EXTRACTION OF BIO-ACTIVE COMPONENTS FROM FRUIT AND VEGETABLE PROCESSING WASTES: USING GRAPE WASTE FROM THE WINE PROCESSING INDUSTRY AS A MODEL

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EXTRACTION OF BIO-ACTIVE COMPONENTS FROM FRUIT AND VEGETABLE PROCESSING WASTES: USING GRAPE WASTE FROM THE WINE PROCESSING INDUSTRY AS A MODEL

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CHAPTER I

INTRODUCTION

According to the 1997 USDA Economic Research Service, about 96 billion pounds of food or 27 % of 356 billion pounds was lost to human use (Lipton and others 1999). A significant quantity of this waste arises from the fruit and vegetable processing industries due to variability of input, high standards of production and amount of non-usable materials in fresh produce. The US Council for Agricultural Science and Technology reported 136 – 410 kg of waste is produced for every ton of input material in these industries (UNEP 2002). The waste consists of a significant amount of solid organic material and includes peels, rinds, seeds, stems, fruits, twigs and other rejected raw material (Stabnikova and others 2005). The by-products from these industries generally have high moisture content (Garcia and others 2005) and are thus prone to microbial spoilage. The high moisture content of the wastes also leads to increased drying and storage costs. Thus, waste plant material is typically used as a fertilizer or in feed in order to minimize the economic impact of its treatment and stabilization (Esteban and others 2007). Food processors also use composting as a low cost method of waste management (Schaub and Leonard 1996). All the above factors, have led to the importance of efficient, economic and environment friendly methods to deal with food processing by-products: in other words, the possible conversion of by-products to co-products.

To illustrate my point, let's look at an upcoming industry in Oklahoma: the wine making industry. The end products of wine processing consist of seeds, skins and stems. This refuse known as 'pomace' is obtained on pressing juice from the grapes. The amount and composition of waste varies and is dependent on the type of process (batch or semi-batch) and also processing conditions (Musee and others 2007; Andres-Lacueva and others 2008; Baydar and Ozkan 2006). A challenge faced by the wine making industry is to add value to the waste by extracting the tannins and polyphenolics already present in the grapes. This implies that the byproducts of the wine making industry can be used to develop nutraceuticals and functional components such as antioxidants, antimicrobials and biofuels. The focus of this project was hence to conceive a method to add economic value to fruit and vegetable waste by developing an efficient method to screen for valuable bioactive components. Waste from the wine industry was used to evaluate extraction conditions suitable for the screening process.

The **objectives** of this work were therefore:

- To develop extraction procedures which can be commercially replicated with ease.
- To test different extraction conditions by evaluating their ability to extract antimicrobial compounds from wine waste. Antimicrobial activity was tested against both Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli* 0157:H7) pathogens commonly encountered in foods.

The long term goal of the project is to apply the selected screening protocol to other fruit and vegetable processing industries in Oklahoma thus turning their wastes into revenue.

CHAPTER II

REVIEW OF LITERATURE

2.1 Importance of Bio-Active Components

The present day consumer is focused on dietary interventions to improve health. This includes foods with functional ingredients that provide additional health benefits (Teratanavat and Hooker 2006). Organic and natural foods are in great demand because as far as the consumer is concerned this is the 'best' form of food (Hughner and others 2007). Needless to say, our market is consumer driven and innovation in food manufacturing practices is a prerequisite for the industry to survive. Researchers have aided the industry in this process and a significant amount of literature is available on the use of plant metabolites in food showing their impact on human health. However, the plant food processing industries produce large quantities of by products and these pose disposal problems (Hui and others 2006). The extraction of functional or bioactive components would therefore be an alternative to other disposal methods. Several foods like cereals, legumes, flax seed, sweet cherry, banana, red onion, echinacea flower head and root, purple potato, ginseng, buck wheat, apple, pears, horseradish, white and red grapes, green tea, black tea, wine, coffee, beer, etc (Andres-Lacueva and others 2008; Velioglu and others 1998; Sembries and others 2006) possess phytochemicals. Thus, phytochemicals (plant extracts) are commonly found and easily available in everyday fruits, vegetables, snack foods as well as breakfast foods. These phytochemicals are plant derived compounds that possess antimicrobial and antioxidant properties. Plants and derived oils that possess antimicrobial properties include garlic (Kyung and others

1996), hops (Larson and others 1996), ginger, turmeric (Gupta and Ravishankar 2005), rosemary (Krajcova and others 2008), sage, cocoa (Andres-Lacueva and others 2008), oregano etc. Tamarind seeds show the presence of flavanoid compounds such as cathechin and epicatechin (Murga and others 2000). Theivendran and others (2006) showed that Grape Seed Extract (GSE) and green tea extract showed antimicrobial activity both alone as well as with nisin. Thus, as mentioned above, plant derived extracts or phytochemicals are obtained from a variety of sources and can be divided into the following general categories as described by Goldberg and others (2003).

1. Phenolic compounds

   Phenolic compounds are characterized by at least one aromatic ring and have one or more hydroxyl groups. Phenolics are classified based on the number and arrangement of carbon atoms and are normally conjugated with sugars or organic acids.

