STUDY AND CHARACTERIZATION

OF ANTIBACTERIAL COMPOUNDS OF ARABICA

COFFEE BERRY PULP

By

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

Background 1
Objectives 3

II. REVIEW OF LITERATURE 4

By-product of agricultural industry 4
Phenolic compounds in plants 5
Phenolic compouns in agricultural by-products 5
Potential application of by-products 6
Coffee pulp 7
Chemical compounds in coffee pulp and antimicrobial activity of coffee 8
Antimicrobial properties of phenolics from coffee 11
Mode of antibacterial actions of phenolics 11
Membrane damage 11
Leakage of intracellular constituents 12
Inhibition of enzymes activities 13
Inhibition of nucleic acid formation 13
Effect of oxygen and heat on the chemical reaction of phenolic in the presence of food matrices 14
Extraction of phenolic compounds 15
Determination of phenolic content 18
Folin Denis 18
Folin Ciocalteau 18
Prusian Blue 19
Characterization of phenolic compounds 20
References 22

III. STUDY AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS OF ARABICA COFFEE BERRY PULP 32

Abstract 33
Introduction.....35
Materials and Methods.....37
Coffee pulp.....37
Solvents and reagents.....37
Methods.....37
Water extraction of antibacterial compounds 37
Heating method.....37
Agitation method 38
Organic solvent extraction of antibacterial compounds.....38
Single extraction 38
Effect of methanol extraction.....38
Effect of reflux time.....38
Fractionation 39
Amberlite resin column.....39
Bacterial cultures 40
Antibacterial activity test 40
Turbidity method 40
Paper disc method.....41
HPLC analysis of coffee pulp fractions.....41
Comparison of antibacterial activity of extracted fractions and commercial Phenolic acids 43
Statistical analysis.....43
Results and Discussion 44
Water extraction of antibacterial compounds by heating 44
Water extraction of antibacterial compounds by agitation at room temperature 45
Effect of methanol concentration on extraction of antibacterial compounds from Arabica coffee berry pulp 47
Fractionation of coffee beryy pulp extracts 48
Antibacterial activity of coffee berry pulp fractions.....49
Separation and analysis of phenolic by HPLC 51
Summary of findings.....61
References.....62

APPENDICES

A. Effect of water extraction of coffee pulp on the growth of *E.coli* 1472 66

B. Fractionation of coffee pulp extract 71

C. Antibacterial activity of coffee pulp fractions.....74

D. Histograms of phenolic compounds of coffee pulp fractions 77

LIST OF TABLES

Table Page

1. Comparison of time needed to reach 0.2 units of absorbance growth of *E.coli* 1472 in the presence of coffee pulp extracted by heating 53

2. Comparison of time needed to reach 0.2 units of absorbance growth of *E.coli* in the presence of coffee pulp extracted by agitation 54

3. Influence of methanol concentration and water during extraction of antibacterial compounds from coffee berry pulp on their inhibitory effect against *E.coli* 1472.....55

4. Antibacterial activity of coffee berry pulp extracted fractions against foodborne pathogens and spoilage bacteria 56

5. Phenolic content in coffee berry pulp extracted fractions 57

A.1. Absorbance reading of *E.coli* 1472 growth in the presence of coffee berry pulp water extract by heating 67

A.2. Absorbance reading of *E.coli* 1472 growth in the presence of coffee berry pulp water extract by agitation 68

A.3. Effect of reflux time during bacterial compounds extraction from coffee berry pulp on their bacterial inhibition against *E.coli* 1472.....69

A.4. The effect of pH adjustment of reflux extraction on the antibacterial activity.....70

B.1. Dry extract before and after fractionation and their recovery.....72

B.2. Dry extract yield obtained after fractionation 73

C. Antibacterial activity of coffee berry pulp extracted fractions against foodborne pathogens and spoilage bacteria 76

D.1. Retention time and R^2 of phenolic standards of calibration curves 79

LIST OF FIGURES

Figure Page

1. Industrial processing of coffee berries 8

2. Chemical structure of some phenolics present in coffee pulp 10

3. Schematic diagram of coffee pulp fractionation 58

4. Comparison of antibacterial effect of methanol extract with initial pH and pH 6.5 59

5. Influence of reflux time during extraction of antibacterial compounds from coffee berry pulp on their inhibitory effect against *Escherichia coli* 1472 60

C.1. Inhibition activity of water soluble coffee pulp fractions toward *E.coli* O157:H7 ATCC 933 (A), *P. fluorescens* (B), *L. monocytogenes* Scott A (C), *S. aureus* 13565 (D); Inhibition activity of ethyl acetate fractions against *B.subtillis* (E) and chloroform fraction against *S.* Typhimurium (F) 75

D.1. HPLC chromatogram of mixture of phenolic standards 78

D.2. HPLC chromatogram of chloroform fraction pH initial 80

D.3. HPLC chromatogram of chloroform fraction pH 6.5 81

D.4. HPLC chromatogram of ethyl acetate fraction pH initial 82

D.5. HPLC chromatogram of ethyl acetate fraction pH 6.5 83

D.6. HPLC chromatogram of water fraction pH initial 84

D.7. HPLC chromatogram of water fraction pH 6.5 85

CHAPTER I

INTRODUCTION

Background

In recent years, there has been an increasing interest in the use of natural antimicrobial compounds instead of synthetic ones. This interest is due to the increasing demands of consumers for foods with fewer synthetic additives and increased safety, quality and shelf-life. The concern over the rise of antimicrobial resistance of bacteria against synthetic antimicrobials has also sparked interest in investigating natural antimicrobials (Smid and Gorris 1999). Historically, plants have provided good sources of natural antimicrobial materials and they synthesize limitless aromatic substances most of which are phenols (Cowan 1999). The potential of using agricultural products, wastes and by- products as natural source of antimicrobial has been explored recently. Plant sources such as coffee, tea, grapes, sorghum, and some herbs such as oregano and spices have been shown to have antibacterial activity due to their content of phenolic compounds (Shetty and others 1994; Rojas and others 2003; Khadambi 2006).

Coffee is the most popular beverage in the world, especially in western countries. In the Arabica coffee industries, large amount of by-products are generated during processing. The disposal of these by-products, such as coffee pulp, is an environmental

concern. Coffee pulp is rich in organic compounds such as sugar, protein and nitrogen although application as livestock feed is very limited (Mazafera 2002). This is due to its high content of anti-nutrient phenolics such as pectin and tannin, and some phenolic acids that are toxic (Pandey and others 2000).

In the last few decades, research interest has been focused on the antibacterial properties of coffee bean extract; however, investigation of possible antibacterial activity of coffee by-products including pulp is very limited. Studies have shown that an antimicrobial activity of coffee extract is due to phenolics since phenolic acid is the major class of phenols identified in coffee species. In addition, chlorogenic acid was found to be degraded to phenolic compounds in coffee species including Arabica (Campa and others 2005). Therefore, coffee berry pulp might also have valuable compounds similar to those found in coffee bean.

Antibacterial compounds, if extracted, would add value to the coffee industry. It may also help reduce environmental pollution by providing livestock producers a better by product for use as feed because the anti-nutritional factors such as phenolic compounds would be removed. In addition, the extract may be useful as a natural food preservative. Preliminary research in our laboratory has indicated that the pulp of coffee Arabica from Hawaii has a potential to reduce the growth of generic *Escherichia coli* and *E.coli* 0157:H7. Therefore, there is the possibility of using Arabica coffee pulp as a source of natural antibacterial compounds and the active components might be used as food additives to prolong the shelf-life of foods.

Objectives

The objectives of this research were to determine the presence of antibacterial compounds, develop an efficient extraction method, analyze phenolic compounds and contrast their inhibitory effect against foodborne pathogens and spoilage bacteria.

CHAPTER II

REVIEW OF LITERATURE

By-products of agricultural industry

Agricultural products can be categorized as being from temperate, tropical, or subtropical zones. In temperate zones, they are usually characterized by moderate amounts of waste material such as peels, seeds and stones. In contrast, tropical and subtropical fruit processing release considerably higher by-products to edible portion ratios. Due to increased production, disposal of wastes and by-products represents a growing problem. Agro-industrial wastes are often utilized as feeds or fertilizer (Schieber and others 2001) however; certain by-products of agricultural industry can not be used directly as a feed stock due to high content of anti-nutritional substances such as phenolic compounds.

Studies have shown that agricultural by-products such as grape pomace or grape seed, old tea leaves, and cocoa by-products can be used as sources of antibacterial compounds (Jayaprakasha and others 2003; Baydar and others 2006; Ozkan and others 2004; Michiyo and others 2004). Pomace is the solid remains of grapes, olives, or other fruits after pressing them for juice or oil. It contains the skins, pulp, seeds, and stems of the fruit.

Phenolic compounds in plants

Phenolics are synthesized by plants as secondary metabolites (Balasundram and others 2006) and occur as phenolic acids, flavonoids, tannins (condensed and hydrolyzable), or other phenolics (Naczk and Shahidi, 2006). They are responsible for pigmentation (Bravo 1998), disease resistance of plants against bacteria, fungi and viruses (Nicholson and Hammerschmidt 1992; Lyon 1989; Friedman 1997), and certain classes such as tannins, provide a defense mechanism against herbivores by precipitating proteins in their digestive tracts (Hagerman and others 1987).

Phenolics are not uniformly distributed in plants (Williams 1957). Most phenolics are commonly stored directly in the vacuole for sequestration from there to the cell (Beckman 2000). Based on solubility, phenolics are divided into two classes: soluble and insoluble. According to Nzack and Shahidi (2006), insoluble phenolics are found in cell walls. Compounds such as ferulic acid and p-coumaric acid were found in cell walls esterified with an insoluble polymer (Fry 1982) or in cell wall polysaccharides (Smith and Harris 2001). Ferulic acid also binds to primary cell walls of monocotyledonous and dicotyledonous plants (Carnachan and Harris 2000). This compound contributes to the toughness of plant texture (Rodriguez-Arcos and others 2004) and strengthens cell walls by limiting cell wall biodegradability (Hartley and Ford 1998).

Phenolic compounds in agricultural by-products

Some by-products of agricultural industries contain high levels of valuable compounds including phenolics, which exhibit antimicrobial activities. Lafka and others (2007) identified hydroxytyrosol, tyrosol, cyanidin glycosides and various phenolic acids

such as gallic acid, catechin, epicathecin, caffeic, syringic, vanillic, *p*-coumaric and *o*-coumaric acids in grape waste. A study by Rubilar and others (2007) indicated that red and white grape contain glycosylated flavonols (quercetin and kaempferol), while almond hulls contain hydroxybenzoic and cinnamic acid derivatives, with small amount of flavan-3-ols. Cocoa beans contain proanthocyanidins (Wollgast and Anklam 2000) and high amount of catechins which amount to 21.89 - 43.27 mg/g of dry defatted samples (Kim and Keeney 1984). Some phenolics, mainly gallic, ellagic acid, and gallates were also reported in mango kernels. Ethanolic extracts of mango seed kernels were composed of 79.5 % of polyphenol and displayed a broad antimicrobial spectrum; however, these compounds were more effective against Gram-positive than Gram-negative bacteria (Kabuki and others 2000).

Potential application of by-products

Nutritional values of food and taste, as well as other sensory properties are influenced by their phenolic content (Bravo 1998). These compounds can also affect the oxidation of food (Nzack and Shahidi 2006). It has been well documented that phenolics in agricultural products or by-products have the ability to act as antioxidants. Antioxidant activities were identified in cocoa beans (Othman and others 2007), wine and grape pomace (Larrauri and others 1996), and dried apple peels (Wolfe and Liu 2003). In addition to providing antibacterial effects, phenolics may also interfere with intake of nutrient absorption (Rojas and others, 2003).

By-products also might exhibit antibacterial activity. By-products of tea contain catechin and polyphenols which showed antibacterial activity. Study by Harkenthal and

others (1999) showed that Australian tea tree oil exhibited antibacterial activity against some foodborne pathogens such as *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Listeria monocytogenes.* Tree tea oil also was found to be effective in reducing the growth of *Pseudomonas aeruginosa* (Papadopoulus and others 2006).

Coffee pulp

One of the most important agricultural commodities in the world is coffee. There are 40 varieties of coffee. Among them, Arabica and Robusta coffee are the two most popular varieties cultivated all over the world for commercial production (Pandey and others 2000). Arabica coffee is most commonly used variety for beverage, especially in western countries. Industrial processing of coffee berries can either be dry or wet (Figure 1), and coffee pulp is a by-product produced from these processes (Pandey and others 2000). In coffee processing, the exocarp, mesocarp and endocarp of the coffee berry are removed. In wet processing, the coffee berry is pulped, fermented, washed, and sun or artificially dried. The pulping process removes the exocarp and most of the mesocarp, thereby removing the pulp from the bean (Pandey and others 2000; Mazzafera 2002). The by-products and waste resulting from wet processing of coffee berries are the pulp, the mucilage, and the waste water (Rolz and others 1980).

