could explain their antimicrobial activity, consequently would reduce the risk of food born outbreak.
- Combination of natural plant extract with other hurdles would improve the safety and the quality of food products.

1.4 Project objectives

- To evaluate and compare the antibacterial activity, and the minimum inhibitory concentration of selected natural plant extracts against some of the most common pathogens cause foodborne illness *in vitro*.
- To determine the phenolic contents of selected extracts by using a colorimetric technique.
- To evaluate their mechanism of action against the selected bacteria, by measuring the ATP concentration, cell constituent release and scanning electron microscopy observation.
- To evaluate the mathematical models which can assist for the determination of the growth, survival and death of tested bacteria under various physical conditions?

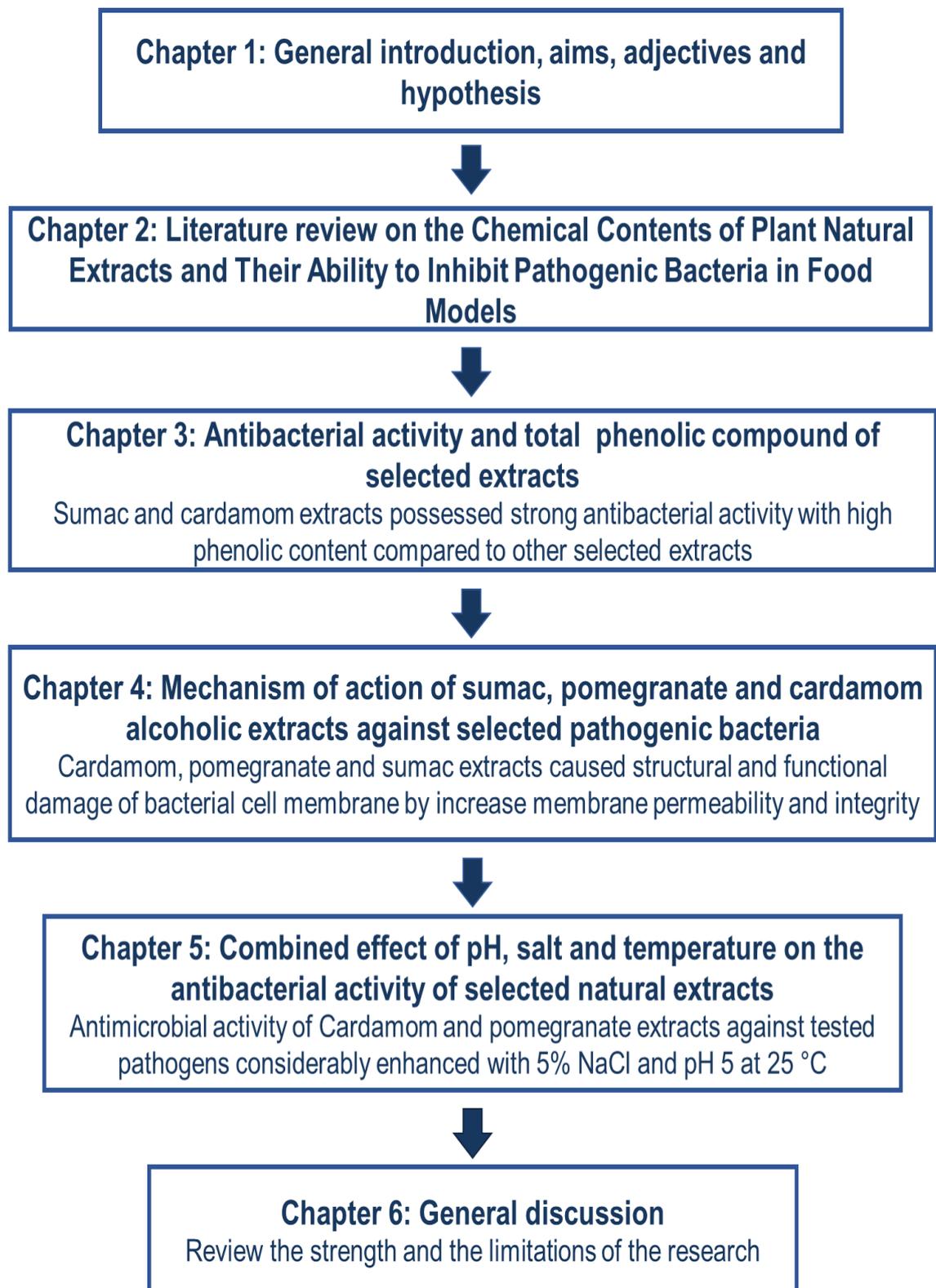


Figure 1. 1: the schematic outline of the thesis

2 Chapter 2 Chemical Contents of Plant Natural Extracts and Their Ability to Inhibit Pathogenic Bacteria in Food Models

2.1 Historical overview of plant antibacterial properties

Since antiquity, plant extracts have been applied in food for their flavouring and preservative properties (Caffin et al. 2011). Early Egyptians applied the natural extract for the same purposes, and have been used for centuries in the Fertile Crescent region as well as, different parts of Asia including India and China. Natural extracts in particular during the 9th century were used for medical application in Arabia, while by the 13th century the pharmacological influences of some natural extracts were described in pharmacopoeias (Surburg and Panten 2006), however their use does not spread widely in Europe until the 16th century. The preparation of essential oil is well known in the 17th century and around 10-15 essential oils were stocked in the pharmacies. Burt (2004) indicated that in 1881 Dela Croix carried out the first experiment regarding the bactericidal property of plant extracts. Moreover, in 1910s cinnamon and mustard were showed strong antimicrobial effect. Meanwhile other natural extracts (such as allspice, oregano, cumin, coriander, caraway, rosemary, bay leaf and thyme) have been demonstrated to have significant antimicrobial properties against pathogenic bacteria, (Burt 2004; Jayaprakasha et al. 2007; Sofia et al. 2007; Kwon et al. 2008).

Recently both pharmaceutical and food industries have shown great interest in the antimicrobial effect of natural plant extracts, thus the use of natural additives are necessary to maintain food quality and safety and to reduce the bacterial resistance towards the antibiotics. (Okoh et al. 2010).

2.2 Antibacterial activity of natural extract and their chemical contents

Several studies have been carried out *in vitro* and *in vivo* to determine the antibacterial activity and the minimum inhibitory concentration (MIC) of natural extracts against a wide range of G^{+ve} and G^{-ve} bacteria. Plant extracts such as oregano, clove, cinnamon, garlic, coriander, rosemary, parsley, sage, and vanillin have been reported to have strong antimicrobial properties (Kim et al. 2006; Lopes-Lutz et al. 2008; Gaekwad and Trivedi 2013; Todd et al. 2013; Kovač et al. 2014; Witkowska et al. 2014). While other extracts, such as ginger, black pepper, red pepper, chilli, cumin and curry showed lower antimicrobial activity (Holley and Patel 2005). Some studies regarding the antimicrobial activity of some plant extracts are listed in Table 2.1.

Numerous of factors influence the antimicrobial efficacy of natural plant extracts. Firstly, chemical composition, which influenced by geographic origin, could considerably affect the activity of plant extracts (Burt 2004). Secondly, the bacterial targets, form (vegetative cell or spore), genus, species, strains, and stage of growth are important factors that affect the antimicrobial spectrum (Naidu 2000). Finally, the type of assay methods could influence the antimicrobial outcome, for example disk diffusion method may not be appropriate for evaluating the activity due to the hydrophobic property of some natural extracts (Burt 2004; Gutierrez et al. 2008; Viuda - Martos et al. 2008).

Several type of methods have been used for evaluating the antibacterial potency and the minimum inhibitory concentration (MIC) of natural extracts *in vitro* including Disk diffusion, agar wells, broth dilution, survival curve and scanning electron microscopy. The most recommended and frequently used methods to determine the antibacterial properties are agar well diffusion and broth dilution (CLSI 2009b; a). The former is a preliminary standard used when

large number of extracts and/ or large number of pathogens to be screened, characterized by a great simplicity and cost-effectiveness, which is based on diameter of the inhibition zone (DIZ) (Lalpuria et al. 2013). In this technique, the natural extracts quantity and the diameter of the inhibition zone are crucial parameters. However, this method is unable to evaluate the MIC values of the antimicrobial agents (CLSI 2009a).

In contrast the broth dilution method can overcome some of the limitations of the agar well diffusion method, by determining the MIC values for the antimicrobial agents through growth curve analyses by comparison with the control culture. In this method the efficacy of the antimicrobial activity is verified by the changes on viable determination or by optical density (Faleiro 2011). Kim and Kim (2007) reported that using of broth dilution assay can draw a quantitative conclusion compared to qualitative data generated by agar well diffusion.

Natural plant extracts contains a variety of compounds with antimicrobial activity. The antibacterial activity of plants, herbs and spices are due to the presence of oils (EOs) and other extract compounds such as tannins, saponins, glucosides, alkaloids, organic acids and other components (Bajpai et al. 2008).

Identifying the antimicrobial compounds of Eos is cumbersome due to their complex mixture, which can contain of up to 45 components at quite different concentrations (Djenane et al. 2011; Espina et al. 2011). The major components can constitute about 20-70 %, while other compounds are present only as trace (Bakkali et al. 2008).

In many cases the activity of natural extracts results from the complex interaction between the various classes of compounds for example polyphenol,

aldehydes, ketones, alcohol, ethers. However, in some cases, the antimicrobial activities of the natural extracts are mainly due to the activity of the major components of natural extracts. Considerable antimicrobial properties of these compounds were exhibited when tested separately (Wijekoon et al. 2013).

It has been reported that the phenolic component and aldehydes are chiefly responsible for the antimicrobial properties of natural extracts. Natural extract that contains phenols or aldehydes, such as cinnamaldehyde, carvacrol, eugenol, citral and thymol as major components displayed the highest antibacterial activity, followed by extracts containing terpene alcohols. While, weak activity were exhibited when natural extracts containing ketones or esters (Dorman and Deans 2000; Ait-Ouazzou et al. 2011).

The phenolic compounds of oregano, clove, thyme, rosemary, sage, basil, ginger, garlic, mace and fennel have been tested against a variety of food-borne pathogens, and they exhibit significant antibacterial activity (Burt 2004; Viuda - Martos et al. 2008). Strong antibacterial effect of *Thymus* and *Origanum* species has been related to their phenolic components such as thymol and carvacrol (Oussalah et al. 2007; Hazzit et al. 2009; Soković et al. 2009). The antimicrobial activity of *Cinnamomum zeylanicum* has been related to its cinnamaldehyde content (Unlu et al. 2010), though in basil both of eugenol (19%) and linalool (54%) were attributed for its strong antibacterial activity (Rattanachaikunsopon and Phumkhachorn 2010). Phenol di-terpenoids is considered to be the principal active component of *Rosmarinus officinalis* (Okoh et al. 2010). The antimicrobial activity of some plant origin phenolic compounds are presented in Table 2.2

The composition of particular natural extracts may vary depending on the harvesting season, geographical sources, and parts of the plants and methods of extraction (Angioni et al. 2006; Celiktas et al. 2007; Saidana et al. 2008).

As reported by Burt (2004) and Bakkali et al. (2008), extracts obtained from natural plant harvesting during or immediately after flowering have the strongest antimicrobial activity. Differences in the composition of natural plant extracts observed when different parts of the same plant are used (Delaquis et al. 2002). Moreover, the compositions of the natural extracts obtained by distillation differ widely than solvent extraction. This can be explained the greater antimicrobial activity of natural plant extracted by hydrodistillation than the corresponding hexane (Danh et al. 2013). Thus, all these factors should be considered when the antimicrobial activity of natural extract was determined.

Table 2. 1: Antibacterial activity and the MIC of most common plants extracts against some of pathogenic bacteria.

Plant essential oil	Bacterial spp.	MIC	Source
Cinnamon	<i>Staph. aureus</i>	0.1-0.4mgml ⁻¹	Lu et al. (2011)
	<i>E. coli</i>	0.1-0.4 mgml ⁻¹	
	<i>S. Typhimurium</i>	0.1-0.4 mgml ⁻¹	
Thyme	<i>E. coli</i>	0.6 mgml ⁻¹	Ivanovic et al. (2011)
	<i>S. enteritidis</i>	0.6 mgml ⁻¹	
Rosemary	<i>E. coli</i>	1.2 mgml ⁻¹	Ivanovic et al. (2011)
Lemon-grass	<i>E. coli</i>	0.6 mgml ⁻¹	(Naik et al. 2010)
	<i>Staph. aureus</i>	0.6 mgml ⁻¹	
Pomegranate	<i>S. enteritidis</i>	4 mgml ⁻¹	Al-Zoreky (2009)
	<i>L. monocytogenes</i>	16.5 mgml ⁻¹	
Clove	<i>E. coli</i>	5 mgml ⁻¹	Pundir et al. (2010)
	<i>Staph. Aureus</i>	2.5 mgml ⁻¹	
Oregano	<i>E. coli O157:H7</i>	0.6 mgml ⁻¹	Shekarforoush et al. (2007)
	<i>Staph. aureus</i>	0.2 mgml ⁻¹	
Mustard	<i>E. coli</i>	10 mgml ⁻¹	Sofia et al. (2007)
	<i>Staph. aureus</i>	30 mgml ⁻¹	

Table 2. 2: The antibacterial activity and the MIC of most common natural extract phenolic contents against pathogenic bacteria.

Eos components	Pathogens	MIC	Reference
Eugenol	<i>E. coli</i>	1.6 mgml ⁻¹	Pei et al. (2009)
	<i>S. Typhimurium</i>	0.1 mgml ⁻¹	Devi et al. (2010)
Carvacrol	<i>E. coli</i>	0.3 mgml ⁻¹	Guarda et al.
	<i>S. Typhimurium</i>	0.5 µgml ⁻¹	(2011)
	<i>L. monocytogenes</i>	0.5 µgml ⁻¹	Tatjana et al. (2011)
Thymol	<i>E. coli</i>	0.2 mgml ⁻¹	Guarda et al.
	<i>S. Typhimurium</i>	0.5 µgml ⁻¹	(2011)
	<i>L. monocytogenes</i>	1 µgml ⁻¹	Tatjana et al. (2011)
α-Terpineol	<i>E. coli</i>	0.9 mgml ⁻¹	Cosentino et al.
	<i>L. monocytogenes</i>	0.9 mgml ⁻¹	(1999)
	<i>Staph. aureus</i>	0.9 mgml ⁻¹	
Citral	<i>E. coli</i>	0.5 mgml ⁻¹	Kim et al. (1995)
	<i>Staph. aureus</i>	0.5 mgml ⁻¹	
	<i>L. monocytogenes</i>	0.5 mgml ⁻¹	

2.3 Mechanisms of action of essential oils against microorganisms

The antibacterial activity of natural extracts has been detected against numerous species of bacteria, while the mechanism of action has not been discussed in detail (Hyldgaard et al. 2012).

Natural extracts consist of several types of chemical compounds, and their activity is not attributed to one mechanism. It has been suggested that natural extracts have Different mechanism of action as follows: pass through the cell wall, disruption the phospholipid bilayer of the bacterial cell membrane, influencing the bacterial DNA/RNA and disrupting of bacterial enzyme systems (Burt et al. 2007; Silva et al. 2007; Proestos et al. 2008). Goñi et al. (2009) reported that the natural extracts act by causing structural and functional damage for bacterial cell wall which can increase the permeability of cell membrane. The mechanism of action of natural extracts and their derivatives is summarised in Table 2.3 and 2.4.

Natural extracts could be characterised by their hydrophobicity, this property enable them to penetrate the cell wall and the lipid molecules of the cell membrane, disturbing the structure of their different layer, increasing the permeability of cell membrane, which may then lead to leakage of ions (Silva et al. 2007; Proestos et al. 2008). The penetrability of *S. typhi* cell membrane was increased by eugenol (Devi et al. 2010), and mustard extract influenced *E. coli* O157:H7 and *S. typhi* cell membranes and reduced the intracellular ATP and pH (Turgis et al. 2009).

Burt (2004) suggested that the viability of bacterial cells is depending on the amount of leakage, as excessive cell constituent release will lead to cell death. The cell death of *E. coli* occurred before cell lysis, due to the effect of tea tree oil. Moreover, the antimicrobial effects of natural extracts against food-borne

bacteria are mainly due to their high level of phenolic compound such as thymol, eugenol and carvacrol. Carvacrol exhibited significant antibacterial activity against *E. coli* O157:H7 by inhibiting the flagellum synthesis. It has been observed that the plant-based phenolic compounds can cause disruption of the cytoplasmic membrane, depletion of proton motive force and finally coagulation of cell contents (Lambert et al. 2001; Shan et al. 2007; Tiwari et al. 2009).

As reported by Burt (2004), the chemical structure of some compounds has a strong effect on their mechanism of action. The hydroxyl group plays an important role in the antibacterial activity of phenolic compounds. Ultee et al. (2002) and Ben Arfa et al. (2006), confirmed the importance of hydroxyl groups in the phenolic compounds of thymol and carvacrol. The strong antibacterial activity of gallic acid was related to the presence of one more hydroxyl group in their chemical structure (Vaquero et al. 2007). While, Veldhuizen et al. (2006) indicated that the antibacterial activity of carvacrol is not mainly attributed to their hydroxyl group.

There are two suggestions related to the mechanism of cyclic hydrocarbon molecules. Neither, the interaction of protein-lipid can be disrupted; due to the accumulation of these lipophilic molecules in the lipid bilayer or that the lipophilic hydrocarbon can interact directly with the hydrophobic proteins. The ATPase activity was inhibited by thymol, eugenol and carvacrol. The loss of membrane integrity and disruption of lipid structure was observed, due to the accumulation of terpenes in the membrane, (Burt 2004; Oussalah et al. 2006; Negi 2012). Lipopolysaccharides are present in the outer membrane of G^{-ve} bacteria, which had shown less sensitivity towards natural extracts than G^{+ve} bacteria. Thus, antibacterial activity of natural extracts is might reduce against G^{-ve} due to their hydrophobic properties (Stefanello et al. 2008).

Table 2. 3: The target site of some plant origin extracts and their mechanism of action.

Natural extracts	Species of bacteria	Mechanisms	Methods	References
Cumin	<i>B. cereus</i>	Mild changes in cytoplasm; cell envelope intact	TEM	Pajohi et al. (2011)
Mustard	<i>E. coli</i> <i>S. typhi</i>	Affected membrane integrity; released cell content; decreased intracellular ATP and pH, increased extracellular ATP .	Measurement of cell constituents release, Measurement of intra- and extracellular ATP concentrations, pH measurements	Turgis et al. (2009)
Oregano	<i>Staph. aureus</i> <i>P. aeruginosa</i>	Dissipated potassium gradient, depolarized membranes, permeabilised membranes, inhibited cell respiration, affected cell structure, and coagulated cytoplasmic material and loss of potassium ions.	Measurement of potassium leakage, Measurement of membrane permeability(staining methods), potassium ions efflux Assay, using of confocal scanning laser microscope or SEM	De Souza et al. (2010) Bouhdid et al. (2009),
Savory	<i>E. coli</i> , <i>L. monocytogenes</i> <i>Staph. aureus</i> <i>S. Typhimurium</i>	Increased extracellular ATP; reduced intracellular pH; affected membrane integrity; structural damages; and cell lysis	TEM, Measurement of cell constituents release, Measurement of intra- and extracellular ATP concentrations, pH measurements	Oussalah et al. (2007), De Oliveira et al. (2011)
Garlic	<i>E. coli</i>	Induced leakage from the bacterial cell.	Atomic force microscopy	Perry et al. (2009)
Cinnamon	<i>E. coli</i> , <i>L. monocytogenes</i>	Morphological changes, Increased extracellular ATP, depolarised and permeabilised membranes, leakage and coagulation of cytoplasmic content	Measurement of extracellular and intracellular ATP, confocal laser scanning microscopy	Gill and Holley (2006), Bouhdid et al. (2010)
Cranberry	<i>E. coli</i> O157:H7	Cell damage, disintegration of the outer membrane	TEM	(Lacombe et al. 2010)

Table 2. 4: The mechanism of action of some natural extract compounds and methods of study.

Compounds	Pathogens	Mechanisms	Methods	References
Limonene (orange, melon, and mandarin)	<i>E. coli</i>	Changed membrane fatty acid composition, Damaged cell morphology.	Analysis of the Fatty Acid Composition, detection of sublethal injury	Di Pasqua et al. (2006); (Espina et al. 2011)
	<i>Staph.aureus</i>			
Linalool (basil and citrus oils)	<i>B. cereus</i>	Destabilised and Permeabilised cell membranes.	Measurement of propidium iodide cell uptake, Detection of sublethal injury	Fisher and Phillips (2006); Ait-Ouazzou et al. (2011)
	<i>E. coli</i>			
	<i>L. monocytogenes</i>			
	<i>Staph.aureus</i>			
Cinnamaldehyde (cinnamon)	<i>S. Typhimurium</i>	Concentration dependent ATPase inhibition, lost cell motility.	Analysis of fatty acid composition, SEM, measurement of protein content, measurement of extracellular and intracellular ATP	Gill and Holley (2006); Di Pasqua et al. (2007)
	<i>B. cereus</i>			
	<i>E. coli</i>			
	<i>L. monocytogenes</i>			
Thymol (thyme)	<i>Staph.aureus</i>	Permeabilised cell membranes, Leaked H ⁺ and K ⁺ ions and ATP, change of cell morphology, Inhibited enzymes involved in ATP synthesis,	Potassium ions efflux assay, cytometric analysis, changes in fatty acid composition (GC analysis), SEM, cell constituent release test	Di Pasqua et al. (2006); Shapira and Mimran (2007); Xu et al. (2008)
	<i>B. cereus</i>			
	<i>E. coli</i>			
	<i>L. monocytogenes</i>			
Vanillin (vanilla)	<i>Staph.aureus</i>	Stimulated ATP production in some, Inhibited cell respiration, Permeabilised cell membranes Dissipated potassium and pH gradients		Fitzgerald et al. (2004); Hyldgaard et al. (2012)
	<i>S. Typhimurium</i>			
Carvacrol (oregano and thyme)	<i>E. coli</i>	Permeabilised cell membranes, depleted intracellular ATP, ATPase inhibition, damaged cell morphology	Atomic force microscopy analysis, measurement of outer membrane protein, measurement of extracellular and intracellular ATP	Gill and Holley (2006); Horváth et al. (2009); La Storia et al. (2011)
	<i>L. monocytogenes</i>			
	<i>Staph.aureus</i>			
	<i>S. Typhimurium</i>			

2.4 Application of natural extracts to food

Many food products are perishable by nature and require protection from pathogenic and spoilage growth during their preparation, storage, and distribution. Plant extracts have shown a considerable promise in a range of application in the food industry and several plant extracts generally recognized as safe (GRAS) (Food Standards Agency 2011b). Plant-base antimicrobials can be applied in food products as an alternative to chemical and synthetic preservative, to control pathogenic and spoilage bacteria (Perumalla and Hettiarachchy 2011; Lucera et al. 2012; Negi 2012). it has been reported that *Nandina domestica* Thumb extract could be used in foods as an alternative to synthetic preservatives (Bajpai et al. 2008). In general, ascending order of antibacterial impact of most effective natural extract in food are: cilantro < mustard < rosemary < mint < thyme < cinnamon < coriander < clove < oregano (Burt 2004). in another study, mustard had stronger antibacterial activity than mint (Sofia et al. 2007).

Despite several plant extracts possessing bioactive properties (Sarikurkcu et al. 2010; Viuda-Martos et al. 2010), it has generally been observed that a greater concentration is required to obtain the same effect in food, as foods are very complex system consisted of different interconnecting microenvironments (Negi 2012). According to Smid and Gorris (1999); Moreira et al. (2007), higher concentrations of the extract are required to attain the same inhibitory effect in food than *in vitro* trials. Higher concentration of tea tree and clove extract were needed to control *E. coli* O157: H7 in minced cooked beef and blanched spinach than in *in vitro* trial (Moreira et al. 2007).

Small groups of natural extracts have been applied in food products. Therefore, there is more need for studies concerning plant base natural extracts and the effects of food components on their antimicrobial activity.

2.4.1 Application to meat and poultry products

Several studies have confirmed the antibacterial activity of plant extract in meat products (Grosso et al. 2008). According to Yin and Chao (2008), the growth of both antibiotic resistant and susceptible strains of *Campylobacter* was inhibited in ground beef, by the influence of roselle extract (*Hibiscus sabdariffa*). The combined effect of clove and oregano showed strong inhibitory effect at the level of 5-20 $\mu\text{l g}^{-1}$ when applied against *L. monocytogenes* and *Aeromonas hydrophilla* in meat products (Burt 2004). The antimicrobial potential of pomegranate extract was tested against food-borne bacteria in meat pate stored at 4°C during 46 days, pomegranate extract inhibited *L. monocytogenes* growth by 4.1 log CFU/g compared to control, which had reached log 9.2 CFU/g after 18 days (Hayrapetyan et al. 2012). In another study, the shelf life of chicken chilli and chicken lollipop was prolonged 5-8 days in sample treated with pomegranate extract than the untreated sample (Gulmez et al. 2006).

It has been observed that the application of winter savory extract combined with preservative techniques such as, high pressure magnetic field, pulsed electric or reduced temperature, controlled the bacterial growth and enhanced quality of minced pork meat (Carraminana et al. 2008). *Pseudomonas spp* and *L. monocytogenes* were inactivated on the meat surface by using milk protein edible film containing oregano, pimento extract (Mosqueda-Melgar et al. 2008). In another study a mixture of rosemary extract and liquorice had shown great antibacterial potential against four types of pathogens (*E. coli*, *L. monocytogenes*, *Lactobacillus sake* and *Pseudomonas*) in vacuum-packaged

ham slices and atmosphere-packaged fresh pork stored at 4°C (Zhang et al. 2009).

The report of European Food Safety Authority (2009) revealed that the occurrences of food-borne outbreaks were increased due to the influence of meat products contaminated by *Salmonella*. Cinnamaldehyde extract at 0.1% had reduced the population of *S. enterica* by 5 log CFU/g on day 3 compared to control (Ravishankar et al. 2010). The inoculated lamb meat with *Salmonella* was treated with 0.1% thyme and 0.3% oregano extract, the combined effect of extracts and the modified atmosphere packaging reduced *Salmonella* population up to 2.8 log cfu/g on day 9 of storage (Karabagias et al. 2011). However, some research have recorded low effect of the extracts against pathogens contaminated meat products (Grosso et al. 2008).

2.4.2 Application to fish

Fish products as in meat can be contaminated by numerous species of pathogens. However, significant influences of some natural extracts had been detected against bacteria in fish (Hayouni et al. 2008). Mahmoud et al. (2004), Revealed that the combined effect of 0.5% of cinnamaldehyde and 0.5% of thymol had extended the shelf-life of carp fillets from 4 to 12 days at 5°C. As well as, oregano oil at 0.1% v/v was inhibited *Photobacterium phosphoreum* growth in cod fillet (Mejlholm and Dalgaard 2002; Tajkarimi et al. 2010). The mixture of 0.5% eugenol and 0.5% linalool in edible solution maintained the freshness of the tuna slices compared to the control (Abou-Taleb and Kawai 2008). Regarding to Yano et al. (2006) hurdle technology has been significantly implemented by using basil, clove and garlic extracts in order to protect seafood from *Vibrio parahaemolyticus* contamination, the population of bacteria were reduced by 1-log after the incubation at 5°C

2.4.3 Application to dairy products

According to Abdalla et al. (2007) mango seed kernel extract had affected *E. coli* and extend the shelf life of pasteurised milk. In comparative studies, cardamom and cinnamon extracts were more effective than mint extract to inhibit the growth of a yoghurt starter culture (Tajkarimi et al. 2010). It was reported that *Satureja cilicica* can be applied in butter as aromatic and antioxidant agent (Ozkan et al. 2007). The significant effect of thyme, oregano, sage, rosemary and cumin was detected against four bacteria used as a starter and two of spoilage bacteria (Hayouni et al. 2008; Viuda-Martos et al. 2010). Hayouni et al. (2008), reported that the oils of *Melaleuca armillaris* showed great effect against lactic acid bacteria, by extending the lag phase from 0.69%–97.5% after 72 h of incubation, which may prolong the shelf life of the products.

2.4.4 Application to vegetable and fruits

The antibacterial activities of natural extract and essential oils have been confirmed against food-borne pathogens and spoilage flora in vegetable and fruits. Oregano oil at 21 $\mu\text{l g}^{-1}$ reduced the population of *E. coli* O157:H7 more than 1 log CFU/g in eggplant salad (Burt 2004; Tajkarimi et al. 2010). Goñi et al. (2009) and Cava et al. (2007), revealed that both of cinnamaldehyde and thymol oil were successfully reduced the population of six salmonella serotypes more than 3 log CFU/g on alfalfa seed, compared with the control (1.9 log CFU/g) after treatment for 7 h. Increasing the temperature reduced the effectiveness of the treatment, this may be due to the volatility of the antibacterial compounds. Cinnamaldehyde oil at 40% and eugenol at 80% in combination with mild heat and acidic pH prolonged the lag phase of *Alicyclobacillus acidoterrestris* in apple juice (Bevilacqua et al. 2010b). Furthermore, combined effect of clove, lemongrass and cinnamon oils at 0.7%

and the alginate-based edible coating reduced the population of *E. Coli* O157:H7 more than 4 logs CFU/g in fresh cut Fuji apple (Rojas-Graü et al. 2007; Raybaudi-Massilia et al. 2008). According to previous information the application of natural extracts alone and in combination with other hurdle technique may prolong the shelf life as well as enhance food quality.

2.5 Factors influencing antibacterial activity of natural extracts in food

Most studies related to the antimicrobial activity of natural extracts have been carried out *in vitro* using microbiological culture media. Consequently, there is less understanding regarding to their efficacy when applied to food systems. Further knowledge is needed for optimised application of natural antimicrobials in food, by studying the influences of these compounds on the activity of any proposed antimicrobial. Foods are mainly composed of water, carbohydrates, fats, proteins and NaCl, which can influence the antibacterial activity of natural extracts. Stoicov et al. (2009) and Gill et al. (2002), reported that certain food components may reduce the antibacterial activity of natural extracts, as well as greater availability of nutrients in foods may enable bacteria to repair damaged cells faster.

It has been supposed that high levels of fat or protein in food protect bacteria from the action of natural extract, in contrast carbohydrates in food had no role in protection of bacteria from the action of natural extracts (Burt 2004; Gutierrez et al. 2008). According to Smith-Palmer et al. (2001) and Mejlholm and Dalgaard (2002), fat can surrounds the bacteria by a protective layer, or the antibacterial agents can be absorbed by the lipid fraction, consequently reducing their activity and concentration in the aqueous phase.