2. Sulphur containing compounds and derivatives
3. Chlorophyll and its derivatives

Of these, ones most commonly found in plants are polyphenols and flavanoids which are derivatives of phenolic compounds (Shi and others 2005). Fig 1 portrays the structures of some basic phenolics. Many of these commonly occur in grapes.

2.2 Wine Processing Industry

Grapes (*Vitis vinifera)* are the second most abundant crop, after oranges, with an annual world production of more than 60 million tons (Schieber and others 2001). According to Mazza and others (1998) about 80 % of this is utilized for wine making, 13% is sold as table grapes and the balance is consumed as raisins, juice and other products. The United States produces about 7.2 million tons (USDA 2008). About 89%

of the grapes produced in the US are from California. Wine type grape production in California is expected to be 53% of the California production i.e. 3.4 million tons (NASS 2008). In Oklahoma, wine production is a forthcoming industry and a 2002 study by USDA showed total wine sales in OK to be nearly 2 million gallons a year. A report on the Oklahoma Grape Growers survey (Stafne 2006) showed a total acreage between 232 - 242 acres.

Grapes can either be table or wine grapes and they are further classified based on the region of growth and climatic conditions. The European Union (EU) is the largest producer of grapes and European grapes are known to thrive in a Mediterranean climate. This climatic condition is also prevalent in California and is encompassed by mild winters and variable summer temperatures. Rainfall in these regions is more prominent in the colder months. Hence, *Vitis vinifera* is a common grape varietal in EU and California. Other American states experience more severe variations in temperature such as multiple frosts and windy weather in spring followed by heavy rainfall in the fall especially September. This results in pollination problems as well as damaged grapes (NASS 2008). Applequist and others (2008) elucidate that American vineyards rely on American varieties of grapes such as Norton and Cynthina, native muscadine varities (*Vitis rotundifolia* and *Vitis labrusca*) and other French hybrids that thrive in Missouri and are capable of producing high quality wines. Specifically in Oklahoma, *Vitis* vinifera dominates with nearly 80%, hybrids less than 15%, American species roughly 7% and muscadine with less than 1% of the total (Stafne 2006). Table 1 shows a comprehensive view of the Oklahoma wine industry and its components.

Fig 2 shows the major steps involved in wine-making. Depending on the type of wine to be produced, further changes are made to the process.

Also of importance is that waste consists of about 20% of the weight of processed grapes (Mazza and others 1998). This is because waste is produced from almost every step of the wine making process. About 3% of the original grapes consist of stems and 20% consists of pomace (skins and seeds). Skins constitute 75% of wet pomace while the remaining comprises seeds (Rabak and Taylor 1921).

Byproducts from the wine processing industry are disposed either by burning or are used as cattle feed. An alternative is the production of grape seed oil for human consumption due to the high level of unsaturated fatty acids (Gomez and others 1996). Some researchers have also studied the possibility of producing functional compounds from grape waste, as has been described in the 'Extraction Methods' of this thesis.

2.3 Grape Seed Composition

Grape seed is a complex matrix that consists of 40% fiber, 16% oil, 11% protein and 7% complex phenols and tannins (CPT) besides sugars, mineral salts etc. Natural phenolic compounds such as proanthocyanidins (PA) are present in CPT and are responsible for the properties of GSEs (Murga and others 2000). It has been found that fresh red *Vitis vinifera* grapes contain about 4 mg/kg of phenolic material. This is normally present in the skin and seeds in the form of gallic acid, caftaric acid, anthocyanins, and oligomeric proanthocyanidins. Even white grapes contain phenolic material in the seed (Goldberg 2003). Hence, the majority of phenolics in grapes are present in the seeds. As a result, seedless grapes have a lower phenolic content. Oligomeric proanthocyanidins are present abundantly in grape seeds, tea leaves and pine

tree barks (Shi and others 2005). They consist of proanthocyanidin monomers which are chemically bonded. The proanthocyanidin monomers found in GSEs are the catechins and epicatechins. Fuleki and others (1997) also found that grape seeds are a rich source of catechins and procyanidins. Besides the monomers (+) - catechin, (-) - epicatechin and their esters with gallic acid, 14 dimeric, 11 trimeric and one tetrameric procyanidins have been identified in grape seeds (Fuleki and others 1997; Baydar and others 2004; Jayaprakasha and others 2003). A total of 11 monomers, dimmers and trimers were extracted by reverse phase HPLC analysis by Fuleki and others (1997) from the seeds of red grape. Ozkan G and others (2004) studied the presence of the total phenolics present in two different Turkish grape cultivars. The yield of total phenolics in grape pomace was found to be 3.5% to 4.5% respectively for the two varieties. Several studies have determined the active component to be gallic acid in the grape seeds (Jayaprakasha and others 2003; Veluri and others 2006; Nawaz and others 2006; Shi and others 2005). Veluri and others (2006) studied the effect of GSEs on growth inhibition and apoptotic death of human prostate cancer cells and identified gallic acid as a major constituent. They substantiated the presence of gallic acid by selectively removing it from the gel filtration column. Thus in many studies on grapes, the phenolic contents are expressed in terms of gallic acid (Cortell and others 2006; Veluri and others 2006; Baydar and others 2004).