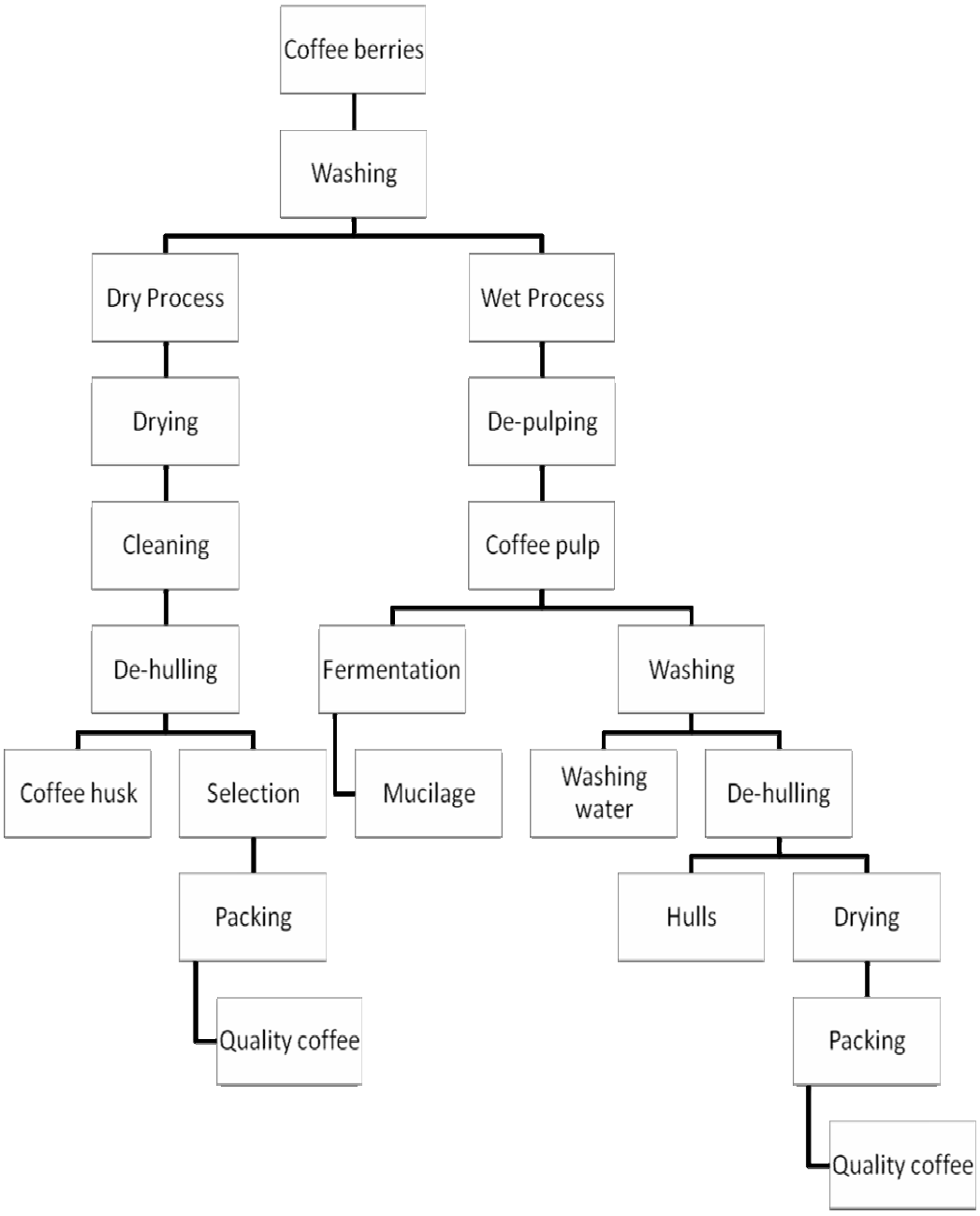


Figure 1. Industrial processing of coffee berries (Pandey and others, 2000)

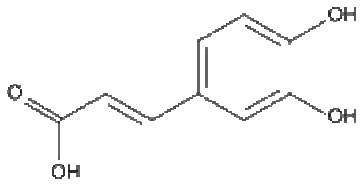
Wet processing in the Arabica coffee industry contributes to a large amount of by-products: approximately 41-45 % of the weight of the coffee berry or 6.3 million tones worldwide production annually (Rojas and others 2003; Moreau and others 2003; Scholer, 2006).

Chemical compounds in coffee pulp and antimicrobial activity of coffee

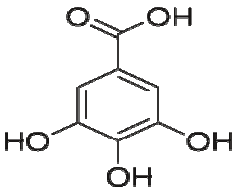
Coffee pulp contains some macromolecules such as proteins, lipids, carbohydrates (fiber, reducing and non-reducing sugars), and minerals (ash). The pulp also contains anti-nutritional factors such as polyphenolics like tannin and some phenolic acids such as chlorogenic acid and caffeic acid. It also contains caffeine. On a dry matter basis coffee pulp consists of 1.3 % caffeine and 4.5 % tannin. Phenolic content varies depending on the cultivar and the processing. Dry processed Ethiopian coffee has about 2.2% phenolics while wet processed coffee pulp has about 24.6%. Phenolics in the cultivar of coffee range between 15.9 - 40.6 % of dry matter (Mazzafera 2002; Rojas and others 2003). According to Elias (1987) as cited by Mazzafera (2002), coffee pulp contains 23.3 % of dry matter which include 3.4% fiber, 2.1 % protein, 1.5 % ash, non-protein and organic components like tannins, sugars, caffeine, chlorogenic acid and caffeic acid. Additional phenolic compounds such as flavan-3-ols (monomer and proanthocyanidins), hydroxynamic acid (caffeolquinic acid, caffeolquinic acid derivates and p-coumaroylquinic acid), flavonols and anthocyanidins were also reported in the pulp (Coronell and others, 2004).

According to Velaz and others (1985), the polyphenols of coffee pulp extracted using ammonium hydroxide solvent had higher binding percentages with proteins than

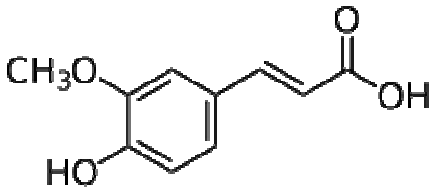
those extracted with methanol-water. This phenomenon may cause deficient protein utilization (Moreau and others, 2003). Tannins were reported to interfere with protein digestibility and inhibit digestion by some enzymes such as proteases, amylases and lipases (Mazzafera, 2002). Phenolic acid is the major class of phenols identified in coffee species. Some phenolics in coffee pulp are presented in Figure 2.



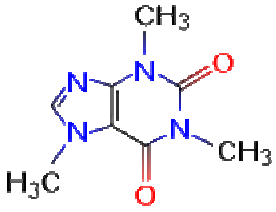
Caffeic acid



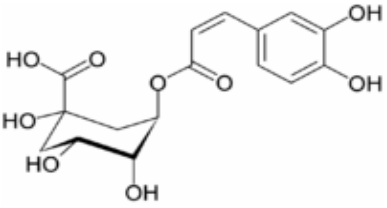
Gallic acid



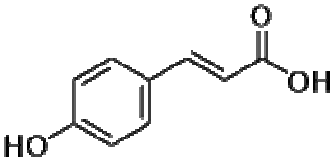
Ferulic acid



Caffeine



Chlorogenic Acid



p-coumaric acid

Figure 2. Chemical structure of some phenolics present in coffee pulp (Balasundram and others, 2006)

Antimicrobial properties of phenolics from coffee

Shetty and others (1994) reported that coffee (*Coffea arabica*) showed bactericidal activity against *Vibrio cholerae, Salmonella* Typhimurium *and S*.Typhi. Tannins and caffeine from coffee were found to inhibit *Staphylococcus aureus, Vibrio parahaemolyticus, V. cholerae,* and *Salmonella* (Michels 2000; Toda and others 1989). Almeida and others (2006) reported that coffee extracts and selected coffee chemical compounds such as caffeine, chlorogenic acid and protocatechuic exhibited antibacterial activity against Enterobacteria. The presence of caffeine in coffee beans also inhibits *Aspergillus parasiticus* and the formation of aflatoxin. According to Mazzafera (2002), the presence of caffeine at 0.1 % also reversibly inhibited protein synthesis in bacteria and yeast. However, some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid.

Mode of antibacterial action of phenolics

Membrane Damage. Various studies have reported the action of phenolics against Gram positive and negative bacteria. Prigent (2005) proposed several mechanisms for the inhibition such as disintegration of cell membrane due to binding of phenolics to protein membrane bilayers, or outer membrane protein (OMP), either by non-covalent or hydrogen binding (reversible), and covalent or hydrophobic interaction (irreversible). Cytoplasmic membrane has been used as a target of biocide for antimicrobial test (Maillard 2002). In a study by Shapiro and Gugenheim (1995) the interaction of phenolics to membrane bilayers will change the permeability of the membrane and lead to membrane disruption. The effect of carvacrol on the cell

membrane of *B.cereus*, a Gram positive bacterium, also was reported by Burt (2007). In this study the carvacrol apparently dissolved in the phospholipid bilayer and causes distortion, expansion and destabilization of the cell membrane. Sanchez and others (2007) reported the action of phenolic compound, oleuropein, against phospatidylglycerol (PG), a model membrane of Gram-positive bacteria and phosphatidylethanoalamine (PE), a model membrane of Gram negative bacteria. In this study, Gram positive bacteria were more susceptible than Gram negative bacteria to oleoropein. This might be because Gram negative bacteria have three essential layers (cytoplasmic, peptidoglican and an outer membrane layer) while Gram positive bacteria have two layer (Lugtenberg and Alphen 1983).

Leakage of intracellular constituents. Antimicrobial actions of phenolic compounds also are due to the leakage of ion and other intracellular constituents (Walsh and others 2003). Phenolic compounds caused membrane leakage in bacteria resulting from the presence of protons, phosphate, and potassium in the growth medium *(*Prigent, 2005; Kaur and Singh, 2008). Lambert and others (2001) reported that carvacrol and thymol caused membrane leakage as indicated by the presence of protons, phosphate, and potassium in the growth media of *S. aureus* ATCC 6538. Loss of potassium from cells of *E.coli* and *S. aureus* which were treated with thymol and eugenol also was observed (Walsh and other 2003). A study by Nohynek and others (2006) showed that phenolic extracts from berries exhibited disintegration of the outer membrane which led to the leakage of lipopolisaccharide (LPS) into the growth medium of Gram negative bacteria *S.* Typhimurium.

Inhibition of enzyme activities. Phenolic compounds can exhibit antimicrobial activities through enzyme inhibition. According to Pimia and others (2005), phenolics extracted from berries exhibited antibacterial activity through inhibition of extracellular microbial enzymes. In *E. coli,* chlorogenic acid inhibited β-ketoacyl-ACP reductase (FabG), an enzyme responsible for fatty acid synthesis in bacteria (Prigent 2005). In the study conducted by Li and others (2008), chlorogenic acid competitively inhibited the binding of NADPH in fatty acid synthesis (FAS) which could lead to suppression of growth of bacteria. Flavonoids such as quercetin inhibited DNA gyrase which functions as catalyst of DNA supercoilling in prokaryotes (Cushnie and Lamb 2005). Another phenolic, sophoraflavone G and (-) epigallochatechin also inhibited energy metabolism (Maxwell 1997).

Inhibition of nucleic acid formation. Inhibition of nucleic acid formation was proposed as another mechanism of antibacterial action of phenolics (Cushnie and Lamb 2005). Caffeine inhibited protein synthesis by causing damage to bacterial DNA, inhibiting DNA repair and synthesis of protein in bacteria (Prigent 2005). According to Kaur and Singh (2008), phenolics from the *Livistona chinensis* fruit extract inhibited the growth of *S. aureus* through DNA, enzyme, and protein denaturation. Li and Trush (1994) studied a group of phenolics that consisted of biphenol, catechol, 1, 2, 4-benzenetriol, 2-methoxyestradiol, 2-hydroxyestradiol, diethylstilbestrol, butylated hydroxytoluene, butylated hydroxyanisole, fert-butylhydroquinone, and some phenolic acids such as ferulic acid, caffeic acid, chlorogenic acid, and eugenol caused strand breaks of model DNA.

Effect of oxygen and heat on the chemical reaction of phenolics in the presence of different food matrices

Organoleptic and nutritional qualities of agricultural products can be influenced by their phenolic components. Any reaction of phenolics that occurs such as oxidation might change the product quality (Damodaran 1996). It also can lead to the development of off flavor, undesirable color and loss of important nutrients. According to Amiot and others (1997), discoloration was considered to be an effect of phenolic oxidation, although it prevented lipid deterioration.

Oxidation of phenolic compounds can occur in the presence of O_2 to form *0*-quinones, unstable compounds which are highly reactive (Pierpoint 1969). Quinones tend to react through covalent reactions with other molecules in the food matrices including proteins, carbohydrate and lipids (Prigent 2005). Quinones are formed enzymatically by polyphenoloxidase (PPO) and peroxidase (Matheis and Whitaker 1984). The formation of quinones may also occur in the absence of enzyme and is known as non-enzymatic oxidation or chemical oxidation (Cheyner and others 1988). Polyphenoloxidase is an enzyme that is involved in the oxidation of phenolic compounds (Ayaz 2008). It induces oxidation of caftaric acid to form caftaric acid *0*-quinone which can be chemically oxidized to regenerate caftaric acid (Cheyner and others 1988). Other phenolic compounds such as chlorogenic acid and caffeic acid and related *o*-diphenol could be oxidized rapidly by o-diphenol oxidases to form chlorogenicquinone and caffeoquinone (Pierpoint 1969). Chlorogenicquinone also could be formed by oxidation of chlorogenic acid by PPO. This quinone forms covalent modifications with food matrices such as protein which strongly decreased its solubility (Prigent 2005).

Increasing the temperature (heat) could weaken the interaction between phenolic compounds and protein because they are dominated by hydrogen binding. Hydrogen binding and hydrophobic interactions are the main driving forces in phenolic compounds and protein interactions. An increase in temperature causes decrease in hydrogen bonding; but increase in hydrophobic interactions. For example, a simple phenol such as chlorogenic acid (CQA), a polar phenolic compound, will decrease its binding to protein with increasing temperature. Heating, in addition will decrease the quantities of phenolic compounds associated with carbohydrate (Deshpande and Salunkhe, 1982). In the presence of oils such as in canola, heating will reduce the phenolic sinapine, a soluble phenolic ester.

Extraction of phenolic compounds

The extraction of a phenolic compound depends on its solubility in water. Generally, water soluble antimicrobial compounds can be released or extracted from plant tissue by rupturing the tissue during sample preparation. Other methods such as juicing, steam distillation, or solvent extraction have also been employed (James 2007). Steam distillation is used to extract phenolic compounds in essential oils. Cowan (1999) reported that water was used to extract active compounds from plant and herbs.

According to Cowan (1999) and Zhong and others (2008), solvent extraction, either single or combination, has been used frequently for extraction of active compounds. Methanol (Taylor and others 1996), ethanol (Nakahara and others 1993), propanol (Fu and others 1998), acetone (Afolayan and others 1997), diethyl ether (Elzaawely and others 2006), ethyl acetate (Kallithraka 2009), chloroform (Perret and

others 1995; Ayafor and other 1994), or their combinations with water in different proportions have been used to extract phenolic compounds (Variyar and others 2003; Naczk and Shahidi 2006). In addition, extraction of phenolic compounds can be modified using hot extraction or superheated extraction (Palma and others 2001).