Al-Kutby et al. (2010), highlighted that the antimicrobial efficacy of cinnamon oleoresin, rosemary oleoresin and Sumac extract at 2% against *L.*

monocytogenes in high fat meat product (ready to eat Doner kebab) was decreased when spice extracts applied before cooking in comparison to application of spice after cooking. The possible explanation of that is due to either the high level of fat in Doner kebab that can provide a protective layer around the bacteria or the lipid solubility of spice extract that minimized their antimicrobial effect.

The high level of protein in food restricted the activity of natural extract (Bevilacqua et al. 2010a). The antimicrobial activity of clove and oregano extracts was reduced in the present of protein in the growth medium at levels ≥ 1 g/100 ml. The populations of *L. innocua* or *E. coli* treated with clove extract at MIC (0.125ml/100ml) increased by 1.94 or 1.33 log CFU/ml respectively, when protein added to the medium at 5 g/100 ml compared with samples containing extract only. In the case of oregano oil at MIC (0.0625ml/100ml), *L. innocua* or *E. coli* increased by 2.32 or 1.85 log CFU/ml respectively, demonstrating a possible antagonistic effect of added protein. (Witkowska et al. 2014)

Burt (2004) reported that the interaction between carvacrol and protein reduced their activity against *B. cereus* in milk. Protein content played an important role as a limiting factor for the antibacterial activity of clove oil against *S. enteritidis* in cheese. Combination of oregano and nutmeg was exhibited strong antimicrobial effect against *E. coli* O157:H7 in broth culture, however, there was no effect detected against the same bacteria in barbecued chicken breast (Shekarforoush et al. 2007). The likely explanation of reduce the antimicrobial activity of natural extracts in present of protein that peptones with hydrophobic properties might display interactions with natural antimicrobial extracts to enable their dissolution in high protein food.

The pH is considered an important factor influencing the effectiveness of the natural extracts. Li et al. (2014b) reported that low pH can enhance the antimicrobial effect of some natural extract, by increasing their hydrophobicity and then facilitate their dissolution in the lipid phase of the bacterial membrane. The antibacterial activity of cinnamon at pH 5 was enhanced when applied against *S. Typhimurium* and *L. monocytogenes* in ready to eat food Al-Kutby. In addition, acidic pH 5% increased the activity of oregano and thyme oil, prolonged the lag phase and reduced the growth rate *B. cereus* and *L. monocytogenes* in ready to eat high protein foods (Gutierrez et al. 2009).

In general, the high level of salt facilitates the action of natural extract (Holley and Patel 2005). the synergistic effect of black zira (*Bunium persicum*) oil and 4% NaCl was significantly reduced the growth rate of *L. monocytogenes* in fish model system in comparison to control (Rabiey et al. 2013). Similarly, Angienda and Hill (2012), reported that NaCl 1.2% significantly enhanced the antimicrobial activity of clove extract against *E. coli*. A positive influence of NaCl at concentration of 5g/100ml on the antimicrobial property of clove and oregano extracts was observed against *L. innocua* and *E. coli* (Witkowska et al. 2014). Increase the level of NaCl can affect the amino compounds of the bacterial cell wall proteins and allow the penetration of extracts (Juven et al. 1994) and/ or increased the cell membrane permeability, consequently growth inhibition due to salt action on intracellular enzymes.

2.6 The sensitivity of pathogens against the natural extracts

The sensitivity of G^{+ve} and G^{-ve} bacteria towards natural antimicrobial effects are different, mainly due to their different cell wall structures. The cell wall of G^{+ve} bacteria consists of a single layer, whereas the G^{-ve} cell wall is a multi-layered structure bounded by an outer cell membrane (Mulyaningsih et al. 2010). In

general, the antibacterial studies demonstrated that the G^{+ve} bacteria are more sensitive to the natural extracts than G^{-ve} bacteria (Matasyoh et al. 2009; Mulyaningsih et al. 2010; Naik et al. 2010). The G^{-ve} pathogens have an outer membrane, therefore the diffusion of hydrophobic compounds is restricted through the lipopolysaccharide of their membranes (Burt 2004; Stefanello et al. 2008).

B. cereus and *Staph. aureus* showed higher sensitivity to black pepper extracts than *E. coli* and *S. typhi* (Karsha and Lakshmi 2010). *Staph. aureus* exhibited significant difference in their sensitivity to feverfew extract in comparison to *E. coli* (Izadi et al. 2010). Al-Reza et al. (2010), reported that *L. monocytogenes* were found to be more sensitive to the *Zizyphus jujuba* extracts than *S. Typhimurium*. The G^{+ve} pathogens had greatest susceptibility towards the lemon, orange, bergamot and *Eugenia chlorophylla* extracts compared to G^{-ve} pathogens (Fisher and Phillips 2006; Stefanello et al. 2008).

On the other hand, some studies have concluded that according to the type of extract G^{-ve} bacteria can be more susceptible to natural extracts than G^{+ve} pathogens. In one study, *Thymus vulgaris* showed the maximum activity against *E. coli* with a MIC 0.33 mgml^{-1} , while poor activity was detected against *Staph. aureus* and *Streptococcus* spp with a MIC 1.33 mgml^{-1} (Imelouane et al. 2009). Derwich et al. (2010) revealed that *E. coli* exhibited greater sensitivity to *Cedrus Atlantica* extract than *B. sphericus*. Moreover, *S. enteritidis* were significantly reduced when treated with mint extract compared to *L. monocytogenes* (Tassou et al. 1996). Lopes-Lutz et al. (2008), reported that the MIC of thyme against *E. coli* and *P. aeruginosa* were lower than that for *L. monocytogenes*, *Staph. aureus* and *B. cereus*.

However, some studies demonstrated that no differences in the sensitivity were detected between *Lactobacillus curvatus*, *Lactobacillus sake*, *Pseudomonas fluorescens* and *Serratia liquefaciens* after 24 h of treatment with the natural extract. While, the inhibitory effect was extended up to 48 h with G^{+ve} pathogens (Ouattara et al. 1997). The antibacterial assay of black pepper (*Piper nigrum*) and clove (*Syzygium aromaticum*) against *E. coli*, *S. pullorum*, *B. subtilis* and *Staph. aureus* found no proof for a variance in the sensitivity between gram-negative and gram-positive pathogens. Nevertheless, the same previous pathogens were tested later against fresh distilled natural extracts using the same method, the result revealed that the *E. coli*, *S. pullorum* were more resistance to the natural extracts than *B. subtilis* and *Staph. aureus* (Dorman and Deans 2000; Burt 2004). Therefore, the difference in the degree of sensitivity between the two groups may be due to the variability in the natural extracts compounds (Burt 2004; Celiktas et al. 2007; Saidana et al. 2008; Tajkarimi et al. 2010).

2.7 Food-borne pathogens

2.7.1 *Campylobacter spp.*

Campylobacter infection is one of the most common causes of food-borne gastroenteritis in developed countries including the UK. *Campylobacter spp.* are widespread in the intestinal tract of warm-blooded animals used for food production. Animals originating foods in particular poultry meat are important sources of campylobacter infections (Forbes et al. 2009). Transmission of *Campylobacter* organisms to humans usually occurs via infected animals and their food products. Most human infections result from the consumption of improperly cooked or contaminated foodstuffs. Chickens may account for 50-70% of human *Campylobacter* infections. (Kapperud et al. 2003). Most of infect cases are caused by two species *Campylobacter jejuni* and *Campylobacter coli*. *Campylobacter spp.* are Gram negative, spiral, fragile organisms. They are sensitive to heating (pasteurisation/cooking), freezing (Chan et al. 2001, Zhao et al. 2003), drying, acidic conditions (pickling), disinfectants and irradiation. They survive poorly at room temperature (21°C) and in general survive better at refrigeration temperatures. They can grow on moist foods at temperatures ranging between 30°C and 45°C, with an optimum temperature of 42°C (Food Safety Authority of Ireland 2002). Food containing low number of *Campylobacter spp.*, ranging from 500 to 10,000 cells is sufficient to cause illness. Thus, the fact that the organism cannot multiply very effectively in most foods does not prevent it from causing food-borne illness.

2.7.2 *Clostridium perfringens*

This bacteria is characterised by their rod shape, G^{+ve} , anaerobic and spore forming bacteria. More than 13 toxins are produced by this bacteria, and

according to their production of four major lethal toxins (Alpha, beta, epsilon and iota) it is divided into five groups A, B, C, D and E (Carman et al. 2008). *Clostridium perfringens* is commonly presented in water, soil, air and gastrointestinal tract of humans and animals, which can play an important role in food contamination (processed and raw) especially meat products. Consumption of contaminated food with huge number of *Cl. perfringens* (10^6 - 10^7 cells/g) type A usually causes food-borne disease. The most common characteristic symptoms of the diseases are start after 8-12 h of eating of contaminated food such as abdominal pain, nausea and diarrhoea.

More than 75% cases of *Cl. perfringens* food poisoning are attributed to meat products and poultry (protein-rich foods), because this bacteria has ability to lack the genetic machinery to produce 13 essential amino acid (Johnson et al. 1997). Furthermore, the competing flora are killed by the effect of mild heat treatment of food which cause food contamination, in the meantime the spores of *Cl. perfringens* survive and germinate (Byrne et al. 2008; Juneja et al. 2009).

2.7.3 ***Staphylococcus aureus***

G^{+ve} , cocci and can grow between 7- 45°C (Lockary et al. 2007). *Staph. aureus* is usually considered as a public health problem, which associated with a hospital acquired and serious community illnesses (Pesavento et al. 2007). Additionally, this pathogen is classified into several strains; some of these strains have the ability to produce enterotoxins (SEs) and other virulence factors. The SEs are heat-stable enterotoxins, which considered as most notable virulence factors of the microorganism, that can cause food poisoning during their massive growth in foods or food-borne outbreaks and decrease the immune response of infected host as the effect of toxic shock syndrome toxin 1 (Vancraeynest et al. 2006; K erouanton et al. 2007). Moreover, SEs have been

divided into two serological types SEA and SEE, and then presence of new types of toxins has been detected (Chiang et al. 2008).

Symptoms such as nausea, severe vomiting, abdominal cramps and diarrhoea started after 1-8 h of ingestion of contaminated food with *Staph. aureus*. However, at least one of the following can approve food poisoning of *Staph. aureus*: Recovery of 10^5 CFU/g in food, isolation of the same bacterial phage from both the infected host and food and detection of bacterial enterotoxins in food (Bryan et al. 1997)

2.7.4 *Listeria monocytogenes*

One of most virulent food-borne fatal, G^{+ve} aerobic to facultative anaerobic pathogens (Moltz 2005; Kohn 2012) Due to their ubiquitous nature, *L. monocytogenes* usually isolated from foods, soil, water and sewage, animal origin foods are the main sources of infection (Schuchat et al. 1991). These bacteria can be also found in other products such as vegetable and salads. *L. monocytogenes* is a causative agent of listeriosis, which is characterised by high mortality rate (30%), severe cases of abortion, meningoencephalitis and septicemia (nonenteritic disease) may also observe. Moreover, the most susceptible people for this disease are immunocompromised patients, elderly people, pregnant women and new-borns. (McLauchlin et al. 2004; Allerberger and Wagner 2009). However, Ingestion of contaminated foods with *L. monocytogenes* is the major path of transmission, while direct transmission from human to human or from animal to human can rarely occur (Allerberger and Wagner 2009).

L. monocytogenes can continue to multiply slowly in low temperature and allowing for growth even in well-refrigerated foods, as well as it can survive in processed foods that depend on acidic and salty condition. Therefore, this

pathogen has successfully caused serious food-borne disease (Allerberger and Wagner 2009).

2.7.5 *Escherichia coli*

Generally, *E. coli* is rod shape, aerobic and G^{-ve} bacteria and their strains are commonly normal inhabitant of the gut of the warm blooded animals and humans. Most of *E. coli* strains are harmless and as part of intestine microflora which preventing the formation of pathogenic bacteria in the intestine and creating vitamin K2. Whereas, food poisoning can be occurred due to the effect of particular strains such as O157:H7 (Shiga-toxin-producing organisms), and consequently causes food product recall (Kohn 2012). Moreover, *E.coli* O157:H7 strains are identified as most common pathogenic strains which is responsible for many intestinal and extra-intestinal infections, and can cause gastroenteritis, haemorrhagic colitis or nosocomial septicemia, haemolytic uremic syndrome and neonatal meningitis (Lockary et al. 2007; Werber et al. 2007). However, it has been observed that pathogenic *E. coli* is implicated with food-borne disease with increasing frequency over the last two decades (Abong'o and Momba 2009).

The main sources of Shiga-toxin-producing organisms are domestic and wild animals, whereas the ruminants are considered a natural reservoirs of the bacteria (Werber et al. 2007). Direct contact between humans and animals or human to humans mainly cause the transmission of infections (Caprioli et al. 2005; Cho et al. 2006). Moreover, most of the outbreaks related to this pathogen have been associated with foods such as meat and dairy products, unpasteurized fruit juices and water, ground beef, raw milk and vegetables (Battu and Reddy 2011).

2.7.6 ***Salmonella* spp**

Presence of *Salmonella* in food products still globally considered a main concern of public health, also many food-borne disease outbreaks occurred due to *Salmonella* (Carrasco et al. 2011). Annually Millions of people have infected with Salmonellosis, and economic consequences have occurred due to the diseases. *Salmonella* is flagellated, rod shape G^{-ve} bacteria with more than 2400 serovars which identified most commonly associated with human illnesses (Lockary et al. 2007). Moreover, the bacteria are drying and freezing resistant and can grow and multiply under both aerobic and anaerobic conditions within the pH 4-8. Whereas, it was observed that heating of contaminated foods with *salmonella* for 15 min at 71°C and pH below 4 can lead to bacterial death (Lockary et al. 2007).

Salmonella serotypes are ubiquitous in nature, therefore it can be found in the gastrointestinal tract of most domestic and wild animals (Gerber et al. 2002). Therefore, production technique of animal origin foods and slaughterhouse practice can increase the prevalence of the bacteria between food producing animals. Consumption of contaminated foods particularly poultry meat and egg with *salmonella* is the important cause of infection (Thong et al. 2002). Additionally, Padungtod and Kaneene (2006) reported that the antibacterial resistance of *Salmonella* has increased, which leads to failure in the treatment of human Salmonellosis.

According to data of European Food Safety Authority (2009), *Salmonella* spp, still a world wide serious food-borne disease risk.

2.7.7 ***Bacillus cereus***

Aerobic and facultative anaerobic, rod shape, G^{+ve} and spore forming pathogen (De Jonghe et al. 2008). The bacteria is ubiquitous in nature and can multiply in

a wide range of condition (Fangio et al. 2010). Therefore, it is usually found in water, soil, foods (raw and processed) and even in an additive (Abriouel et al. 2007; Ki Kim et al. 2009). Adams and Moss (2007), stated that some of food-borne disease in Europe has been attributed to *B. cereus*, and different strains of the bacteria associated with food poisoning and food-borne disease. However, two types of *B. cereus* food poisoning have been detected in diarrheal and emetic syndrome. The symptoms that related to diarrheal syndrome is started after 8-16 h of eating of contaminated foods, which characterised by the occurrence of abdominal pain and watery diarrhea (Granum and Lund 2006). While in the emetic syndrome, nausea and vomiting occurred within 6 h of ingestion of contaminated food (Rajkovic et al. 2006). In general, the occurrence of food-borne illnesses that associated to *B. cereus spp* occurred when the enterotoxigenic strains proliferate to reach up to 10^6 CFU/g (Valero et al. 2003). In the meantime, contaminated food with 10^3 CFU/g is unsafe for consumption, due to the considerable difference of the infective dose (Granum and Lund 2006). Moreover, the lack of competition in refrigerated storage room can help the activated spore to grow out, re-germinate and producing toxin in food (Rajkovic et al. 2006). *B. cereus* spores can also survive in processed and ready to eat foods with mild heat treatment, their capability of resistant to low pH can enhance the persisting condition in stomach then pass into intestine , germinate, multiply and producing toxins (Granum and Lund 2006). Regarding to Vilas-Bôas et al. (2007), It is commonly impossible to prevent the contamination of processed food with *B. cereus*, due to the wide range occurrence of it is spores.

2.8 Conclusions

Different types of plant natural extracts showed significant antibacterial activity against food related pathogens. The application of natural extracts in the food system is more preferable than synthetic preservative. However, Data concerning application of natural extracts in food systems compared to synthetic additives is still limited. The antibacterial activities of natural extracts are mainly due to the presence of phenolic compounds in their contents, which acts by attacking of cell wall, increasing the permeability of the cytoplasmic membrane. The sensitivity of the bacterial groups towards the natural antimicrobial is different. *Gram positive* bacteria are generally more sensitive than *gram negative* groups. Higher concentrations of the extract are needed to achieve the same inhibitory effect in food than in cultured media. Two main factors should be considered when natural extracts applied in food, the sensory properties and the cost of the extract. Moreover, further studies are needed for optimisation of natural antibacterial in food, by investigating the influences of food components and other environmental factors on the activity of any proposed extract.

3 Chapter 3: Antibacterial activity and the phenolic contents of selected extracts

3.1 Introduction

Since antibacterial agents from synthetic additives have been used for several years to inhibit pathogenic bacteria, there is therefore a great interest in developing new natural and nontoxic antibacterial agents from natural compounds (Negi 2012). Food safety is still a concern for the scientist and public health, in spite of all the current preservation methods such as modified-atmosphere packaging, irradiation of food and using of artificial additives (WHO 2012). Food quality and safety can be affected by pathogenic and spoilage bacteria (Celiktas et al. 2007; Jacob et al. 2010). According to an estimation that carried out by WHO (2012), more than 2.2 million deaths occurred worldwide, due to food-borne diarrheal illness especially in young children. However, long term ingestion of some synthetic preservative compounds can cause respiratory and other health problems, in addition to their carcinogenic impact (Fleming-Jones and Smith 2003; Hernández-Hernández et al. 2009).

Naturally occurring compounds of numerous plants, spices and herbs have been shown to have antibacterial potential against different types of pathogenic bacteria. Significant biological effects of plant natural extracts with their phytochemical compounds such as alkaloids, phenols, peptides, tannins and flavonoids have been detected. As well as their effect as an antioxidant, anti-inflammatory and anticancer have been confirmed (Rahman and Kang 2009; Oyedemi et al. 2010)

Spices and herbs have been usually used in food not only as flavouring and aromatic agents, but also as folk medicine and food preservatives. Demand for these materials has been growing as a result of increasing consumption of

minimally processed ready-to eat foods, which contain herbs and spices as ingredients (Witkowska et al. 2011). In addition to imparting characteristic flavours, specific type of spices and herbs extend the shelf life of foods by preventing rancidity through their antioxidant activity or through inactivating or inhibiting pathogenic and spoilage growth (Patrignani et al. 2008). Fruits, spices and herbs and their extract are commonly recognised to be safe, because of their traditional use without any documented detrimental influence and due to the dedicated toxicological studies (Burt 2004).

Sumac (*Rhus*): generally, the common name of the *Rhus* (*R*) genus is sumac, which can grow in non-cultivated viable area. The species of *R. coriaria* (Sicilian sumac) is usually used in the Middle East for traditional treatment and as a food additive, it is characterised by its antiseptic, antidiarrheatic, analgesic, anorexic and antihyperglycaemic activities (Rayne and Mazza 2007) . *R. coriaria* showed significant antioxidant and antibacterial effect (Adwan et al. 2006). On the other hand, *R. typhina* (Staghorn sumac) grows in North America and is used to produce a specific type of beverage called “sumac-ade” or “rhus juice” and serves also as a folk medicine which has diuretic, anti-haemorrhoidal, antiseptic and tonic properties (Moerman 1998).

Pomegranate (*Punica granatum*): fruit is usually cultivated in the Mediterranean and other Asian regions. For many years, several parts of this fruit have been traditionally used for treatment. Pomegranate is prescribed as an anti-inflammatory against urinary tract infection and for digestive tract disorders (Lansky and Newman 2007). Moreover, an anticancer effect of their peel and juice essential oils has been reported (Kim et al. 2002). Balasundram et al. (2006), revealed that the biological activity of peel and seed fractions is higher than the pulp fractions. Pomegranate is rich in tannin of the ellagitannin group

that has shown a strong antioxidant effect in the methanolic extract. The ability of pomegranate peel to prolong the shelf life of chicken products has been also investigated (Damašius et al. 2009; Yang et al. 2010). Furthermore, their antibacterial activity has been exhibited against numerous types of G^{+ve} and G^{-ve} bacteria (Kanatt et al. 2010).

Star anise (*Illicium verum*): is a star shaped pericarp growing in certain part of Asia such as China and Vietnam; because of their diverse biological activity therefore it is applied traditionally in case of stomach ache, skin inflammation and as pain relief in the case of rheumatism. Recently, over 50 compounds have been determined in star anise and seco-prezizaane type sesquiterpenes are the main constituents among them (Yang et al. 2010; Wang et al. 2011). Some of these compounds have shown to have strong biological activity as neurotrophic and neurotoxic (Yokoyama et al. 2002). Moreover, star anise is rich with shikimic acid that acts as an antiviral and particularly used against bird flu virus (Yokoyama et al. 2002).

Cardamom (*Elettaria cardamomum*): is known as a queen of all spice which is cultivated in different part of Asia and Africa such as south India, Tanzania, Srilanka and Morocco. Cardamom is dried plant of the Zingiberaceae family, which commonly grows to a height of eight feet (Kapoor 2000). In general, this cardamom is used as flavouring and aromatic agent in food and for treatment in case of digestive disorders and as medicine for intestinal gas (Ravindran and Madhusoodanan 2002). In recent years, an interest towards its natural extracts have been developed, therefore the antibacterial activity of cardamom has been detected against several bacterial species such as *E. coli* (Zhang et al. 2009), *Staph. aureus* (Arora and Kaur 2007; El Malti and Amarouch 2009), *S. typhi* (Singh et al. 2008) and *P. aeruginosa* (Alemdar and Agaoglu 2009). Furthermore, Singh et al. (2008) reported that the use of different solvents may lead to obtain of extracts with various chemical profiles and activities.

Cranberry (*Vaccinium oxycoccos*): fruits are considered as an important source of bioactive compounds, such as organic acids and phenolic compounds, which may have antibacterial potential. The antibacterial activity of cranberry has been detected against several types of pathogenic bacteria, including *E. coli*, *Staph. aureus*, *Campylobacter* and *Salmonella* (Côté et al. 2011). It has been found that the berries phenolic compounds played an important role in preventing of particular infectious diseases such as urinary tract disorders, dental decay as well as reduced the risk of cardiovascular diseases and tumors. Cranberry phenolic compounds specially the proanthocyanidins has been shown to reduce the adhesion of *E. coli* to the urinary tract (Heinonen 2007). Moreover, Narwojsz and Borowska (2010) reported that berry phenolic compounds have shown to have strong antioxidant activity. Nevertheless, factors such as, extraction method and preservation temperature can affect the antibacterial and

antioxidant capacity of cranberry, therefore interpretation and comparison of results are complicated (Côté et al. 2011)

3.2 Material and methods

3.2.1 Natural extracts

Cardamom oleoresin and star anise aquaresin were provided by Kalsec (Kalsec Mildenhall, UK), cranberry powder were obtained from Healthy Supplies (Healthy Supplies Ltd. UK), sumac from (Green Cuisine Food Products (Green Cuisine Watton, UK) and pomegranate from Navitas Natural (Navitas Natural. USA). Moreover, two types of extracts were prepared (aqueous and alcoholic extracts).

Table 3. 1: Natural extract types, parts, volatile oil and code number

Natural extracts	Types	Part of plant	Volatile oil	Code number
Cardamom	oleoresin	Seed	80-100%	28-02
Star anise	aquaresin	pericarp	45-65%	42-01-19
Sumac	powder	Berries	N/A	993709
Pomegranate	Powder	Whole fruit	N/A	110802
Cranberry	Powder	Berries	N/A	A699

N/A: not applicable

3.2.1.1 Preparation of pomegranate extract

According to Al-Zoreky (2009), alcoholic and water extract of pomegranate were prepared.

3.2.1.1.1 Alcoholic extract

Pomegranate powder (10 g) was dissolved in 80% (v/v) of methanol : water, dissolving ratio of powder to solvent was 1:10 (w/v) (Zhouyong et al. 2008). The mixture was left to shake for 24 h and then filtered with Watman filter paper no. 41. The filtrate sample was centrifuged at 8654 g for 10 min (MSE-Europa 24 B

UK), membrane filter then used to percolate the clear extract, clear extract was kept at $4\pm 1^{\circ}\text{C}$ in the dark (Williams Refrigeration and Air-conditioning, laboratory of nutrition, Plymouth University). The fruits residue that was present in watman filter papers and centrifuge tubes was re-suspended into the solvent and centrifuged as mentioned previously, the solution of first and second extraction then pooled together and concentrated in a vacuum rotary evaporator at 40°C (Buchi, Switzerland). The hydro alcoholic extracts were kept in refrigerator at $4\pm^{\circ}\text{C}$.

3.2.1.1.2 Water extract

The previous extraction protocol was followed in order to prepare pomegranate water extract with an exception, water was used as solvent instead of methanol 80% (Al-Zoreky 2009).

3.2.1.2 Preparation of sumac extracts

3.2.1.2.1 Alcoholic extract

Ethanol 80 % (v/v) was used to prepare hydro alcoholic sumac extract. At the ratio of 1:10, a ten g of ground sumac was added up to 100 ml of ethanol 80%, then the mixture was left in shaker for 24 h, followed by filtration through Watman filter paper no. 41. Extracting method was carried out according to the method described by (Kossah et al. 2011). The extract was concentrated by using a rotary evaporator (Buchi, Switzerland) and the extracted material then stored in cold and dry place.

3.2.1.2.2 Water extract of sumac

According to the method of (Nasar-Abbas and Halkman 2004) a water extract of sumac was prepared, by macerating 5 gm of ground sumac in 95 ml of distilled water (w/v) and keeping for 1 h with occasional stirring followed by gently boiling for 2 min on a hot plate equipped with a magnetic stirrer.

Collected extract was chilled and filtered through Watman filter paper no. 41 and then kept in the dark.

3.2.1.3 Preparation of cranberry extract

The previous protocol (pomegranate extraction method) was followed to prepare alcoholic and water extract of cranberry.

3.2.2 Bacterial cultures

A six food related bacteria were used to determine the antibacterial activity of selected extracts as follows: *E. coli* K12, *Staph. aureus* ATCC 6821, *L. monocytogenes* 5105, *S. Typhimurium* DT104, *Cl. perfringens* NCTC 8237 and *B. cereus* NCIMB 11925. All these strains obtained from the microbiological collection from the school of biomedical and biological science at the University of Plymouth. These bacteria were selected according to potential hazard in food and also they considered as a common food borne disease in the UK (Food Standards Agency 2014)

3.2.3 Preparation of normal saline

Bacterial cultures were diluted by using sodium chloride solution, 0.85% (Oxoid-limited, Basing stoke, Hampshire, UK), to reach a concentration of 10^7 - 10^8 CFU/ml.

3.2.4 Preparation of standard (McFarland's) opacity tube

A specific amount (0.05, 0.1, 0.2, 0.3 and 0.4) ml of 1% barium chloride was added to 1% sulphuric acid (9.95, 9.9, 9, 9.7 and 9.6) ml respectively in order to prepare McFarland turbidity standard tubes (Harrigan 1998).

3.2.5 Preparation of brain heart infusion agar (BHI)

BHI agar was used to prepare agar plates for agar well diffusion method. One litre of agar was obtained by dissolving 37 g of brain heart infusion agar in one litre of microwaved (10 min) distilled water. Then distributed in Universal Eppendorf tubes (Eppendorf AG, 22331, Hamdarg, Germany), and were sterilized by autoclaving for 15 min at 121°C.

3.2.6 Preparation of brain heart infusion broth (BHI)

To prepare 1L of BHI broth (Oxoid Ltd. Basingtoke, Hampshire, UK), 37 g of media was dissolved in 1 L of distil water then distributed in 10 ml aliquots in Universal Eppendorf dispenser tubes (Eppendrof AG, 22331, Hamdarg, Germany), these tubes autoclaved for 15 min at 121°C. Stock cultures of all bacteria were prepared by growth in BHI broth.

3.2.7 Total Phenol Content

According to Viuda-Martos et al (2010), 3 mM Gallic acid (Sigma, Poole, UK) was prepared as standard) and Folin–Ciocalteu’s reagent (Sigma, Poole, UK) was used to determine the total phenolic compound (TPC) of selected natural extracts. A volume of 25 µl of different concentration (5, 10, 20, 30, 40 and 50) mgml⁻¹ of each extracts (alcoholic and water) was dispensed into Eppendorf tubes followed by 625 µl Folin– Ciocalteu’s reagent (diluted with water 1:10) and 500 µl of sodium carbonate (7.5% w/v), all tubes were vortexed and then incubated at 50°C for 5 minutes. Then 200 µl of the mixture was dispensed into a clean 96-well microplate to measure the absorbance at 760 nm using a plate reader spectrophotometer (Spectra Max 340PC, Molecular Devices Corporation, Sunnyvale CA, USA). (Sigma, Poole, UK). The results were expressed as mg Gallic acid equivalentents (GAE) /ml sample. Each assay was carried out in triplicate.

3.2.8 Antibacterial activity of natural extracts

The agar well diffusion method was employed to evaluate the inhibitory effect of selected extracts against six pathogenic bacterial strains (Kuri et al. 1998; Fernandez-Lopez et al. 2005; Al-Zoreky 2009). Stock cultures of all tested bacteria were incubated for 18 h in BHI broth (Oxoid Unipath Ltd., Basingtoke, Hampshire, UK) for culture. Final cell concentration was standardized at 10^7 - 10^8 cfu/ml using the McFarland standards, 200 μ l of inocula was transferred into each plate containing brain heart infusion (BHI) agar (20 ml) (Oxoid Unipath Ltd., Basingtoke, Hampshire, UK). The agar was left to solidify for 30 min, and then a sterilized Cork borer was used to create six wells (8mm) in each plate. Concentrations of extract (Cardamom oleoresin, star anise aquaresin, Sumac, pomegranate and cranberry water and alcoholic) 100, 10, 1 (mgml^{-1}) were added into the wells (40 μ l), plates then left for 15-20 min at room temperature to allow diffusion of extract. Bacterial strains were then incubated as follows, *Cl. perfringens* was incubated anaerobically at 37°C and *B. cereus* was incubated aerobically at 30°C, whereas all other strains were incubated aerobically at 37°C. Similar to amount of extract, control (samples were prepared and sterile distilled water was used as control instead of extract. The inhibition diameter in millimetres (mm) was measured in three directions by using callipers, the average then was calculated. The experiment was performed in triplicate.