Some of the bio-active properties of (GSE) are presented in the following statements. Baydar and others (2004) showed antimicrobial activity of GSEs at concentrations of 4% and 20%, and find that it may be used an effective antimicrobial to prevent bacterial contamination in foods. They also reported that gram positive bacteria

were more inhibited than the corresponding gram negative ones. GSE of the variety Ribier showed significant antilisterial activity through 2 distinct types of compounds: skin derived (pigmented) and seed derived (non – pigmented) polymeric phenolics (Rhodes and others 2006). Inhibitory effect of GSE against lipases is due to the synergistic activity of multiple components as against a single component. Presence of a variety of health promoting compounds in GSE has been shown by many studies. Some of them include flavonoids that prevent obesity (Moreno and others 2003) and antimicrobial effects by various proanthocyanidins (Theivendran and others 2006; Baydar and others 2004; Jayaprakasha and others 2003). Moreno and others (2003) have indicated a lack of toxicity and hence support the use of GSE in foods.

2.4 Extraction Methods

Extraction is defined as the process of obtaining a compound from a mixture by physical or mechanical means. It is an indispensable step to isolate the active ingredients in plant material. Many techniques have been developed to extract the bio-active components of grape pomace. The core foci of extraction methods are choice of solvents and addition of thermal or mechanical energies to increase the efficiency of the process. This is achieved by varying the rate of mass transfer in the system. Also of importance is that many components are thermally unstable as well as photosensitive. Thus, care must be taken to not only increase the amount of extracted compound but also to obtain them in an active form.

The first step in an extraction process is sample preparation. Studies conduct preparation of the sample in a multitude of ways depending on the state of the raw material and the components of interest. Oszmianski and others (1989) removed the

grape seeds, froze it as well as ground it under liquid nitrogen. The stage of evolution of the grapes was of interest in this study as earlier studies showed that phenolic content in an unripe fruit is higher than a fully ripe grape. Baydar and others (2006) studied wine by products such as grape seed, bagasse (skin and stem) and pomace (skin, seed, and stem) to determine the tocopherol contents. In this case, the samples were air dried at room temperature in the dark and were crushed in a grinder. Gomez and others (1996) treated grape seed under different particle size and drying times. In their case, they used a fraction grape seed size of 0.75 mm and a relative humidity less than 0.35%.

2.4.1 Traditional Methods

Traditionally, extraction of the compounds used organic solvents such as hexane (Gómez and others 1996) . Baydar and others (2006) evaluated two traditional methods of extraction: hot and cold. For cold extraction, 2g samples were extracted with 10 mL of hexane by triple extractions at room temperature. Rotary evaporation under vacuum was used as means to remove the solvent and the extract was filtered and resuspended in a mixture of heptane: tetrahydrofuran (95:5 v/v). For hot extractions, a Soxhlet apparatus was used. Temperature was 65 °C with 1 g of ground sample and 50 mg of pyrogallol as an antioxidant. The extraction apparatus was completely covered in foil and thus protected from light. Baydar and others (2004) used dried and powdered grape seeds wherein fatty materials were removed by Soxhlet extraction with petroleum ether (60 °C for 6 h). The defatted grape seed (50 g) was further extracted with a mixture of acetone: water: acetic acid (90:9.5:0.5, 200 mL for 8 h) and ethyl acetate: methanol: water (60:30:10). It was found that the former extraction yielded more phenolics than the latter. This agrees with results obtained by Jayaprakasha and others (2003). In Soxhlet

extractions, a variety of combinations of solvents can be used depending on the presumed chemical composition of the grape seed. Another group (Lafka and others 2007) studied different extraction solvents, times, concentration and pH to determine phenolic content of winery wastes. Phenolic extraction was carried out on samples defatted with hexane (10:1 v/w). Extraction solvents tested were methanol, ethanol, methanol: ethanol (1:1), isopropanol and ethyl acetate (3:1 to 12:1 v/w) under extraction times (30 min to 24 h) in an orbital shaker at room temperature. Extract was filtered through a Buchner funnel and dried using a rotary evaporator. The residue was re-dissolved in methanol and stored at -20 ^{o}C until further analysis. The highest phenolic content (95.9%) was extracted using ethanol: waste (1:1) while isopropanol gave the least (4.2%). Pinelo and others (2005) studied the effect of temperature (25 ^{o}C to 50 ^{o}C), solvent interaction time (between 30 min and 90 min) and solvent to solid ratio (1:1 to 5:1) on grape pomace extracts. The grape pomace (10 g) was subjected to batch extraction in a rotary shaker (140 rpm) and methanol, 96% ethanol and distilled water were used as pure solvents. On filtration, the solids were separated and extracts were analyzed for total phenolic content and antiradical activity. It was deduced that extraction with ethanol as the solvent was the most suitable in the given conditions. Also optimum conditions of temperature (50 ^{o}C) and solvent: solid ratio (1:1) maximized antiradical activity of the phenolic extracts. Thus, the commonly used organic solvents, in conjunction with a polar solvent like water, are ethyl acetate, acetic acid, petroleum ether, methanol, ethanol and acetone (Jayaprakasha and others 2003; Baydar and others 2004; Fuleki and others 1997; Ozkan and others 2004).