The extraction of phenolics is influenced by factors such as solvent polarity, ratio of solvent to sample and extraction time (Shahidi and Nazck 2004). Different polarity of solvent might influence the type of chemical or phenolic compounds recovered. Polyphenol (Lapornik and others 2005), phenolic acids such as chlorogenic, gallic, protocatechuic, and caffeic and essential oil (Manjang 2001; Kulisic and others 2004; Baydar and others 2004; Cimanga and others 2002; Solomakos and others 2008) have been extracted with water by distillation or hydrodistillation. Methanol was used to extract essential oil (Sagdic and others 2002), phenol, proantocyanidins and flavonoids (Skerget and others 2005), cathechol and resolcinols (Ortigoza and others 2002), and polyphenols (Turkmen and others 2005), while ethanol was used to extract some phenolic acids (Chun and others 2005), and flavonoids (Bros and others 2009). A non polar solvent such as diethyl ether combined with ethyl acetate was used to extract phenolic compounds of grape seeds (Kalithraka and others 1995). Nilasari (1998) reported that chloroform also was used to extract essential oil, phenol, and alkaloid.

Many extraction methods have been tested by modifying the ratio of solvents. Horax and others (2005) used 80 % methanol to extract the phenolic compound from bitter melons. Baydar and others (2004) extracted phenolic compounds of defatted grape material in a soxhlet apparatus using acetone: water: acetic acid (90:9.5:0.5 ratio) at 60^{o}C. Wen and others (2005), extracted phenolic acids from herbal medicines by dissolving the

materials in methanol/water/TFA (50:50:0.1) followed by sonication and centrifugation. In coffee, methanol is the preferred solvent to extract phenolic compounds (Variyar and others 2003; Coronell and others 2004). Hydroxynnamic acids of coffee were extracted with 40 % methanol (Casal and others 1999; Risso and others 2007). Moreover, a study by Variyar and others (2003) used 80 % methanol to extract some phenolics such as caffeine and chlorogenic acid from raw monsooned Arabica coffee. Methanol too was used to extract phenolic compounds in Arabica coffee pulp prior thiolysis HPLC (Coronel and others 2004).

Recovery of phenolics was influenced also by the extraction period (Shahidi and Naczk 2004). Variyar and others (2003) reported that caffeine and chlorogenic acid were extracted by soaking the sample in 80 % methanol for 2 h. Extraction of lichi fruit pericarp with 60 % ethanol for 3 h resulted in the highest recovery of phenolics (Zhong and others 2008). Extraction method also influenced the extraction time. Markom and others (2007) reported that 3 hours extraction using supercritical fluid extraction (SFE) in 10 % methanol and 10 % ethanol yielded the highest hydrolyzable tannins, although, combination of 50 % ethanol and water resulted in the same amount of tannins after 2 hours extraction. A study conducted by Lardy and others (2002) showed that extraction of water soluble phenolic compounds was done by maceration for 24 h. Hydroxynnamic acids also was extracted completely with 40 % methanol by maceration for 24 h (Casal and others 1999).

Determination of phenolic content

Reaction of phenolic compounds with a colorimetric reagent has been used frequently as a method to study a phenolic content (Vermerris and Nicholson 2006). Phenolic content or total phenolic can be determined using method of Folin Dennis (FD), Folin Ciocalteau (FC), and Prussian blue (PB) assay (Shahidi and Nazck 2004).

Folin Denis. The Folin Denis assay (FD) was used to determine phenolic contents (Shahidi and Nazck 2004) such as tannin. In 1912, it was claimed that FD was the surprising sensitive assay which detected blue color reaction (Folin and others 1912). In this study, the color was formed by reduction of phosphotungstic and phosphomolibdic reagent by polyphenolic compound and its reaction to uric acid. Schlesinger and Hasey (1981) reported that FD assay could determine total soluble phenolic content as tannin. Folin Denis was reported as the best assay available for phenolic determination. This assay was used widely for determination of total phenolic in plant materials and beverages (Sahidi and Nzack 2004). According to Singleton and Rossi (1965), there were some limitations in using FD assay, such as unreliable result if the samples are inadequately mixed or not following proper order of reactant addition, unstable blue color formation, and difficulties experiment using large sample. White and dense precipitates were formed in the presence of high concentration of FD reagents which interfere in the quantification of color intensity.

Folin Ciocalteau. Folin Ciocalteau assay is a substitution of the FD assay (Shahidi and Nzack 2004). Folin and Ciocalteau (1927) modified the FD assay by addition of lithium sulfate and bromine to the reagents which prevented any precipitation. According to Singleton and Rossi (1965) and George and others (2005), this assay

involves reaction of the Folin Ciocalteau (FC) reagent with phenolic compounds under alkaline solution. In the assay, the FC reagent oxidizes phenolic compounds through their hydroxyl sites (transferring electron from phenolic compounds) resulting in the production of complex molybdenum (Mo_8O_{23})-tungsten blue ($W8O_{23}$) complex which is known by phosphomolybdic /phosphotungstic acid complexes (Ainsworth and Gillespie 2007). The intensity of the blue color formed is directly proportional to the amount of phenolic compounds (s) present.

The FC assay was claimed to be the easiest and the most consistent method to yield total phenolic content in brown algae dissolved in 80 % methanol (Alstyne 1994). Singleton and Rossi (1965) mentioned that the FC assay had more advantages for total phenolic determination compared to the FD assay. However, Shahidi and Nazck (2004) reported that FC assay reacted with not only phenolic compound but also with those found in the extractable protein including ascorbic acid. The FC was used widely for determination of total phenolic of agricultural products. The assay has been used to quantify total concentration of phenolic hydroxyl groups present in wine by-products (Alonso and others 2002), bitter melon (Horax and others 2005), tomato extract (Luthria and others 2006), vegetables and fruit waste (Peschel and others 2006), red wine grape (Thimothe 2007), green coffee powder (Ramalaksmi and others 2007).

Prusian blue. Prusian blue assay also was used to quantitate polyphenolic compounds as tannin (Despandhe and Cheryan 1987). The reaction involved in Prusian blue assay was based on the formation of ferricyanide-ferrous ion complex by reduction of ferric to ferrous ion by polyphenolic compounds (Shahidi and Nzack 2004). This assay gave a higher total phenolic content of dry bean than did the FC assay (Despandhe and

Cheryan 1987). Carmona and others (1991) reported that although FD gave similar result as Prusian blue assay, Prusian blue assay was recommended for tannin determination due to its convenience.

Characterization of phenolic compounds

Several methods have been widely used to separate and identify specific chemical composition of phenolic compounds such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography (Vermerris and Nicholson 2006). Although TLC is the simplest, least expensive and fastest method for the separation of phenolic compounds (Vermerris and Nicholson 2006), Nazck and Shahidi (2004) reported that HPLC and GC were the most common methods used. A HPLC method was employed commonly to identify phenolic acids in berries, fruit and beverages (Matilla and others 2006), and herbal medicines (Wen and others 2005). It also was used to identify simple phenolics present in coffee (Casal and others 1999; Nardini and others 2002; Variyar and others 2003; Monteiro and others 2007; Perrone and others 2008; Urakova and others 2008).

Gas chromatography was applied by Kuwatsuko and Shindo (1973) to determine 13 phenolic acid compounds in rice straw and its decayed product. The study claimed 90 % recovery of phenolic acids such as *p*-coumaric, p-hydrobenzoic, vanillic and syringic acids, while ferulic was recovered only about 70 %. Gas chromatography-mass spectrophotometry was able to separate 8 phenolic acids and 4 flavonoids in methanolic and aqueous plant extracts (Fiamegos and others 2004). The GC-MS also was used

commonly for determination of phenolic compounds of essential oil in cortex cinnamomi (Gong and others 2004), and commercial essential oil (Baratta 1998).

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CHAPTER III

STUDY AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS OF ARABICA COFFEE BERRY PULP

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Abstract

Antibacterial compounds present in Hawaiian Arabica coffee berry pulp were extracted using water and methanol. The antibacterial activity was determined using a turbidity method. Antibacterial activity of 80 % methanol extract was higher when compared to water extract. Methanol extraction and 2 h reflux showed the highest antibacterial effect and this extract were further fractionated using chloroform, followed by ethyl acetate, then water and finally acidic methanol. Fractions were tested for antibacterial activity using disc method. Cultures used were foodborne pathogens and spoilage bacteria consisting of *E.coli* O157:H7 ATCC (35150, 933, 43890, and 43894), *Salmonella* Typhimurium 29631, *Pseudomonas aeruginosa*, *P. fluorescens*, *Listeria monocytogenes* Scott A, *L. monocytogenes* 39-2. *Staphylococcus aureus* 13565 and *Bacillus subtilis*. Phenolic were identified using Reverse Phase-High Performance Liquid Chromatography.

Fractionation of coffee pulp with methanol recovered 85 % of the dry extract. Water soluble fraction exhibited significantly higher antibacterial activity than chloroform and ethyl acetate fractions. Water fraction showed similar inhibition activity against *E. coli* O157:H7 strains. *Pseudomonas fluorescens* was the most sensitive bacteria to water fractions. *Listeria monocytogenes* strains were more susceptible to antibacterials recovered by chloroform than all other bacteria tested. Caffeine was observed as a dominant phenolic in Hawaiian Arabica coffee pulp. Chloroform was able to extract the highest amount of caffeine (705 µg/g wet pulp). Chlorogenic acid, caffeic acid, and *p*-coumaric acid were detected in ethyl acetate and water soluble fractions. In

conclusion, results indicated there were antibacterial compounds present in Arabica coffee berry pulp. It is possible that other antibacterial compounds were present in these fractions. Hawaiian Arabica coffee berry pulp may useful as a food preservative.

Introduction

Addition of synthetic chemical food preservatives is a means to inhibit microbial growth and control food spoilage and foodborne diseases (Gould 1995; Sinell 1995). However, consumers currently are worry about such chemical preservatives and the impacts it may have on their personal health (Naidu 2000). As a result, there is an increasing interest in the development of naturally derived antimicrobials as alternatives to synthetic chemical food preservatives (Roller 1995; Smid and Gorris 1999). A rich source of natural antimicrobial compounds is agricultural by-products (Chusnie and others 2005).

Arabica coffee is one of the products that were identified as a source of natural antimicrobial compounds due to its phenolic content (Toda and others 1989; Shetty and others 1994; Michels 2000; Mazzafera 2002). Research has been increasingly focused on the antibacterial activity of Arabica coffee bean against some pathogenic bacteria such as *Staphylococcus aureus, Vibrio parahaemolyticus, V. cholerae, Salmonella* Typhimurium, *S.* Typhi and *Escherichia coli,* (Toda and others 1989; Shetty and others 1994 and Michels 2000; Yuki and others 2003), however, investigation of coffee by-products including extraction of coffee berry pulp for antimicrobial agents has been very limited.

Extraction of phenolic compounds depends on their solubility in water. Various solvents, either singly or combined have been utilized for phenolic extraction from plant products (Zhong and others 2008). Examples include methanol, ethanol, ethyl acetate, chloroform at different combinations and concentration in water have been used to

extract phenolic compounds (Variyar and others 2003; Naczk and Shahidi 2006). However, methanol in water appears to be the preferred solvent to extract phenolic compounds from the coffee bean (Casal and others 1999; Variyar and others 2003; Coronell and others 2004; Risso and others 2007).

Due to the possibility of coffee berry pulp containing valuable compounds similar to those found in coffee bean, if extracted, these compounds may add extra value to coffee industry. Furthermore, it can be used as alternative natural food antibacterials. Therefore, the objectives of this research were to determine presence of antibacterial compounds, develop an efficient extraction method, analyze of phenolic and measure their inhibitory effect against foodborne pathogens and spoilage bacteria.

Materials and Methods

Coffee pulp

Coffee berry pulp was obtained from an Arabica coffee plantation in the Kona District, Hawaii, USA. Wet coffee berry pulp was obtained before coffee berry fermentation process and was transported to the food microbiology lab at the Robert M.Kerr Food and Agricultural Products center in Stillwater, Oklahoma. Coffee pulp was immediately frozen and kept at -20°C. Samples were thawed prior to the start of each experiment.

Solvents and reagents

Methanol, ethanol, chloroform, and ethyl acetate were obtained from Pharmaco-Aaper (Brookfield, CT), Amberlite resin XAD-2 (Supelco, Bellefonte, PA), Folin Ciocalteau reagent (Sigma Aldrich, St Louis, MO), sodium carbonate (EM Science, NJ), and acetonitrile (Fischer Chemicals, NJ). Phenolic acid standards consisted of caffeine, caffeic acid, gallic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, epicatechin (Sigma Aldrich, St Louis, MO), Trypticase Soy Broth, Bacto Peptone Agar (Becton, Dickinson Company, Franklin Lakes, NJ), and silicon bath oil (Foss Tecator AB, Hoganas, Sweden).

Methods

Water extraction of antibacterial compounds

Heating method. A water soluble extract of Arabica coffee pulp was prepared by following the procedure of Lardy and others (2002). Twenty grams of thawed coffee

pulp was added to 100 ml of distilled water. Sample was blended in a sterilized blender and poured into a sterilized bottle. Then, samples were heated at 100 ^{o}C for 30, 60 and 90 minutes. All samples were vacuum filtered through Whatman filter paper # 1 followed by centrifugation at 10,000 x g for 10 minutes at 2-4 ^{o}C. Supernatants were collected and filter sterilized through 0.45 μm filters and extracts were stored at 4 ^{o}C until antibacterial activity analysis was performed.

Agitation method. Twenty grams of pulp were blended with 100 ml of sterilized distilled water and poured into a sterile glass beaker covered with aluminum foil to protect it from light. Samples were stirred for 0, 24, 48, and 72 hours at room temperature on a stir plate. All samples were vacuum filtered, centrifuged and filter sterilized as described above. Extracts were stored at 4 ^{o}C until antibacterial activity test were performed.

Organic solvent extraction of antibacterial compounds.

Single extraction.

Effect of methanol concentration. Twenty grams of pulp were blended with 200 mL of solvent (50%, and 80% methanol). Slurry was refluxed for 2 hours at 65 °C in a Soctec silicon oil bath system (Tecator Soctec System HT 1046) (Tecator, Fairfiled, NJ), filtered and centrifuged as previously described. Extracts were adjusted to pH 6.5 and evaporated using RapidVap Labconco evaporator at 30°C for 40-43 h (Data was reported from one replication).