3.2.9 Determination of the minimum inhibitory concentration (MIC)

According to (Okoh et al. 2010), a standard colorimetric broth microdilution method was employed to determine the minimum inhibitory concentration of selected extract against each susceptible pathogens. Additionally, a flat bottom transparent 48 well plate (Corning, fisher Scientific) was used and sequential twofold dilutions with sterile brain heart infusion broth of the antibacterial natural extracts were done. 850 μ l aliquots of each dilution in addition to 50 μ l of test bacteria were added into each well. Moreover, controls of test bacteria were performed by adding 50 μ l of bacteria into 850 μ l of brain heart infusion broth. The final concentration of extracts ranged from 1.5-50 mgml^{-1} . Each assay was employed in triplicates and the turbidity of the culture was measured by micro-plate automated reader (TECAN, Infinite 200, Reading, UK). Each culture plate was incubated at 37°C for 24h, the MIC was determined as the lowest concentration that eliminated the bacterial growth. However, a media plate count method was used to confirm the results.

3.2.10 Statistical analysis

All experiments were carried out in triplicate; the standard statistical method was applied to calculate mean and standard errors. Analysis of variance (ANOVA) was conducted and one-way ANOVA with Tukey's were performed using Minitab version 16 (Minitab Ltd, Coventry, UK), to analyse data and to identify the significant difference between the compared mean respectively. Additionally, the statistical difference was considered to be significant when $P < 0.05$.

3.3 Results

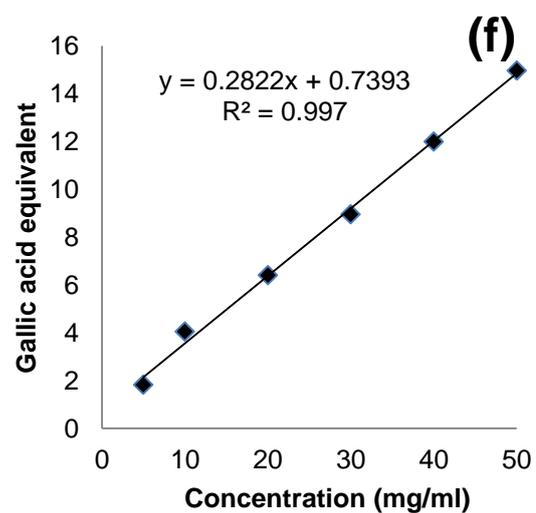
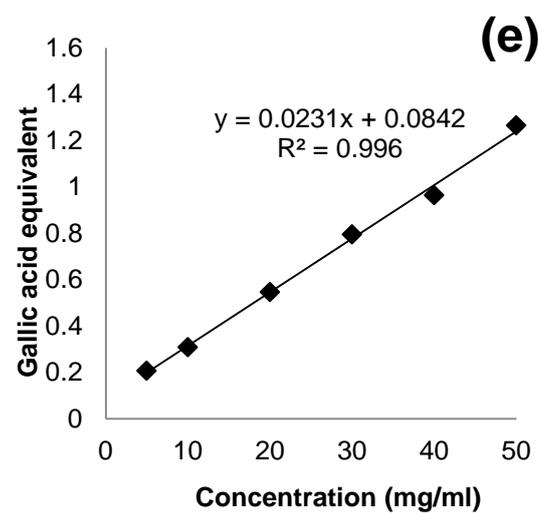
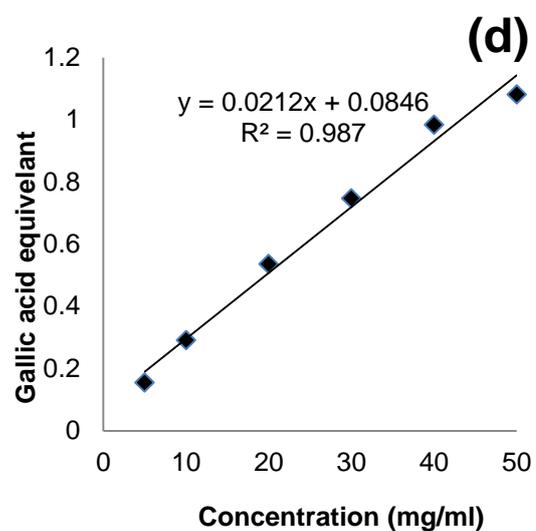
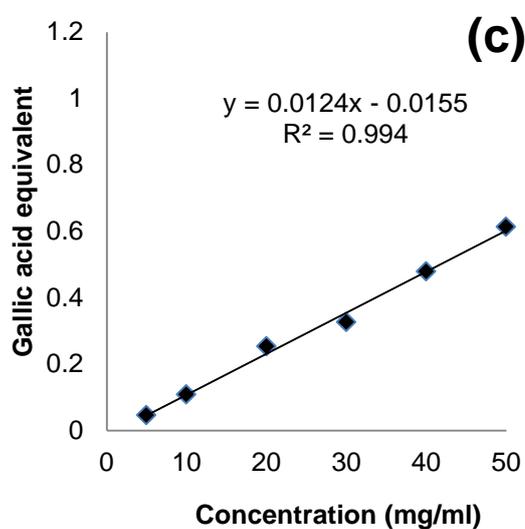
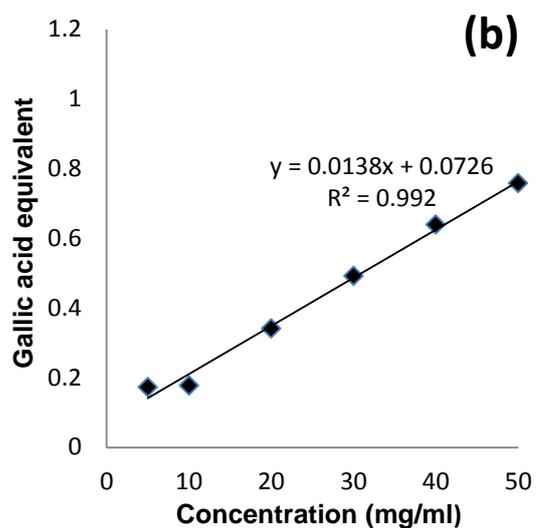
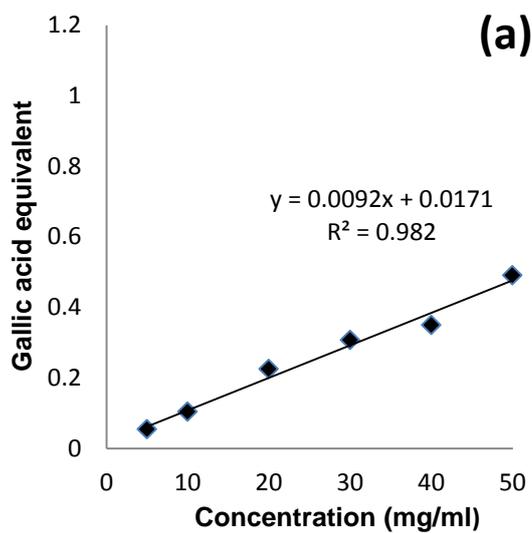
3.3.1 Total phenolic compound

The total phenolic content of natural extract (TPC) mg Gallic acid equivalent (GAE) /ml sample was affected by both, types of natural extract and the extraction solvent used. Generally, alcoholic extracts had significantly higher ($P < 0.05$) TPC compared to water extracts (Table 3.2). The TPC of natural extracts was concentration dependant and followed a linear trend with a positive correlation between the concentration and the TPC of all natural extracts. The regression equation could be useful to predict the TPC at any concentration (Figure 3.1).

Table 3. 2. Total phenol compounds of natural extract Gallic acid equivalent at different concentration. Result represented as mean \pm SE (n=3).

Natural extracts	Concentration mgml ⁻¹					
	5	10	20	30	40	50
Ca O*	1.07 \pm 0.00 ^a	1.97 \pm 0.01 ^{ab}	3.75 \pm 0.01 ^{abc}	6.41 \pm 0.00 ^{abc}	8.85 \pm 0.01 ^{bc}	11.0 \pm 0.00 ^c
Cr Mt♦	0.15 \pm 0.01 ^a	0.29 \pm 0.01 ^{ab}	0.54 \pm 0.02 ^{abc}	0.75 \pm 0.04 ^{abc}	0.98 \pm 0.06 ^{bc}	1.08 \pm 0.09 ^c
Cr W♦	0.05 \pm 0.00 ^a	0.11 \pm 0.00 ^{ab}	0.25 \pm 0.01 ^{abc}	0.33 \pm 0.00 ^{abc}	0.48 \pm 0.00 ^{bc}	0.61 \pm 0.00 ^c
Po Mt♦	0.17 \pm 0.01 ^a	0.18 \pm 0.00 ^{ab}	0.28 \pm 0.02 ^{abb}	0.49 \pm 0.00 ^{abc}	0.64 \pm 0.00 ^{bc}	0.76 \pm 0.0 ^c
Po W♦	0.05 \pm 0.00 ^a	0.10 \pm 0.00 ^{ab}	0.23 \pm 0.01 ^{abc}	0.31 \pm 0.01 ^{abc}	0.35 \pm 0.00 ^{bc}	0.49 \pm 0.01 ^c
St A♦	0.11 \pm 0.00 ^a	0.20 \pm 0.00 ^{ab}	0.62 \pm 0.04 ^{abc}	1.11 \pm 0.02 ^{abc}	1.62 \pm 0.04 ^{bc}	2.03 \pm 0.00 ^c
Su Et*	1.80 \pm 0.00 ^a	4.10 \pm 0.02 ^{ab}	6.40 \pm 0.03 ^{abc}	9.00 \pm 0.04 ^{abc}	12.0 \pm 0.02 ^{bc}	15.0 \pm 0.02 ^c
Su W♦	0.20 \pm 0.03 ^a	0.30 \pm 0.01 ^{ab}	0.55 \pm 0.00 ^{abc}	0.70 \pm 0.03 ^{abc}	0.96 \pm 0.00 ^{bc}	1.26 \pm 0.01 ^c

Natural extracts: Ca: cardamom, Cr: cranberry, Po: pomegranate, St: star anes, Su: sumac, O: oleoresin, A: aquaresin, Mt: methanolic extracts, Et: ethanolic extracts, W: water extracts. *♦: Natural extracts within a row with different symbols indicate significant differences ($p < 0.0$), ^{a-c}: mean within a column with different letters are significantly different ($p < 0.05$).



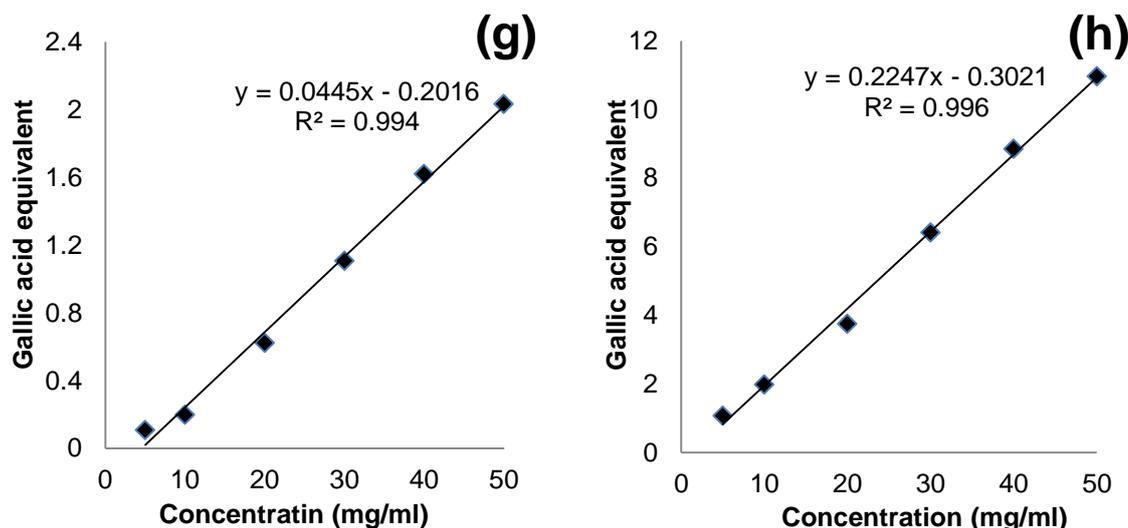


Figure 3. 1: Total phenolic compound of natural extracts (Pomegranate water (a), Pomegranate alcohol (b), Cranberry water (c), Cranberry alcohol (d), Sumac water (e), Sumac alcohol (f), Star Anis (g), and cardamom (h)).

3.3.2 Antibacterial activity of natural extracts

The antibacterial activities of natural extracts against selected bacteria are presented in (Table. 3.2, 3.3). Different antibacterial activities have been demonstrated against the six bacteria depending on the natural extract, extraction solvent and the concentration used. Alcoholic extracts of sumac, cardamom, pomegranate and star anise had a greatest activity at the concentration of 100 mgml^{-1} compared with water extracts. Sumac water extract and cranberry were ineffective against all tested bacteria. A significant ($P < 0.05$) variation in the antibacterial activity diameter inhibition zone (DIZ) of natural extracts was exhibited against the tested bacteria.

Positive correlation between the antibacterial activity (DIZ) and the phenolic content were observed for Cardamom, Pomegranate and Sumac (Figures 3.2 and 3.4).

Table 3. 3. Inhibition zone (mm) of different natural extracts^a at the concentration of 100 mgml⁻¹, the diameter of each well is 8 mm.

Natural extracts	Bacterial groups					
	<i>E. coli</i>	<i>Staph. aureus</i>	<i>B. cereus</i>	<i>S. Typhimurium</i>	<i>Cl. perfringens</i>	<i>L. monocytogenes</i>
Ca O	13.9	14	14.5	11	10	11
Cr Mth ^a	–	–	–	–	–	–
Cr W	–	–	–	–	–	–
Po Mth	12.8	11.9	12.4	12	10	10.5
Po W	11.8	10.8	10.8	–	–	–
St A	12	10	12.5	11.5	10	10
Su Eth	16	15.3	15.3	15.8	13.8	13.5
Su W	–	–	–	–	–	–

^a Natural extracts: Ca: cardimom, Cr: cranberry, Po: pomegranate, St: star anes, Su: sumac, Mth: methanolic extracts, Eth: ethanolic extracts, O: oleoresin, A: aquaresin, W: water extracts, –: no inhibition (> 8mm).

..

Table 3. 4. Inhibition zone (mm) of different natural extracts at the concentration of 10 mgml⁻¹, the diameter of each well is 8 mm.

Natural extracts	Bacterial groups					
	<i>E. coli</i>	<i>Staph. aureus</i>	<i>B. cereus</i>	<i>S. Typhimurium</i>	<i>Cl. Perfringens</i>	<i>L. monocytogenes</i>
Ca O	9.4	9.4	9.6	9.3	9.1	9.3
Cr Mth	–	–	–	–	–	–
Cr W	–	–	–	–	–	–
Po Mth	–	–	9	–	8.5	–
Po W	–	–	–	–	–	–
St A	–	–	–	–	–	–
Su Eth	11.8	11.1	11.3	11.4	11.5	10.6
Su w	–	–	–	–	–	–

Natural extracts: Ca: cardimom, Cr: cranberry, Po: pomegranate, St: star anes, Su: sumac, O: oleoresin, Mth: methanolic extracts, Eth: ethanolic extracts, W: water extracts, A: aquaresin.

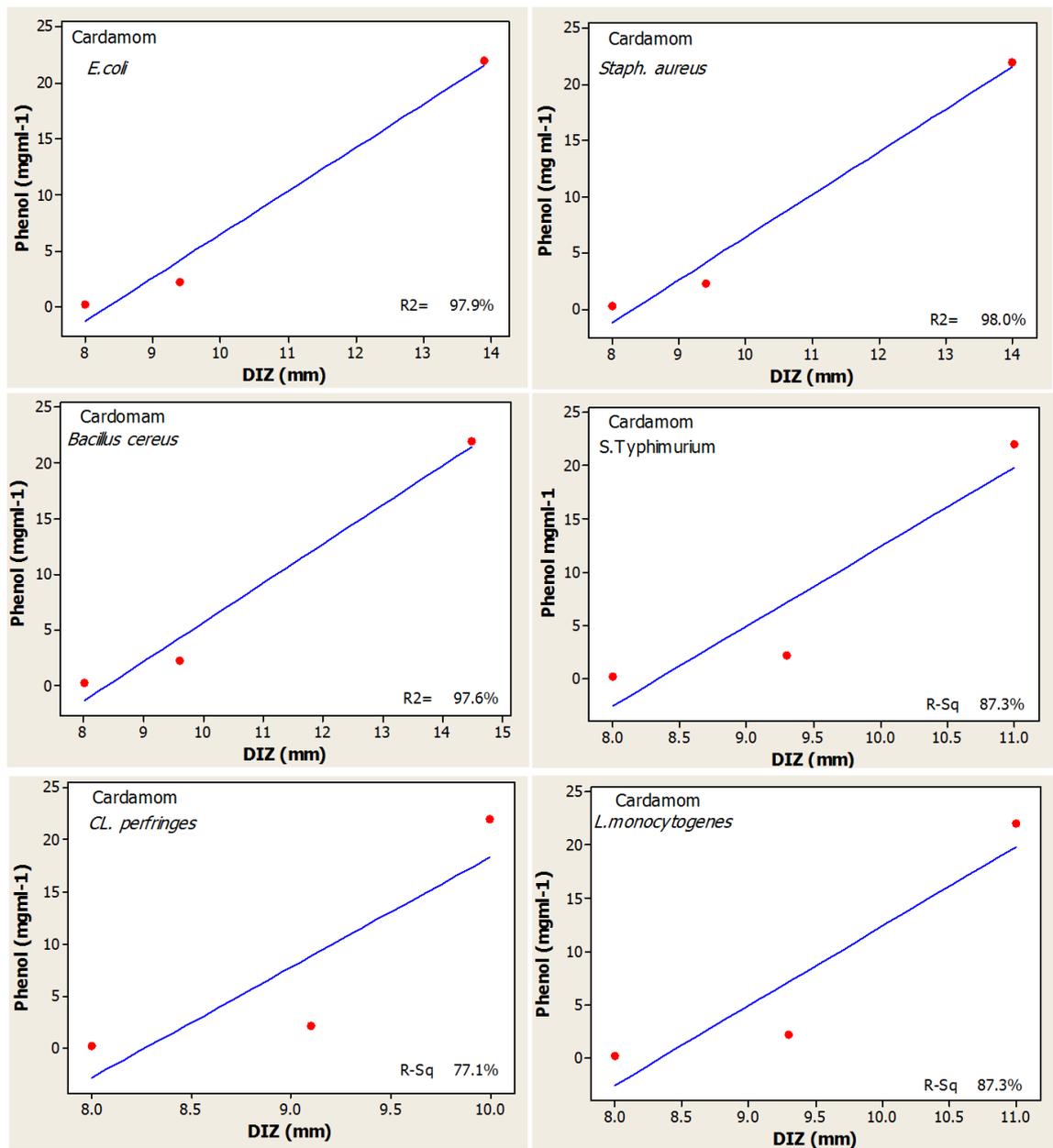


Figure 3. 2: Correlation between phenol concentrations and antibacterial activity against selected bacterial groups treated with cardamom extracts.

DIZ: diameter of inhibition zone

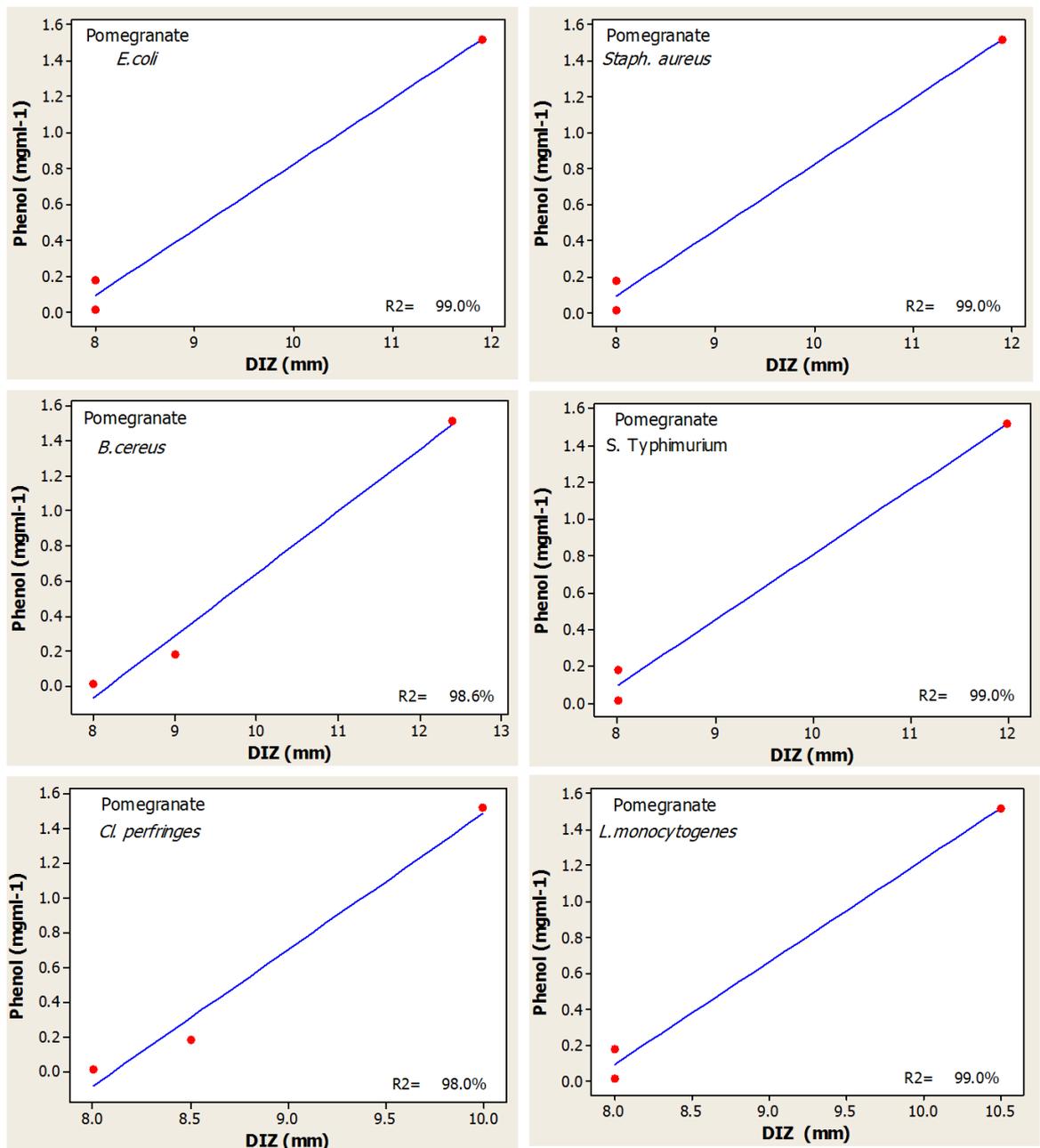


Figure 3. 3: Correlation between phenol concentrations and antibacterial activity against selected bacterial groups treated with pomegranate extracts.

DIZ: diameter of inhibition zone

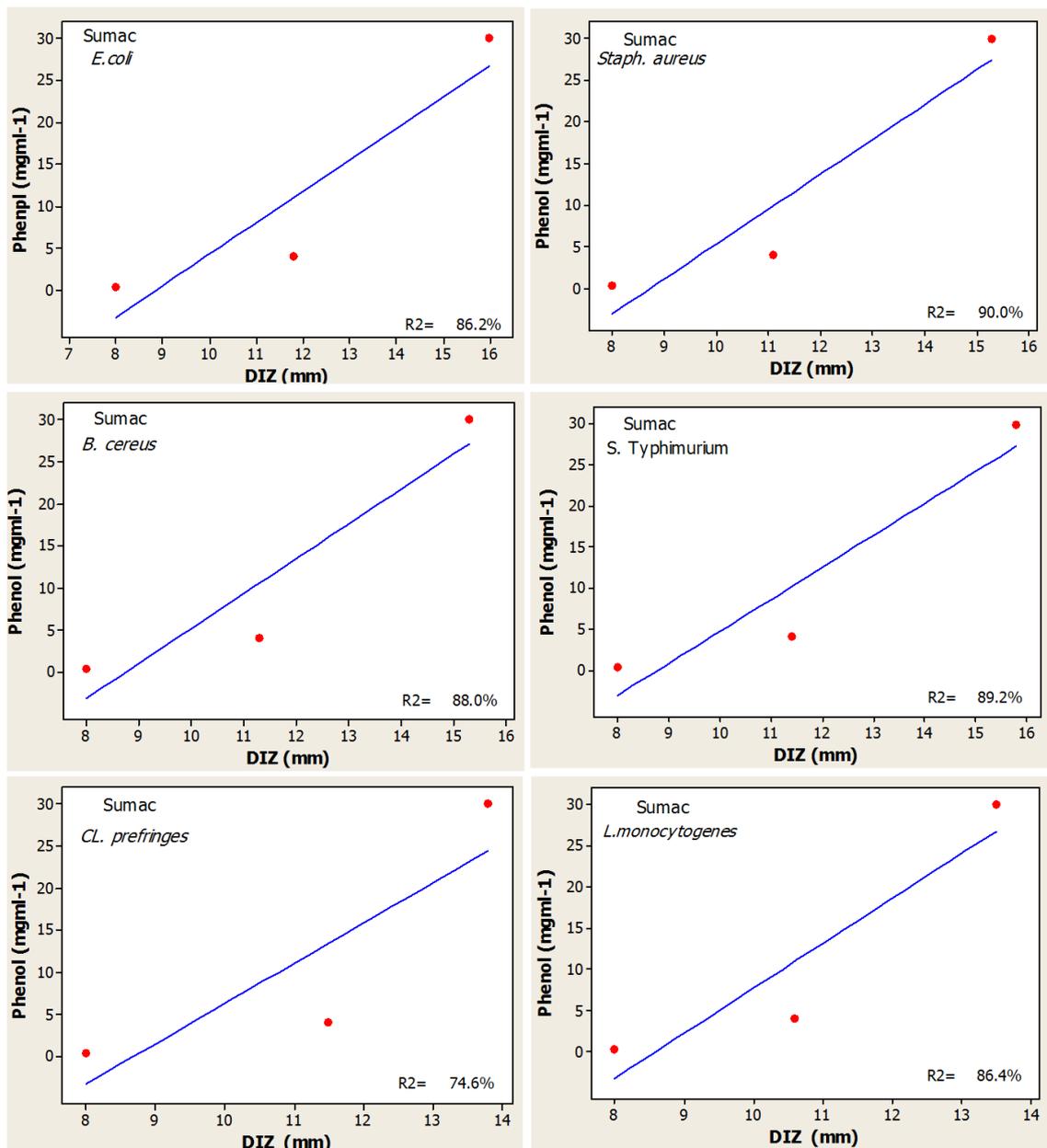


Figure 3. 4: Correlation between Phenol concentrations and antibacterial activity against selected bacterial groups treated with sumac extracts.

DIZ: diameter of inhibition zone

3.3.3 Minimum inhibitory concentration (MIC)

The MIC was defined as the lowest concentration of an antimicrobial that inhibit bacterial growth .In this study broth microdilution method was used to determine the MIC of selected natural extract and plate count method was performed to confirm the result. The MIC values of selected natural extracts obtained at 24 h are presented in table 3.4. As shown in the table all tested bacteria strains (*L. monocytogenes*, *B. cereus*, *E. coli*, *Staph. aureus*, *Cl. perfringens* and *S. Typhimurium*) show different sensitivity against the natural extracts (Table 3.6). Sumac and cardamom demonstrated the higher effectiveness against selected pathogens with low concentration followed by pomegranate with moderate activity. Star anise exhibited low activity with high concentration. Sumac and cardamom inhibited pathogenic growth of *L. monocytogenes* and *B. cereus* at an MIC (3.1, 6.2 mgml⁻¹) respectively. Cardamom inhibited *E. coli*, *Staph. aureus*, *S. Typhimurium* and *Cl. Perfringens* growth at an MIC (6.5, 3.1, 25, 14 mgml⁻¹) respectively, while sumac extract inhibited them at an MIC (8, 6.2, 6.2, 12 mgml⁻¹).

Pathogenic sensitivity toward natural extracts measured as MIC followed this descending order:

E. coli: cardamom > sumac > pomegranate > star anise.

L. monocytogenes: sumac =cardamom > pomegranate > star anise.

Staph. aureus: cardamom > sumac > pomegranate > star anise.

B. cereus: sumac =cardamom > pomegranate > star anise.

S. Typhimurium: sumac > pomegranate > cardamom > star anise.

Cl. perfringens: sumac > cardamom > pomegranate > star anise

Table 3. 5: The MIC (mgml⁻¹) and phenol equivalent of selected natural extract against pathogenic bacteria.

Natural extracts	Bacterial groups											
	<i>E. coli</i>		<i>Staph. aureus</i>		<i>B. cereus</i>		<i>S. Typhimurium</i>		<i>Cl. perfringens</i>		<i>L. monocytogenes</i>	
	<i>MIC</i>	<i>phenol</i>	<i>MIC</i>	<i>Phenol</i>	<i>MIC</i>	<i>Phenol</i>	<i>MIC</i>	<i>Phenol</i>	<i>MIC</i>	<i>Phenol</i>	<i>MIC</i>	<i>Phenol</i>
Ca O	6.5 ^a	1.43	3.1 ^a	0.68	6.3 ^a	1.38	25 ^c	5.50	14 ^c	3.08	3.1 ^a	0.68
Po Mth	12.6 ^c	0.30	12.5 ^c	0.25	6.3 ^a	0.12	12.5 ^b	0.25	7.5 ^a	0.15	12.5 ^b	0.25
St A	100 ^d	4.00	100 ^d	4.00	100 ^b	4.00	100 ^d	4.00	100 ^d	4.00	25 ^c	1.00
Su Eth	8 ^b	2.40	6.3 ^b	1.89	6.3 ^a	1.89	6.3 ^a	1.89	10 ^b	3.00	3.1 ^a	0.93

Natural extracts: Ca: cardamom, Cr: cranberry, Po: pomegranate, St: star anise, O: oleoresin, Su: sumac, Mth: methanolic extracts, Eth: ethanolic extracts. ^{a-d}: means (n=3) within a column with different letters are significantly different (p<0.05).