As research in the food industry focuses on action of the extracted compounds in food systems, organic solvents are coupled with water. In this process the catechins, which are lipid soluble are extracted with organic solvents while the aqueous procyanidins are extracted with water. In 2006, Nawaz and others extracted the polyphenols with ethanol: water (1:1). This solvent mixture improved the solubility of the bioactive components in the diluted ethanol. The principle behind the use of solvent extraction is the preferential action of the solvents as some materials are more soluble in one than in another. Single, double and triple extractions were carried out in order to increase the efficiency of extraction and to concentrate the extracts. The optimum conditions (maximum polyphenols 11.4% of total seed weight) they determined were 0.2 g/mL (solid to liquid ratio), and a double stage extraction with a 0.2 μm pore size.

2.4.2 Modern Technologies

Some modern methodologies are microwave extraction, ultrasonic techniques, ohmic heating and ultrasonic techniques. Microwave assisted extraction was used to obtain phenolic alkaloids in a traditional Chinese plant (Lu and others 2008). In comparison with traditional methods, interaction time was reduced from 2 h to 90 s and varying the ionic liquid influenced the extracted compounds. Hong and others (2001) used Microwave Assisted Extraction to obtain phenolics from grape seed. They found no effect of variation in extraction time (20 - 200 s) and power (150 – 300 W) on yield of phenolics. However changing the polarity of the solvent increased the total polyphenolics content. Ultrasonic extraction acoustically breaks down the cell membranes releasing the intracellular components and thus enhances penetration of solvent into plant materials. Velickovic and others (2008) used spent sage plant waste (after extraction of essential

oils) to obtain possible bioactive compounds using ultrasonic extraction. Extraction using super critical fluids is another alternative and is used widely at laboratory, pilot plant and commercial scales. The most commonly used solvent is carbon dioxide as it is non toxic, environmentally safe, cheap and a selective solvent (Murga and others 2000). Besides being cheap and non toxic, carbon dioxide is readily available with high purity and has a low critical temperature. Carbon dioxide is suitable for aqueous extractions and hence Murga and others (2000) also used co-solvents like ethanol to extract the phenolics from grape seed. Solubility was higher for gallic acid than for the catechins at 313 K and 20 MPa, and also increased with use of ethanol as co-solvent. Thus, each type of extraction has its own advantages and disadvantages. Though super critical extraction is a commonly used modern method, it is not practical to use if the total phenolics are of interest as this deals with preferential extraction. It can be used for individual compounds and has the advantage of increasing efficiency of extraction by varying operating conditions. Though traditional methods pose a time constraint, it may be selected depending on the need of the process. Extractability of the bio-active components also depends on the variety of grape on which the extraction is carried out (Shi and others 2005; Baydar and others 2006; Cheynier and others 2006; Gachons and others 2003; Ozkan and others 2004).

2.5 Microorganisms

2.5.1 *Escherichia coli* 0157:H7

Characteristics

Escherichia coli 0157:H7 or enterohemorrhagic *E. coli* is a rod shaped pathogenic bacterium. It is a gram negative, catalase positive, facultative anaerobe which has been

linked to many outbreaks in the recent past. Conditions that affect the growth of the organism are temperature, pH and water activity. The organism has a temperature range of 10 °C to 45 °C with an optimum growth temperature of 37 °C. It can also survive low pH conditions of about 4 – 4.5 (Ukuku and others 2001) and is hence common in many low pH foods such as apple juice and sausages. The organism has a doubling time of about 20 - 30 mins. The above factors cause a major concern not only in fresh foods such as spinach and salads, ground beef, milk as well as the present day 'Ready to Eat' foods.

Implications

It is associated with acute hemorrhagic colitis and haemolytic uraemic syndrome. In 1982, there was a recall of ground beef patties associated with the former disease (CDC 2002). This microorganism is responsible for the production of a deadly toxin and is hence associated with high morbidity and mortality rates especially in immune-compromised individuals, elderly and children. The infections are spread through fecal and water contamination, unhygienic conditions, unpasteurized (raw) milk or use of the same in cheese (CDC 2008) .This microorganism is hence one of the major causes of concern to the food industry.

Dose factors

Tilden and others (1996) reported an outbreak of the *E. coli* 0157:H7 in salami and the infectious dose was reported as low as about 2 - 7 cells for the susceptible populations. Chang and others (2007) reported an infectious dose as low as 10 - 100 CFU/g. The mechanism of pathogenicity of *E. coli 0157:H7* is the ability of the organism to attach to the wall of the intestine as well as produce Shiga toxins that are analogous to the verotoxins (Mora and others 2005).

Activity of Phenolics Extracts

Ozkan and others (2004) reported that 'Emir' pomace extract developed an inhibition zone of 23.67 mm at a 20% concentration against *E.coli* 0157:H7. They also studied the effect of the extracts at different concentrations (0.5%, 1% and 2.5%) on the organism for 96 h at 37 ºC. At 1% and 2.5%, the extracts had bactericidal effects against the organism. At the end of 24 h and 48 h the same concentrations of the extract had inhibitory effects against *E. coli* 0157:H7. Rhodes and others (2006) showed a narrow spectrum of antimicrobial activity against the microorganism. Only a 1-log reduction was obtained in numbers when tested against phenolic fractions of juice, seed and skin at the end of an hour exposure time. However, researchers have also stated that these effects are cultivar specific.