Effect of reflux time. Eighty grams of pulp were blended with 80% methanol and adjusted to 400 mL into a 500 mL round bottom conical flask. Samples were refluxed

at 65 °C for 1, 2, 3 and 4 hours and then filtered, centrifuged and solvents were evaporated as described above. Dried extracts were kept at 4°C in a closed container until antibacterial analysis was performed.

Fractionation

Extract previously obtained from 80% methanol and 2 hours reflux extraction was subsequently fractionated (Figure 3.1) using a method described by Zhang and others (2008). After reflux, sample was filtered and centrifuged. Supernatan was collected and evaporated in a Buchi Rotavapor R-3000 (Buchi Coorporation, New Castle, DE) at 40^{o} C for 90 minutes to a final volume of 80 mL. Forty milliliters chloroform was added, mixed well, poured into a separatory funnel and allowed to separate for several hours. A good separation of chloroform fraction and water solution was observed overnight. The chloroform fraction was collected. The water solution was further fractionated by adding 40 mL of ethyl acetate, shaking well and allow in the mixture to separate in a separatory funnel for 24 hours. The ethyl acetate fraction was collected. The aqueous fraction was further absorbed on an Amberlite (XAD-2) column, rinsed with distilled sterilized water followed by acidic methanol (adjusted to pH 3 with HCl). The water and acidic methanol fractions were collected. The fractionation method is shown in Figure 3.

All collected fractions were adjusted to pH 6.5. Water soluble fractions were freeze dried in a Labconco Freeze drier system (Freezemobile 12EL, Virtis, NY) for 72 hours whereas, chloroform, ethyl acetate and methanol acidic fractions were evaporated using a rotary evaporator at 40 ^{0}C. Each fraction was re-suspended in 2.0 mL of its solvent for antimicrobial activity test.

Amberlite resin column preparation. Twenty four (24) grams of XAD-2 (Amberlite Resin, Supelco, Bellefonte, PA) was added with 30 mL of 80 % methanol to cover a resin bed of 1-2 inches (2.5-5 cm), stirred carefully and let stand for 15 minutes. Methanol was discarded and replaced with sterile water and allowed to separate for 10 minutes. A dry and clean of column (20 cm x 1.5 cm) was filled up to approximately 1 inch (2-5 cm) with deionized water before adding the resin slurry. The slurry was poured slowly into the column, and excess water drained, maintaining the water flow until all the air bubbles were dislodged.

Bacterial Cultures

Several strains of *Escherichia coli* O157:H7 ATCC (933, 35150, 43890 and 43894), *Pseudomonas aeruginosa, P. fluorescens, Salmonella* Typhimurium 29631, *Listeria monocytogenes* Scott A, *L.monocytogenes* 39-2, *Staphylococcus aureus* 13565, and *Bacillus subtillis* were obtained from the culture collection of Dr. Stanley E. Gilliland at the Food Microbiology Laboratory – Robert M. Kerr FAPC, Oklahoma State University. Stock cultures were maintained at -70 ^{o}C. Prior to the antibacterial activity test, all strains were subcultured at least three times in Tryptic Soy Broth (TSB). Cultures were incubated at 37 ^{o}C for 16 to 18 h, except Pseudomonas that were incubated at room temperature.

Antibacterial activity test.

Turbidity method. Inhibitory effect of coffee berry pulp water extracts were determined by a turbidity method following modification method provided by Gilliland

and Walker (1990). One hundred millimeters of sterile Trypticase Soy Broth (TSB) was inoculated at 1% *E.coli* 1472 and 5 mL were dispensed into test tubes containing different volumes (0, 1, 2, 3, 4 and 5 mL) of coffee berry pulp extract. Final volumes were adjusted to 10 mL with distilled sterilized water. All tubes were incubated at 37^{o} C in a water bath. Culture growth was monitored using a spectrophotometer Spectronic 21 D (Spectronic Instruments, Rochester, NY). Although different concentrations of extract were tested (Appendix Table A.2) only results from 3 mL extract were reported due to the medium absorbance reading score collected. Absorbance readings at 620 nm were obtained every hour for 6 hours. Growth curves were constructed by plotting increases in absorbance reading versus incubation times. The number of hours required to increase the absorbance by 0.2 U in each sample was used to compare the antibacterial activity of coffee pulp extract against bacteria tested.

Paper disc method. Potential antibacterial activity of each extracted fraction was tested using paper disk method. Volumes of 75, 100 or 200 µL of each re-suspended fraction sample was added to a 1.0 or 1.3 cm diameter paper disc on the surface of 5 mL of agar inoculated with 1% of specific culture. Plates were incubated at 37 ^{o}C for 18 h and diameters of clear inhibition zones were measured. Antibacterial activities of the extracts were reported as diameter (mm) of clear zones (coffee pulp fraction-control).

HPLC analyses of coffee pulp fractions

All collected fractions were analyzed for presence of phenolics by high performance liquid chromatography (HPLC) using a modified method of Thimothe and others (2007). Dried extracts of water soluble fractions were resuspended in sterilized

distilled water to a final concentration of 100 mg/mL, whereas, dry extracts of chloroform fractions and ethyl acetate fractions were dissolved in methanol (HPLC Grade) to a final concentration of 10 mg/mL. All samples were filtered through 0.2 µm filter prior to analysis by HPLC. Volumes of 50 µL ethyl acetate and water soluble fractions, 20 µL chloroform fractions; and 20 µL standards were injected (concentration stated below).

The phenolics were analyzed using a Reverse Phase- HPLC system (Alliance Waters 2690, Waters, Ireland) with a photodiode array detector (PDA, Waters 2996). Phenolic compounds were separated by a gradient elution system on a Sun Fire^{TM} C_{18} column (5µm particle size, 4.6 x 250 mm i.d.) including a guard column (5µm particle size, 4.6 x 30 mm) at 25^{o}C. The flow rate was adjusted to 1.0 mL/min. For gradient elution mobile phases A and B were employed. Solution A contained 0.1 % of H_3PO_4 in deionized water, and solution B contained 0.1 % H_3PO_4 in acetonitrile (HPLC grade) and these were applied (for 65 minutes) as follows: 0-50 min, from 95% A and 5% B to 65% A and 35% B linear gradient; 50-60 min from 65% A and 35% B to 95% A and 5% B; 60-65 min held at 95% A and 5 % B. The PDA was set at 210-600 nm and chromatograms were extracted at λ_{280} and λ_{320} nm (Mattila and others 2006). Individual phenolics were identified by comparison of UV-Vis spectra and retention time, and were quantified using millennium^{32} (Waters) software base on standard calibration curves.

$$\frac{\text{Phenolic acid from HPLC data } \left(\frac{mg}{mL}\right) x \left(\frac{\text{dry fraction (mg)}}{\text{Concentration of fraction } \left(\frac{mg}{mL}\right)} \right) x \frac{\text{Injection volume of standards } (\mu L)}{\text{Injection Volume of fraction } (\mu L)}}{\text{weight of pulp (g)}}$$

A mixture of 1mg/mL of each standard phenolic (gallic acid, caffeine, chlorogenic acid, caffeic acid, epicathecin, *p*-coumaric acid and ferulic acid) was prepared and diluted to obtain 0.25, 0.50, 0.75 and 1.0 mg/mL solutions to build calibration curves. All calibration curves showed an r^2 of 0.974 to 0.999 (Appendix Table D.1).

Statistical Analysis

Analysis of data from three replicated experiments was performed using analysis of variance (ANOVA) with General Linear Model (GLM) procedure in SAS/STAT ^{R} Software, version 9.1.3. (SAS Institute 2002-2003). The main and interaction effects were considered significant if $p \leq 0.05$.

Results and Discussion

Previous studies indicated presence of phenolic acids with antibacterial properties in coffee bean (Shetty and others 1994; Michels 2000). Few studies have been done with coffee berry pulp. Due to the large amounts of pulp (coffee waste) that are produced annually, there is an urgent need to find any potential application or extracting any potential valuable compound present in this coffee waste. Thus, Hawaiian Arabica coffee pulp was studied for the presence of antibacterial compounds. Adawiyah (1998) and Moreira and others (2001) reported that aqueous extraction was used in preliminary studies because of its lower cost, easy of application, and because no extra hazardous waste is generated.

Water extraction of antibacterial compounds by heating

Several soluble phenolic extraction systems using water and/or organic solvents have been previously described in the literature (Variyar and others 2003; Naczk and Shahidi 2006). Heating processes inhibit polyphenoloxidase, thereby reducing phenolic acid oxidation (Moreira and others 2001). Effect of heating time on the extraction of antibacterial compounds was determined by the time required for the absorbance for each to increase 0.2 units during the growth of *E.coli* 1472 at 37^{o}C (Table 1; Absorbance growth is listed in Appendix Table A.1).

Inhibition activity of the extract against *E. coli* 1472 was indicated by longer time (ranged from 2.28 hours to 5.67 hours) required for the absorbance to reach an increase of 0.2 units during growth of *E.coli* 1472 compared to control (1.89 hours). Data analysis

showed that *E. coli* culture in the control group grew significantly faster than in the coffee extract group ($p<0.05$). Significant differences were also observed at different heating times ($p<0.05$). Coffee pulp extracted by heating 30 and 60 minutes exhibited strongest inhibition activity which was indicated by significantly slower growth (5.59-5.67 minutes) compared to 0 and 90 minutes of heating time. Significant increase in bacterial growth also was observed in samples extracted after 90 min of heating compared to 30 and 60 minutes. Deshpande and Salunkhe (1982) reported that increasing the extraction time at high temperature will weaken hydrogen bonding and decrease the phenolic interaction. The antibacterial activity of coffee berry pulp extracts declined as the temperature and the heating period increased.

Water extraction of antibacterial compounds by agitation at room temperature

Concentration of phenolic compounds extracted from coffee berry pulp was influenced by agitation at room temperature or maceration. Maceration was defined as a preparation of extract by soaking the raw materials in water or organic solvents (Adawiyah, 1988). According to Kelebek and others (2006), maceration conditions greatly impacted the extraction of the phenolic compounds. Extraction by agitation at room temperature for 0h, 24h, 48h and 72 h was conducted. Results are shown in Table 2. Data analysis indicated a significant effect of agitation time on antibacterial activity ($p<0.05$). Coffee berry pulp extracts significantly decrease culture growth. The most significant effect was observed with extracts obtained after 48 h agitation time which significantly different to other agitation time and was indicated by the longest time taken to increase 0.2 units of absorbance (Culture growth is listed in Appendix Table A.2).

Time required for the culture to increase the absorbance units in the presence of extracts ranged from 3.88 to > 6.00 hours.

Results showed that antibacterial compounds present in coffee berry pulp can be extracted with water. Similar results were obtained by Arora and others (2009) after boiling coffee powder in water. Distillation or hydrodistillation extracted active compounds such as polyphenol (Lapornik and others 2005), phenolic acids and essential oils (Solomakos and others 2008); however, Cowan (1999) and Zhong and others (2008) frequently used combinations of water with other solvents to extract active compounds. To confirm that antibacterial activity was not due to low pH (3.8) of water extracts, a new antibacterial analysis was conducted by a turbidity method with extracts which pH was adjusted to 6.5. No differences on absorbance readings were observed between control and pH 6.5 extracts (Figure 4). This may be due to the de-protonization of hydroxyl groups of phenolic compounds which eventually lead to the formation of quinones (Nazck and Shahidi 2004). Yabuta and other (2001) also reported that NaOH solutions commonly used for increasing the pH value will oxidize phenolic to form quinones. Furthermore, this treatment decreased phenolic's ability as antibacterial. In our study the inhibitory effects of phenolics recovered from water extracts, when adjusted to pH 6.5, resulted in higher levels of *E.coli* (8.76 log cfu/mL) after 6 h incubation when compared to unadjusted pH 3.8 (6.73 log cfu/mL). The control had 8.81 log cfu/mL of *E.coli* 1472. Based on the turbidity method above, antibacterial activity of phenolics recovered from coffee pulp extracted with water was highly dependent on pH. However results obtained from the plate count method suggest even with adjusted pH, there was antibacterial activity still present.

Effect of methanol concentration on extraction of antibacterial compounds from Arabica coffee berry pulp

Extraction of phenolic compounds is influenced by solvent polarity, ratio of solvent to sample, and extraction time (Shahidi and Nazk, 2004). Different polarity of solvent might influence the type of chemical or phenolic compound recovered. A polar solvent such as water has been used to extract polyphenol, phenolic acid and essential oils (Lapornik and others 2005; Manjang 2001). However, not all phenolic compounds can be extracted with water due to their diverse polarities and the variability of raw material. In coffee extractions, methanol in different ratio to water is the preferred solvent to extract phenolic compounds (Casal and others 1999; Variyar and others 2003; Coronell and others 2004, Horax and others 2005; Risso and others 2007).

Coffee berry pulp was mixed with 50% and 80% v/v methanol solutions and extracts were obtained after 2h reflux at 65°C. Antibacterial effect of methanol extracts was tested against *E. coli* 1742 by disc method and compared with water extracts (Table 3.0). Methanol solvent was used as a control on the disc. Methanol extracts showed higher inhibition against *E. coli* 1472 than water extract and no inhibition was observed from control treatment. The water extracted fraction produced a turbid zone around a paper disc instead of a clear halo indicating some antibacterial or potential bacteriostatic effect but not as strong as methanol extracted fractions. This result suggested that methanol was more efficient in extracting higher concentration of antibacterial compounds. Therefore, methanol was used for further experiments.

The antibacterial activity of coffee berry pulp extracts was influenced by extraction time. Extraction periods, usually varying from 1 min to 24 hours have been

reported (Naczk and Shahidi, 2004). Effect of reflux time during methanol extraction of antibacterial compounds was studied (Figure 5 and Appendix Table A.3). Methanol extracts obtained after 2 h reflux showed higher inhibition activity than reflux for only 1h. Heat application during reflux improved the extractions of phenolic compounds, therefore higher inhibition was observed. Adawiyah (1998) reported that temperature is one of the factors that influence the speed of extraction. High temperature will increase diffusion coefficient of solvent into the sample; furthermore, it will increase the yield and shorten the extraction time. However, Deshpande and Salunkhe (1982) reported that increasing the extraction time also will reduce antimicrobial activity.