3.4 Discussions

3.4.1 Total phenolic compounds

Of all six natural extract tested in this study, A significantly ($P < 0.05$) higher significant differences were found in sumac, cardamom alcoholic extracts in contrast to pomegranate (water, alcoholic), cranberry (water, alcoholic), star anis aquaresin and sumac water extracts. The greatest TPC in alcoholic extract of sumac was perhaps due to either their high contents of polyphenolic compounds (Zalacain et al. 2003; Kosar et al. 2007; Tian et al. 2009) or due to the extraction solvent used. According to Sultana et al. (2009), the ethanol extract of sumac had great TPC.

Sumac water extract exhibited low TPC, while a comparative study which carried by Bursal and Köksal (2011), revealed that the TPC of the sumac water extracts were higher than ethanol extracts. There were significant difference ($P < 0.05$) between Sumac, cardamom alcoholic and pomegranate (water, alcohol), cranberry (water, alcoholic), star anise alcoholic and sumac water extracts. As well as, cardamom showed high TPC and our result were similar to the result obtained by Kong et al. (2010) identified cardamom as having a great phenolic contents. According to Amma et al. (2010), the high scavenging activity of cardamom can be attributed to their high phenolic content. On the other hand, pomegranate (water/alcohol), cranberry (water/alcohol), star anise alcohol and sumac water extracts showed low TPC.

Al-Zoreky (2009), revealed that water extract of pomegranate exhibited low TPC compared to alcoholic extract. Additionally, the activity of methanolic extracts was attributed to their high TPC. In a previous study the result showed that the antioxidant potency of pomegranate alcoholic extract was correlated to their high phenolic content (Elfalleh et al. 2011).

Berries are fruits rich in phenolic compounds such as phenolic acids, flavonoid and anthocyanins (Türkben et al. 2010). Cranberry water and methanol extracts showed high TPC (Côté et al. 2011). Moreover, Sariburun et al (2010) demonstrated that higher contents of berry fruits in TPC, flavonoid and anthocyanin contributes to their significant antioxidant activity.

In this study a low level of phenolic content was detected in star anise, while high amounts of both TPC and flavonoids were evaluated in the same fruits (Padmashree et al. 2007). Similar results have been reported by Dzamic et al (2009).

According to previous studies, the differences in the result of TPC concentration could be due to several factors such as environmental characteristics, period of harvesting, cultivar variability, fruit maturity, and extraction solvent and procedure (Sariburun et al. 2010).

3.4.2 Antibacterial activity

In this study, selected natural extract displayed variable antibacterial activity against selected bacteria. According to the diameter of inhibition, a significant difference ($P < 0.05$) was detected between alcohol and water extracts. Alcoholic extracts possessed higher antimicrobial activity than water extracts. This may be due to the concentration of the volatile oil (essential oil) being higher in alcoholic extracts than in water extracts.

Sumac alcohol extract showed strongest activity ($P < 0.05$) compared to cardamom, pomegranate and star anise extracts. This may be due to their high contents of phenol, tannins and other compounds such as organic acid that play a critical role as an antibacterial (Gulmez et al. 2006).

Sumac extracts: alcohol extract of sumac showed activity against both G^{+ve} and G^{-ve} bacteria with MIC ranged from 3.1-10 mgml⁻¹. This result concurred with the result of (Wu et al. 2008; Bursal and Köksal 2011), who reported that hydro alcohol extract of sumac had strong activity towards different strains of G^{+ve} and G^{-ve} bacteria. The study that conducted by (Kossah et al. 2011) , revealed that alcoholic extract of sumac showed strong antimicrobial activity, a concentration dependence and broad spectrum antibacterial activity.

Nasar-Abbas and Halkman (2004) observed that among all tested G^{+ve} bacteria *B. cereus* was found to be the most sensitive, while *L. monocytogenes* showed less sensitivity than the others. In contrast to the present study, *L. monocytogenes* exhibited high sensitivity with MIC 3.1 mgml⁻¹ followed by *B. cereus* and *Staph. aureus* with MIC 6.3 mgml⁻¹. This may be due to either different strains used or solvents applied for sumac extraction.

On the other hand, sumac water extract was ineffective against selected pathogenic bacteria. Whereas such result differs from those reported by other studies (Nasar-Abbas and Halkman 2004; Gulmez et al. 2006). The results obtained by Gulmez et al. (2006), revealed that psychrotrophic, mesophilic Enterobacteriaceae and coliform bacteria in a broiler wing modal showed higher sensitivity towards sumac water extract. Nasar-Abbas and Halkman (2004), indicated that sumac water extract has strong activity against *B. subtilis*, *L. monocytogenes*, *E. coli*, and *Salmonella* spp.

Pomegranate extracts: In this study, pomegranate alcohol extract exhibited a moderate antibacterial activity against selected pathogens, except against *Cl. perfringens* had low activity. this is in agreement with the experiments that conducted by Al-Zoreky (2009) who reported that the pomegranate methanol extract had a potent inhibitory effect against *L. monocytogenes*, *S.aureus* and

E.coli. Panichayupakaranant et al. (2010), demonstrated that pomegranate extract exhibited a bacteriostatic effect against a G^{+ve} anaerobe and G^{+ve} facultative anaerobic bacteria.

On the other hand, this study revealed that the Pomegranate water extract showed lower activity against the selected pathogenic bacteria than alcohol extract, which is similar to the results that obtained by (Prabuseenivasan et al. 2006) who indicated that pomegranate methanol extract had greatest antibacterial activity than water extracts against several strains of G^{+ve} and G^{-ve} pathogens. Pomegranate extracts are rich with gallic and ellagic acid, hence the antibacterial activity of the extracts may be due to their effect (Prabuseenivasan et al. 2006). However, among studies the difference in the activity and in MIC could be clarified by the differences in antimicrobial techniques used in tests, TPC of extracts and strain sensitivity (Al-Zoreky 2009)

Cardamom extract: cardamom extract showed an inhibitory effect against all selected pathogens with a mean MIC ranged from 3.1-25 mgml⁻¹. In line with this result El Malti and Amarouch (2009) revealed that both of G^{+ve} and G^{-ve} had shown strong sensitivity towards cardamom extract. Singh et al. (2008), reported that cardamom essential oil exhibited strong antibacterial activity against *E. coli*, *Staph. aureus*, *B. cereus* and *S. typhi*. Cardamom fruits are rich with alkaloids, glycoside, steroids, protein, carbohydrates, terpenoids, tannins and phenolic compound, their antibacterial effect may be due to the presence of these compounds (Kumar et al. 2010). However, cardamom extract exhibited low activity against *Cl. perfringens* compared to sumac, which is in agreement with the result conducted by Si et al. (2009) who indicated that *Cl. perfringens* showed low sensitivity to the cardamom extract.

On the other hand, Orak et al. (2011) indicated that cardamom was ineffective against *E. coli*, whereas in this study cardamom extract was effective against *E. coli*.

Star anise extract: star anise in this study showed low antibacterial activity in comparison to other extracts with a high MIC with low TPC. Moderate activity of extract has been detected against *L. monocytogenes* in comparison to other selected pathogens. In line with this study, the result of Shan et al. (2007) emphasised the importance of phenolic compounds in the antibacterial activity of natural extract and might considerably contribute their antibacterial activity. However, Si et al. (2009) reported that star anise volatile oil and extract rich in trans-anethole, exhibited a broad spectrum of antimicrobial activity. Additionally, star anise showed strong activity against *Staph. aureus* and *B. cereus*. Significant activity of star anise was exhibited against 67 clinical drug resistant isolates (Yang et al. 2010). Additionally, the antibacterial activity of star anise water extract was demonstrated against *Staph. aureus* (Allerberger et al. 2002).

Cranberry extract: both alcohol and water extracts of cranberry were ineffective against selected bacteria and there was a low TPC observed in their contents, this might be due to the extract used. In contrast, Wu et al. (2008) indicated that cranberry concentrate exhibited antibacterial activity against four food-borne bacteria (*E. coli* O157:H7 and *S. Typhimurium*, *L. monocytogenes* and *S.aureus*). Berry fruits are rich with bioactive compounds such as organic acids, phenolics, and anthocyanins, their antibacterial activity was demonstrated against *E. coli* O157:H7 (Si et al. 2009). Padmashree et al. (2007) reported that *Staph. aureus* and *E. coli* showed strong sensitivity towards water soluble phenolic compounds of cranberry extract. Moreover, *L. monocytogenes*

was entirely inactivated within 30 min of exposure with pure neutralised cranberry juice.

It is worth referring to the factors that can help to determine the presence of different concentrations of antimicrobial compounds in the final product, such as the nature of extracts, production technique, purity and preservation level. In contrast, numerous assays have been employed on synthetic growth media with dilution or diffusion in a solid media. Therefore, in this type of study It is difficult to perform a full comparison with the results obtained by other authors (El Malti and Amarouch 2009).

3.5 Conclusion

The natural extracts of sumac, cardamom, pomegranate and star anise exhibited variable antibacterial activity *in vitro*. However cranberry was ineffective, their inhibitory effects augmented with increase in extracts concentrations. Sumac and cardamom alcoholic extract showed a highest inhibitory effect with a broad-spectrum range against tested bacteria. Different efficacy of extracts was observed against selected bacteria, and both of G^{+ve} and G^{-ve} bacteria exhibited sensitivity towards the selected extracts.

The total phenolic contents (TPC) of alcohol extract were higher than water extract, sumac and cardamom alcohol extracts showed high TPC in comparison with the other alcohol extracts. Moreover, positive correlation was detected between the TPC and the antibacterial activity. Therefore, using of these potent natural antibacterial instead of synthetic compounds in food could be more useful to obtain safe and good quality foods.

4 Chapter 4: Mechanism of action of sumac, pomegranate and cardamom extracts against selected bacteria

4.1 Introduction

Natural extracts consist of several compounds and their action on bacterial cell targets is not well defined. Several interaction reactions take place simultaneously, therefore it is difficult to identify particular action sites (Burt 2004; Hyldgaard et al. 2012). The major components of natural extracts are phenol, aldehyde, ketones and terpenes, which mainly act against the cytoplasmic membrane of the microorganism. Phenolic compounds act by attacking cell wall, interacting with cell membrane, changing cell membrane function and influences protein and lipid ratios in the membrane and inducing efflux of potassium ions (Negi 2012). Moreover, the hydrophobic property of natural extracts (carvacrol and thymol) enables them to accumulate in the cell membrane, disturbing the structures and causing an increase of permeability (Carson et al. 2002; Goñi et al. 2009). The disrupting of the membrane can cause leakage of intracellular constituents and impairment of metabolic enzymes or dissipate cellular energy in form of ATP and also affecting the nucleic acid of the pathogens (Tiwari et al. 2009; Lv et al. 2011).

Sumac, cardamom and pomegranate are rich in phenolic compound (Table 3.1), volatile and organic compounds such as tannins, gallotannin, ellagitannin, gallic acid, ellagic acid, terpinen, limonene, 1,8-cineole, terpenoids, flavonoid, methyl gallate and anthocyanin (Gil et al. 2000; Agaoglu et al. 2005; Kosar et al. 2007; Reddy et al. 2007; Chen and Chen 2011). The antibacterial activity of these compounds had been observed against several pathogens. Methyl gallate from sumac showed a strong inhibitory effect against *Staph. aureus* and *S. Typhimurium* (Rayne and Mazza 2007). Bialonska et al. (2009), reported that

the antimicrobial activity of pomegranate against pathogenic *E coli*, *Cl. perfringens* and *S aureus* was attributed to ellagitannin. Also, gallic acid was showed a strong inhibitory effect against *Salmonella* growth (Puupponen-Pimiä et al. 2005).

Raybaudi-Massilia et al. (2009), reported that the application of eugenol, carvacrol and thymol against pathogens caused disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents. The intracellular ATP of *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* was decreased due to the effect of cinnamaldehyde, while no apparent change was detected on their cell membrane (Oussalah et al. 2006). Santoro et al. (2007), revealed that scanning and transmission electron microscope demonstrate the ultrastructure changes in different compartments of the bacterial cell such as the plasma membrane, cytoplasm (swelling, shrivelling, vacuoles, leakage) and the nucleus. Also, scanning electron microscopy demonstrated structural alterations in the envelope of bacterial cells that treated by natural extract (Di Pasqua et al. 2007).

There is a lack of information about the mechanism of action of sumac, cardamom and pomegranate extracts. Therefore, the aim of this study is to evaluate the effect of these extracts against selected bacteria by measuring:

- The intra and extra cellular bacterial ATP
- The morphology of the cell membranes using electron microscopy
- The release of bacterial cell constituents such as DNA release.

4.2 Material and method

4.2.1 Sumac, pomegranate and cardamom extracts

Cardamom oleoresin was provided by Kalsec (Kalsec Mildenhall, UK), Sumac and pomegranate powder were obtained from Green Cuisine Food Products as details in Section 3.2.1, pomegranate and sumac extracts were prepared as detailed in Sections 3.2.1.1.1 and 3.2.1.2.1 respectively.

4.2.2 Bacterial culture

All five bacteria (*E. coli* K12, *Staph. aureus* ATCC 6821, *L. monocytogenes* 5105, *S. Typhimurium* DT104, and *B. cereus* NCIMB 11925) were obtained from the school of Biological sciences / University of Plymouth liquid nitrogen culture collection.

4.2.3 Preparation of normal saline

Normal saline was prepared as described in Section in 3.2.3 and used to dilute the bacterial culture to reach a concentration of 10^7 CFU/ml.

4.2.4 Preparation of brain heart infusion broth (BHI)

Brain heart infusion agar was prepared as in Section 3.2.6.

4.2.5 Scanning electron microscopy method (SEM)

The mechanisms of action of selected extracts were examined against selected bacterial cells, SEM studies were carried out as previously reported by Lv et al. (2011) and Turgis et al. (2009). the incubated overnight cultures (18 h) at 37°C for all tested bacteria except *B. cereus* at 30°C (approximately 10^7 CFU/ml) were treated with selected extract combinations at MIC and MTC values and a control (bacteria in PBS) which incubated at room temperature for 3 h (Turgis et al. 2009). After the incubation, cells were harvested by centrifugation for 10 min at 5000 g (Rotina 46 centrifuge, Hettich Zentrifuge, Germany) and washed twice

with 0.1 M phosphate buffer solution (PBS, pH 7.0), and then were resuspended in PBS containing 2.5% glutaraldehyde and kept for 24 h at 8°C to fix the cells. The bacterial cells were filtered through a cellulose filter 0.21mm (Watman-Etch Membrane Filtration Products UK), and then dehydrated in water–alcohol solutions at various concentrations (30, 50, 70, 80, 90 and 100%) for 10 min each. Finally, the samples were fixed on SEM support, and then sputter-coated with gold under vacuum, followed by microscopic examinations using a scanning electron microscope (JEOUL 5600LV, Tokyo, Japan)

4.2.6 Cell constituents release

The release of cell constituents into the supernatants was measured according to the method described by Turgis et al. (2009) and Rhayour et al. (2003). Cell from working culture (100 ml) of selected bacteria were centrifuged 3000g for 15 min (MSE-Europa 24B uk), collected and washed three times, then resuspended in PBS. One hundred millilitres of cell suspension were incubated for 1 h at 35 ±1°C at environmental incubator shaker (Thermo scientific-4334 USA) in the presence of three concentrations of extracts of sumac, cardamom and pomegranate (control (bacteria in (PBS), MIC, MTC). Then a 10 ml of incubated sample were filtered through 0.45 µm pore-size filter (Millex-Merek Millipore Ltd Germany). The concentration of the constituents released was estimated by UV absorbance measurements of each filtrate using a UV-spectrophotometer (Unicam UV4-100 UK) at 260 nm.

4.2.7 Intra- and extracellular ATP concentrations

The intra and extracellular ATP assay was performed by the method described by Turgis et al. (2009) adapted as follows. Each working cultures that contained 10^7 CFU/ml of each tested strain was centrifuged for 10 min at 1000 g (Rotina 46 centrifuge, Hettich Zentrifuge, Germany). The cell pellets were washed 3 times with PBS, and centrifuged under the same conditions. A cell suspension (10^7 CFU/ml) was prepared with 4 ml PBS, then 0.9 ml of the suspension was added into the Eppendorf tubes. Cell suspensions were treated with different concentrations of sumac, cardamom and pomegranate extracts (control (bacteria in PBS), MIC, MTC), and kept at room temperature (20-22 °C) for 30 min. Treated samples were centrifuged for 5 min at 2000 g (Rotina 46 centrifuge, Hettich Zentrifuge, Germany), and then placed on ice to protect the samples from ATP loss until testing. The ATP concentration was determined by using an ATP assay kit (Biothema BA, Handen, Sweden), ATP standard was used as a reference. The ATP concentration of each preparation was measured in the upper layer (supernatant) which represents the extracellular ATP, while the lower layer (cell pellet) was represented the intracellular concentration.

The ATP Biomass kit HS consisted of three components:

- a) ATP reagent (consisting of lyophilised luciferase/ luciferin)
- b) ATP standard- 10^{-7} mol/L ATP
- d) Extractant - for extraction of ATP

Twenty μ l of the extract and 160 μ l of ATP reagents were added to 20 μ l of each supernatant. The sample was measured for the first time by using a Pi-102 tube luminometer (Hygiena International Ltd., Watford, UK). A 10 μ l of 10^{-7}

mol/l ATP standard was added to the mixture and the sample measured for the second time.

Calculation of sample ATP content was according to the equation 4.1:

$$ATP\ sample = \frac{I\ sample\ (1^{st}\ reading)}{(I\ sample + standard\ (2^{nd}\ reading) - I\ sample\ (1^{st}\ reading))} \dots (4.1)$$

The concentration of intracellular ATP was determined by washing the cell pellets three times with PBS. The samples were centrifuged for 5 min at 1000g and the microorganisms were disrupted by adding 60 μ l of extractant, and kept at room temperature for 15 min and then centrifuged. After that, a 160 μ l of ATP reagent was added into a 20 μ l of the sample. The sample was measured by the luminometer, followed by adding a 10 μ l of the ATP standard and measured for the second time. The same previous steps and equation was followed to determine the intracellular ATP. The experiment was carried out in triplicate and the concentration of ATP was pmol/ml.

4.3 Results

4.3.1 Scanning electron microscope

The SEM microstructures of both untreated (control) and extracts treated bacterial cells are presented in Figures (4.3 to 4.8). The control cell groups had a typical structure of G^{+ve} and G^{-ve} , showing an irregular and striated wall for *E. coli*, *S. Typhimurium*, *B. cereus* and *L. Monocytogenes* Figures (4.1 to 4.6) and smooth wall for *Staph. aureus*. While, an alteration in the cell morphology had occurred when strains were treated with selected natural extracts. The electron micrographs of the treated cells with the natural extracts at MIC showed severe damage of bacterial cell wall, which may consequently cause disruption, lysis of the membrane integrity Figures (4.1 to 4.6). In addition, deformed shape was also observed in treated cells, accompanied by the lack of cell walls Figures (4.5 and 4.6). Further observation revealed presence of vacuoles in the treated cells, these presumably containing the extracts within them (Figure 4.6).

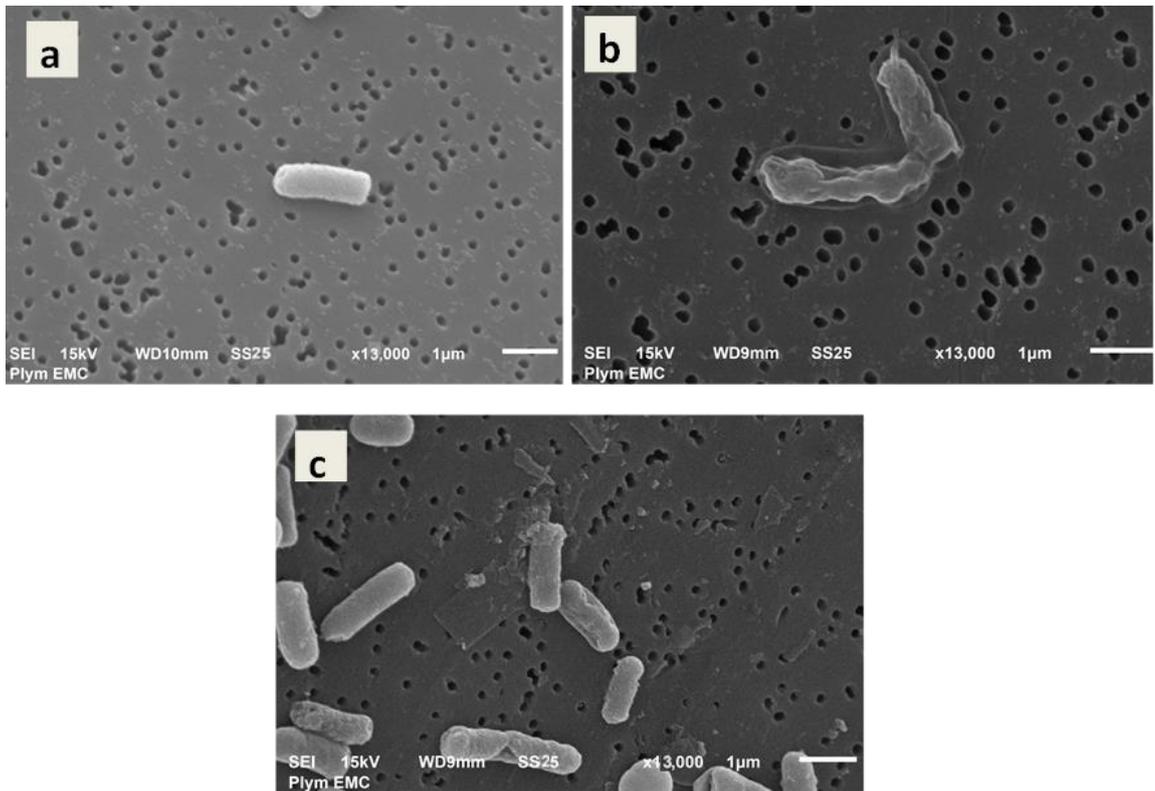


Figure 4. 1: Scanning electron microscope observation of *E. coli* treated with sumac extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

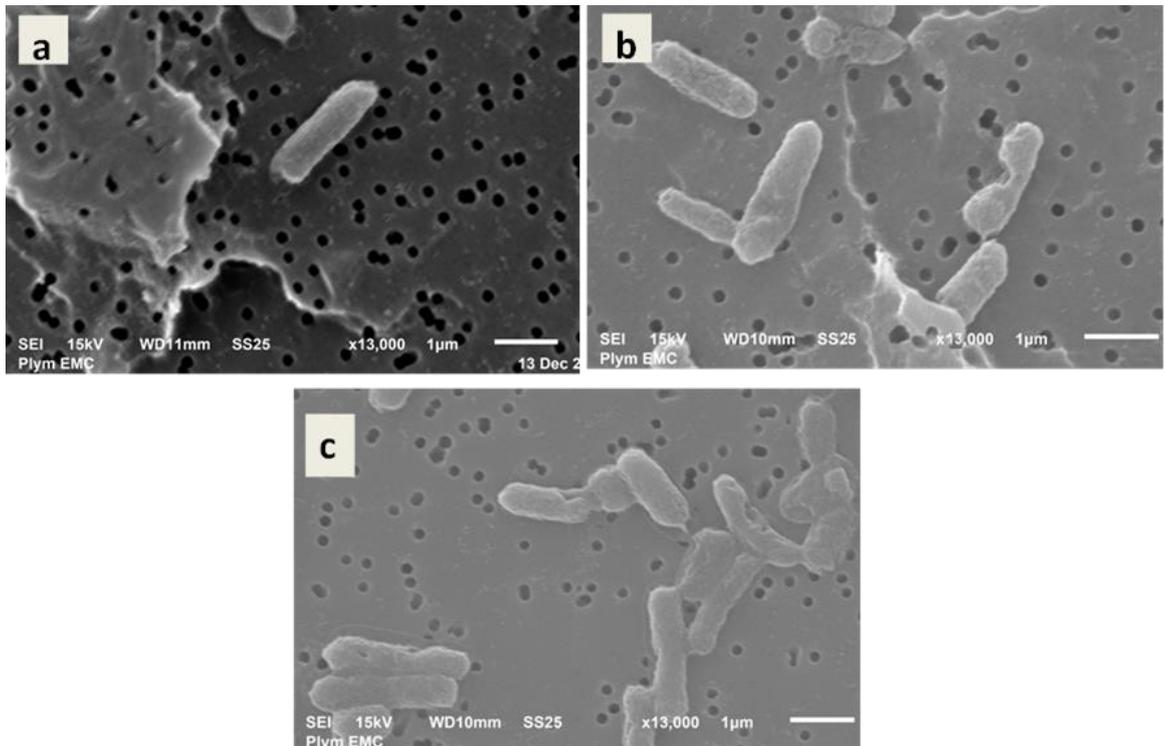


Figure 4. 2. Scanning electron microscope observation of *L. monocytogenes* treated with cardamom extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

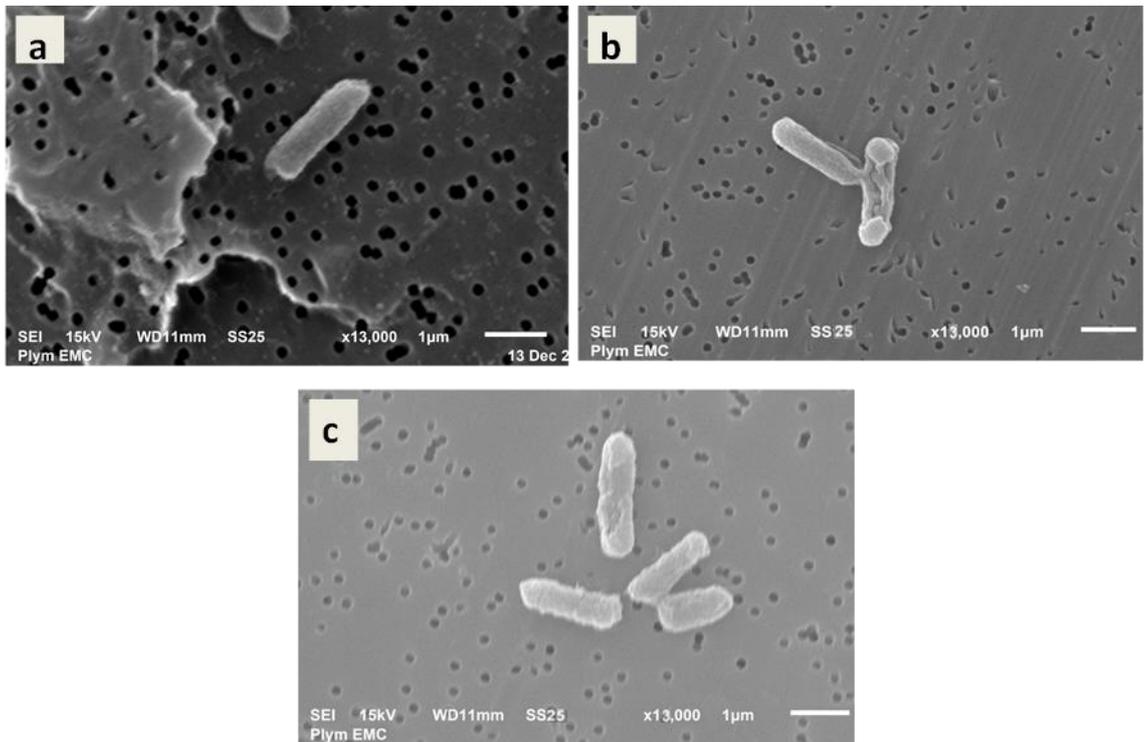


Figure 4. 3. Scanning electron microscope observation of *L. monocytogenes* treated with pomegranate extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

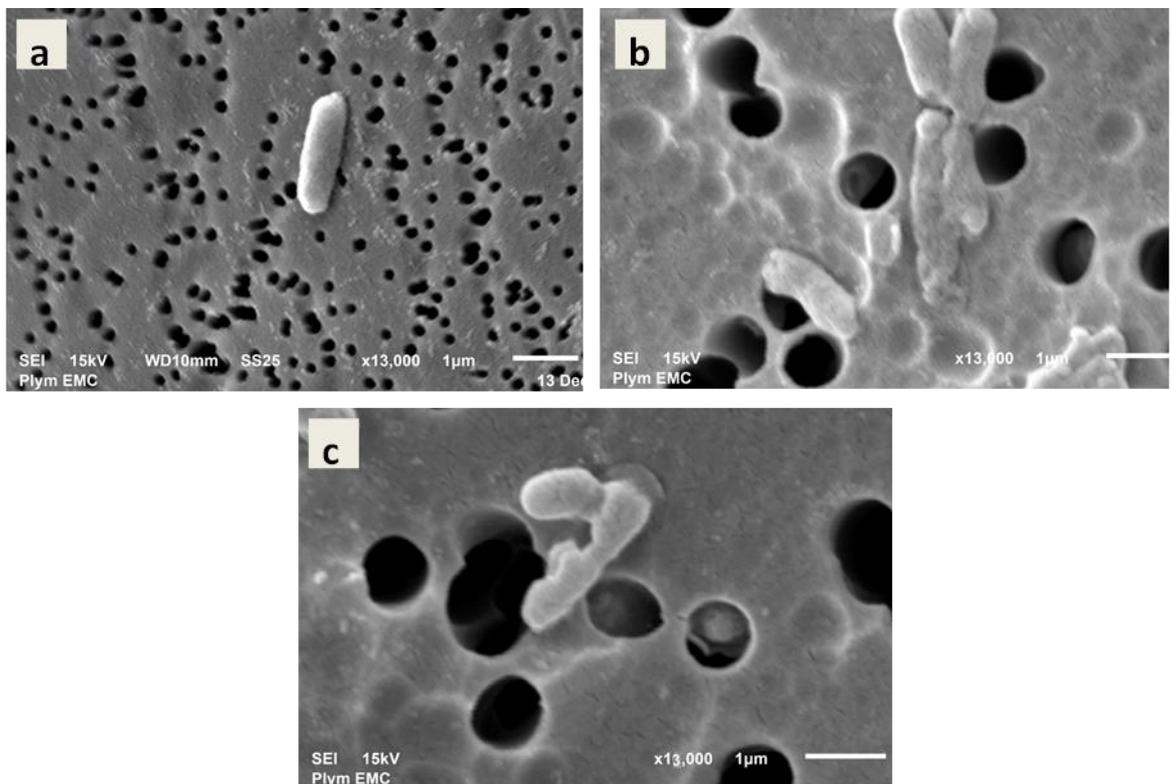


Figure 4. 4. Scanning electron microscope observation of *S. Typhimurium* treated with cardamom extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