2.5.2 *Staphylococcus aureus*

Characteristics

Staphylococcus aureus (commonly referred to as staph) is a cocci or spherical shaped microorganism which occurs singly, in pairs or grape like clusters. It is a gram positive, catalase negative, facultative anaerobe and is non-spore forming. The organism can survive over wide ranges of water activity and osmotic concentrations, hence aptly called osmotolerant. Lotter and others (1978) found that *S. aureus* can grow at a water activity as low as 0.864. Although a mesophile, the organism grows over a wide temperature range, from 6.5 ºC to 46 ºC, the optimum being 30 ºC to 37 ºC (Prescott 2005). Also, the organism can grow in a pH range from 4.2 to 9.3 (Baird-Parker 1965). Many strains of *S. aureus* grow in 7-10% salt concentrations (NaCl) while some strains can grow in as high concentrations as 20%. Various factors such as water activity (a_w),

temperature, pH and red-ox potential determine the maximum permissible salt concentrations (Jay 1992).

Reservoirs

The primary reservoirs of *S. aureus* are the skin and mucous membranes, in particular the nasopharyngeal region of birds and mammals (Atanassova and others 2001). The largest numbers of the organism are found near body openings, wherein numbers per sq. cm may reach $10^3 – 10^6$ in moist habitats and $10 – 10^3$ in dry habitats (Jay 1992).

Implications

The mode of action of the organism is through production of enterotoxins and the enterotoxin producing *S. aureus* is a major cause of food intoxication. The staphylococcal enterotoxin is a heat stable protein with a molecular weight of approximately 27-31 kDa. The effects caused can be minor (pimples, boils or rash) or sometimes severe (pneumonia or blood infections (Atanassova and others 2001). It is usually spread from person to person contact through open wounds, nasal discharge or improper hand washing. Thus, it is not transferred through the food itself but as a mode of post-production contamination (Rauha and others 2000). In 2005, the Center for Disease Control estimated about 94,000 drug resistant life threatening staph infections and about 19,000 deaths (CDC 2007).

Dose factors

The implicated foods are ham, beef, poultry, warmed over foods, cream filled pastries and egg products to name a few. Staphylococccal food poisoning is associated with unhygienic food treatment like improper holding temperatures and is frequently allied to manually handled foods. The minimum infectious dose of the staph entertoxin

has been reported by Jay (1992) as 20 ng from an outbreak traced to 2% chocolate milk. The minimum number of cells of staphylocci to produce enough enterotoxin to cause food poisoning is 10^7 (Lotter and Leistner 1978).

Activity of Phenolics

Baydar and others (2006) studied the antibacterial effects of GSE against *S. aureus*. Extracts of three different varieties at concentrations (0.5%, 1%, 2.5% and 5%) showed bactericidal effects against the organism at the end of 48 h interaction period. Researchers studied the antimicrobial activity of Ribier variety of grape juice, skin and seed phenolics at a pH of 3.5. At the end of a 60 min exposure period all phenolic fractions were inhibitory to *S. aureus*. However, this reduction was small and accounted for a maximum of one log reduction in cell numbers (Rhodes and others 2006). Baydar and others (2004) found that GSEs exhibited antibacterial activity *against Staphylococcus aureus* COWAN1 at a 20% concentration. They also determined that acetone: water: acetic acid as a solvent was more effective than its methanol counterpart. These results are in conformation with other studies (Jayaprakasha and others 2003).

2.6 Mechanism of mode of action of phenolics against the microorganisms

The site and number of hydroxyl groups are related to the level of toxicity and are directly proportional to each other. Research on this is limited and is contradictory. Some researchers have described the mechanism of simple phenols such as catechol and epicatechin to be substrate deprivation and membrane disruption respectively. Other mechanisms of action noted by the study of phenols in general and subclasses of phenolic acids, flavonoids and tannins involve enzyme inhibition, enzyme inactivation, formation of complexes with cell walls and metal ions (Cowan 1999).

CHAPTER III

EXTRACTION OF BIO-ACTIVE COMPONENTS FROM FRUIT AND VEGETABLE PROCESSING WASTES: USING GRAPE WASTE FROM THE WINE PROCESSING INDUSTRY AS A MODEL TO EXTRACT ANTIMICROBIALS

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Wastes generated from the fruit and vegetable processing industries cause significant disposal problems. A simple method to recover bio-active components from the waste would be economically advantageous. Therefore the objective of our research was to evaluate the effectiveness of different extraction methods in recovering antimicrobial components from processing wastes. Grape pomace from the wine making industry was used as a model to evaluate the extraction efficacy.

Grape waste was ground under liquid nitrogen to a uniform particle size (<3.6mm). Influence of process parameters such as solvents utilized (100% petroleum ether, 70% methanol, 50% acetone and 0.01% pectinase), solvent: waste ratio (2:1 or 4:1) and interaction time (1, 2, 4 and 8 h) were studied. Efficacy of the treatment parameters were studied based on antimicrobial testing of the extracts against common Gram negative and Gram positive food borne pathogens: *Escherichia coli* 0157:H7 and *Staphylococcus aureus*. Antimicrobial activity was measured based on time taken to increase absorbance of bacterial growth by 0.5U.