Fractionation of coffee berry pulp extracts

Extracts from coffee berry pulp previously obtained from 80% methanol extraction and 2 h reflux were further fractionated. Multi step extraction or fractionation consisted of four steps started with a non polar solvent (chloroform). It was followed by semi polar fractionation with ethyl acetate, then polar fractionations with water and finally acidic methanol. Dried extracts obtained from 80 g coffee berry pulp after extraction with 80% methanol and 2h reflux averaged 6.28 g. After fractionation, total dried extracts averaged 5.03g indicating about 85% recovery (Appendix Table B.1). Water soluble fraction yield was significantly higher than other fractions (92.97%) (Appendix Table B.2). Similar results were obtained by Yen and others (2005) testing the yield of water extracts of roasted coffee residue compared to those extracted with methanol, ethanol and hexane.

Antibacterial activity of coffee berry pulp fractions

Several foodborne pathogens and food spoilage bacteria were selected as indicators for antibacterial activity of all coffee berry pulp extracted fractions. Antibacterial activity of each coffee berry pulp fraction adjusted to pH 6.5 was studied using a paper disc method. Antibacterial activity was indicated by a clear inhibition zone around the disc after incubation for 18 hours at 37 ^{o}C. Results are shown as diameter of inhibition halo (mm) in Table 4. Wider inhibition halo indicated stronger antibacterial activity of the fractions.

Since acidic methanol fractions showed none inhibition activity against *E.coli* 1472 in previous experiments, only chloroform, ethyl acetate and water soluble fractions were analyzed. Inhibitory effect of coffee berry pulp extracts was significantly influenced by bacterial species and type of fractions ($p<0.05$) (Table 4) (Appendix Table C and Figure C.1). In general, all fractions produced some inhibitions against all tested cultures. However, water soluble fraction exhibited significantly higher antibacterial activity than chloroform and ethyl acetate fractions ($p<0.05$).

Water soluble fraction showed similar inhibition activity against *E.coli* O157:H7 strains. It seems that the fraction was more effective in reducing the growth of *E.col*i O157:H7. Similar inhibition activities also were exhibited by chloroform and ethyl acetate against these strains except for *E.coli* O157:H7 35150. Chloroform fraction showed similar antibacterial activity against salmonella strains. It had significantly higher inhibitory effect on *L. monocytogenes* than ethyl acetate fraction which showed similar effect. Among the group of Gram negative bacteria, *Pseudomonas fluorescens* was the most sensitive bacteria to water soluble fractions. *L. monocytogenes* strains (Gram

positive bacteria) were more susceptible to chloroform fraction than all others bacteria tested.

Similar results using coffee extracts were reported by Shetty and others (1994), and Michels (2000). The growth of Gram positive bacteria such as *B. subtilis,* and *S. aureus,* were inhibited by ethyl acetate and chloroform fractions of coffee residue (Nishina and others 1994). Their study showed that ethyl acetate fractions exhibited the strongest antibacterial activity followed by chloroform fractions, and no inhibition was found in water fractions. Antibacterial activity of ethyl acetate was similar to the study conducted by Daglia and others (1994). In the study, compounds extracted from roasted coffee bean with ethyl acetate exhibited inhibition activity against *S. aureus*. Our study indicated that *S. aureus* was inhibited strongest by both ethyl acetate fraction and water soluble fraction. A Study by Arora and others (2009) also showed inhibition activity of water extract of coffee Arabica against Gram positive bacteria such as *S. aureus* MTCC 87 and against Gram negative bacteria such as *P. aeruginosa* (MTCC 1457) and *S*. Typhi (MTCC 531). *Salmonella* Typhimurium also was found to be inhibited by Arabica coffee extract as reported by Shetty and others (1994).

All fractions of coffee pulp exhibited inhibition activity against all Gram positive and Gram negative bacteria but in general, the Gram positive bacteria tested were more susceptible coffee pulp fractions than Gram negative bacteria .This finding might be attributed to the difference in the cell wall composition of the bacteria. Gram positive bacteria have two layer consisting of peptidoglican and cell wall while Gram negative bacteria has three layers consisting of cytoplasmic, peptidoglican, and outer membrane layer which act as a barrier for antimicrobial compounds (Lugtenberg and Alpen 1983).

Separation and analysis of phenolic by HPLC

The phenolic content of coffee berry pulp extracted fractions was analyzed using Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and concentration of phenolics was calculated based on calibration curves (Appendix Figures D1-7 and Table D.1). Phenolic found in Hawaiian Arabica coffee berry pulp extracted fractions are shown in Table 5. Caffeine was present in all fractions. However, the amount of caffeine found in chloroform extract was significantly higher ($p < 0.05$) when compared to ethyl acetate and water soluble fractions. Caffeine was the only phenolic detected in the chloroform fraction.

Chlorogenic acid, caffeic acid and *p*-coumaric acid were detected in ethyl acetate and water soluble fractions. Ethyl acetate was more non polar than water; however it was able to extract some phenolic acids that also dissolve in water such as chlorogenic acid, caffeic acid and *p*-coumaric acid. This may be due to the polarity of ethyl acetate. Byers (2003) reported that ethyl acetate has 4.4 of polarity index and has solubility of 8.7 % (w/w) in water. Therefore, it might allow ethyl acetate to dissolve some polar compounds that also dissolve in water soluble fractions.

Table 5 shows that Hawaiian Arabica coffee pulp extract contained 0.849 mg of caffeine/gram pulp (wet matter) which is similar to 1.1 % (dry matter basis) of the coffee pulp. This is strongly in agreement to the study reported by Pandey and Soccol (1988) as cited by Mazafera (2002). The study reported the amount of caffeine as about 1.3 % of coffee pulp (dry matter basis). Similar results also identified 1.2 % (w/w) of caffeine in Brazilian Arabica coffee husk (Brand and others 2000). Caffeine is one of the major

compounds in coffee species. Higher amount of caffeine (1.89 %, w/w) was observed in coffee bean roasted (Bohsain and others 1999).

Caffeine also was found as a dominant compound in the ethyl acetate fractions. Some phenolic identified in this experiment were also reported by Martinez (1988). He reported phenolic acids of Venezuelan-Arabica coffee pulp extracted using ethyl acetate included protocathecuic acid, cathecin, chlorogenic acid, epicathecin, caffeine, isochlorogenic acid (I, II, and III), and ferrulic acid with chlorogenic acid as predominant (42.2%). However, chlorogenic acid in Hawaiian Arabica coffee pulp extracted with ethyl acetate was 0.011 mg/g pulp and small amounts of caffeic acid, coumaric acid and ferrulic acid. A water soluble fraction contained caffeine (0.043 mg/g pulp) and small amounts of chlorogenic acid, caffeic acid and *p*-coumaric acid.

Table 1. Comparison of time needed to reach 0.2 units of absorbance growth of *E.coli* 1472 in the presence of coffee pulp extracted by heating

Extraction time at 100 °C (minutes)[1]	Hours[2]
Control[3]	1.89[d]
0	2.28[c]
30	5.59[a]
60	5.67[a]
90	4.70[b]

[1] 20 g of coffee pulp in 100 mL deionized water heated in bath at 100° C; assay tubes contained 3 mL extract, 2 mL deionized water and 5 mL TSB

[2] Time for A620nm of culture to increase by 0.2 units; each values is an average from three experiments

[3] No extract added in control trial

[abcd]Means with the same letter are not significantly different

Table 2. Comparison of time needed to reach 0.2 units of absorbance growth of *E.coli* 1472 in the presence of coffee pulp extracted by agitation

Extraction time (hour)[1]	Hours[2]
Control[3]	1.79[c]
0	3.88[b]
24	4.31[b]
48	>6.00[a]
72	4.48[b]

[1] 20 g pulp in 100 mL deionized water blended and agitated; assay tubes contained 3 mL extract, 2 mL deionized water and 5 mL TSB

[2] Time for A620nm of culture to increase by 0.2 units; each values is an average from three experiments

[3] No extract added in control trial

[abcd]Means with the same letter are not significantly different

Table 3. Influence of methanol concentration and water during extraction of antibacterial compounds from coffee berry pulp[1] on their inhibitory effect against *Escherichia coli* 1472.

Extraction	Diameter of inhibitory zone (mm)		
	100 µL[1]	150 µL[1]	200 µL[1]
80 % methanol[2]	13.0	15.7	22.7
50 % methanol[2]	11.5	13.5	22.0
Water[3]	14.0[4]	18.0[4]	21.0[4]

[1]Volume of extract adjusted to pH 6.5 placed on disc, extracts were resuspended in water
[2]Refluxed for 2 hr at 65 ^{o}C; 20 g sample + 200 mL of 80 % and 50 % methanol
[3]Extracted (20 gram sample + 200 mL deionized water) for 0 hr at room temperature
[4]Turbid, not clear inhibitory zone

Table 4. Antibacterial activity of coffee berry pulp extracted fractions against foodborne pathogens and spoilage bacteria

Culture	Diameter of inhibition halo (mm)[1]		
	Chloroform[2] B	Ethyl acetate[2] C	Water[2] A
E.coli O157:H7 933	6.0^{Bfg}	4.7^{Bcd}	15.0^{Ade}
E.coli O157:H7 35150	4.3^{Cg}	18.7^{Aa}	16.0^{Bde}
E.coli O157:H7 43890	6.0^{Bfg}	5.0^{Bc}	15.3^{Ade}
E.coli O157:H7 43894	5.3^{Bg}	4.7^{Bcd}	14.7^{Ade}
Salmonella Typhimurium	17.7^{Abc}	3.0^{Bde}	18.3^{Ac}
S. Typhimurium 29631	19.3^{Ab}	18.0^{Aa}	14.3^{Be}
P. aeruginosa	8.0^{Bfg}	2.7^{Ce}	15.0^{Ade}
P. fluorescens	15.0^{Bcd}	11.3^{Cb}	24.0^{Aa}
L.monocytogenes 39-2	21.7^{Aab}	18.3^{Ba}	15.7^{Cde}
L.monocytogenes Scott A	24.0^{Aa}	17.3^{Ba}	20.0^{Ab}
S. aureus	10.0^{Bef}	17.3^{Aa}	16.3^{Ad}
B. subtilis	13.3^{Ade}	3.0^{Bde}	15.3^{Ade}

[1] Data shown as average of three experiments
[2] Volume of 75 µL extract adjusted to pH 6.5 was placed on discs
[abcde]Means in the same column with the same letter are not significantly different
[ABC]Means in the same row with the same letter are not significantly different

Table 5. Phenolics content in coffee berry pulp extracted fractions

Fractions	Phenolic acid content (µg/g pulp)[1]			
	Caffeine	Chlorogenic acid	Caffeic acid	*p*-coumaric acid
Chloroform	700.0[a]	ND	ND	ND
Ethyl acetate	42.2[b]	0.4	1.8	1.1
Water soluble	107.2[b]	31.7	11.1	6.0
Total phenolic acid/g pulp	849.4	32.1	12.9	7.1

[1] Data shown as average of three replicated experiments; caffeic acid was only present in one sample of water soluble fraction;. ND= non detected