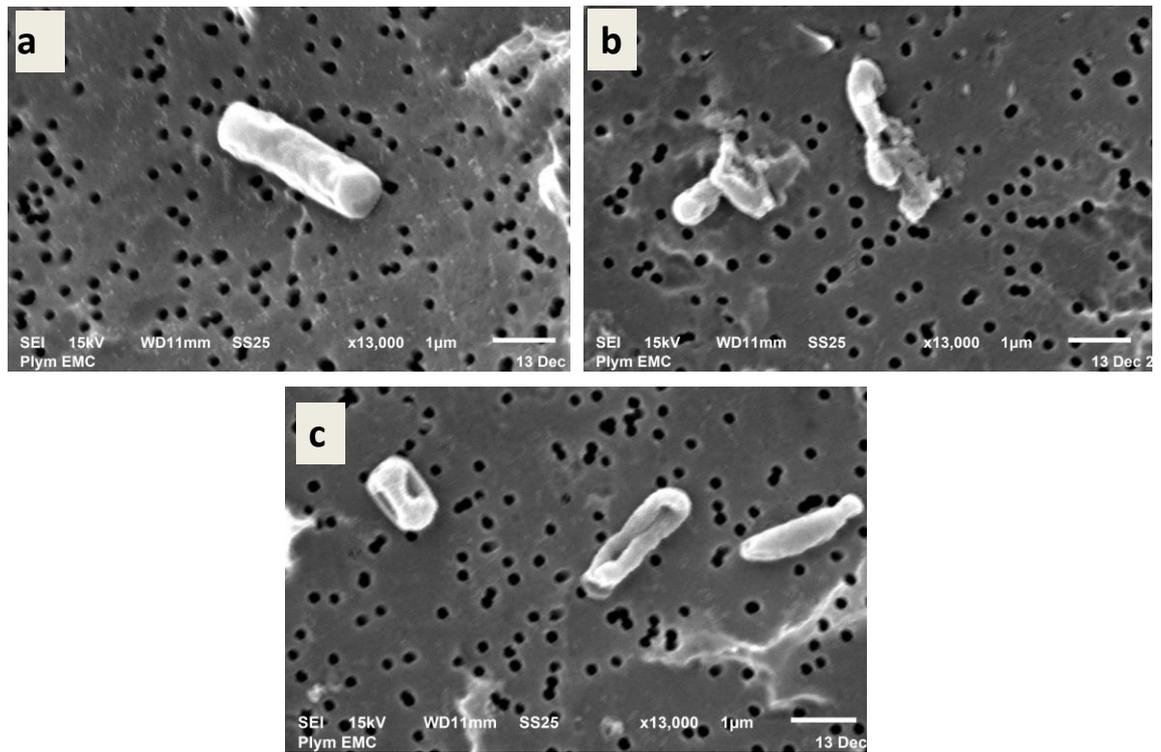


Figure 4. 5. Scanning electron microscope observation of *B. cereus* treated with cardamom extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

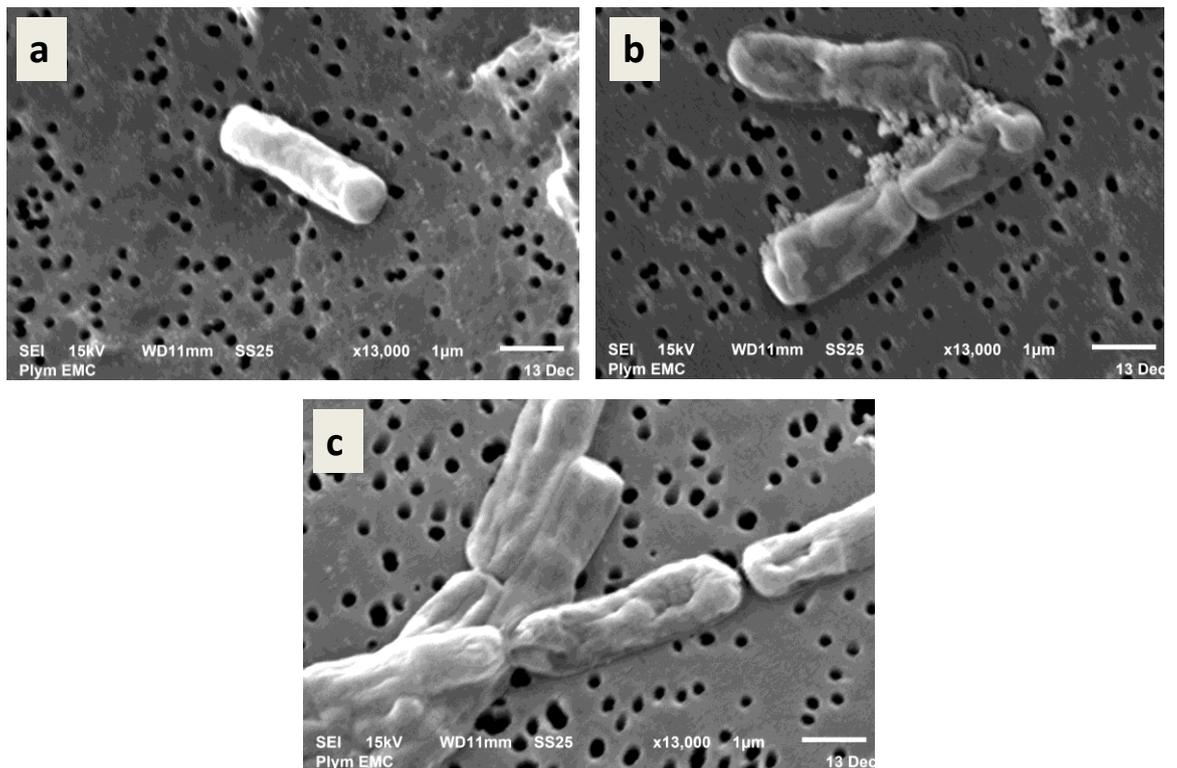


Figure 4. 6. Scanning electron microscope observation of *B. cereus* treated with pomegranate extract (MIC (b), MTC (c)) and untreated bacterial cell (a, control).

4.3.2 **Bacterial cell constituent release**

The cell constituents released of pathogenic bacteria, was determined by the measurement of the Absorbance at 260-nm of the suspension. Table 4.1 shows the result of cell constituents release when the target bacteria were treated with both minimum and maximum inhibitory concentrations (MIC, MTC) of plant extracts.

Generally, the result demonstrates that the extracts of sumac, cardamom and pomegranate had a significant effect ($P < 0.05$) on the cell integrity when compared to a control sample.

The cell constituents release of the tested bacteria increased progressively ($P < 0.05$) by the increase of the natural extract concentration, thus further increase of cell constituent release was observed when tested bacteria treated with MIC of sumac, cardamom and pomegranate extracts than MTC (Table 4.1).

According to the type extracts different efficacy of the bacterial cell constituents' release of tested pathogens was observed.

Table 4. 1: Effect of natural plants extract at MTC and MIC levels (mgml^{-1}) on DNA release.

Bacterial strains	Control	Plant extracts					
		Sumac		Cardamom		Pomegranate	
		MTC*	MIC**	MTC	MIC	MTC	MIC
<i>E. coli</i>	0.076 ^f	0.197 ^e	0.348 ^b	0.168 ^f	0.265 ^c	0.222 ^d	0.377 ^a
<i>S. Typhimurium</i>	0.066 ^d	0.180 ^c	0.337 ^a	0.185 ^c	0.310 ^a	0.227 ^b	0.332 ^a
<i>Staph. aureus</i>	0.033 ^f	0.239 ^c	0.361 ^a	0.215 ^d	0.289 ^b	0.174 ^e	0.218 ^d
<i>B. cereus</i>	0.082 ^f	0.209 ^d	0.376 ^a	0.273 ^c	0.389 ^a	0.177 ^e	0.347 ^b
<i>L. monocytogenes</i>	0.065 ^e	0.250 ^d	0.435 ^c	0.481 ^b	0.697 ^a	0.095 ^e	0.219 ^d

MTC* : Maximum tolerance concentration, MIC** : Minimum inhibitory concentration, For Sumac extracts MIC= 3.1mgml^{-1} for all tested bacteria except *E. coli* =4; MIC= 6.3mgml^{-1} for all tested bacteria except *E. coli*= 8mgml^{-1} and *L. monocytogenes*= 3.1mgml^{-1} ,

For Cardamom extracts MTC= 3.1mgml^{-1} for *E. coli* and *B. cereus* except *S. Typhimurium*= 12.5, *L. monocytogenes* and *Staph. aureus* = 1.5; MIC= 6.3mgml^{-1} for *E. coli* and *B. cereus* except *S. Typhimurium* = 25mgml^{-1} , *L. monocytogenes* and *Staph. aureus* = 3.1mgml^{-1}

For Pomegranate extracts MTC = 6.3mgml^{-1} for all tested bacteria except *Bacillus cereus*= 3.1mgml^{-1} ; MIC= 12.5mgml^{-1} for all tested bacteria except *Bacillus cereus*= 6.3mgml^{-1} .

a-f: Means (n=3) with the different letters in a row are statistically different (P<0.05).

4.3.3 The concentration of Intra and extracellular ATP

The effect of cardamom and Pomegranate (MIC, MTC) on the intra and extracellular ATP concentration of the bacterial cells was presented in Figures (4.7 and 4.8).

The intracellular ATP concentration of tested bacteria was significantly reduced ($P < 0.05$) when treated with cardamom at (6 mg ml^{-1}) MTC and (3 mg ml^{-1}) MIC (Figure. 4.7). Under the same condition the extracellular ATP concentration increased significantly ($P < 0.05$) compared to the control.

A significant difference ($P < 0.05$) in the intracellular ATP concentration was detected between the two different concentrations (MTC, MIC) of cardamom, when used against *E.coli*, *L. monocytogenes* and *Staph. aureus* (Figure 4.7). While, no significant ($P > 0.05$) difference in the intra ATP concentration between the MTC and MIC was observed for *B. cereus* and *S. Typhimurium*. The extracellular ATP concentration of all bacteria significantly ($P < 0.05$) increased by increasing the concentration of extract from MTC to MIC, except in *S. Typhimurium*.

Similar to the results of cardamom, the intra and extracellular ATP concentration was significantly ($P < 0.05$) affected by using of pomegranate against the same pathogens. However, no significant difference ($P > 0.05$) in the intracellular ATP concentration of *E. coli* was detected between the MTC and MIC (Figure 4.8). Moreover, the extracellular ATP concentration was reduced when pomegranate extract was used at high concentration against *L. monocytogenes*, *B. cereus* and *Staph. aureus*. Pomegranate at the MIC had the same effect when used against *S. Typhimurium* (Figure 4.8). The ATP result of sumac was excluded due to the interference with luminometer reading.

Cardamom and pomegranate extract demonstrated a great effect by increasing the extracellular ATP concentration and decrease the intracellular concentration. It's assumed that the envelope damage caused by the antibacterial agents increased the loss of the cell nucleic acid and the other contents (Table 4.1); and causes the release of ATP from the bacterial cells and induction of the intracellular ATP depletion figures (4.7 and 4.8).

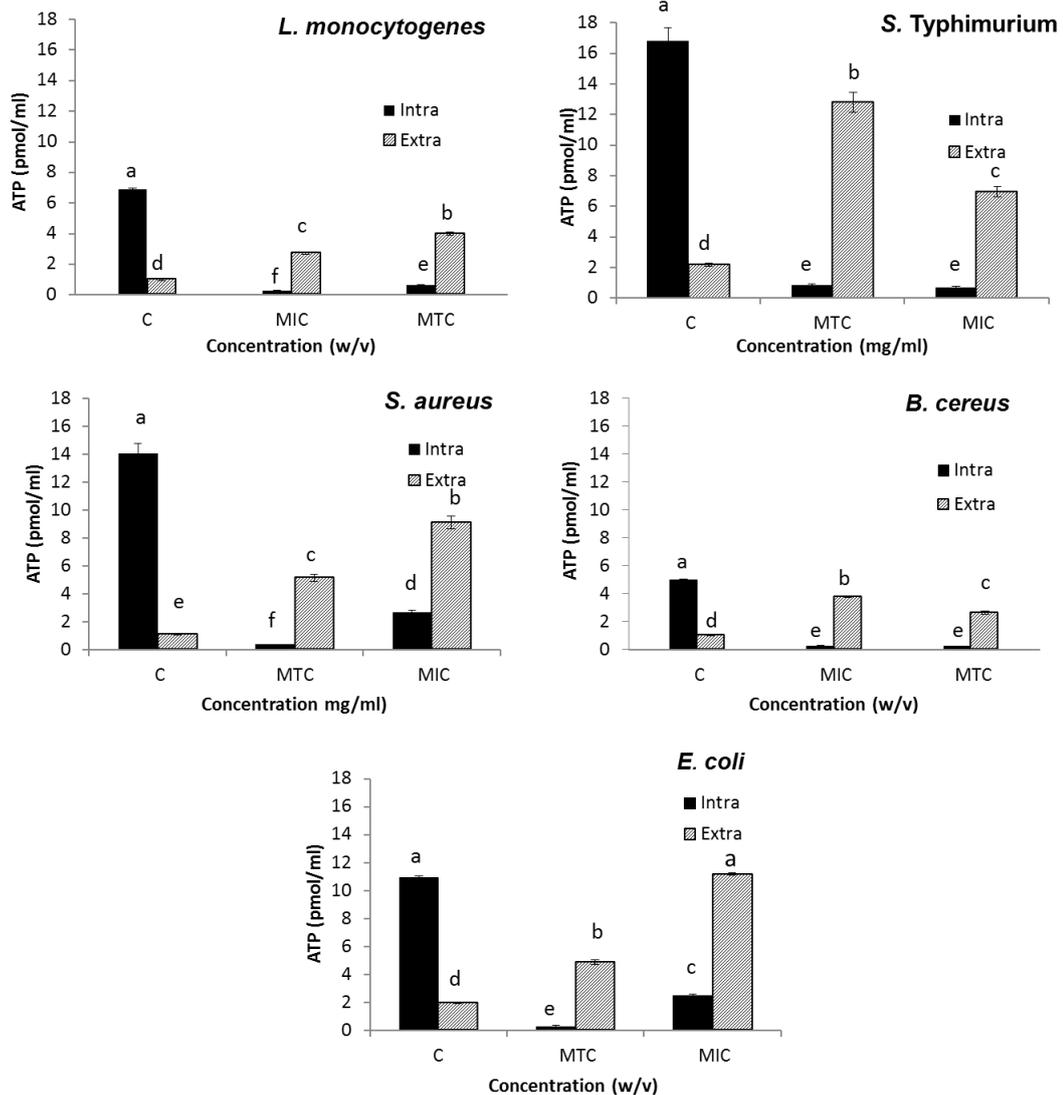


Figure 4. 7: Effect of cardamom extracts on intracellular and extracellular ATP concentrations of tested pathogens.

C: control, MTC*: Maximum tolerance concentration, MIC**: Minimum inhibitory concentration, For Sumac extracts MIC= 3.1mgml⁻¹ for all tested bacteria except *E. coli* =4; MIC= 6.3 mgml⁻¹ for all tested bacteria except *E. coli*= 8mgml⁻¹ and *L. monocytogenes*= 3.1mgml⁻¹,

For Cardamom extracts MTC= 3.1 mgml⁻¹ for *E. coli* and *B. cereus* except *S. Typhimurium*=12.5, *L. monocytogenes* and *Staph. aureus* = 1.5; MIC=6.3mgml⁻¹ for *E. coli* and *B. cereus* except *S. Typhimurium* = 25 mgml⁻¹, *L. monocytogenes* and *Staph. aureus* = 3.1mgml⁻¹

For Pomegranate extracts MTC = 6.3 mgml⁻¹ for all tested bacteria except *Bacillus cereus*= 3.1mgml⁻¹; MIC= 12.5mgml⁻¹ for all tested bacteria except *Bacillus cereus*= 6.3 mgml⁻¹.

a-f: Means (n=3) with the different letters in a column for each microorganism are statistically different (P<0.05).

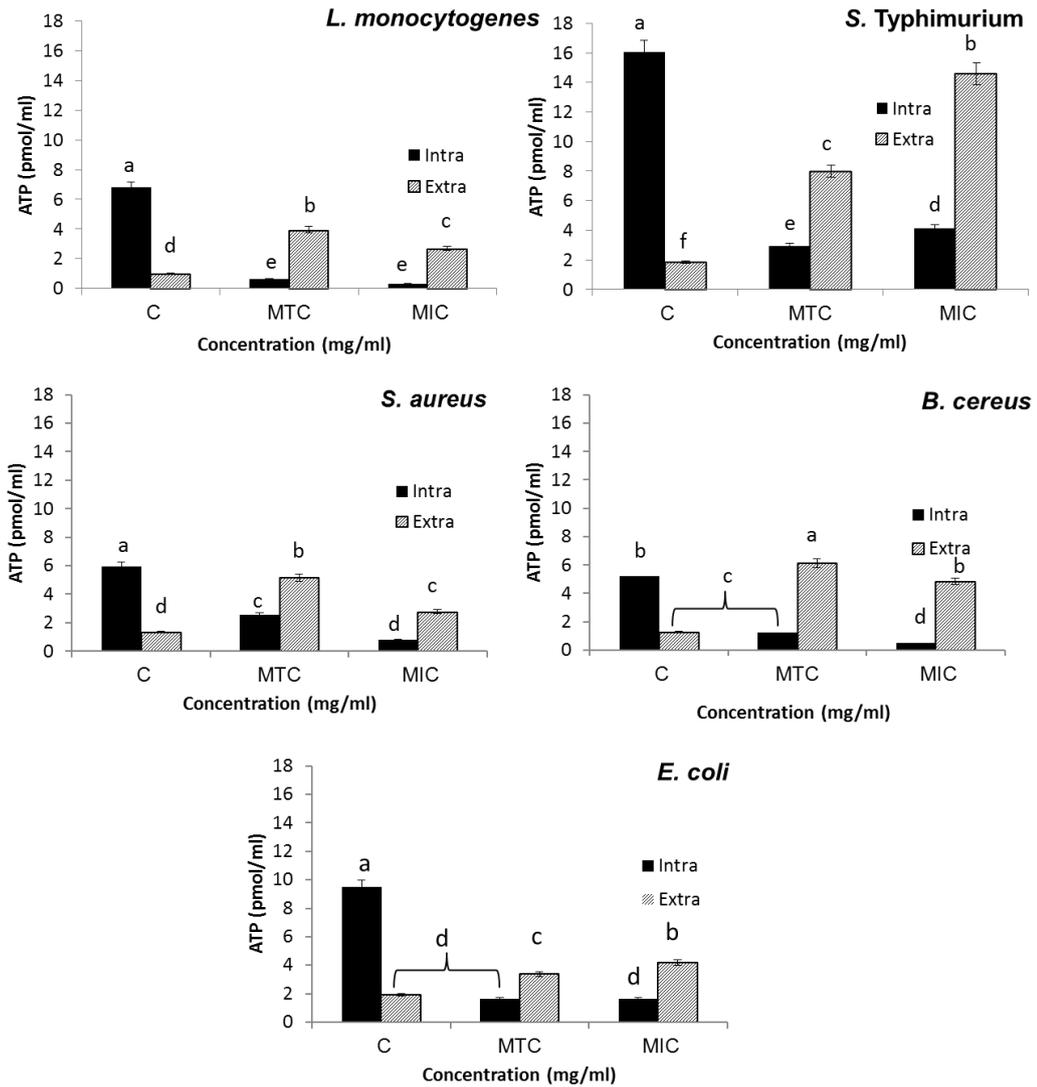


Figure 4. 8: Effect of pomegranate extracts on intracellular and extracellular ATP concentrations of tested pathogens.

C: control, MTC^{*}: Maximum tolerance concentration, MIC^{**}: Minimum inhibitory concentration, For Sumac extracts MIC= 3.1mgml⁻¹ for all tested bacteria except *E. coli* =4; MIC= 6.3 mgml⁻¹ for all tested bacteria except *E. coli*= 8mgml⁻¹ and *L. monocytogenes*= 3.1mgml⁻¹,

For Cardamom extracts MTC= 3.1 mgml⁻¹ for *E. coli* and *B. cereus* except *S. Typhimurium*=12.5, *L. monocytogenes* and *Staph. aureus* = 1.5; MIC=6.3mgml⁻¹ for *E. coli* and *B. cereus* except *S. Typhimurium*= 25 mgml⁻¹, *L. monocytogenes* and *Staph. aureus* = 3.1mgml⁻¹

For Pomegranate extracts MTC = 6.3 mgml⁻¹ for all tested bacteria except *Bacillus cereus*= 3.1mgml⁻¹; MIC= 12.5mgml⁻¹ for all tested bacteria except *Bacillus cereus*= 6.3 mgml⁻¹.

a-f: Means (n=3) with the different letters in a column for each microorganism are statistically different (P<0.05).

4.4 Discussion

4.4.1 Scanning electron microscope

The SEM observation of this study revealed that the wall structure of the bacterial cells was impaired by the addition of the natural extracts. Consistent with this result, the cell constituents have been leaked through the membrane and the ATP concentration was changed. Figures (4.1 to 4.6) showed the disruption of some treated cell wall, although an increase in their permeability. Lv et al. (2011) and Carson et al. (2002), reported that the hydrophobic characteristic of natural extracts enables them to accumulate in the cell wall, disturbing of membrane structures, increase of the permeability and then Leakage of intracellular constituents. The alteration of *Bacillus subtilis* morphology when treated with gallotannins was attributed to the inhibition of the enzymes involved in cell separation (Engels et al. 2011). The SEM observation exhibited severe damage of *staph. aureus* membrane after the treatment with terpinen (Carson et al. 2002).

On the other hand, Bajpai et al. (2009), presumed that natural extract phenolic compounds play an important role by passing through the cell wall, increase membrane permeability, release of intracellular constituents and interfere with membrane functions and then changing the morphology. Nevertheless, Fisher and Phillips (2009), suggested that partial damage to the cell wall may occur after being treated with the natural extract, which may then interact with cell membrane, increase permeability, causing an increase in the cell constituents release.

The SEM observation of this study also showed different type of damages and deformities in the shape of treated cells Figures (4.5 and 4.6). Which is similar

to the study that conducted by Oussalah et al. (2006), reported that morphological changes and deformed shape were observed, when *L. monocytogenes* and *E. coli* were treated with Spanish oregano extract. Moreover, the degenerative change in the cell wall of *L. monocytogenes* occurred after the treatment with Thyme extract (Rasooli et al. 2006). However, the type of damage could be varied according to the type of the pathogen (Rhayour et al. 2003). (Turgis et al. 2008), indicated that clove and oregano extract caused different type of damage to *E. coli* and *B. subtilis* cells such as presence of vacuoles in *E. coli* cell wall. Though, the deformity in the cell shape was detected in *B. subtilis*.

Vacuoles were also observed in the cell wall of treated cells (Figure 4.6). It has been suggested that the presence of vacuoles in the cell wall may be due to the coagulation of the membrane, and this can be detected when the extract is trapped within the cell (Fisher and Phillips 2008). The presence of vacuoles were observed in the cell wall of *E. coli* and *L. monocytogenes* after the treatment with cinnamon and savory (Oussalah et al. 2006). The same changes were detected when *E. coli* and *Staph. aureus* were treated with cinnamon and oregano extract (Becerril et al. 2007). This study confirmed that the physiological and morphological changes in the bacterial cells occurred due to the activity of the selected extracts.

4.4.2 **Bacterial cell constituent release**

Generally, sumac, pomegranate and cardamom extracts passed through the cell wall, disrupt the multilayer of the cell, which may then interact with the cell membrane and increased their permeability, and consequently caused a considerable loss of cytoplasmic constituents (Tables 4.1). Carson and Hammer (2011), indicated that the release contents of the bacterial cells is an indicative

of gross and irreversible damage to the cytoplasmic membrane; moreover, different type of natural extracts and their contents that attack the cell membrane such as α -pinene and tea tree oil induce the release of cell contents, consequently loss of nucleic acid through a damaged cell membrane.

Heinonen (2007), observed that tannin inhibited *E coli* and *Staph. aureus* growth by destabilisation of the cytoplasmic membrane, increase the permeability of the cell membrane, inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth such as iron and zinc, whose depletion can severely limit bacterial growth.

This study suggested that the hydrophobic properties and high phenolic contents of selected extracts as confirmed in chapter 3 are responsible for the disruption of bacterial wall structures which leads to increased membrane permeability due to an inability to separate the extracts from the bacterial cell membrane. Partial hydrophobic activity of the gallic acid would allow it to pass through the cell wall of *E coli* and *Staph. aureus*, act efficiently on bacterial membranes destabilizing them. It has been observed that the plasma membrane of *E. coli* and *L. monocytogenes* was disrupted when treated with ellagic acid, by localised hyper-acidification and disruption of electron transport (Puupponen-Pimiä et al. 2005). Terpenes showed strong activity to disrupt and penetrate the lipid structure of the *S aureus* cell wall, leading to denaturing of proteins and destruction of cell membranes, which leading to cytoplasmic leakage, cell lysis and eventually cell death (Oussalah et al. 2006). Gallotannins exhibited strong activity against *B. cereus*, *L. monocytogenes*, *Staph. aureus* and *E. coli*. Engels et al. (2011), indicated that the antibacterial activity of gallotannins are attributed to their strong affinity for iron and likely additionally relate to the inactivation of membrane-bound proteins. Furthermore,

Pomegranate extract inhibits *Staph. aureus* growth by interfering with bacterial protein production and secretion (Braga et al. 2005).

This study also demonstrated a positive correlation between the concentration of the natural extracts and the cell constituent release. Bacterial constituents release was increased when treated with MIC than the MTC. Similarly, Carson et al. (2006), reported that the release of potassium ions from *Staph. aureus* increased with increasing concentration of tea tree extract. It has also been observed that the leakage of bacterial nucleic acid significantly increased with the increase of *Peltophorum ferrugineum* extract concentration (Dandapat et al. 2012).

4.4.3 The concentration of Intra and extracellular ATP

Other studies have demonstrated that plant natural extracts act by inducing changes in the permeability of the bacterial cell membrane. The results have suggested that The Interaction between the selected extracts compound when passed through the cell wall and the cytoplasmic membrane (outer and inner), causes damaging of the cell membrane protein, depletion of the intracellular ATP pool via decreased ATP synthesis Figures (4.7 and 4.8) and augmented hydrolysis that is separate from the increased membrane permeability and reducing the membrane potential via increased membrane permeability (Table 4.1). Therefore, it is assumed that the rate of ATP synthesis was reduced or that the rate of ATP hydrolysis was increased.

There are no studies related to the activity of both cardamom and pomegranate on the ATP level of bacterial cells. However, It has been observed that thymol could take part in the up or down regulation of genes involved in outer membrane protein synthesis and inhibition of enzymes involved in the synthesis of ATP (Radulovic et al. 2013). According to Hyldgaard et al. (2012), the

inhibition of ATPase enzyme and perturbation of the cell membrane is the main antimicrobial action of cinnamaldehyde. Carson and Hammer (2011), reported that carvacrol has shown to cause collapse of the proton-motive force and depletion of the ATP pool in *L. monocytogenes*. On the other hand, Burt (2004), reported that the intracellular ATP concentration decreased while there was no proportional increase in the extracellular level when *B. cereus* treated with carvacrol.

However, this study showed that the bacterial cell membrane was the main target for cardamom and pomegranate extract Figures (4.7 and 4.8). The extracellular ATP level was drastically increased when the linoleic acid applied against *B. cereus* and *Staph. aureus* (Lee et al. 2002).

The extracellular ATP of treated *E. coli* with eugenol and carvacrol was significantly increased due to the disruption of the cell membrane (Gill and Holley 2006). A higher decrease in the intracellular ATP level was observed when mustard extract used against *S. typhi* and *E. coli O157:H7* (Turgis et al. 2009).

A significant difference ($P < 0.05$) between the intracellular and extracellular ATP concentration was detected, when both extracts were applied against the bacteria, This result concurred with the result of (Chen and Cooper 2002). The membrane structure of tested bacteria was impaired by the extracts; therefore, it is presumed that the intracellular ATP leaked through the imperfect membrane. On the other hand, the result showed a significant reduction ($P < 0.05$) in the extracellular ATP of *L. monocytogenes*, *B. cereus* and *Staph. aureus* at high concentration (MIC) of pomegranate in comparison to MTC. Which is similar to the result that was obtained by Turgis et al. (2009) this suggested a loss in the production level of ATP in *S. typhi* when treated with

mustard. The same result was observed when cardamom used against *S. Typhimurium*.

The molecular weight (507 g/mol) of ATP may play a role in determining its inability to pass through the damaged pore by extract treatment. Moreover, the internal ATP may be strongly reduced in the presence of a weak ATP efflux suggests that it is hydrolysed inside sensitive cells. Two mechanisms could explain the hydrolysis: a shift in the equilibrium of the ATP hydrolysis reaction as a consequence of inorganic phosphate loss through the membrane with impaired permeability (Abee, Klaenhammer, & Letellier, 1994) or an accelerated hydrolysis due to the attempt of cells to regenerate the electrochemical gradient by proton extrusion driven by the ATPase energy-consuming pump (Chen & Montville, 1995). According to Chen and Montville (1995), this second mechanism was concomitant with cell death in bacteria.

4.5 Conclusion

The mechanism of action of sumac, cardamom and pomegranate extracts was investigated against tested bacteria. This study verified that selected extracts act by causing structural and functional damage to the bacterial cell wall, and the type of damages was varied depending on the type of bacteria. In general, sumac, cardamom and pomegranate extracts significantly affected the cell constituent release compared to bacterial control. The cell constituent's release of selected bacteria increased progressively with the increase of the extract concentration. The bacterial cell wall was the main target of the selected extracts. Therefore, the result presumed that the release of ATP was due to the cell envelope damage, and interaction of extracts with cell membrane, consequently increase their permeability. Consistent with this result the SEM

observation showed that the bacterial cell wall was impaired by the extracts. However, more studies are needed to elucidate the particular mechanism of action of natural extract constituents on the metabolic pathway of pathogens.

5 Chapter5: Combined effect of pH, salt and temperature on the antibacterial activity of selected natural extracts

5.1 Introduction

Conventionally the microbial safety of food has been relied on microbial tests. However, these tests have been criticised as an expensive, labour demanding, time consuming and non-cumulative research tool. Therefore, several methods have been developed to determine the microbial growth in food. Predictive microbiology is an area of study in food microbiology that has gained significant scientific awareness as a tool for risk analysis and shelf life prediction. Predictive microbiology is based on mathematical models that are allowed to anticipate the behaviour of microorganisms (growth, survival and inactivation) under several physical and chemical conditions, such as temperature, pH and salt concentrations (McMeekin et al. 2008; Pin and Baranyi 2008; Leroi et al. 2012; Saucedo-Reyes et al. 2012).