Probit models were fit to the increase in absorbance (A_{620}) data and inverse predictions were used to identify time required for a 0.5U increase. The time taken to increase microbial concentrations by 0.5U was about 4.2 h for *E.coli* (control 3.6 h) and 3.9 h for *S.aureus* (control 3.3 h) with 50% acetone as a solvent. This is much higher than the time to reach equivalent microbial concentrations for the other solvents. Thus, it appears that this extract contains higher amounts of bioactive components. For both the organisms, significant inhibition ($p<0.05$) was obtained at lower interaction times and longer extraction times of 8 h were not significant.

In conclusion, results indicated the optimum interaction times and extraction ratios for each solvent in recovering antimicrobial bioactive components from fruit and vegetable processing wastes was effective.

Key words: grape pomace, antimicrobial, extraction

INTRODUCTION

The US Council for Agricultural Science and Technology has reported that fruit and vegetable processing industries produce about 136-410 kg of waste for every ton of input material. This plant waste material is disposed by burning or used as cattle feed (Esteban and others 2007). A challenge faced by these industries is to add value to the waste.

An emerging industry in Oklahoma is the wine making industry. A study conducted by USDA in 2002 showed that total wine sales in the state of Oklahoma were approximately 2 million gallons. Also, data from the Oklahoma Grape Growers Survey (Stafne 2006) illustrates that total acreage is between 232-242 acres. With this growth in the industry, a cause of concern is the large quantities of by-products produced. By-products from the wine making industry consist of seeds, stem and skin. It constitutes about 20% of the weight of processed grapes (Mazza and others 1998) and is produced from almost every step of the wine making process. Grape seeds are rich sources of polyphenolic compounds which have antioxidant and antimicrobial properties (Garcia-Marino and others 2006; Nawaz and others 2006; Postescu and others 2007; Schieber and others 2001; Shi and others 2005; Baydar and Ozkan 2006; Baydar and others 2007; Baydar and others 2004). Thus, a solution to the problematic waste production in this industry can be achieved by extracting the polyphenols from the grape waste.

Extractions of the polyphenols from grapes are carried out both by traditional methods as well as modern technologies. Traditional methods include solvent extraction using a variety of organic solvents like hexane (Gómez and others 1996), methanol,

acetone and ethyl acetate (Baydar and others 2004; Garcia-Marino and others 2006; Jayaprakasha and others 2003; Ozkan and others 2004; Pinelo and others 2005). Modern technologies include microwave extraction(Hong and others 2001), supercritical fluid extraction (Murga and others 2000) and ultra filtration (Nawaz and others 2006; Shi and others 2005).

The focus of this project was to develop a screening procedure which can add economic value to the waste from fruit and vegetable processing industries. Waste from the wine making industry was used as a model to evaluate extraction procedures for the screening process. The objectives of the study were hence to evaluate extraction conditions which can be replicated with ease commercially. Also, different extraction conditions were evaluated based on their ability to extract antimicrobial compounds from winery waste. Antimicrobial activity was tested against common food borne pathogens: *Escherichia coli* 0157:H7 and *Staphylococcus aureus*.

MATERIALS AND METHODS

Sample Collection

About 800 pounds of grape waste (pomace) were obtained from Summerside Winery, Vineta, OK. The grapes were of the Cynthiana variety with small berries. This is a red grape and hence the pomace was obtained after fermentation of the grapes. The pomace was at the winery for about a week. Upon arrival the pomace was stored overnight at 4-6°C and then vacuum packaged (Multivac C500, Multivac Inc., Kansas City, MO). Approximately 3 lbs of pomace was placed in each Cryovac vacuum package bag (Type 540, 8x14 cm, Cryovac Division., W.R. Grace and Co., Simpsonville, SC). Pomace was stored in over 250 bags. Vacuum package bags were initially stored for 3d at 0°C and then transferred to a -20°C storage.

Sample Preparation

A total of 32 vacuum bags were randomly selected and allowed to thaw overnight at 0°C. Each bag was then opened; woodchip and stem residues were removed. The contents were then mixed and a 10 lb sample was obtained for further processing. The pomace was then transferred to a cold room (4°C), half pound batches were taken in a metal strainer and were frozen rapidly using liquid nitrogen (-196°C). The sample was then immediately ground for 30s bursts in a cold room using a Warring Blender (model 51BL31, Torrington, CT). The blender jar was allowed to equilibrate at -20°C prior to use. The powder obtained was screened for particles less than 3.36 mm using Tyler Sieve Size 6. This was then stored in large Ziploc bags at -20°C until further extraction and analyses were performed.

Bioactive Compound Extraction

Preliminary investigations were conducted to determine optimum solvent: waste ratios. The following ratios were tested: 1:1, 2:1, 3:1, 4:1 and 5:1. Extraction ratios were selected based on % recovery of extracts which were optimum at 2:1 and 4:1. Two process parameters for extraction of bio-active compounds from the powdered pomace were investigated for solvents differing in degree of hydrophobicity (petroleum ether, 70% methanol, 50% acetone and 0.01% pectinase).