[ab]Means with the same letter are not significantly different

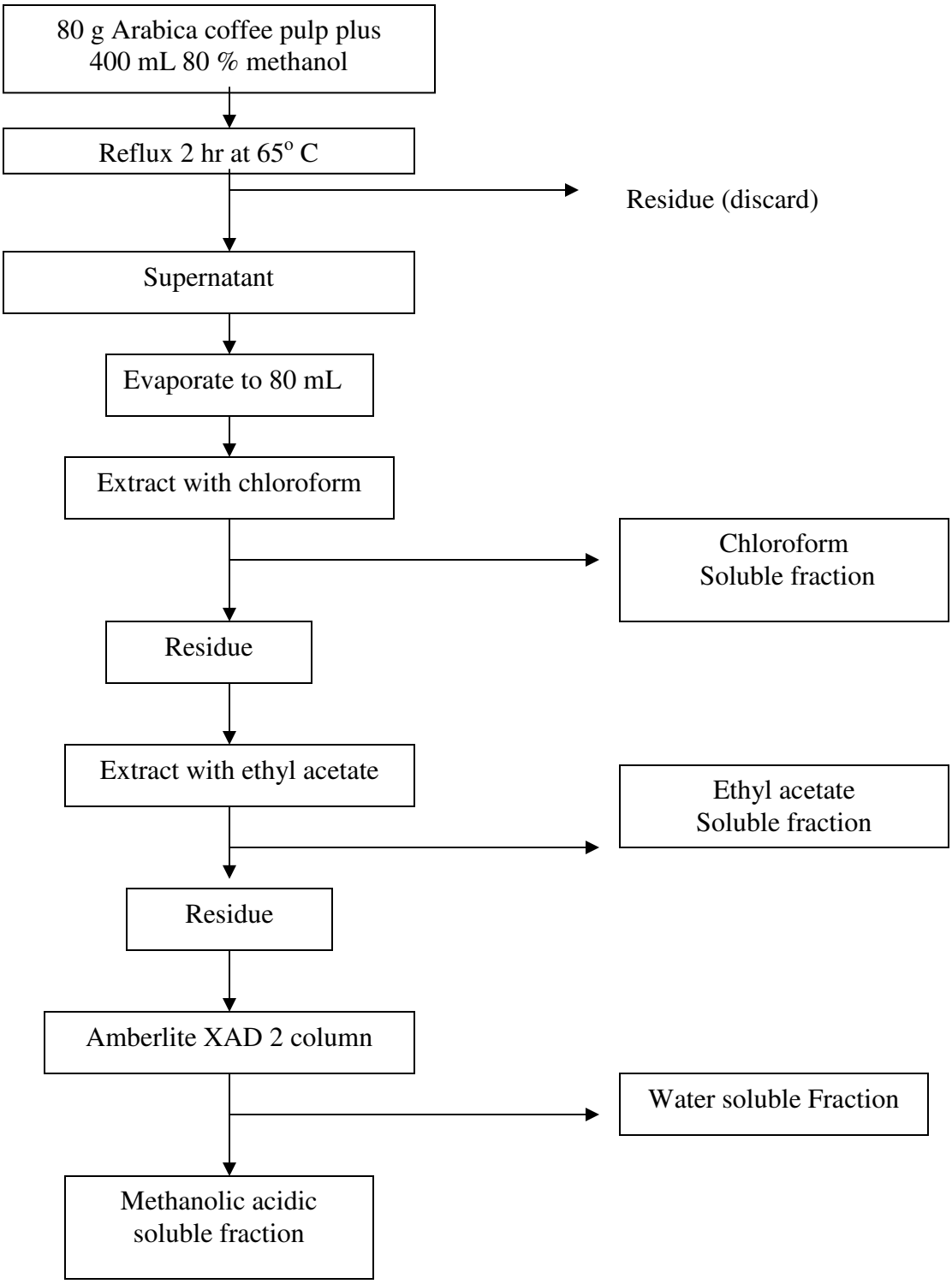

Figure 3. Schematic diagram of coffee pulp fractionation

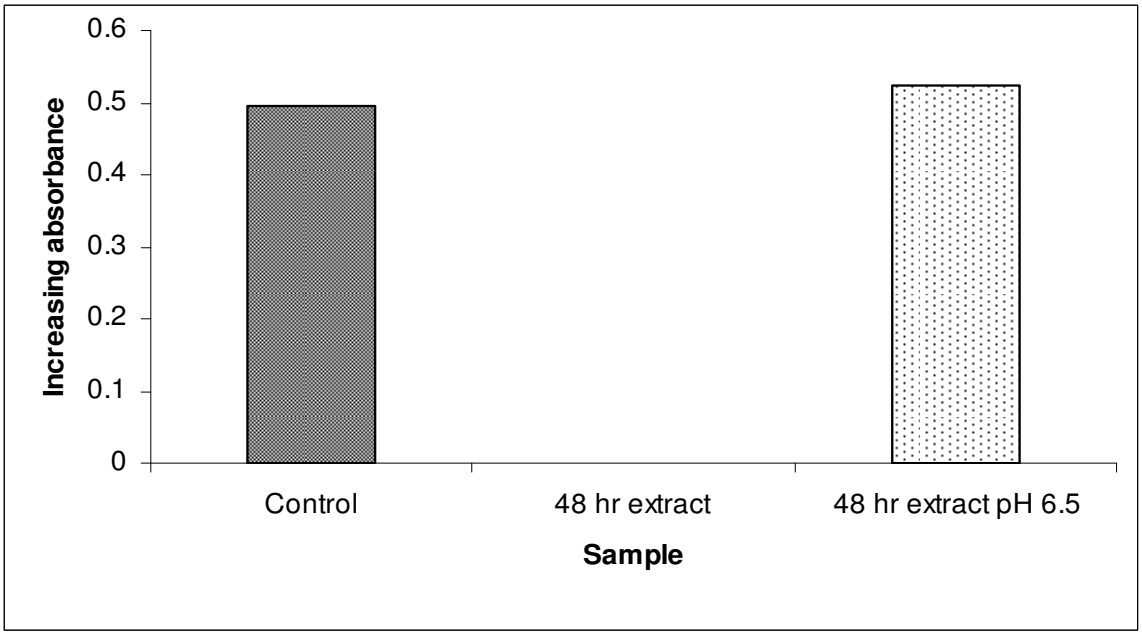

Figure 4. Comparison of antibacterial effect of methanol extract with initial pH and pH 6.5

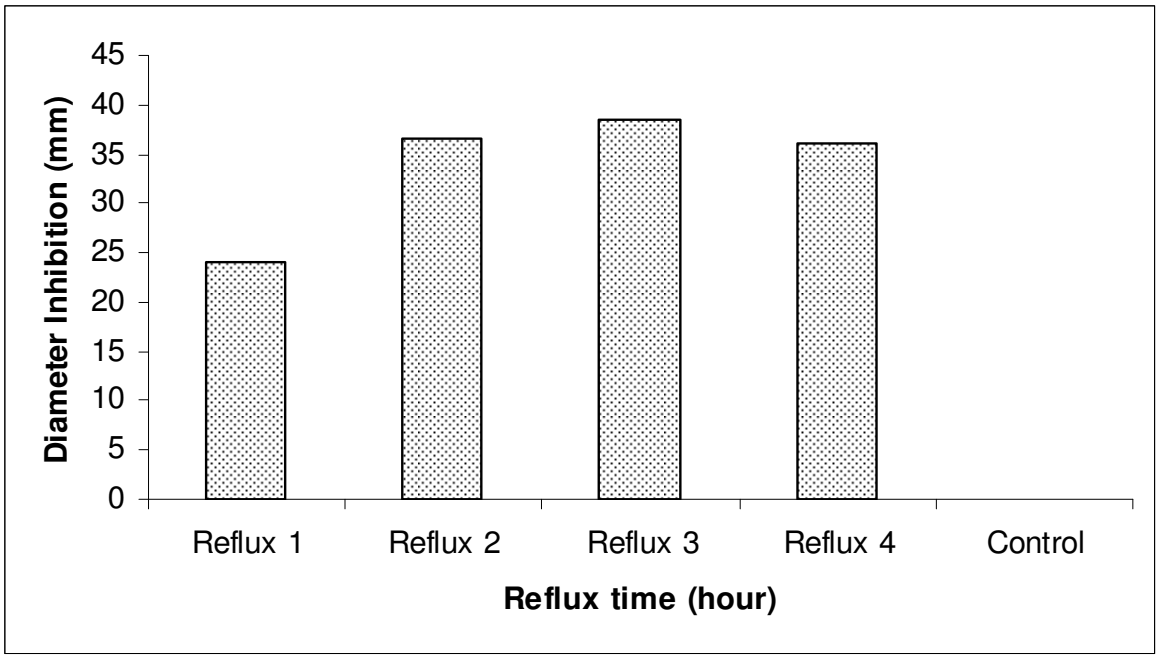

Figure 5. Influence of reflux time during extraction of antibacterial compounds from coffee berry pulp on their inhibitory effect against *Escherichia coli* 1472

Summary of findings

Antibacterial compounds present in coffee berry pulp can be extracted with water. Extraction through heating at 100 ^{o}C and agitation at room temperature significantly reduced bacterial growth ($p<0.05$). Extracts obtained after heating samples for 30 and 60 minutes showed significantly slower growth of *E.coli* 1472 compared to control and also most significant effect was observed with extracts obtained after 48 h agitation time. Higher inhibition of *E.coli* 1472 was exhibited by 80 % methanol extract compare to water extract. Thus, methanol was more efficient extracting higher concentration of antibacterial compounds. Methanol extracts obtained after 2 h reflux showed higher inhibition than reflux for only 1 h.

Fractionation of coffee berry pulp resulted 85 % of fraction recovery. Water fraction exhibited significantly higher antibacterial activity than chloroform and ethyl acetate fractions. Water fractions showed similar inhibition activity against *E.coli* O157:H7 strains. *Pseudomonas fluorescens* was the most sensitive bacteria to water fractions. *Listeria monocytogenes* strains were more susceptible to chloroform fractions than all others bacteria tested. Caffeine was observed as a dominant phenolic in Hawaiian Arabica coffee pulp. Chloroform was able to extract the greatest amount of caffeine (705 μg/g wet pulp). Chlorogenic acid, caffeic acid, and *p*-coumaric acid were detected in ethyl acetate and water soluble fractions. In conclusion, results indicated there were antibacterial compounds present in Arabica coffee berry pulp. It is possible that other antibacterial compounds were still present in these fractions. Results indicated that extracts of Hawaiian Arabica coffee berry pulp may useful as a food preservative.

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APPENDIX A

EFFECT OF WATER EXTRACT OF COFFEE BERRY PULP ON THE GROWTH OF *E.coli* 1472

Table A.1. Absorbance reading of *E.coli* 1472 growth in the presence of coffee berry pulp water extract by heating

Treatment	Time (minute)	Extract (mL)	A_{620} nm [3]					
			1	2	3	4	5	6
Control[1]		0	0.027	0.250	0.458	0.479	0.484	0.487
Heating[2]	0	5	0.021	0.024	0.024	0.026	0.025	0.030
		4	0.017	0.021	0.026	0.039	0.052	0.075
		3	**0.035**	**0.183**	**0.368**	**0.434**	**0.453**	**0.454**
		2	0.037	0.197	0.367	0.423	0.442	0.453
		1	0.044	0.274	0.433	0.479	0.492	0.502
	30	5	0.134	0.139	0.137	0.140	0.140	0.145
		4	0.109	0.113	0.113	0.118	0.123	0.134
		3	**0.104**	**0.115**	**0.150**	**0.211**	**0.277**	**0.318**
		2	0.087	0.202	0.359	0.417	0.432	0.442
		1	0.165	0.294	0.448	0.492	0.499	0.508
	60	5	0.156	0.160	0.160	0.162	0.162	0.168
		4	0.125	0.128	0.131	0.134	0.135	0.146
		3	**0.105**	**0.119**	**0.153**	**0.210**	**0.276**	**0.315**
		2	0.102	0.219	0.371	0.428	0.450	0.463
		1	0.084	0.303	0.447	0.485	0.496	0.505
	90	5	0.204	0.207	0.207	0.210	0.211	0.217
		4	0.171	0.175	0.178	0.186	0.193	0.208
		3	**0.150**	**0.170**	**0.218**	**0.305**	**0.368**	**0.404**
		2	0.263	0.266	0.415	0.476	0.480	0.488
		1	0.099	0.318	0.461	0.506	0.505	0.523

[1] No coffee pulp extract added in the treatment
[2] Heating was conducted at 100 ^{o}C for 0, 30, 60 and 90 minute
[3] Each value is the mean from 3 trials

\

Table A.2. Absorbance reading of *E.coli* 1472 growth in the presence of coffee berry pulp water extract by agitation

Treatment[1]	Time (hour)	Extract (mL)	A_{620} nm						
			0	1	2	3	4	5	6
Control		0	0.009	0.065	0.258	0.369	0.389	0.402	0.408
Agitation	0	5	0.054	0.057	0.059	0.062	0.063	0.064	0.072
		4	0.049	0.056	0.061	0.078	0.104	0.128	0.169
		3	**0.043**	**0.052**	**0.092**	**0.162**	**0.258**	**0.306**	**0.330**
		2	0.039	0.068	0.198	0.323	0.363	0.375	0.387
		1	0.028	0.075	0.251	0.349	0.386	0.392	0.403
	24	5	0.044	0.040	0.046	0.048	0.054	0.051	0.061
		4	0.036	0.036	0.044	0.056	0.074	0.087	0.125
		3	**0.032**	**0.044**	**0.075**	**0.130**	**0.219**	**0.277**	**0.311**
		2	0.026	0.061	0.193	0.317	0.360	0.370	0.380
		1	0.021	0.072	0.245	0.350	0.384	0.384	0.399
	48	5	0.083	0.093	0.091	0.092	0.097	0.093	0.094
		4	0.077	0.087	0.085	0.088	0.091	0.096	0.103
		3	**0.053**	**0.066**	**0.081**	**0.111**	**0.154**	**0.196**	**0.240**
		2	0.440	0.072	0.159	0.272	0.340	0.359	0.370
		1	0.031	0.077	0.244	0.349	0.381	0.388	0.402
	72	5	0.040	0.047	0.046	0.044	0.048	0.051	0.051
		4	0.034	0.043	0.045	0.051	0.061	0.075	0.086
		3	**0.027**	**0.040**	**0.067**	**0.116**	**0.199**	**0.261**	**0.297**
		2	0.024	0.056	0.167	0.292	0.351	0.359	0.373
		1	0.025	0.074	0.248	0.356	0.390	0.386	0.404

[1] No coffee pulp extract added in the treatment
[2] Agitation was conducted at room temperature for 0, 24, 48 and 72 hours
[3] Each value is the mean from 3 trials

Table A.3. Effect of reflux time during bacterial compounds extraction from coffe berry pulp on their microbial inhibition against *E.coli* 1472

Reflux time[1] (hr)	Zone Inhibition (mm)[2]		
	100 µL	150 µL	200 µL
1	18.0	18.0	24.0
2	20.0	27.0	36.5
3	19.0	29.5	38.5
4	20.0	29.5	36.0

[1] Reflux was conducted at 65^0C in soctec silicon bath oil
[2] Paper disc diameter was 10 mm; Tryticase soy agar was inoculated with 10^7 cfu/mL bacteria

Table A.4. The effect of pH adjustment of reflux extraction on the antibacterial activity

Sample	pH		Zone Inhibition (mm)[1]		
	Liquid Extract	Adjusted	100 µL	150 µL	200 µL
Methanol 80 %	5.05	6.51	13.0	15.7	22.7
Methanol 80 %	5.05	-	22.0	28.7	35.0
Methanol 50 %	4.56	6.54	11.5	13.5	22.0
Methanol 50 %	4.56	-	19.0	25.0	33.7
Water	3.94	6.54	14.0*	18.0*	21.0*
Water	3.94	-	13.0	18.0	19.0

*Inhibition with turbid zone. [1] Tryticase soy agar were inoculated with 10^7cfu/mL bacteria

APPENDIX B

FRACTIONATION OF COFFEE PULP EXTRACT

Table B.1. Dry extracts before and after fractionation and their recovery

Extract	Dry Extract (g)[1]	Total Fractions (g)[2]	Recovery Fractions (%)[3]
I	5.76	4.91	85.17
II	6.22	5.35	85.98
III	5.75	4.83	83.97
Average	5.91	5.03	85.04

[1] Dry extract obtained from 80 g coffee berry pulp after extraction with 80% methanol and reflux for 2h
[2] Total dry extract obtained after fractionation
[3] Recovery fractions calculation were total fractions (g) / dry extract (g)

Table B.2. Dry extract yield obtained after fractionation

Fractions	Dry extract (g)[1]	Yield/g dry extract (%)	Yield / g pulp (%)
Chloroform	0.158	3.14	0.198
Ethyl acetate	0.099	1.97	0.124
Water soluble	4.673	92.97	58.41
Methanolic acidic	0.096	1.91	0.120