As indicated by Huang (2013) and Isabelle and André (2006) , Predictive models were classified into primary and secondary models. Primary models such as Gompertz, Logistic and Baranyi model are commonly used for fitting microbial growth data, which describe the population growth curve over time, and generate information about the microorganism such as lag time duration and generation time. Whereas, the secondary models including Belehradek-type models, Response surface model and Arrhenius-type model are used to predict microbial growth under dynamic conditions. The influence of environmental conditions on the parameters of the primary model are described by the secondary model (Zhou et al. 2012),. Thus, the extent of microbial proliferation can be predicted by gathering a detailed knowledge of the growth rate response to the dominant environmental parameters.

Natural extracts are aromatic and volatile oily liquids obtained mainly from plant material. They exhibit strong activity against several types of microorganisms such as viruses, bacteria, parasite and fungus (Vukovic et al. 2007; Gutierrez et al. 2008). Sinigaglia et al. (2008) and Angienda et al. (2010), reported that the Antibacterial activities of natural extract and their components have been exploited in controlling food-borne pathogens and spoilage bacteria. However, a higher concentration of natural extracts is required to attain the same impact in food as in vitro (Burt 2004; Viuda - Martos et al. 2008). Therefore, the organoleptic effect should be considered when natural extract used as food preservative. Gutierrez et al. (2008) and Holley and Patel (2005), revealed that using of natural extract in combination with other preservative may help to reduce the concentrations, and control some bacteria that are known to show consistently high resistance to plant extracts.

Natural extracts have been used in combination with other preservative agents and with a variety of treatments toward several types of food-borne bacteria (Yoon et al. 2011). A number of potential synergistic factors such as, pH, low water activity and sodium chloride were recommended to be used with the natural extracts. Salt (NaCl) has been traditionally used as food preservative, while the evidence of its combination with natural extracts to inhibit the bacterial growth in food is still limited. However, Sodium chloride has been exhibited synergistic and an antagonist effects when applied with clove, oregano and Cinnamon extracts under different circumstance. clove was bactericidal against *Salmonella* Typhimurium, *Escherichia coli* and *Bacillus cereus* at 1.8% NaCl (Angienda et al. 2010). Doyle and Glass (2010), reported that the growth of *Salmonella* Typhimurium, *Escherichia coli* and *L. monocytogenes* was significantly inhibited, when treated with NaCl (2.5-5.5%), pH 6 in broth culture.

Taormina (2010), reported that the growth and toxin of *CL. botulinum* were inhibited in meat and cheese by using of sodium chloride; Although, The combination of sodium chloride and sodium acetate showed a strong activity against *L. monocytogenes* and lactic acid bacteria.

The aim of this study was to evaluate the combined effect of pH, temperature and sodium chloride on the antibacterial activity of natural extracts (cardamom and pomegranate) against selected pathogens.

5.2 Material and method

5.2.1 Natural extracts

Natural extracts were provided by Kalsec and Green Cuisine Food Products, UK as detailed Section 3.2.1, and pomegranate alcoholic extract were prepared as detailed in Section 3.2.1.1.1.

5.2.2 Bacterial strains

Culture collection strains of *Escherichia coli* K12, *Staphylococcus aureus*, *Listeria monocytogenes* 5105, *Salmonella* Typhimurium DT104, and *Bacillus cereus* were obtained from the stocks of the school of Biological Sciences at the University of Plymouth. These bacteria were selected according to their potential hazard in foods (Newell et al. 2010). Inocula were prepared as follows: each bacterial strain groups was maintained on brain heart infusion (BHI) agar (Oxoid Ltd., Basingtoke, Hampshire, UK) at $4\pm 1^{\circ}\text{C}$.

A loop-full of a fresh subculture was used for inoculation into fresh BHI broth and incubated aerobically over night at 37°C . McFarland standard opacity tubes (harrigan 1998) were used to adjust the level of microorganisms in the broth media to 10^7 - 10^8 CFU/ml see chapter 3 section 3.2.4.

5.2.3 Preparation and inoculation of culture media

For the growth and maintenance of different bacteria in this study, Brain heart infusion broth (Oxoid Ltd., Basingtoke, Hampshire, UK) was used. The basal media were prepared as detailed in Chapter 3, Section 3.2.5 and the pH was adjusted to five, six, or seven by using 1M NaOH or 1M HCl. To the basal media 0, 2.5 and 5 % NaCl was added to provide three concentrations of NaCl for each pH conditions. Afterwards, spice extracts were added for all pH conditions and all NaCl concentrations. This pH adjusted media was sterilized by autoclaving at 121°C for 15 minutes, then stored at 4±1°C (Park et al. 2007).

5.2.4 Experimental design

A factorial design was used to investigate the growth kinetics of selected bacterial groups for 24 hours under different combination conditions. Factors were two types of spice extracts (cardamom and pomegranate), three temperatures (25, 30 and 35°C), three NaCl concentrations (0, 2.5 and 5%) and three pH levels (5, 6 and 7).

5.2.5 Growth temperature and growth rate measurements

Samples for determination of growth rates of selected bacterial groups were incubated either at 25, 30 and 35°C. Absorbance measurements were obtained from a Microplate automated reader (TECAN, Infinite 200, Reading, UK) which was linked to a personal computer (Dell, Magellan Standard 6.5, Reading, UK). Flat bottom transparent 48 well plates with cover (Corning, Fisher Scientific, Loughborough, Leicestershire) were used. Microplate wells were topped up with 850 µl of each medium combination of NaCl, spices and pH, and then 50 µl of inoculum containing 10⁵ CFU/ml of the different culture were added. A kinetic cycle of 24h with a shaking duration 5 s performed before each hourly measurement were programmed for every growth experiment. The Absorbance

measurements were done in triplicate at a wavelength of 590 nm and bandwidth of 19 nm (Banja 2010; Al-Kutby 2012).

5.2.6 Primary model (Baranyi and Roberts model)

Growth curve against time obtained from Absorbance measurement for tested bacteria groups were fitted to Baranyi and Roberts model ((Baranyi and Roberts 1994a) using DMFit manual Version 2.0 (Institute of Food Research, Norwich, UK), conditions in which growth was not observed, were not included.

The model generates output parameters including lag time (lag), the maximum growth rate (Rate h^{-1}), initial bacterial density (y_0) and the maximum microbial load (y_{End}). In addition, this model calculated an estimate of the error associated with the parameter value R^2 (Adjusted R-square statistics of the fitting), which was considered as an indicator of the goodness of the fit and provided a more intuitive measure of how well the model fitted the data (Baranyi and Roberts 1994b).

5.2.7 Secondary model of the growth rate

The kinetic parameters obtained from the Baranyi and Roberts model for testing bacterial groups were used to develop the response surface analysis model (RSA) using Minitab (version 16). This model described the variation of the growth rate as a function of the growth conditions.

The dependent variable was the maximum growth rate because it is responds directly to the current growth environment (Rosso et al. 1995; Baranyi and Pin 2001; Muñoz-Cuevas et al. 2010), and the independent variables were temperature, pH, NaCl and spice extract.

The regression analysis of the response surface model was performed by the following second-degree polynomial equation:

5.3 Results

5.3.1 Primary model

The microbial growth curves for selected pathogens were created by fitting the Absorbance measurements to the Baranyi and Roberts model. The growth rates (Rat (h^{-1})) and lag time duration (t-lag) of the bacterial groups and their coefficients of determination (R^2) at different salt concentrations, pH levels and natural extracts types are shown in Tables 5.2 to 5.6.

In general, control samples had the high growth rate in comparison with other treatments (Table 5.1). The control samples (broth media with inoculum with no addition of salt and spice) showed highest Rat (h^{-1}) of *L. monocytogenes*, *E. coli*, *S. Typhimurium* and *Staph. aureus* at 35°C, whereas *B. cereus* showed highest Rate (h^{-1}) at 30°C.

The results of treated samples demonstrated that *B. cereus* was the most sensitive bacteria at different growth conditions used in comparison with other bacterial groups, where no growth was observed for most combinations of treatments especially when cardamom extracts were used (Table 5.3).

For each growth condition, the overall Rate (h^{-1}) of bacterial groups in BHI broth was slower at 5% NaCl concentrations at different temperatures and pH levels used for both cardamom and pomegranate extracts (Table 5.2- 5.6). The highest Rat (h^{-1}) for *L. monocytogenes* was detected when treated with cardamom at 35°C, 0 NaCl and pH 7 (Table 5.2). *B. cereus*, *E. coli* *S. Typhimurium* and *Staph. aureus* treated with pomegranate also showed the highest Rat (h^{-1}) at the same level of NaCl, pH and temperature (Table 5.3- 5.6). The R^2 values obtained from this model were high for all treated samples,

which ranged from (0.83-0.99) for cardamom extracts and (0.81-.099) for pomegranate extracts (Table 5.2- 5.6) indicating a good fit of the model.

Table 5. 1: Predictive growth parameters of untreated bacterial groups (control samples) were determined using Baranyi and Roberts model.

Bacterial groups	Growth conditions			Control sample		
	Temperature (C°)	NaCl (%)	pH	Rat (h ⁻¹)	t-lag	R ²
<i>L. monocytogenes</i>	25	0	7	0.035	0.73	0.98
	30	0	7	0.110	1.749	0.97
	35	0	7	0.144	0.51	0.90
<i>B. cereus</i>	25	0	7	0.065	3.417	0.90
	30	0	7	0.103	1.421	0.91
	35	0	7	0.067	–	0.94
<i>E. coli</i>	25	0	7	0.035	–	0.96
	30	0	7	0.050	–	0.99
	35	0	7	0.059	–	0.99
<i>S. Typhimurium</i>	25	0	7	0.037	–	0.95
	30	0	7	0.046	–	0.96
	35	0	7	0.064	–	0.94
<i>Staph. aureus</i>	25	0	7	0.097	2.957	0.99
	30	0	7	0.205	1.576	0.96
	35	0	7	0.247	2.295	0.97

Table 5. 2: Predictive growth parameters for *Listeria monocytogenes* under the different growth conditions were determined using Baranyi and Roberts model.

Growth conditions			Natural extracts					
Temperature (C°)	NaCl	pH	Cardamom extracts			Pomegranate extracts		
			Rat (h ⁻¹)	t-lag	R ²	Rat (h ⁻¹)	t-lag	R ²
25	0	7	0.019	4.90	0.92	0.21	2.54	0.88
25	2.5	7	0.011	12.07	0.95	0.008	5.82	0.91
25	5	7	0.003	–	0.96	0.002	12.30	0.90
25	0	6	0.020	5.93	0.95	0.009	4.64	0.97
25	2.5	6	0.014	6.23	0.99	0.007	6.04	0.91
25	5	6	0.003	10.60	0.90	0.002	12.27	0.89
25	0	5	0.022	–	0.99	0.008	4.62	0.92
25	2.5	5	0.004	8.16	0.99	–	–	–
25	5	5	0.000	–	0.99	–	–	–
30	0	7	0.026	4.40	0.93	0.023	–	0.92
30	2.5	7	0.011	11.7	0.95	0.010	3.34	0.97
30	5	7	0.004	–	0.96	0.003	15.90	0.94
30	0	6	0.014	6.23	0.99	0.011	12.21	0.93
30	2.5	6	0.009	19.1	0.99	0.008	6.82	0.91
30	5	6	–	–	–	–	–	–
30	0	5	0.023	–	0.96	0.003	17.81	0.99
30	2.5	5	0.004	8.16	0.99	–	–	–
30	5	5	–	–	–	–	–	–
35	0	7	0.033	5.88	0.99	0.028	–	0.93
35	2.5	7	0.006	7.79	0.92	0.014	9.79	0.95
35	5	7	–	–	–	0.004	4.01	0.96
35	0	6	0.023	18.07	0.96	0.013	11.11	0.91
35	2.5	6	0.009	19.4	0.97	0.011	–	0.85
35	5	6	–	–	–	0.008	–	0.91
35	0	5	0.002	–	–	0.009	4.64	0.97
35	2.5	5	–	–	–	0.002	11.87	0.98
35	5	5	–	–	–	–	–	–

Table 5. 3: Predictive growth parameters for *Bacillus cereus* under the different growth conditions were determined using Baranyi and Roberts model.

Growth conditions			Natural extracts					
Temperature (C°)	NaCl	pH	Cardamom extracts			Pomegranate extracts		
			Rat (h ⁻¹)	t-lag	R ²	Rat (h ⁻¹)	t-lag	R ²
25	0	7	–	–	–	0.046	2.65	0.99
25	2.5	7	–	–	–	–	–	–
25	5	7	–	–	–	–	–	–
25	0	6	–	–	–	0.02	10.08	0.89
25	2.5	6	–	–	–	–	–	–
25	5	6	–	–	–	–	–	–
25	0	5	–	–	–	–	–	–
25	2.5	5	–	–	–	–	–	–
25	5	5	–	–	–	–	–	–
30	0	7	–	–	–	0.083	–	–
30	2.5	7	–	–	–	–	–	–
30	5	7	–	–	–	–	–	–
30	0	6	–	–	–	0.002	2.90	0.95
30	2.5	6	–	–	–	–	–	–
30	5	6	–	–	–	–	–	–
30	0	5	–	–	–	–	–	–
30	2.5	5	–	–	–	–	–	–
30	5	5	–	–	–	–	–	–
35	0	7	–	–	–	0.105	1.11	0.98
35	2.5	7	–	–	–	0.103	3.41	0.97
35	5	7	–	–	–	0.022	5.25	0.81
35	0	6	0.026	10.32	0.83	0.047	–	0.98
35	2.5	6	0.016	11.89	0.98	0.030	3.22	0.91
35	5	6	–	–	–	0.005	7.91	0.94
35	0	5	–	–	–	0.030	3.65	0.96
35	2.5	5	–	–	–	0.031	5.18	0.83
35	5	5	–	–	–	0.003	7.11	0.96

Table 5. 4: Predictive growth parameters for *E.coli* under the different growth conditions were determined using Baranyi and Roberts model.

Growth conditions			Natural extracts					
Temperature (C°)	NaCl	pH	Cardamom extracts			Pomegranate extracts		
			Rat (h ⁻¹)	t-lag	R ²	Rat (h ⁻¹)	t-lag	R ²
25	0	7	0.013	–	0.95	0.014	–	0.96
25	2.5	7	0.011	10.06	0.96	0.010	10.20	0.94
25	5	7	0.010	10.07	0.99	0.008	15.48	0.92
25	0	6	0.015	6.05	0.99	0.008	6.38	0.99
25	2.5	6	0.007	6.70	0.98	0.007	10.37	0.89
25	5	6	–	–	–	–	–	–
25	0	5	0.009	13.46	0.89	0.004	–	0.92
25	2.5	5	–	–	–	0.002	–	–
25	5	5	–	–	–	–	–	–
30	0	7	0.011	5.72	0.95	0.035	1.12	0.99
30	2.5	7	0.004	7.40	0.92	0.009	2.72	0.95
30	5	7	0.003	7.22	0.96	–	–	–
30	0	6	0.018	8.40	0.98	0.022	1.91	0.98
30	2.5	6	0.008	9.74	0.97	–	–	–
30	5	6	–	–	–	–	–	–
30	0	5	0.023	–	0.98	0.009	5.80	0.99
30	2.5	5	–	–	–	0.006	7.16	0.92
30	5	5	–	–	–	0.004	11.14	0.98
35	0	7	0.02	7.38	0.98	0.041	1.30	0.97
35	2.5	7	0.010	14.41	0.95	0.022	–	0.99
35	5	7	–	–	–	0.014	5.90	0.97
35	0	6	0.037	10.61	0.90	0.024	2.46	0.97
35	2.5	6	0.008	15.86	0.90	0.010	4.25	0.98
35	5	6	–	–	–	–	–	–
35	0	5	0.021	–	0.98	0.019	4.73	0.91
35	2.5	5	–	–	–	0.009	5.24	0.94
35	5	5	–	–	–	0.004	7.54	0.81

Table 5. 5: Predictive growth parameters for *Salmonella* Typhimurium under the different growth conditions were determined using Baranyi and Roberts model.

Growth conditions			Natural extracts					
			Cardamom extracts			Pomegranate extracts		
Temperature (C°)	NaCl	pH	Rat (h ⁻¹)	t-lag	R ²	Rat (h ⁻¹)	t-lag	R ²
25	0	7	0.024	1.62	0.96	0.034	1.50	0.99
25	2.5	7	0.016	3.83	0.97	–	–	–
25	5	7	–	–	0.99	–	–	–
25	0	6	0.019	1.61	0.90	0.028	2.5	0.82
25	2.5	6	–	–	0.93	0.007	3.54	0.96
25	5	6	–	–	–	–	–	–
25	0	5	0.003	–	0.90	0.013	–	0.98
25	2.5	5	–	–	–	–	–	–
25	5	5	–	–	–	–	–	–
30	0	7	0.032	1.68	0.98	0.039	0.26	0.95
30	2.5	7	0.013	3.65	0.90	0.031	8.34	0.89
30	5	7	0.009	4.10	0.98	0.016	–	0.84
30	0	6	0.025	5.89	0.99	0.024	–	0.94
30	2.5	6	0.009	7.88	0.99	0.012	1.69	0.92
30	5	6	–	–	–	0.007	3.54	0.96
30	0	5	0.024	–	0.94	0.020	5.80	0.91
30	2.5	5	0.010	10.61	0.94	0.009	7.81	0.91
30	5	5	–	–	–	0.001	11.2	0.92
35	0	7	0.038	1.13	0.99	0.057	–	0.94
35	2.5	7	0.019	–	–	0.054	3.67	0.94
35	5	7	0.009	7.88	0.99	0.022	5.97	0.99
35	0	6	0.035	1.57	0.98	0.049	–	0.87
35	2.5	6	0.013	3.89	0.95	0.045	–	0.99
35	5	6	0.006	14.78	0.96	0.011	–	0.93
35	0	5	0.019	–	0.91	0.040	4.07	0.92
35	2.5	5	0.012	8.17	0.97	0.015	–	0.98
35	5	5	0.005	17.79	0.97	0.004	–	0.90

Table 5. 6: Predictive growth parameters for *Staph. aureus* under the different growth conditions were determined using Baranyi and Roberts model.

Growth conditions			Natural extracts					
Temperature (C°)	NaCl	pH	Cardamom extracts			Pomegranate extracts		
			Rat (h ⁻¹)	t-lag	R ²	Rat (h ⁻¹)	t-lag	R ²
25	0	7	0.012	–	0.91	0.045	2.15	0.99
25	2.5	7	0.003	4.05	0.98	0.033	7.60	0.92
25	5	7	–	–	–	0.003	7.90	0.99
25	0	6	0.043	1.96	0.99	0.024	8.66	0.99
25	2.5	6	0.037	1.71	0.99	0.021	12.02	0.99
25	5	6	–	–	–	0.011	18.03	0.99
25	0	5	0.054	–	0.99	0.002	10.23	0.98
25	2.5	5	0.008	–	0.99	–	–	–
25	5	5	0.001	–	0.99	–	–	–
30	0	7	0.019	5.84	0.88	0.079	2.52	0.99
30	2.5	7	0.001	17.50	0.97	0.054	6.70	0.92
30	5	7	–	–	–	0.005	11.37	0.99
30	0	6	0.055	2.35	0.99	0.038	4.55	0.98
30	2.5	6	0.048	2.54	0.99	0.025	4.62	0.99
30	5	6	0.002	8.74	0.97	0.019	6.57	0.99
30	0	5	0.058	1.52	0.99	0.004	7.84	0.89
30	2.5	5	0.022	3.64	0.97	–	–	–
30	5	5	–	–	–	–	–	–
35	0	7	0.042	11.17	0.99	0.101	2.33	0.99
35	2.5	7	0.016	14.94	0.97	0.037	–	0.82
35	5	7	–	–	–	0.030	–	0.86
35	0	6	0.070	–	0.96	0.016	–	–
35	2.5	6	0.042	–	0.98	–	–	–
35	5	6	0.007	–	0.99	–	–	0.95
35	0	5	0.035	1.51	0.99	0.009	1.68	0.98
35	2.5	5	0.024	–	0.98	–	–	–
35	5	5	0.002	12.24	0.90	–	–	–

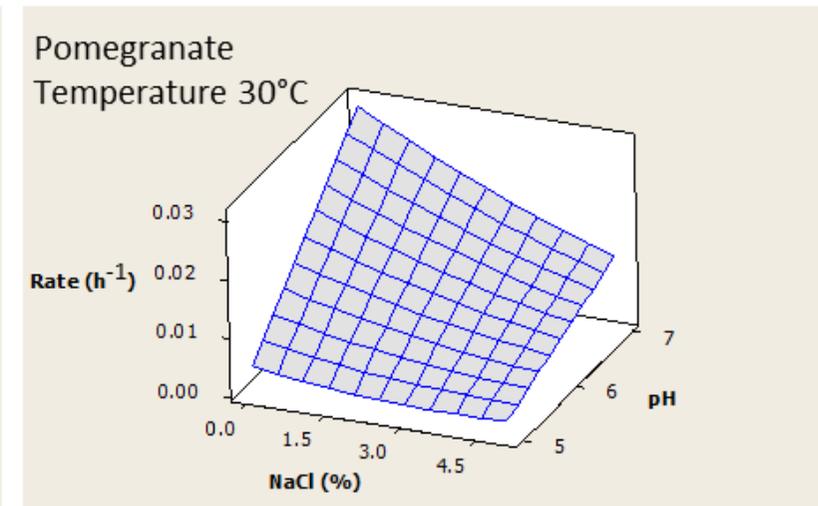
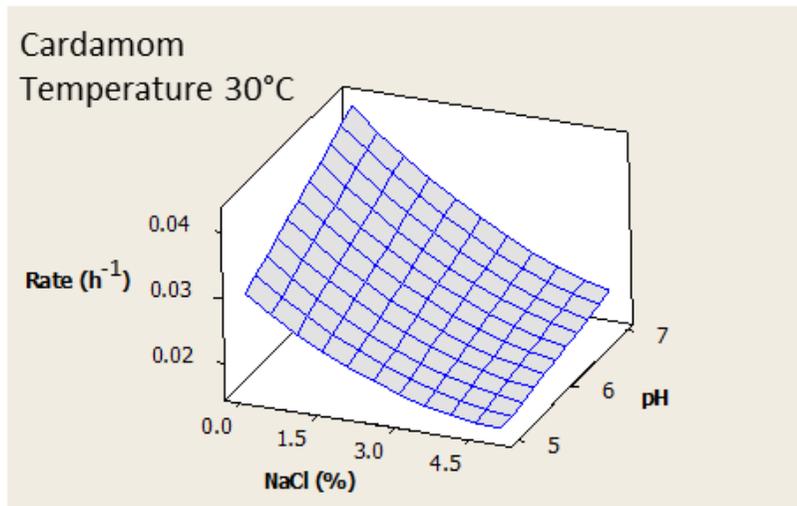
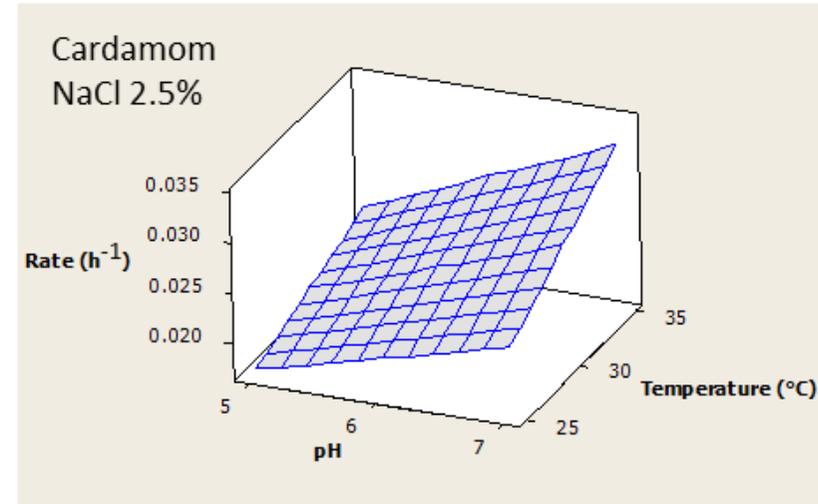
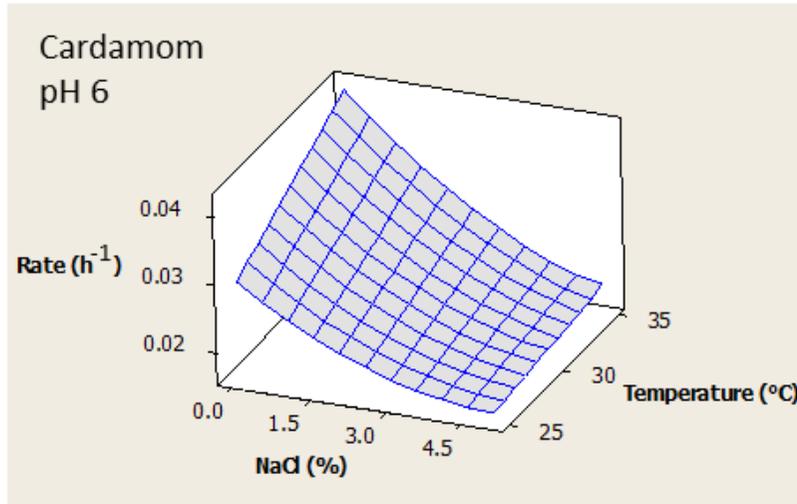


Figure 5. 1: Predicted growth rate of *L. monocytogenes* as a function of combinations of NaCl, pH, temperature and natural plant extracts using response surface analysis model.

Equation 5.4 was obtained, consider only the most significant terms ($p < 0.05$) for *B. cereus* treated with pomegranate extract. *B. cereus* treated with cardamom extracts were excluded from the secondary model, because most of the growth rate results obtained from the primary model showed no growth.

$$\begin{aligned} \text{Rate } h^{-1} = & 1.005 - 0.044 * \text{Temperature} + 0.045 * \text{NaCl} - 0.164 * \text{pH} + 0.0006 \\ & * \text{Temperature}^2 + 0.0113 * \text{pH}^2 - 0.00056 * \text{Temperature} * \text{NaCl} \\ & + 0.002 * \text{Temperature} * \text{pH} - 0.006 * \text{NaCl} * \text{pH} \dots \dots \dots (\text{eq. 5.4}) \end{aligned}$$

The effect of pH on the growth rate of *B. cereus* was very clear, the regression analysis of the RSA model for the maximum growth rate of *B. cereus* against temperature, NaCl concentrations and pH levels has shown that pH had a highly significant effect ($p < 0.001$) on the growth rate of this organism with temperature and NaCl coming second in effect (Figure 5.2) The estimate coefficient for pH were high comparing with temperature, NaCl and their interaction.

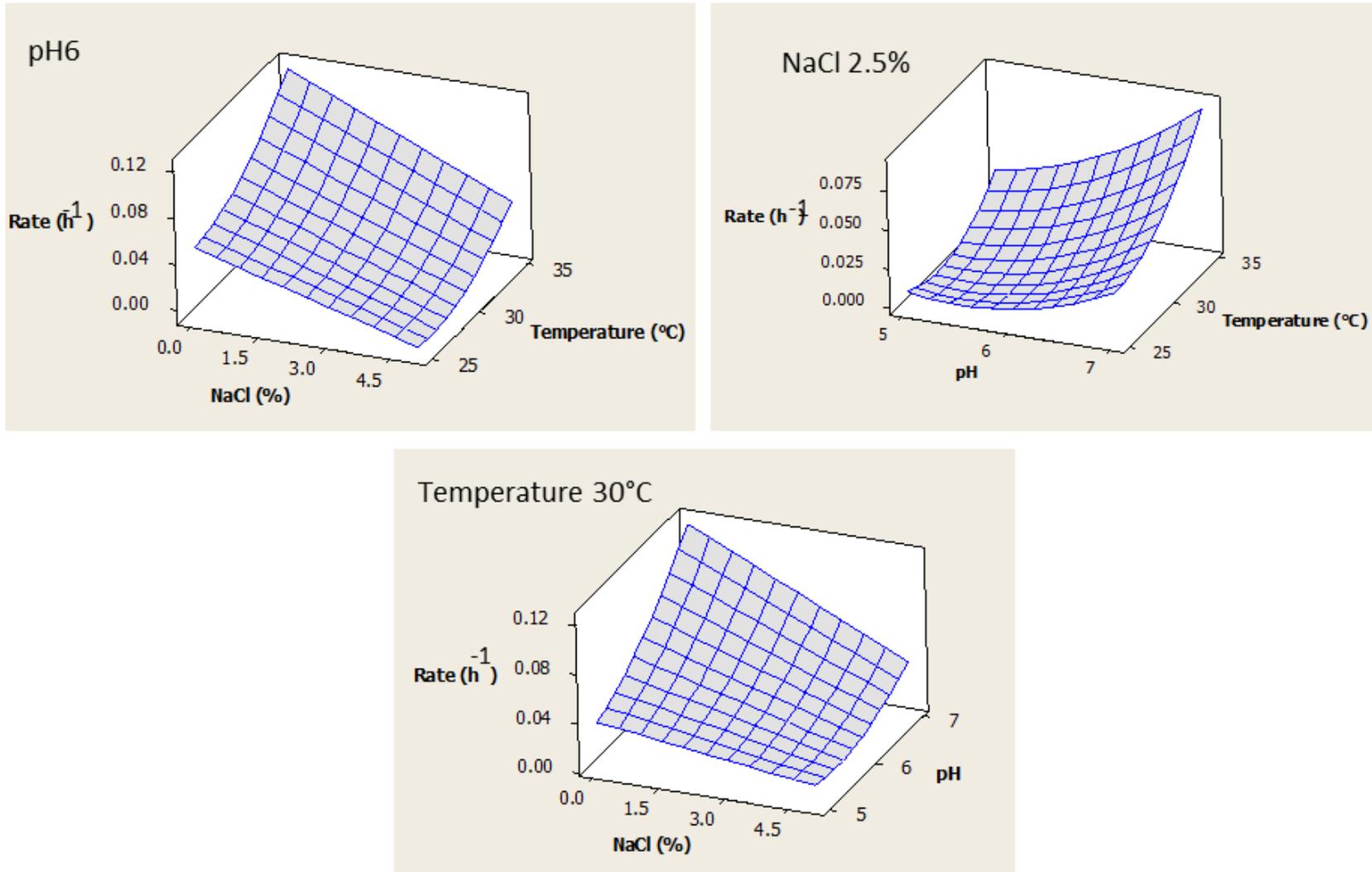


Figure 5. 2: Predicted growth rate of *B. cereus* as a function of combinations of NaCl, pH, temperature and pomegranate extracts using response surface model.