- Solvent: waste ratio 2:1 or 4:1
- Interaction time (h): 1, 2, 4 or 8 h

Extractions were carried out at room temperature. Based on experimental design, 8 possible extraction conditions were evaluated for each of the 4 solvents. All extractions were performed in triplicate. Extractions were conducted by placing 20 g of ground pomace in a 250 mL Erlenmeyer flask. An appropriate amount of solvent was added (40 or 80 mL) and the flask was stoppered with a neoprene type plug. The flask was placed in an incubator shaker (Classic C76, New Brunswick Scientific, Edison, NJ) maintained at 18 °C and 250 rpm for the aforesaid interaction time. The petroleum ether, 50% acetone and 70% methanol samples were filtered through a Whatman #1 filter paper (55 mm No1, Whatman Inc. Ltd., Mainstone, England) while 0.01% pectinase extractions were filtered using Miracloth (Calbiochem, San Diego, USA) . The samples were filtered under vacuum at a flow rate of 4.5-5 liter/min. Filtrate was transferred to a 100 mL volumetric flask and brought to volume with the respective solvent except in the case of petroleum ether. The petroleum ether was first evaporated and the residue was

resuspended in 100% acetone. The resuspended residue was then brought to a 100 mL volume with acetone as stated previously.

Bacterial Cultures

Bacterial cultures namely *Escherichia coli* 0157:H7 ATCC 35150 and *Staphylococcus aureus* ATCC 13565 were obtained from the stock culture collection of Food Microbiology, Robert. M. Kerr Food and Agricultural Products Center, Oklahoma State University. The cultures of both the strains were maintained by subculturing in Tryptic Soy Broth (BD, Sparks, MD) using 1% innocula and 18-20 h of incubation at 37 °C. They were stored at 4 - 6°C between transfers. Also, each culture was subcultured at least three times prior to experimental use.

Antimicrobial Activity

The extracts were compared for their ability to inhibit the growth of *E.coli* ATCC 35150 and *S.aureus* ATCC 13565. The treatments were randomly distributed and 4 treatments per extraction (set) were tested for antimicrobial activity at a given time. All extracts (0.5mL) except for 0.01% pectinase were evaporated using a heating block (set at room temperature) under the influence of nitrogen gas for 30-40 min. To all the test tubes (including pectinase) 0.5 mL of water was added in order to suspend the extracts. The volume was brought up to a total of 5 mL with Tryptic Soy Broth. The effects of the extracts were evaluated by inoculating the treatments at 1% with the culture. The control for each set of test tubes was 0.5 mL of water brought up to 5 mL with TSB and inoculated at 1% with the culture. The test tubes were incubated at 37 °C. Growth was monitored by measuring the A_{620} every hour for 6 h. Growth curves were constructed by plotting increases in absorbance (increase in absorbance from 0 h readings) versus

incubation time. Growth curves of the action of the extracts against the bacteria are shown in the appendices. The number of hours required to increase the absorbance by 0.5 U in each sample was used to compare the activity of the microorganisms. Probit analysis was conducted and the time taken to reach an absorbance of 0.5 U was obtained by inverse predictions. Analysis of each of the extractions was carried out in duplicate and against *E.coli* 0157:H7 and *S. aureus.* The above mentioned method is a modification from a previous study by Gilliland and others (1985). Gilliland and others (1985) compared bile tolerance of cultures of *L. acidophilus* isolated from fecal samples of pigs using time taken to increase the turbidity by 0.3 U. Their analysis in this case was carried out using least significance difference mean separation techniques.

Statistical Analysis

Probit models were fit to the increased absorbance data for each sample and each solvent. Estimates of time resulting in a 0.5 increase in absorbance units were obtained using inverse prediction models. Probit analyses for all solvents indicated 0.50 increases in absorbance units occurred between 3 and 4 h. In order to reduce the number of treatment (sample) comparisons within solvent, comparisons were also made at these 3 and 4 h times using analyses of covariance (ANCOVA) where baselines (h=0, Sample – control) and GLMM (Generalized Linear Mixed Model) with random effects of trial and set. Hourly measurements were analyzed using repeated measures methods for modeling covariance. In the case of *Escherichia coli* 0157:H7, responses were modeled by a beta distribution, and the repeated measures were modeled using a heterogeneous covariance structure (early measurements were less variable than the latter: 5 h and 6 h measurements). In the case of *Staphylococcus aureus*, responses followed a gamma

distribution, and the repeated measures were modeled using a first order autoregressive heterogeneous covariance structure. Each sample and hour combination means was adjusted for the covariate and pair wise sample comparisons were made at 3 and 4 h for each solvent. Statistical Analysis was performed using SAS 9.2 (Cary, NC) version 9.2.

RESULTS

Screening procedures were examined to obtain bio-active components from grape pomace. The extracts (treatments) were analyzed for their ability to inhibit food pathogen growth in Tryptic Soy Broth. Growth of pathogens was monitored by measuring hourly absorbance at 620 nm. The absorbance prediction at 0.5 U generally occurred at the mid range for log growth of bacteria (see Appendices). Inhibition at this absorbance level took place between the 3 h and 4 h period, and hence, closer examination of the action of the solvents at each of these times is also explained. Petroleum ether and 70% methanol were evaluated at a baseline value of 0.02 while 50% acetone and 0.01% pectinase were evaluated at a baseline value of 0.01. These baseline values were representative of the range of 0 h responses for each solvent.