APPENDIX C

ANTIBACTERIAL ACTIVITY OF COFFEE PULP FRACTIONS

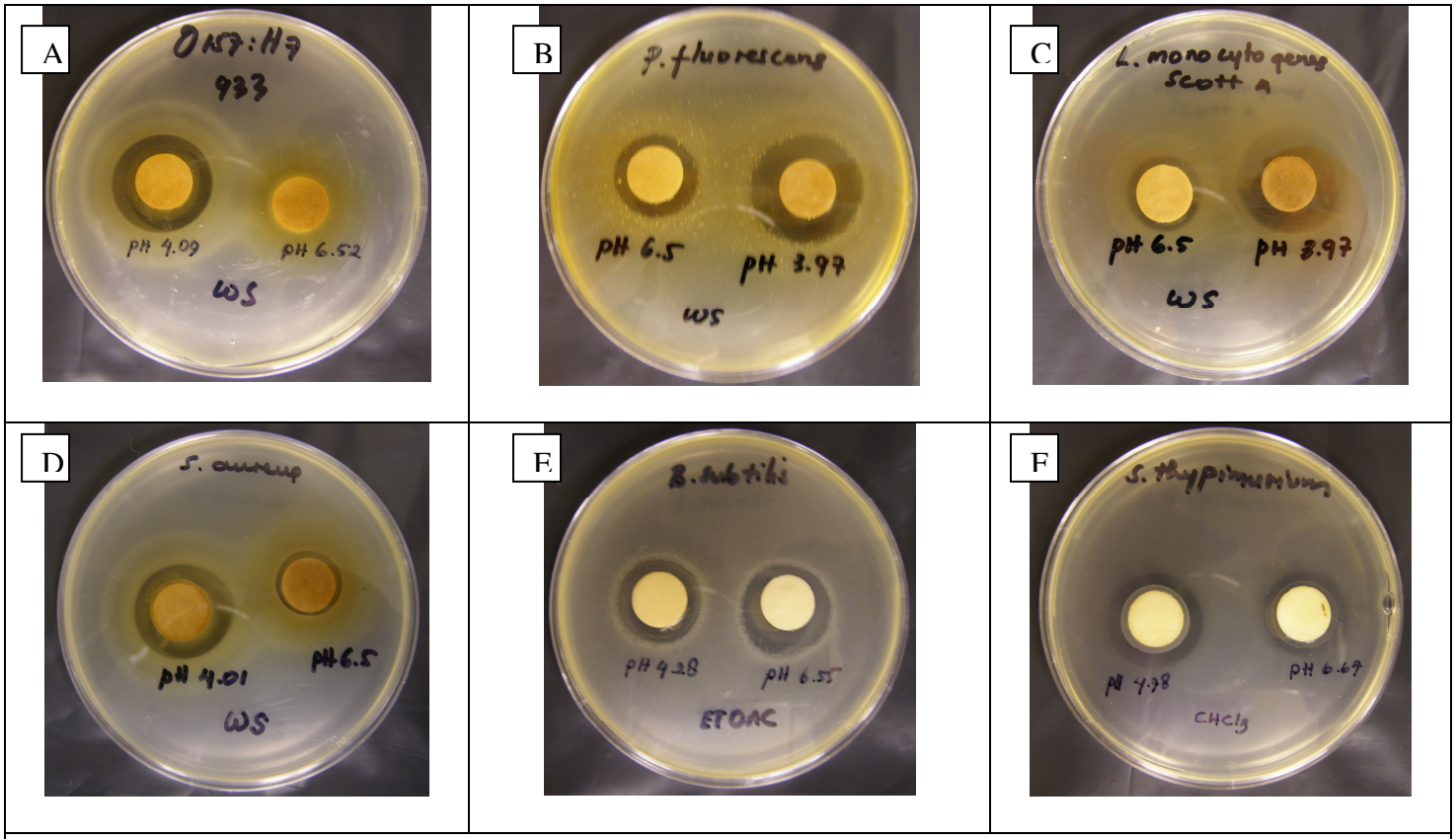


Figure C.1. Inhibition activity of water soluble coffee pulp fractions towards *E.coli* O157:H7 ATCC 933 (A), *P.fluorescens*(B), *L.monocytogenes* Scott A (C), *S.aureus* 13565 (D); Inhibition activity of ethyl acetate soluble fractions against *B.subtilis* (E) and chloroform soluble fraction against *S.*Typhimurium (F)

Table C. Antibacterial activity of coffee berry pulp extracted fractions against foodborne pathogens and spoilage bacteria[1]

Bacteria	Water Soluble Fraction[2]		Chloroform Fraction ($CHCl_3$)[2]			Ethyl Acetate Fraction (EtOAc)[2]		
	pH 4.01	pH 6.50	pH 4.16	pH 6.56	Control $CHCl_3$	pH 4.53	pH 6	Control EtOAc
E.coli O157:H7 933	25.3	15.0	25.0	20.0	14.0	18.0	19.7	15.0
E.coli O157:H7 35150	21.3	16.0	23.3	19.3	15.0	18.7	18.7	0.0
E.coli O157:H7 43890	22.0	15.3	23.0	20.0	14.0	18.0	19.0	14.0
E.coli O157:H7 43494	22.7	14.7	28.0	19.3	14.0	18.7	18.7	14.0
Pseudomonas aeruginosa	22.3	15.0	24.3	22.0	14.0	17.0	16.7	14.0
P. fluoresence	33.7	24.0	34.3	35.0	20.0	25.0	28.3	17.0
Salmonella Typhimurium	28.7	18.3	27.7	31.7	14.0	19.0	18.0	15.0
S. Typhimurium 29631	22.7	14.3	20.7	19.3	0.0	16.3	18.0	0.0
Listeria monocytogenes Scott A	31.7	20.0	24.7	24.0	0.0	16.0	17.3	0.0
L. monocytogenes 39-2	21.0	15.7	22.3	21.7	0.0	17.7	18.3	0.0
S.aureus 13565	24.0	16.3	22.3	24.0	14.0	18.0	17.3	0.0
Bacillus subtilis	24.3	15.3	31.7	28.3	15.0	18.7	18.0	15.0

[1] Antibacterial activity was measured as diameter inhibition zone (mm); diameter of paper disc 13 mm; Tryticase soy agar was inoculated with 10^7 cfu/mL bacteria; [2] Volume of 75 µL extract placed on discs

APPENDIX D

HISTOGRAMS OF PHENOLIC COMPOUNDS

OF COFFEE PULP FRACTIONS

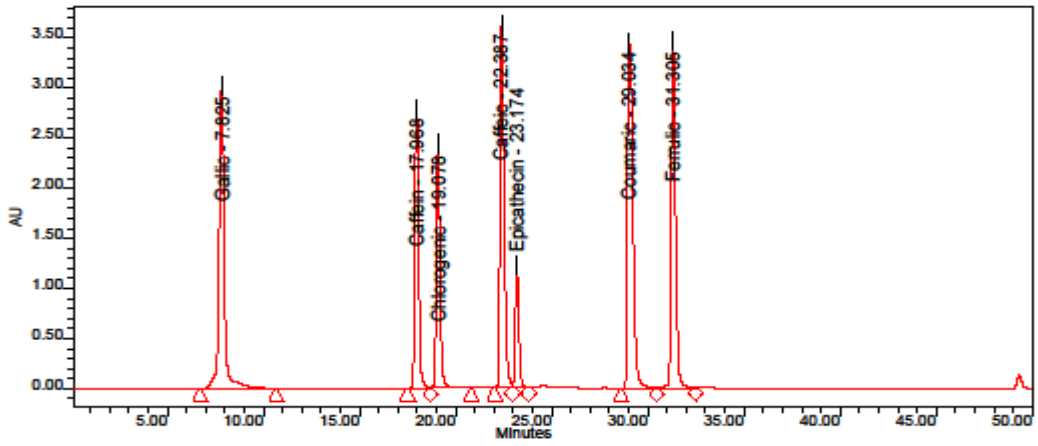
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	Mix 7 standards 1.00 mg/mL	Acquired By:	System
Sample Type:	Standard	Date Acquired:	5/28/09 1:08:38 PM
Vial:	4	Acq. Method Set:	test10
Injection #:	1	Date Processed:	5/28/09 5:25:46 PM
Injection Volume:	20.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	Wvln Ch1
Sample Set Name:	Test10 for mix 7standards	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Gallic	7.825	57416897	3031795	1.000	
2	Caffein	17.968	39690132	2783669	1.000	
3	Chlorogenic	19.078	32391848	2442930	1.000	
4	Caffeic	22.387	55024335	3621180	1.000	
5	Epicathecin	23.174	15874713	1230631	1.000	mg/mL
6	Coumaric	29.034	70677584	3439409	1.000	
7	Ferrulic	31.305	54595652	3479951	1.000	

Report Method: Untitled Printed 12:00:32 PM8/1/09 Page: 1 of 1

Figure D.1. HPLC chromatogram of mixture of phenolic acid standards

Table D.1. Retention time and R^2 of phenolic acid standards of calibration curves

Phenolic acid[a]	Retention Time (minutes)[b]	R^2
Gallic acid	7.825	0.9959
Caffeine	17.958	0.9828
Chlorogenic acid	19.078	0.9998
Caffeic acid	22.387	0.9983
Epicathecin	23.174	0.9996
Coumaric acid	29.034	0.9743
Ferrulic acid	31.305	0.9997

[a] Each value is the mean from 3 replicate trials
[b] Phenolic acid standard curves were prepared with 0.25, 0.50, 0.75 and 1.0 mg/mL

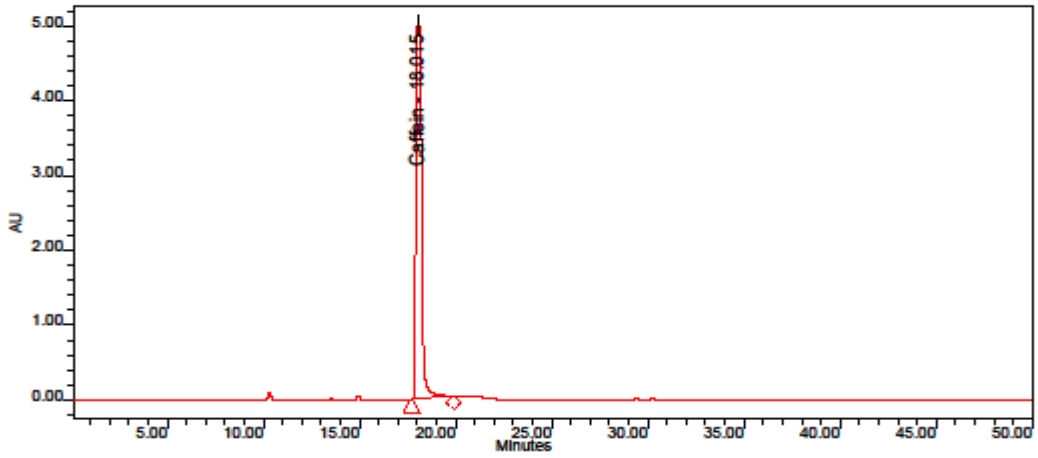
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	chcl3 4 rep 1. 10 mg/mL	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/5/09 2:26:33 PM
Vial:	1	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/8/09 4:27:58 PM
Injection Volume:	20.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	coffee pulp 20 uL injection	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Gallic	8.157				
2	Caffein	18.015	107783704	4994709	2.718	
3	Chlorogenic	19.343				
4	Caffeic	22.711				
5	Epicathecin	23.459				
6	Coumaric	29.479				
7	Ferrulic	31.628				

Report Method: Untitled Printed 4:57:45 PM 6/8/09 Page: 1 of 1

Figure D.2. HPLC chromatogram of chloroform fraction pH initial

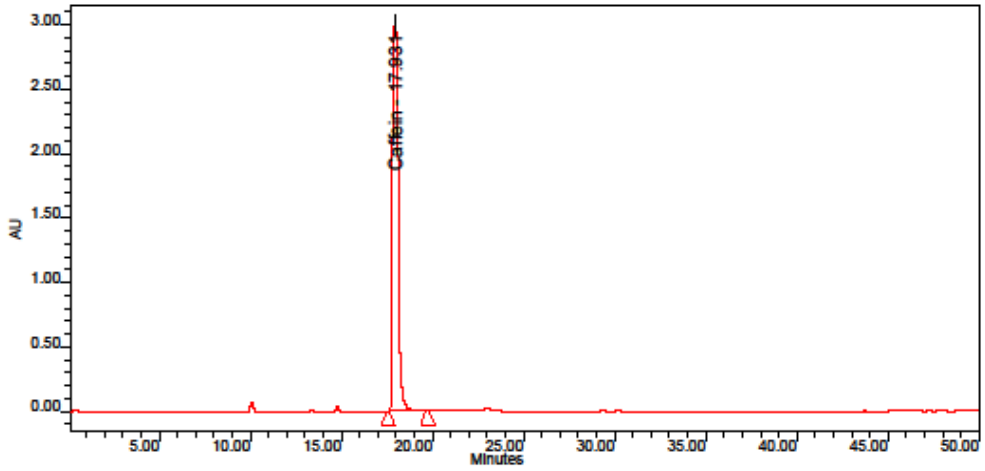
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	chcl3 6.5 rep 2. 10 mg/mL	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	6/5/09 5:57:18 PM
Vial:	5	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/8/09 4:28:06 PM
Injection Volume:	20.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	coffee pulp 20 uL injection	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram

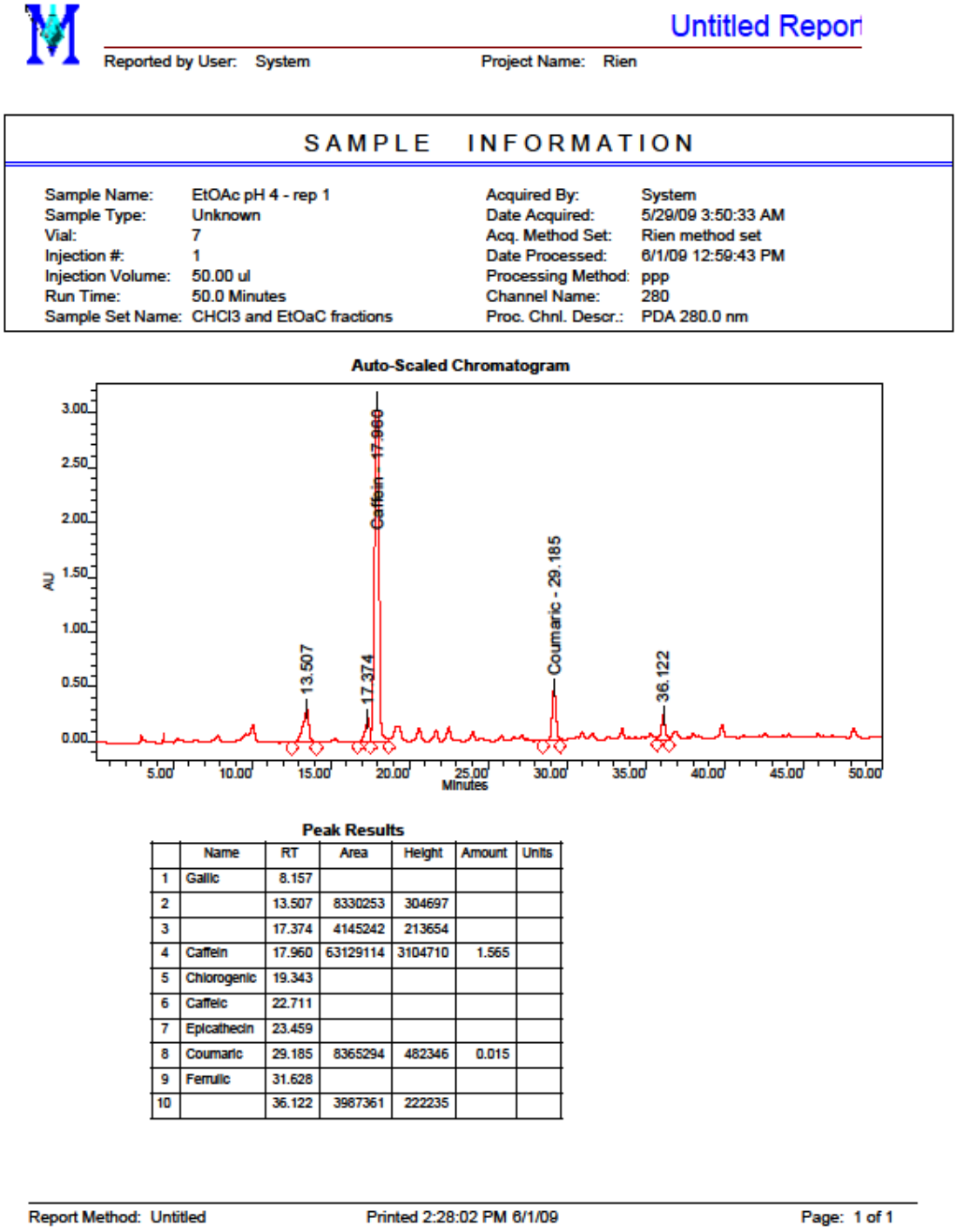


Peak Results

	Name	RT	Area	Height	Amount	Units
1	Gallic	8.157				
2	Caffein	17.931	67666477	2998782	1.682	
3	Chlorogenic	19.343				
4	Caffeic	22.711				
5	Epicathecin	23.459				
6	Coumaric	29.479				
7	Ferrulic	31.628				

Report Method: Untitled Printed 4:54:16 PM 6/8/09 Page: 1 of 1

Figure D.3. HPLC chromatogram of chloroform fraction pH 6.5



Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	EtOAc pH 4 - rep 1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/29/09 3:50:33 AM
Vial:	7	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/1/09 12:59:43 PM
Injection Volume:	50.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	CHCl3 and EtOaC fractions	Proc. Chnl. Descr.:	PDA 280.0 nm

Peak Results

	Name	RT	Area	Height	Amount	Units
1	Gallic	8.157				
2		13.507	8330253	304697		
3		17.374	4145242	213654		
4	Caffein	17.960	63129114	3104710	1.565	
5	Chlorogenic	19.343				
6	Caffeic	22.711				
7	Epicathecin	23.459				
8	Coumaric	29.185	8365294	482346	0.015	
9	Ferrulic	31.628				
10		36.122	3987361	222235		

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Figure D.4. HPLC chromatogram of ethyl acetate fraction pH initial

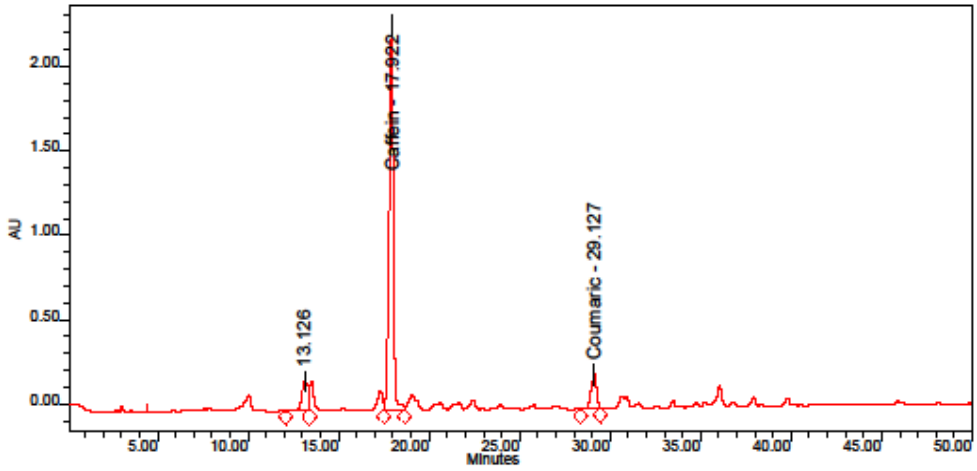
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	EtOAc pH 6.5 - rep 2	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/29/09 7:21:48 AM
Vial:	11	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/1/09 12:59:44 PM
Injection Volume:	50.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	CHCl3 and EtOaC fractions	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Gallic	8.157				
2		13.126	4532856	174642		
3	Caffein	17.922	37055027	2282995	0.892	
4	Chlorogenic	19.343				
5	Caffeic	22.711				
6	Epicathecin	23.459				
7	Coumaric	29.127	4472240	204011		
8	Ferrulic	31.628				

Report Method: Untitled Printed 2:18:09 PM 6/1/09 Page: 1 of 1

Figure D.5. HPLC chromatograms of ethyl acetate fractions pH 6.5

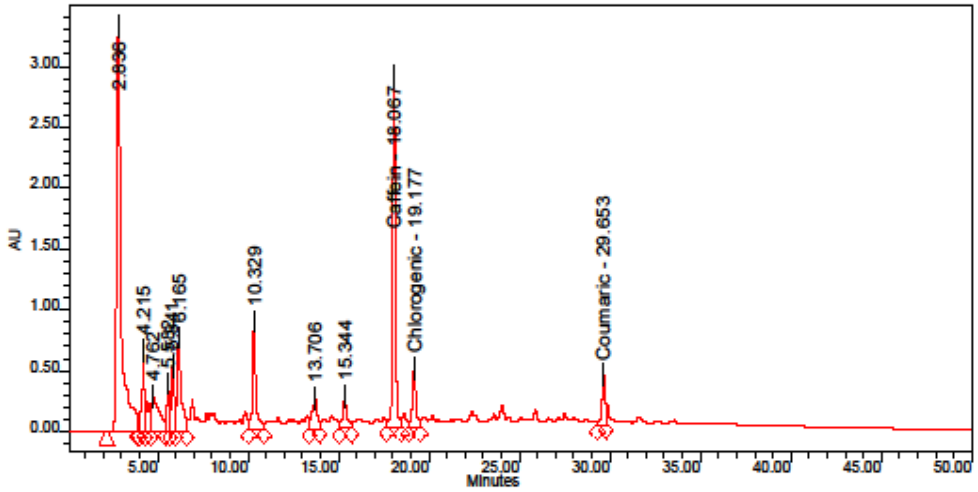
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	WS 4.11 100mg/mL Rep 1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/29/09 3:02:30 PM
Vial:	1	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/1/09 1:29:47 PM
Injection Volume:	50.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	WS pH 4 and 65 100mg per mL	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.836	59062171	3335355		
2		4.215	6038635	663612		
3		4.762	8066713	288514		
4		5.582	3751404	389551		
5		5.841	5788498	530329		
6		6.165	11576205	766142		
7	Gallic	8.157				
8		10.329	11155375	893804		
9		13.706	5012914	253186		
10		15.344	4350273	262403		
11	Caffein	18.067	32828693	2884210	0.783	
12	Chlorogenic	19.177	6970739	491840	0.247	

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Figure D.6. HPLC chromatograms of water soluble fractions pH initial

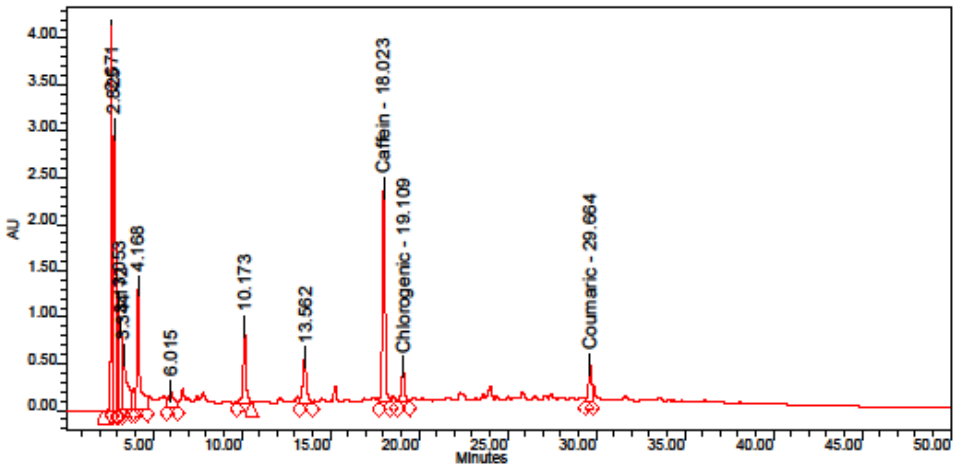
Untitled Report

Reported by User: System Project Name: Rien

SAMPLE INFORMATION

Sample Name:	WS 6.5 100mg/mL Rep 1	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	5/29/09 3:55:18 PM
Vial:	2	Acq. Method Set:	Rien method set
Injection #:	1	Date Processed:	6/1/09 1:29:48 PM
Injection Volume:	50.00 ul	Processing Method:	ppp
Run Time:	50.0 Minutes	Channel Name:	280
Sample Set Name:	WS pH 4 and 65 100mg per mL	Proc. Chnl. Descr.:	PDA 280.0 nm

Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1		2.671	22004520	4122302		
2		2.825	31869947	3056016		
3		3.053	7595854	1173249		
4		3.172	8529971	895541		
5		3.344	10516813	598210		
6		4.168	14000602	1321298		
7		6.015	3844122	174407		
8	Gallic	8.157				
9		10.173	9067029	834231		
10		13.562	7173704	487040		
11	Caffein	18.023	24747024	2319292	0.574	
12	Chlorogenic	19.109	5097217	382299	0.192	

Report Method: Untitled Printed 3:11:00 PM 6/1/09 Page: 1 of 2

Figure D.7. HPLC chromatograms of water soluble fractions pH 6.5

VITA

BAIQ RIEN HANDAYANI

Candidate for the Degree of

Doctor of Philosophy

Dissertation: STUDY AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS OF ARABICA COFFEE PULP EXTRACT

Major Field: FOOD SCIENCE

Biographical:

Personal Data: Born to Saudah SH and L.M. Sirkah in Mataram, West Lombok, West Nusa Tenggara, Indonesia, on November 15, 1968

Education: Received Master of Science in Food Science, from Bogor Agricultural University, Indonesia in 2000. Completed the requirements for the Doctor of Philosophy in Food Science at Oklahoma State University, Stillwater, Oklahoma in July, 2009

Experience: Lecturer at Technology Agricultural Study Program-Agricultural Faculty of Mataram University, West Nusa Tenggara-Indonesia (1994-present); Food processing trainer for small scale Industries and women groups in West Nusa Tenggara-Indonesia (1995-2002)

Professional Memberships:
Member of Institute of Food Technologist (2007-present); Member of American Society for Microbiology (2009-present); Member of The Indonesian Association of Food Technologist (Aug 2000 – present)

Name: BAIQ RIEN HANDAYANI Date of Degree: December, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: STUDY AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS OF ARABICA COFFEE PULP EXTRACT

Pages in Study: 85 Candidate for the Degree of Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study:

The objectives of this research were to determine the presence of antibacterial compounds, develop an efficient extraction method, analyze of phenolic contents, and measure their inhibitory effect against foodborne pathogens and spoilage bacteria. Antibacterial potential present in Hawaiian Arabica coffee berry pulp was determined using various extraction processes with water and methanol. Antibacterial activity of extracts was determined previously using turbidity method. Methanol extracts with 2 h reflux were further fractionated using chloroform, followed by ethyl acetate, then water and finally acidic methanol. All fractions were tested for antibacterial activity using disc method against foodborne pathogens and spoilage bacteria. Phenolics were identified using Reverse Phase-High Performance Liquid Chromatography.
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Findings and Conclusions:

Water extract of coffee pulp showed potential antibacterial effect against *E.coli* 1472. Higher antibacterial activity was provided by 80 % methanol compared to water extraction. Further fractionation of 80 % methanol coffee pulp extract resulted 85 % of fractions recovery. Water fraction exhibited significantly higher antibacterial activity than chloroform and ethyl acetate fractions. Water fractions showed similar inhibition activity against *E.coli* O157:H7 strains. *Pseudomonas fluorescens* was the most sensitive bacteria to water fractions. *Listeria monocytogenes* strains were more susceptible to chloroform fractions than all others bacteria tested. Caffeine was observed as a dominant phenolic. Chloroform was able to extract the highest amount of caffeine (705 µg/g wet pulp). Chlorogenic acid, caffeic acid, and *p*-coumaric acid were detected in ethyl acetate and water soluble fractions. Results indicated there were antibacterial compounds present in the pulp. In conclusion, Hawaiian Arabica coffee berry pulp may useful as a food preservative.
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ADVISER'S APPROVAL: Dr. Stanley E.Gilliland