Equations 5.5 and 5.6 estimated the predicted Rate (h^{-1}) of *E.coli* in combinations of temperature, NaCl, pH, cardamom and pomegranate extracts in BHI broth respectively

$$\begin{aligned} \text{Rate} (h^{-1}) = & -0.136 - 0.0066 * NaCl + 0.046 * pH + 0.001 * NaCl^2 \\ & - 0.004 * pH^2 - 0.0003 * Temperature * NaCl + 0.0015 * NaCl \\ & * pH \dots \dots \dots (eq. 5.5). \end{aligned}$$

$$\begin{aligned} \text{Rate}(h^{-1}) = & 0.09011 - 0.0075 * Temperature + 0.0112 * NaCl \\ & + 0.00011 * Temp^2 + 0.00044 * NaCl^2 - 0.00025 * Temperature \\ & * NaCl + 0.00031 * temperature * pH - 0.0015 * NaCl \\ & * pH \dots \dots \dots (eq. 5.6) \end{aligned}$$

The regression analysis of the RSA model for Rate (h^{-1}) of *E.coli* against temperature, NaCl concentration, pH and cardamom extract has shown that the pH had a highly significant effect ($p < 0.001$) on this organism with NaCl coming second in effect (Figures 5.3).

For pomegranate extracts the regression analysis of the RSA model for the Rate (h^{-1}) of *E.coli* has shown that both temperature and NaCl had a significant effect ($p < 0.001$) on this bacteria in BHI broth (Figure 5.3).

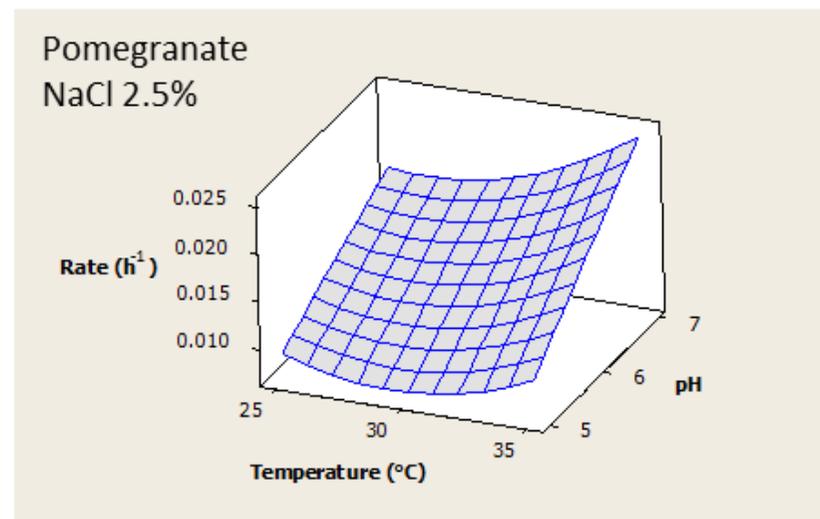
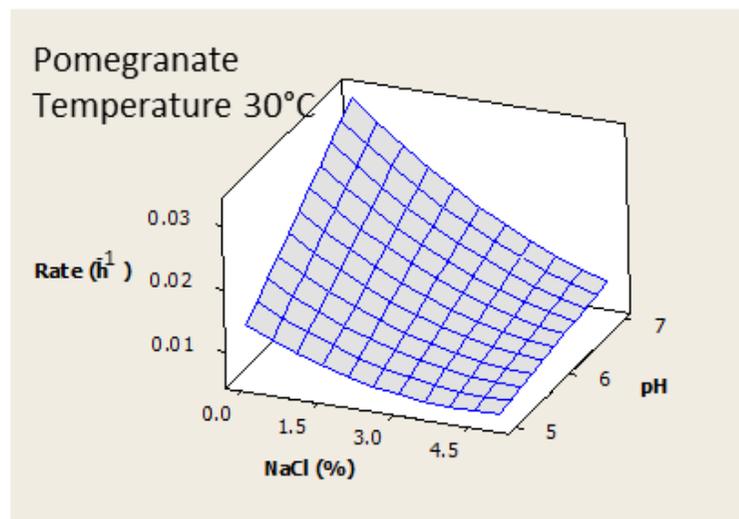
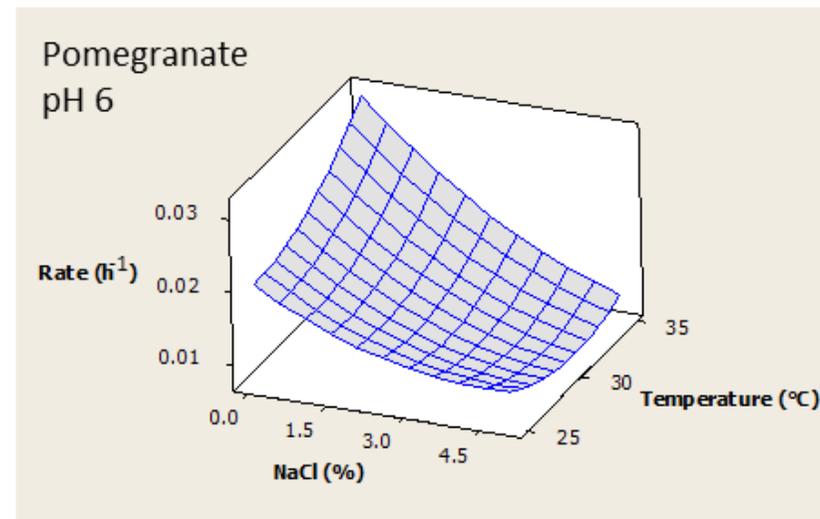
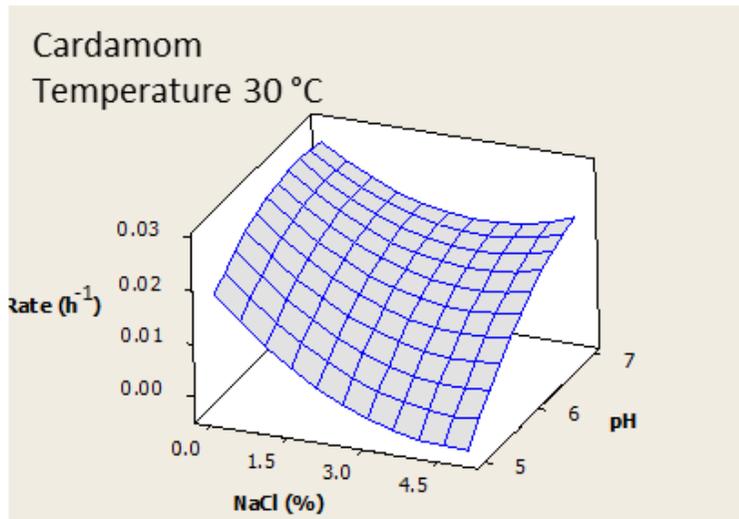


Figure 5. 3: Predicted growth rate of *E.coli* as a function of combinations of NaCl, pH, temperature and natural plant extracts using response surface analysis model.

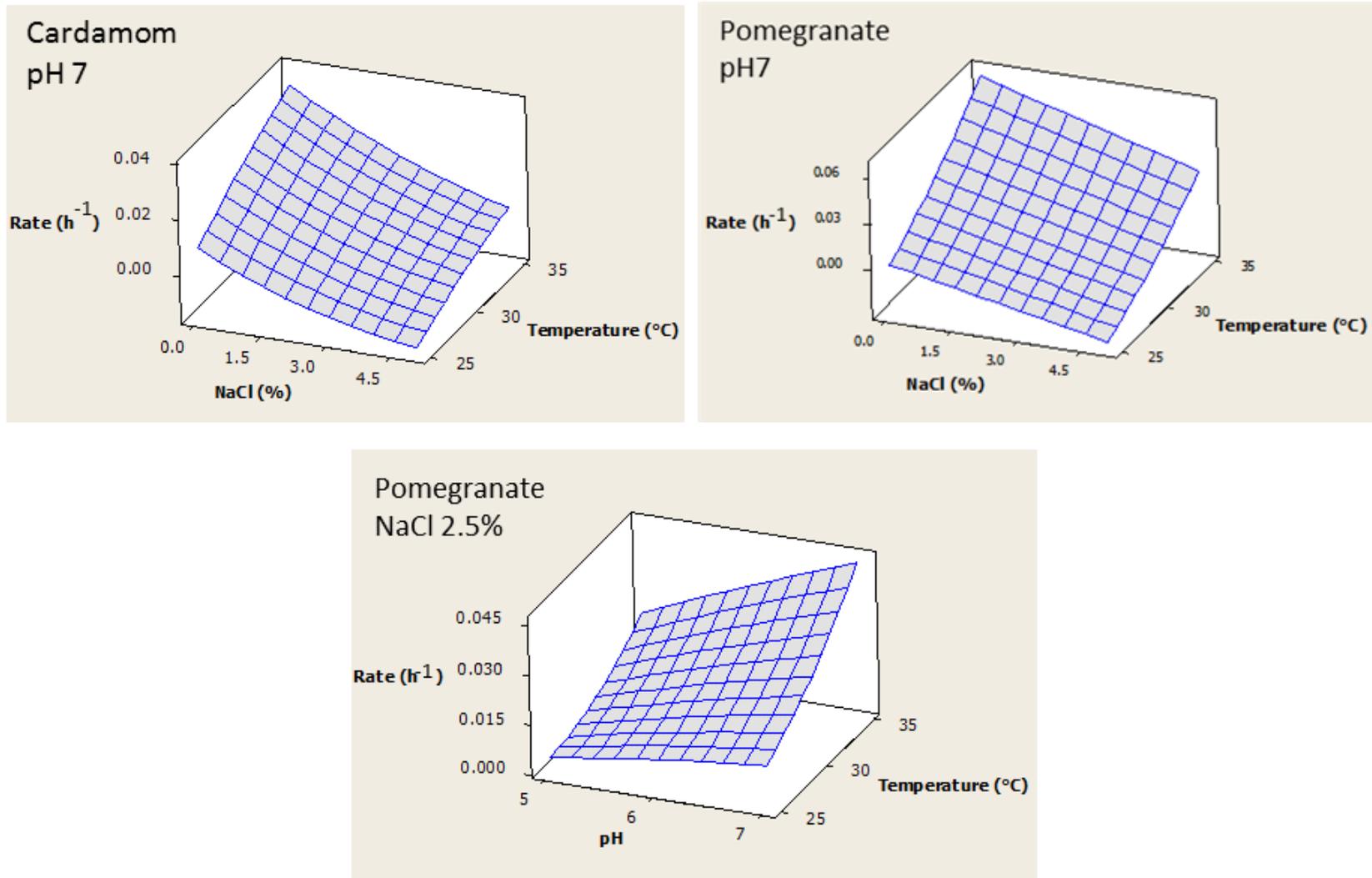


Figure 5. 4: Predicted growth rate of *S. Typhimurium* as a function of combinations of NaCl, pH, temperature and natural plant extracts using response surface analysis model.

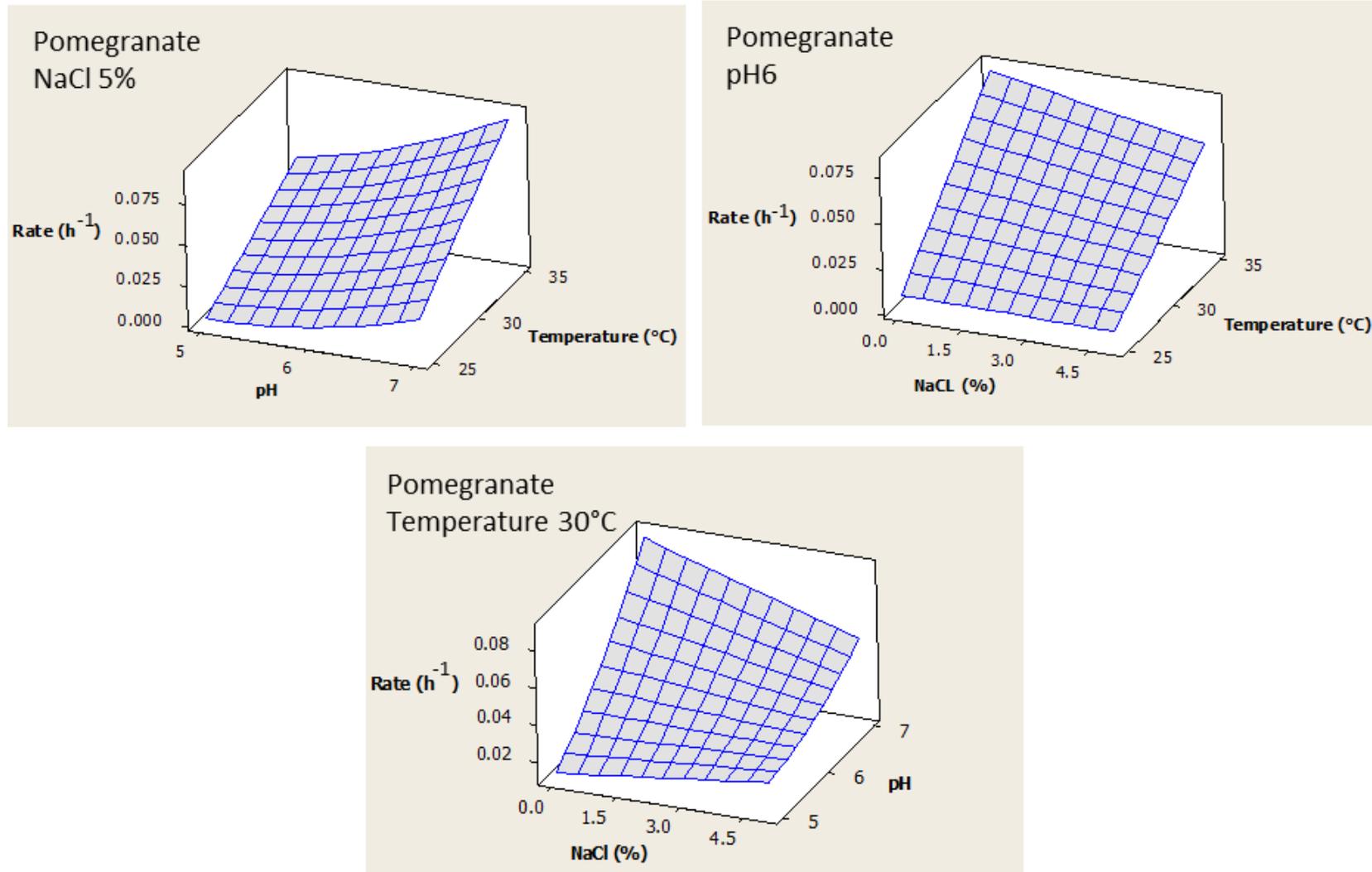


Figure 5. 5: Predicted growth rate of *Staph. aureus* as a function of combinations of NaCl, pH, temperature and pomegranate extracts using response surface analysis model.

5.3.3 Evaluation of model performance

The statistical index of R^2 and the root mean square errors RMSE were used in the evaluation of the model performance (Table 5.7) (Al-Kutby 2012; Tiwari et al. 2014). The R^2 values of the model was ranged from 0.96 to 0.79 for prediction the growth rate of all selected bacterial groups under the combined effects of temperature, pH, NaCl and selected extracts treatments in BHI medium. The root mean square errors (RMSE) of the models were very small for all bacterial groups (Table 5.7).

Furthermore, a graphical comparison was performed to determine the data group trends and to illustrate the goodness of the proposed RSA model by plotting observed values against predictive values (Figure 5.6).

Table 5. 7: Evaluation of response surface model performance of the combined effect of temperature, pH, NaCl and different extracts in the prediction of selected pathogens

Bacterial groups	Cardamom		Pomegranate	
	R^2	RMSE	R^2	RMSE
<i>L. monocytogenes</i>	0.96	0.00004	0.94	0.00002
<i>B. cereus</i>	-	-	0.84	0.00017
<i>E. coli</i>	0.79	0.00002	0.87	0.00002
<i>S. Typhimurium</i>	0.92	0.00001	0.89	0.00004
<i>S.aureus</i>	-	-	0.87	0.00009

-: Data excluded

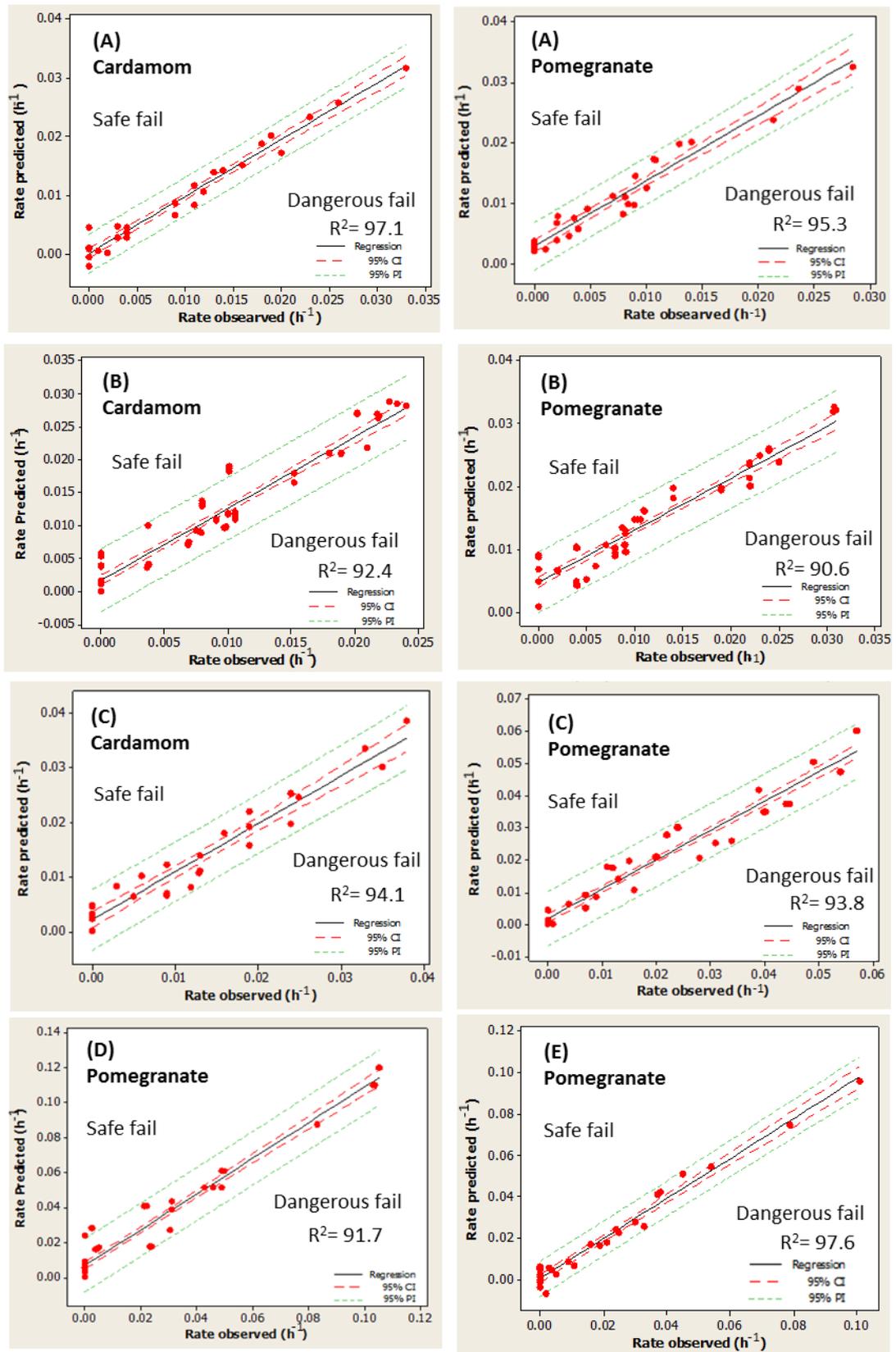


Figure 5. 6: Graphical comparison of growth rate observed and growth rate predicted for tested pathogens as a function of combinations of NaCl, pH, temperature and cardamom or pomegranate extracts, (A): *L. monocytogenes*; (B): *E.coli*; (C): *S. Typhimurium*; (D): *B. cereus*; (E): *Staph. aureus*.

5.4 Discussion

Mathematical models are used to predict the growth of pathogenic and spoilage bacteria in food. Efforts need to improve kinetic models to predict the effect of the extrinsic factors on the growth parameter of selected pathogens. In this study, both primary and secondary models were used to predict the effects of environmental conditions on changes in microbial growth kinetic parameters for five selected bacteria under the effect of natural extracts.

5.4.1 Predict the growth kinetic parameter for selected pathogens from Absorbance measurements by using Baranyi and Roberts model

The Absorbance measurement data of selected pathogens at various environmental conditions were fitted to Baranyi and Roberts model. Whereas, data that showed no growth were excluded from this analysis. The results demonstrated that Baranyi and Roberts model is reliable and appropriate for obtaining precise estimates of microbial growth kinetic (Table 5.1-5.6). Different studies suggested the use of Baranyi predicted models to describe the bacterial growth (Tiwari et al. 2014) more references. The data related to the goodness of fit represented by the coefficient of determination (R^2) indicated that Baranyi and Roberts model fitted well the observed data. The R^2 values were very high and close to one (0.81-0.99). As suggested by Banja (2010) and Tiwari et al. (2014), the higher the R^2 indicated the close fit of the model to the data.

Both maximum growth rate and the lag time duration are mainly used in order to characterise the bacterial growth curve (Baranyi et al. 2009; Al-Kutby 2012). In this study, the higher R_{at} (h^{-1}) of the selected bacterial groups was achieved at 35°C of incubation temperature and in control samples than for treated samples. It has been observed that factor such as temperature, pH and salt have a critical role in controlling bacterial growth (Muñoz et al. 2010; Valero et al. 2010).

Therefore, the higher Rat (h^{-1}) in control sample may be attributed to the presence of salt and the natural extracts in treated samples. Angienda et al. (2010), reported that the synergistic effect of pH, sodium chloride, temperature and natural extracts was achieved against *S. Typhimurium*, *E. coli* and *B. cereus*. The combined effect of low temperature and pH was detected against *E. coli* O157:H7 (Valero et al. 2010). Moreover, in this study the antibacterial of cardamom and pomegranate was confirmed against selected bacterial groups, which is in agreement with previously documented studies (Singh et al. 2008; Al-Zoreky 2009; El Malti and Amarouch 2009; Panichayupakaranant et al. 2010).

The calculated t-lag (h) parameter from the Baranyi and Roberts model showed that the combined effect of natural extracts and high NaCl concentration increased the t-lag parameter of the model at different levels of pH and incubation temperature. The t-lag of *L. monocytogenes* was 12.30 when treated with pomegranate extract and 5% NaCl, whereas at 0% NaCl the t-lag was 2.54 (Table 5.2). The Baranyi and Roberts model expressed the duration of the lag period t-lag as the time required to carry out the work necessary for the cells to overcome an initial hurdle (h_0) and to get ready to divide, which requires adaptation work (Baranyi and Roberts 1994a). Current results indicated that the work to be done for a given NaCl increased when the final NaCl concentration increased, which is in line with the data obtained from (Angienda et al. 2010).

It has been stated that Lag time duration is inherently more difficult to predict than the growth rate because it is influenced by physiological state of the inoculum in addition to the growth condition (Pin and Baranyi 2008; Muñoz et al. 2010). As reported by Robinson et al. (1998), t-lag represents a transition period when the microbial cells adjust to their new environment. However, Robinson et al. (1998) and Mellefont et al. (2003), reported that the t-lag was

the amount of work to be done to adjust to new environmental conditions and the rate at which that work is done. In this study the Rat (h^{-1}) was used to build the secondary model.

5.4.2 Secondary model

The statistical indices represented by R^2 and RMSE value obtained from the secondary model are listed in Table (5.7), The R^2 values were relatively high for most selective bacteria, indicating the adequacy of the data to fit the model.

This study found that the RMSE values were certainly small in comparison with previous studies (Valero et al. 2006; Moh et al. 2011). The RMSE obtained from the RSA model of *L. monocytogenes* was 0.0223 (Valero et al. 2006), while present result showed that the RMSE of the same bacteria was 0.00002.

The efficacy of the extrinsic factors (Temperature, pH, and NaCl) in addition to the cardamom and pomegranate extracts on the growth rate of selected pathogens were analyzed with statistical parameters and with the aid of response surface graphs (Figures 5.1- 5.6).

- **Synergistic effect**

It is generally accepted that no single preservative can act alone against pathogenic and spoilage pathogens in food, as well as, the application of natural extracts at high concentrations has shown to cause organoleptic effects in foods (Burt 2004; Viuda-Martos et al. 2008).

In this study, the MICs of cardamom and pomegranate in combination with pH, NaCl and temperature were tested against selected pathogens and the result showed that selected extracts are active against both G^{+ve} and G^{-ve} pathogens at different degrees of effectiveness depending on the environmental condition and type of pathogens. Similarly, the growth rate of *L. monocytogenes* and *S. Typhimurium* was reduced from 0.44 to 0.18 h⁻¹, when treated with rosemary at 30-15 °C, NaCl 1% and pH 5 (Al-Kutby 2012). *B. cereus* showed the highest sensitivity against extracts at different level of NaCl and pH in comparison with other pathogens. Which is in agreement with the results obtained by Angienda et al. (2010), who reported that *B. cereus* was more sensitive than *E coli* when treated with clove extract at 37°C, pH (5.5-7.3) and NaCl 1.8% in broth culture media.

The recent study showed that the interaction between the natural extracts and the other preservatives demonstrated significant ($P<0.05$) effect against bacterial groups such as the interaction between the extracts with temperature and pH, NaCl and pH or NaCl, pH and temperature alone.

- **Effect of NaCl**

Generally, the results showed that selected extracts act by reducing the R_{at} and prolonging the t-lag when used in combination with 2.5% NaCl at different level of pH and temperature, whereas, no growth was observed above this concentration of NaCl (Table 5.2 - 5.6). The response surface model revealed a significant effect of NaCl ($P<0.05$) against *E coli* and *Staph. aureus* Figures (5.3

and 5.5) and Tables (5.4 and 5.6). The NaCl acts synergistically with selected extracts by rendering the bacterial cells more susceptible to the activity of the extract. In agreement with Temilade (2009), who reported that the growth rate of *Staph. aureus* was reduced when treated with *Eleutherine americana* in the presence of 7.5% of salt; and total elimination of the strain was observed within 24h. The response surface model demonstrated a significant effect of NaCl ($P < 0.001$) against *L. monocytogenes* and *S* Typhimurium in BHI broth; both of maximum growth rate and the t-lag of the pathogens was decreased and increased respectively, when the NaCl concentrations approached the higher levels (5%) (Al-Kutby 2012) . Angienda and Hill (2012), reported that the synergistic impact of NaCl 1.2% with clove extract was detected against *E. coli*. On the other hands, Ultee and Smid (2001), reported that NaCl at 0.125% showed antagonist effect when applied with carvacrol against *B. cereus* in rice. The antibacterial activity of cinnamaldehyde did not improve when combined with NaCl 4% against G^{+ve} and G^{-ve} in agar media (Burt 2004).

It has been suggested that two mechanisms could explain the synergistic effect of NaCl and natural extracts against the bacterial cells: firstly, the NaCl can act by affecting the amino compounds of the bacterial cell wall proteins and allow the penetration of extracts (Juven et al. 1994). Secondly, inhibiting of the bacterial growth by its action on the intracellular enzymes (Burt 2004).

- **Selected extracts effects**

The antibacterial activity of cardamom and pomegranate has been detected against several type of pathogens (Dahham et al. 2010; Husain and Ali 2013). Previous results had demonstrated that cardamom and pomegranate had antimicrobial effect against selected pathogens.

Experiments to determine the effect of selected extracts on the lag time duration and growth rate can be categorised into several responses: no bacterial growth,

increase lag time duration, and decrease in the Rat in comparison to control. The combination of the last two phenomena was commonly observed in this study. The increasing of the lag time may be attributed to the cell damage and need subsequent time to repair it before growth; or could be some cells are killed and only a few survive. While, the combination of last two responses can be occurred when the generation time of the pathogens influenced by the effect of extract. In agreement with Gupta et al. (2012), who reported that the maximum growth rate of *S. Enteritidis* and *L. monocytogenes* was significantly lower in samples treated with 6% (MIC) of seaweed extract.

- **Effect of pH**

The low pH increase the susceptibility of the pathogens to natural extracts (Gutierrez et al. 2009; Seow et al. 2014). The present results showed that all tested pathogens except *B. cereus* could grow at pH 5 in the presence of natural extracts, but produced the long t-lag and short Rat compared to control. this may be due to the fact that low pH increasing the hydrophobicity of natural extract components, and facilitate their dissolution in the lipids of bacterial cell membrane (Rivas et al. 2010; Epanand and Epanand 2011; Li et al. 2014a). Similarly, it has been observed that *B. cereus* in growth media showed high susceptibility to the natural extracts than *E. coli* at pH 5 (Angienda et al. 2010). , Al-Kutby (2012) reported that pH 5 produced strong inhibitory effect against *S. Typhimurium* and *L. monocytogenes* in the presence of natural extracts. The antibacterial activity of natural extracts was strongly affected *L. monocytogenes* at pH 5 (Gutierrez et al. 2008). On the other hands, low environmental pH may be insufficient to affect some pathogens, if they are relatively acid-tolerant species. It has been found that *L. monocytogenes* and *S. Typhimurium* can grow at pH 4.6 and 3.6 respectively (García and Heredia 2009; Abdollahzadeh et al. 2014).

- **Effect of temperature**

Some studies indicated that the antibacterial activity of natural extracts was increased at high temperature, whereas others found to the opposite trend (Kotzekidou et al. 2008; Rivas et al. 2010; Severino et al. 2014). In this study, the temperature significantly ($P < 0.05$) affected the growth rate of *L. monocytogenes*, *E. coli*, *S. Typhimurium* and *B. cereus* when used in combination with pomegranate Figures (5.1 and 5.4) and Tables (5.2 and 5.5). This was in agreement with Moleyar and Narasimham (1992), who reported that the growth of *B. cereus* was completely inhibited by cinnamaldehyde for 30 days at 30°C, while the activity of the extract was decreased at 20°C. Clove extracts showed a strong effect against *E. coli* in broth media at 37°C (Angienda and Hill 2012). The population *L. monocytogenes* in fish model system were reduced by 1.4-1.6 log due to the effect of Black Zira (*Bunium persicum*) extract after 12 days of incubation at 4°C (Rabiey et al. 2013). Moreover, the low temperature ranged from (15-4°C) was the most decisive factor for controlling the growth of *L. monocytogenes* and *S. Typhimurium*, in spite of psychrotrophic features of *L. monocytogenes*; maximum growth rate of *S. Typhimurium* was decreased when treated with cinnamon at low temperature ranged from (15-4°C) (Al-Kutby 2012). According to Valero and Salmeron (2003), the optimum temperature would be influenced by target matrix, the type of natural extract and the optimum temperature of the pathogen.

5.5 Conclusion

The mathematical models developed in this study could be beneficial for predicting the behavior of selected pathogens in growth media under a range of environmental conditions temperature (25-35°C), NaCl (0-5%) and pH 5-7. The Baranyi and Roberts model is recommended due to the precise and reliable estimates of growth kinetic parameters that are given in this study. The favourable environmental factors to reach maximum growth as obtained from the primary models were temperature between 30 and 35°C, 0% NaCl concentration and pH 7 levels.

The experimental data were fitted to the secondary model (RSA), due to the higher R^2 value and smaller RMSE that provided by the model. In other words, the model describes the data precisely for all bacterial groups in culture media. However, validation may be necessary to compare between model data and experimental validation data, and to identify those circumstances where the model can be relied upon and where it could fail.

Cardamom and pomegranate are shown to give antibacterial effects against tested bacteria (chapter 3). The combined effect of these extracts with pH, NaCl and temperature can act synergistically to reduce the bacterial growth rate and increase the lag time under appropriate condition. Furthermore, selected extracts in combination with other preservatives can be used to inhibit the pathogenic growth, or to prolong the shelf life of food since experimentally this has been proven in the present study.

The pathogenic growth rates were determined by using absorbance measurements, which are rapid and inexpensive, compared to the traditional microbiological method, which is labour intensive, time consuming and non-cumulative research tool. While, the drawback of absorbance measurement is that it measures both live and dead cells

6 Chapter 6: General discussion and future work

6.1 General discussion

Concern over food quality and safety is increasing due to the increase of food-borne disease outbreaks. Natural plant extracts are becoming more popular as naturally occurring antimicrobials. For an optimal application of natural extracts in food, more information is needed on their antibacterial properties, and potential to reduce the risk of food borne outbreak. Therefore, this study aimed to establish the factors influencing the survival and growth of pathogenic bacteria by applying novel methods of pathogen inactivation. Another aim was to investigate their mechanism of action against some of the most common food-borne organisms (Food Standards Agency 2011a), which causes the largest number of food-borne illness cases (Figure 6.1) and death in the UK (Food Standards Agency 2013; 2014). However, one of the most common organism, as identified for priority action by the FSA is *Campylobacter* (Figures 6.1 and 6.2).

The antibacterial activity and the phenolic contents of two types of extracts (alcoholic and water) of edible plants and fruit (sumac, cardamom, pomegranate, star anise and cranberry) against six food-borne pathogens were determined and compared (Chapter 3). All extracts except cranberry (alcoholic and water) and sumac (water) demonstrated antibacterial activity against the selected bacterial groups with different efficacy. The result revealed that natural plant extracts had antibacterial influence against both G+ve and G-ve bacteria with different efficacy. Several studies showed that G-ve bacteria are more resistant than G+ve to the action of natural antimicrobial, due to the presence of lipopolysaccharide in their outer membrane.

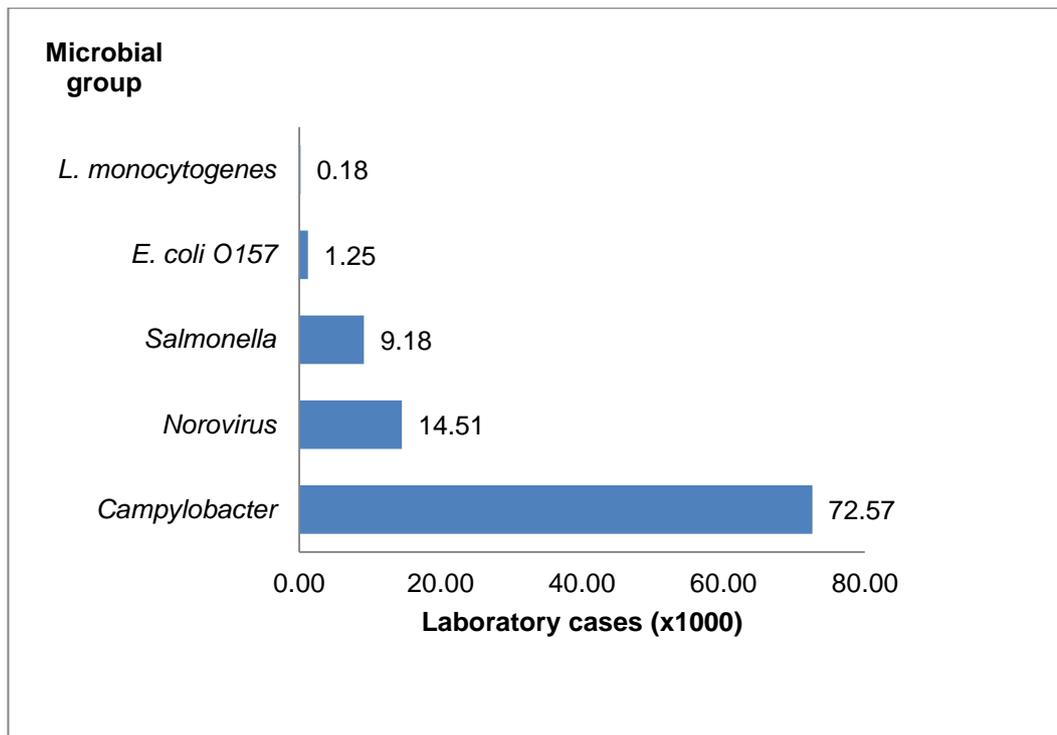


Figure 6.1: Laboratory confirmed cases of Food -borne disease in the UK 2012.

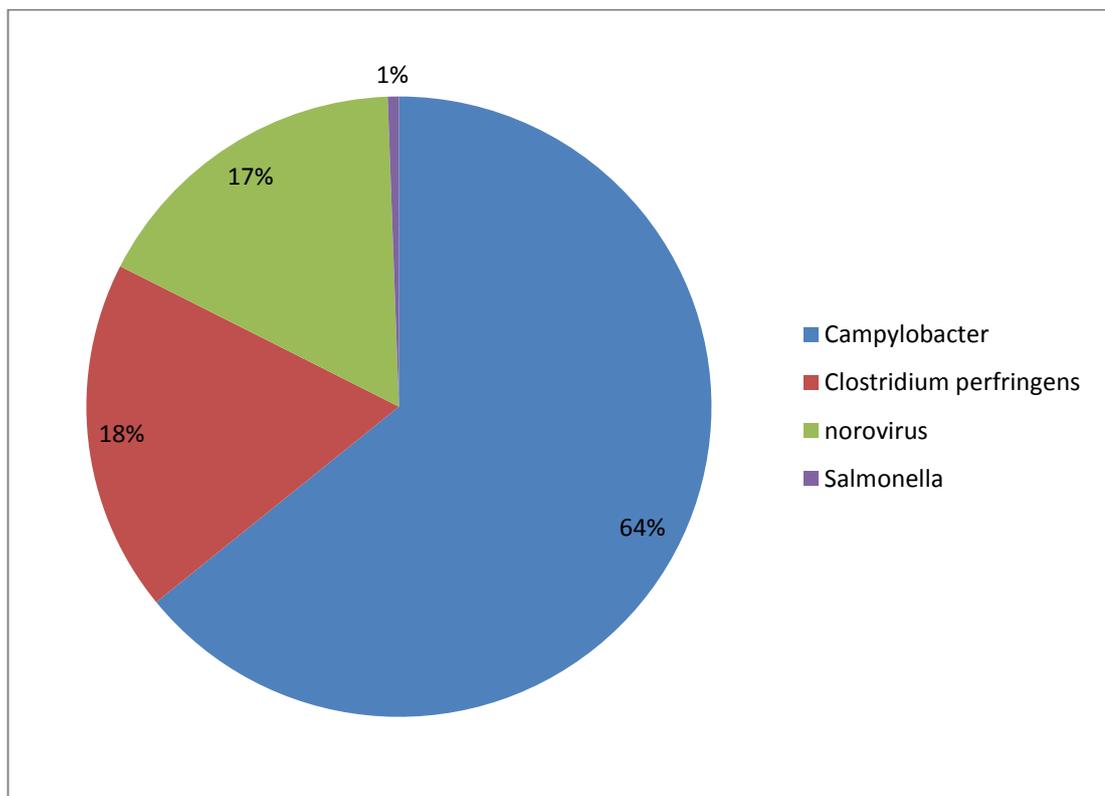


Figure 6.2: Food-borne poisoning reported cases in the UK 2014..

However, Kim et al. (1995) hypothesised that the antibacterial activity did not depend on the type of Gram reaction. The activity can be dependent on the type of natural extracts (Kim et al. 1995; Bakkali et al. 2008). The results obtained in this study support the concept of Kim et al. (1995), were no obvious differences between G^{+ve} and G^{-ve} bacteria was measured in the susceptibility, indicating that selected extract may exhibited different action against the bacterial groups, by interference with the phospholipid bilayer of the cell membrane, impairment of a diversity of enzyme system; and destruction of nucleic acid. The results suggested that selected extract could have the potential to be used in food systems to reduce the risk of food borne outbreak cause by resistant bacteria.

Both the agar well diffusion and broth micro dilution method were used to determine the diameter of inhibition zone (DIZ) and the minimum inhibitory concentration (MIC) of selected spice extracts respectively. The agar well is widely used method for evaluating the activity of plant origin antimicrobials. As reported by Seow et al. (2014) and (Al-Kutby 2012) , a large number of natural extracts can be tested per plate against a single pathogen, and a small amount of sample is needed to obtain the result.

Broth microdilution is quantitative reference method routinely used in laboratories. This method is widely utilized, allowing for the simultaneous testing of multiple antimicrobials with comfort especially when microtiter plates are used. The microdilution method can decrease labor and time. As proposed by Jiang (2011) broth microdilution would be appropriate and fast method for MIC determination, is probably that the natural extract had a closer contact with bacteria growing in the well of microplate in broth microdilution method; thus, the extracts could inhibit the bacteria growth completely and effectively.

Among all the natural extracts tested, sumac and cardamom exhibited a broad spectrum of antimicrobial activity against tested bacteria, with a minimum inhibitory concentration and a highest phenolic content. Total phenolic content of selected extracts were concentration dependant ($R^2 < 0.98$). Positive correlation between the antibacterial activity and the phenolic content were observed for cardamom, sumac and pomegranate extracts (Figures 3.2-3.4). It has been suggested that the antibacterial activity of natural extracts may be attributed to the presence of polyphenolic, volatile and organic compounds such as tannins, gallotannin, ellagitannin, gallic acid, ellagic acid, terpinen, limonene, terpenoids, flavonoid, methyl gallate and anthocyanin (Li et al. 2008; Engels et al. 2009; Kaushik et al. 2010; Wu et al. 2013). possibly due to the combined effects of adsorption of polyphenols to bacterial membrane through membrane disruption and consequent leakage of cell contain (Negi 2012).

Plant natural extract consist of different components, their antibacterial activity is not attributed to specific mechanism chapter 4 focusing on investigation the antimicrobial action of natural extracts that demonstrate promising antibacterial activity against tested bacteria.

The measurement of particular cell leakage markers, such as a 260 nm absorbing material was an indicator of cell wall disruption and membrane permeability and integrity to natural extracts (Oussalah et al. 2006). Table 4.1 showed that severe loss of the bacterial cells constituent occurred due to the activity of extracts, this result was supported by the SEM observation (Figures 4.1 to 4.6). Similarly, Nazzaro et al. (2013) and (Diao et al. 2013) suggested Increasing in the membrane permeability can occur due to the distortion of the cell wall and cytoplasmic membrane, resulting in a leakage of different vital cell constituents by natural extracts .

ATP is necessary for maintaining the activity of all living cells during all phases of growth. It has been observed that the ATP is broken down in dead cells by autolysis within a few minutes (Dostalek and Branyik 2003). Significant ($P < 0.05$) increase of extracellular ATP was detected, when tested cells treated with selected extracts. Selected extracts affected the cell wall and membrane integrity and induced depletion of the ATP concentration. The interaction between the extracts compound and the cell wall presumably increased the membrane fluidity and permeability, which results with an efflux of the ATP. In agreement with this result, Akthar et al. (2014), found that the increase in cell membrane permeability is directly associated with the depletion of the ATP pool, loss of ions and reduction in membrane potential and collapse of the proton pump.

The intra-extracellular ATP was measured using the ATP bioluminescence assay that provides a rapid method to identify the viability of bacterial cells (Hunter and Lim 2010).

The ultrastructure studies have proved to be a useful tool for investigating the cellular changes, as well as providing complementary information about the mode of action of selected extracts against the bacterial cells. The main SEM observation of this study showed the morphological changes in the cell wall of selected bacterial groups treated with cardamom, pomegranate and sumac extracts (Figures 4.1 to 4.6), which have also been observed for several strains of tested pathogens after the treatment with different natural extracts (Kaya et al. 2008; Engels et al. 2011).

The alterations in the morphology of tested bacteria might be attributed to the impact of natural extracts on the cell wall, increase the permeability of the membrane, which might lead to the lysis of the bacterial cell membrane,

subsequently causing the cell death. The cells damage could be varied depending on the type of pathogens such as presence of vacuoles on the cell wall and observation of the deformities cells shape. The SEM observation showed deformity in the cell shape in *B. cereus* treated with cardamom and pomegranate extracts (Figures 4.5 and 4.6). As reported by (Gao et al. 2011; Diao et al. 2014), scan photography showed the presence of vacuole in *E. coli* cell wall when treated with clove and oregano extract, while deformity in the cell shape was detected in *B. Subtilis* after the treatment with same extracts.

The mechanism of action of sumac, pomegranate and cardamom extracts against selected pathogens are summarize in Figure 6.2

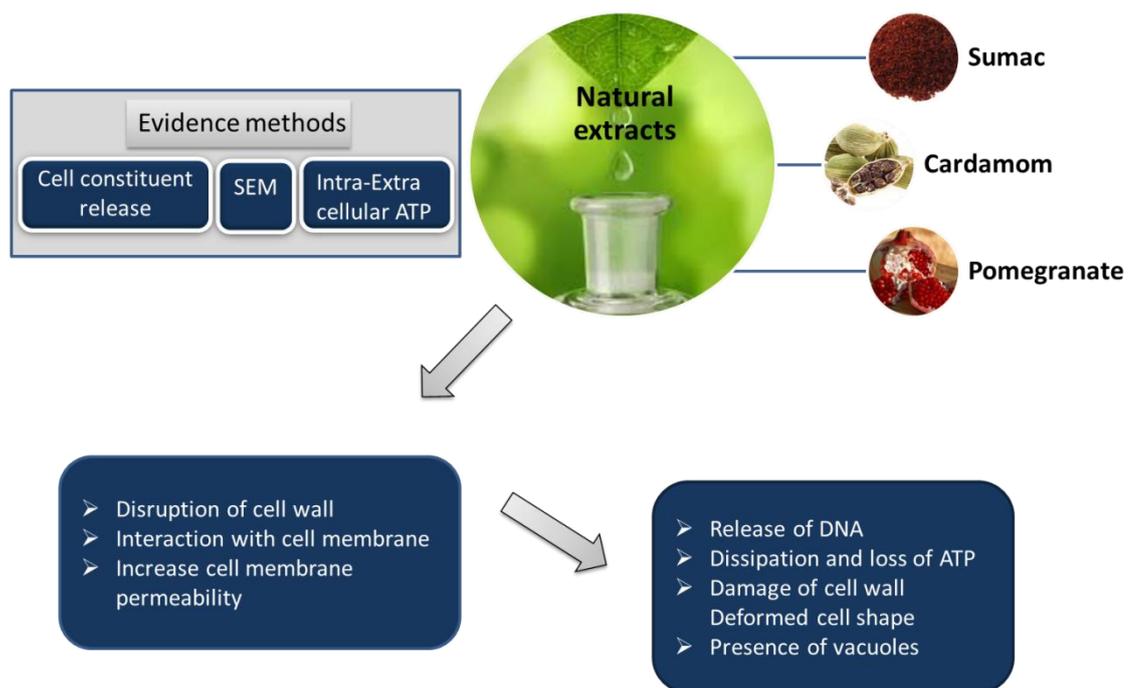


Figure 6. 2: Antimicrobial action of natural extracts against tested pathogens (left box) and their consequence (right box).

Predictive model is a promising field in food microbiology help to design control measures to produce safe and good quality food (Koutsoumanis et al. 2005, Nychas et al. 2008). While, predictive models can propose a cost effective solution by reducing the microbiological testing to determine product safety and quality (Buchanan 1993), some models may not be accurate, as a result of

inconsistent microbial responses and/or variations in the growth media (Food Safety Authority of Ireland 2011). Thus, the use of the model must always be used with caution. In chapter 5, both of primary and secondary models were used to predict the growth of pathogenic bacteria in broth media under various conditions, represented by *L. monocytogenes*, *S. Typhimurium*, *E.coli*, *B. cereus* and *S aureus*. Precise and reliable estimates of growth kinetics parameters for all bacterial groups have been obtained from Baranyi and Roberts model (primary model). The results obtained from primary model confirmed that the use of this model to predict the growth rate for subsequent secondary modelling (RSA) is recommended. Indeed, many studies reported that Baranyi and Roberts model is reliable and precise to predict the growth parameter of pathogenic bacteria (Al-Kutby 2012) (Banja 2010).

The RSA (secondary model) results indicated that the effect of the four tested variables (temperature, pH, NaCl, and spice extracts) was varied depending on bacterial species. The tested extracts (cardamom, pomegranate) demonstrated considerable bacteriostatic and/or bactericidal activity, and their impacts were increased when used in combination with a 5 pH, 5% NaCl concentrations and 25 °C. This indication may offer food products a degree of protection against selected pathogens.

Tecan plate reader was used to determine the growth curve parameters of selected bacterial groups, this system based on the absorbance measurement which generate a considerable volume of microbiological growth data compared to the conventional total viable count method which is time consuming and labour intensive. The drawback for the absorbance measurement method is that it measures the dead and live pathogens. Results obtained from chapter 5 indicated that using of Tecan plate reader was satisfied for measuring

absorbance at 25°C and above which is agreed with (Al-Kutby 2012) and (Banja 2010).

This study provides evidence for the potential use of cardamom, pomegranate and sumac in combination with other hurdle to reduce the risk of survival and growth of food borne pathogens.

6.2 Future work

- Since the antimicrobial and antioxidant activity of natural extracts depends on their chemical composition; thus the extraction, separation and quantitation of these chemicals are challenging. Gas chromatography-mass spectrometry analysis, allows identifying the main contents of selected extracts, consequently assessing their antibacterial and antioxidant activity.
- Pomegranate, Sumac and cardamom extracts showed promising *in vitro* antimicrobial activity against tested pathogens under a range of environmental conditions. Application of selected spice extracts in food could extend the shelf life and maintain the safety of processed food. Accordingly, it might be applicable to determine their efficacy, stability and bioavailability in food model. As well further investigation is important to identify the conditions that improve their activity without detrimental influences on the sensory properties of the food products.
- Further investigation is needed to evaluate the interactions between the selected extracts with other food ingredients such as lipid, carbohydrate and protein.
- Studying the interaction of natural antimicrobials combination can identify their synergistic or antagonistic effect; thereby help to find potent natural

extract blends that can be used in food preservation without simultaneous sensory effects.

- Develop and validate the microbial mathematical models for growth, survival and death of pathogenic and spoilage bacteria in food business operators and food processing establishments, where there is exposure to different environmental conditions. This will help to minimise the risk associated with these microorganisms in food during cooking, cooling, storage, distribution, and adding decision to current control strategies.
- Applying of transmission electron microscope (TEM) to investigate the effect of selected extract on the bacterial cytoplasmic membrane, and also more details can be obtained related to the alteration in cell morphology.
- In response to the public health problem associated with *Campylobacter spp.* in meat and poultry product in UK, further investigation is required for the control and elimination of campylobacter hazards in this type of products. Therefore evaluating the antimicrobial effect (bactericidal and/or bacteriostatic) and the mode of action of sumac, cardamom and pomegranate against this pathogen is important and is a focal area for future research.
- Components of natural extracts also appear to act on Enzymes such as ATPase which is located in the cytoplasmic membrane and bordered by lipid molecules. Study the mechanism of action of cardamom, pomegranate and sumac on such enzyme need to be explored.

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Appendix A

Associated studies and professional development

Professional Courses:

Module code	Module title	credit
BIO 5124	Postgraduate Research Skills and method in biology	10
ENV 5101	Laboratory based teaching method	10
ELC 004	English Summer/Winter program for research students	0
BIO5102	Principles and Applications in Electron Microscopy	10

Conference Contribution and Attendance:

- Young Researchers Event and Biosciences (KTN) conference, 14th of November 2012, Edinburgh
- CARS PG Symposium Plymouth university, 10 December 2012, New Continental Hotel-Plymouth.
- Postgraduates and Early Career Scientists (PECS) Research Conference, 25 October 2012, London, UK.

Appendix B

Poster presentation

Antibacterial activity and phenolic contents of natural extracts against food-borne pathogens

Abass Aal-Tay, Jane Beal, Victor Kuri
School of Biomedical and Biological Sciences, University of Plymouth, UK



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Background

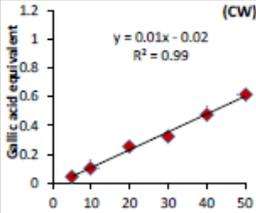
- ❖ Food-borne disease is still recognised as public health threat, resulting from consumption of contaminated food with pathogenic bacteria¹
- ❖ Elimination of pathogenic growth has been the major issue, due to continuous pathogenic resistance toward antibiotic and growing of food-borne outbreaks
- ❖ Plant base natural extracts and their phytochemical compounds (tannins, flavonoids, phenolic diterpenes, and phenolic acids) are considerable source of novel antimicrobial compounds in particular against pathogenic bacteria²

Aims

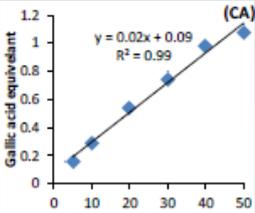
The aims of this study were to evaluate the total phenolic compounds (TPC) of selected natural extracts

To determine the antibacterial efficacy and their minimum inhibitory concentration against a selection of pathogenic bacterial strains (MIC)

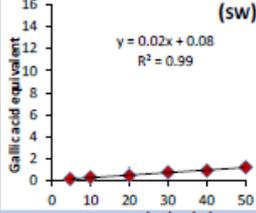
Results



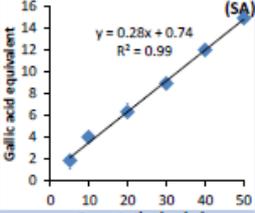
(CW)



(CA)



(SW)



(SA)

Figure 1. Total phenolic compound of Chambery water & alcoholic extracts (CW, CA) & Sumac water and alcoholic extracts (SW, SA)

Materials and methods

Natural extracts

- Cardamom (Ca) and star anise (St) essential oils were provided by (Kalsec, Mildenhall, UK)
- Sumac (S), pomegranates (P) and cranberry (C) were prepared as water (W) & alcoholic (A) extracts from the powder

Bacterial cultures

- Six food-borne pathogens were used as target organisms: *Listeria monocytogenes* 5105, *Staphylococcus aureus*, *Salmonella* Typhimurium DT104, *Escherichia coli* K12, *Bacillus cereus*, and *Clostridium perfringens* NCTC8237.
- Strains were obtained from the School of Biomedical & Biological Sciences microbiological collection (University of Plymouth)

Total phenolic compound

- Folin-Ciocalteu's (colourimetric method)

10 part water + 1 part reagent



25 µl of sample + 62.5 µl Folin reagent + 300 µl of sodium carbonate



Spectrophotometer



Read at 750 nm

Agar wells diffusion method³

Stock cultures of target strains were grown in brain heart infusion broth for 18 h. Final cell concentrations were standardized to 10⁷–10⁸ CFU ml⁻¹ using the McFarland solutions standards (opacity tube⁴). 200 µl of these were added to each plate containing 20 ml brain heart infusion agar and left to set. Then wells (8 mm diameter) were formed in each plate using a sterile cork borer. Forty µl of each extract were applied into each well and left to diffuse for 15–20 min at room temperature prior to incubation at the appropriate conditions.

Conclusions

- Natural extracts have potent antimicrobial activity against pathogenic bacteria.
- Alcoholic extracts possess strong efficacy against most bacterial strains and high TPC comparing with water extracts.
- Positive correlation was observed between the TPC and the antibacterial activity of selected natural extracts.

Table 1. Antibacterial activity^a of natural extracts against pathogenic bacteria

Spice extracts	Inhibition zone (8 mm)					
	<i>E. coli</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>S. Typhi</i>	<i>Cl. perf</i>	<i>L. mono</i>
CaA	13.9	14	14.5	11	10	11
CA	–	–	–	–	–	–
CW	–	–	–	–	–	–
PA	12.8	11.9	12.4	12	10	10.5
PW	11.8	10.8	10.8	–	–	–
StA	12	10	12.5	11.5	10	10
SA	16	15.3	15.3	15.8	13.8	13.5
SW	–	–	–	–	–	–

^a Bacteria were tested at 10⁷ CFU / ml for 24 h, – No inhibition.

Acknowledgments

AAT postgraduate scholarships by the Ministry of Higher Education of Iraq

To all the technicians at the food and nutrition & microbiological laboratories for support with the tests

References

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Predictive Model of *Listeria monocytogenes* and *Salmonella typhimurium* Growth Rate under Different Temperatures, pH, NaCl and Spice Extracts



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RESEARCH
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Background

- **Predictive Model** is a Promising field in food microbiology, it is a **Mathematical models** built with data from laboratory testing and computer software and Offer a **cost effective** solution¹.
- *L. monocytogenes* & *S. typhimurium* are mostly considered as a potential hazard in ready to eat meat products² and also they account for almost 60% of the burden of food-borne disease in UK³.
- **Food poisoning** associated with these types of pathogens is due to either the Pathogen is commonly **present in product ingredients**, **Environmental contamination** or **cross-contamination**.
- Culinary spice extract (**Cinnamon, rosemary and Sumac**) as well known for their **Antibacterial** effect⁴.

Aims

The aims of this study were to investigate the combined effects of temperature, pH, sodium chloride and spice extract addition on the growth kinetics parameters of *L. monocytogenes* and *S. typhimurium* and use data to build a growth model

Materials and methods

Bacterial cultures

L. monocytogenes NCTC7973
S. typhimurium DT104

Natural extracts

- Cinnamon and Rosemary oleoresin were provided by Kalsec company
- Sumac hydro alcoholic extracts was prepared from sumac powder

Preparation & inoculation of culture media

Growth rate measurement
Total viable (TVC) & Turbidity measurement



Growth Data analysis

Primary models

Secondary model
RSA

Results

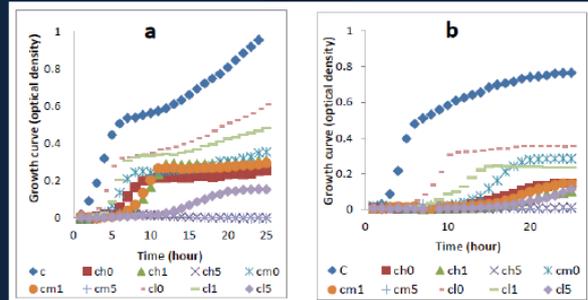


Fig 1: Growth curve of *S. typhimurium* (a) & *L. monocytogenes* (b) in BHI broth (pH=7) at 30°C using plat reader

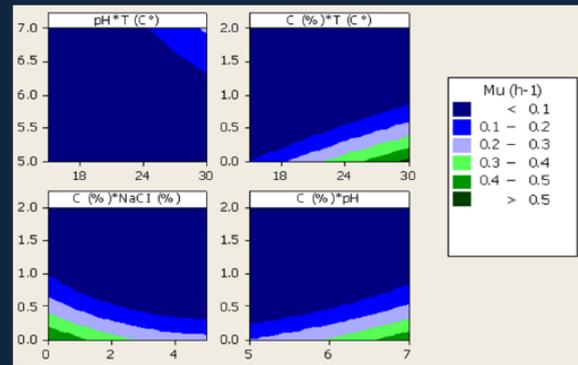


Fig 2: Contour plots showed the predicted growth rate of *L. monocytogenes* as a function of combinations of NaCl, pH, temperature (T) and cinnamon (C) using response surface analysis model. Axis labels (Y & X) above each graph

Conclusions

- The model developed in this study could be useful for **predicting** *L. monocytogenes* and *S. typhimurium* behaviour in **RTE meat products**.
- The different types of model provided different growth information and are thus **complementary**.
- The primary model (**Baranyi and Roberts model**) has given **precise and reliable estimates** of growth kinetic parameters.
- The **minimum growth rate** (μ_{\min} h⁻¹) are temperature of 15°C, 5.5% NaCl concentration, 0.2 % spice extracts and pH 5.
- Spice extracts were a **fundamental factor** to control pathogens in food.

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Acknowledgments

SAK postgraduate scholarship by the Ministry of higher education of Iraq

To all the technicians at the food and nutrition & microbiological laboratories for support with the tests

Appendix C

Certificate of attendance



Chartered
Institute of
Environmental
Health

Level 2 Award in
Food Safety in Catering
1 credit

Abass F Aal-Tay

has successfully completed a programme of training
and an assessment which concluded the course



Course Director



Graham Jukes
Chief Executive
Chartered Institute of Environmental Health

Examination date 04 November 2011

CIEH recommends you refresh your training by 04 November 2014

Centre Number FF23168

Certificate Number 8484803

Original issue date 21 November 2011



Certificate of Participation

This is to certify that

Abass Aal-Tay

Presented at the Centre for Agricultural & Rural
Sustainability (CARS) Postgraduate Symposium
10th December 2012
Plymouth University

Professor Mick Fuller
Head of Graduate School and
Director of Graduate Studies

Ayman Almerei
Symposium Organiser