Petroleum Ether

a. Measurement of Bioactive compounds effective against Gram negative pathogens using *Escherichia coli* 0157:H7 as a model

When looking at the action of petroleum ether using time to inhibition at 0.5 U (**Fig. 3a**), all the extracts were successful in inhibiting E. *coli* except the 4 h 4:1 and 8 h 2:1 extracts. The most effective extracts (those that show higher action of bioactive components) were 2 h 2:1 and 8 h 2:1. Further comparison at 3 h of growth (**Table 2**) shows that, only the 1 h 2:1 extract was significantly different ($p<0.05$) from the control for this pathogen. The comparison of the antimicrobial activity of extracts by measuring microbial concentration after 3 h does not correlate well with conclusions from the time required to increase microbial concentration by 0.5 absorbance units. This suggests that

growth at 3 h is not a good predictor for recovery of antimicrobial compounds from the extracts. However, evaluation of the extracts at 4 h of growth (**Table 3**) indicates that the only effective extract was 2 h 2:1 ($p<0.05$). Based on the above evaluation for petroleum ether, the 4 h growth rate predicts that the 2 h 2:1 extract is the most effective against *E. coli*, which is in agreement with the data evaluated at 0.5U.

b. Measurement of Bioactive compounds effective against Gram positive pathogens using *Staphylococcus aureus* as a model

The 2 h 4:1, 4 h (2:1 and 4:1) and 8 h 2:1(**Fig. 4a**) extracts contained bioactive components in sufficient quantities to be effective against *S. aureus*. These correlate well with our findings at 3 h of growth (**Table 4**) wherein the 4 h (2:1 and 4:1) and 8 h 2:1 extracts are statistically significant ($p<0.05$) from the control. Evaluation of the extracts at 4 h of growth (**Table 5**) shows that 2 h 4:1, 4 h 4:1 and 8 h 2:1 are statistically different from the control. The 8 h 2:1 extract was most effective in recovering bioactive components effective against *S. aureus*. Based on these evaluations, the least time consuming extraction condition for recovery of bioactive compounds that are effective against *S. aureus* using petroleum ether as the extracting solvent is the 2 h 4:1 extract. If time was not a significant factor in cost, the 8 h 2:1 extract would be the most economical.

It appears that the extracts which were most effective in case of *E. coli* were least effective in case of *S. aureus*. Longer interaction times were necessary to extract sufficient antimicrobial compounds for significant inhibition of *S. aureus* as compared to *E. coli* 0157:H7. The recommended extracts for *E.coli* 0157:H7 and *S. aureus* are 2 h 2:1 and 2 h 4:1 respectively. Researchers have used petroleum ether as a solvent for removal

of fatty materials in a Soxhlet Extractor as a preliminary stage for further extractions (Baydar and others 2004; Jayaprakasha and others 2003) and not as an extraction solvent itself. This is one of the first approaches towards using petroleum ether as a solvent to extract antimicrobial compounds.

70% Methanol

a. Measurement of Bioactive compounds effective against Gram negative pathogens using *Escherichia coli* 0157:H7 as a model

Based on the time taken to increase microbial concentrations by 0.5 absorbance units (**Fig. 3b**), all the extracts except 1 h 2:1 and 4 h 4:1 appeared to contain bioactive components in sufficient quantities to inhibit E. *coli* 0157:H7. However, measurement of growth of *E. coli* 0157:H7 at 3 h of growth (**Table 2**) indicated that only the 4 h 4:1 extract was significantly different from the control ($p<0.05$). This was not a good correlation with the findings based on time taken to increase microbial concentrations by 0.5 U. Evaluation of the extracts at microbial growth level of 4 h (**Table 3**) indicated that 2 h (2:1 and 4:1), 4 h 2:1 and 8 h 4:1 are statistically significant ($p<0.05$) from the control. The 4 h growth level results agreed more with the observations at 0.5 U than those at 3h. Based on absorbance at 0.5U and the 4 h growth levels, the 2 h 2:1 extract appears to be the most effective against *E. coli* 0157:H7 with 70% methanol as the solvent.

b. Measurement of Bioactive compounds effective against Gram positive pathogens using *Staphylococcus aureus* as a model

The time taken to increase microbial concentrations by 0.5 absorbance units (**Fig. 4b**) shows that all the extracts contained bioactive components in sufficient quantities to

inhibit the Gram positive pathogen, *S. aureus*. The time taken to increase absorbance by 0.5U was 3.3 h for the control and ranged from 3.53 – 3.75 h for the extracts. Thus, some extracts showed higher antimicrobial activity as compared to the others. Further comparison at 3 h of growth (**Table 4**) shows that all the extracts except the 2 h 4:1 and 8 h 2:1 are statistically significant from the control ($p<0.05$). However, the screening method which was most effective is the 1 h 4:1 extract. Evaluation of extracts at 4 h of growth (**Table 5**) also indicated that all extracts contain bioactive components in sufficient quantities to inhibit *S.* aureus. These results correlate with our findings at 3 h of growth as well as with the time taken to obtain an increase of 0.5 absorbance units.

In conclusion, our findings for 70% methanol shows that a 1 h 4:1 extract was sufficient to achieve statistically significant inhibition for the *S. aureus* pathogen while 2 h 2:1 extract was suitable for the *E. coli* pathogen. Jayaprakasha and others (2003) studied the effect of grape seed extracts by a methanol: water: acetic acid assay and found that both these bacteria were inhibited by the extracts. Similar findings were also reported by other researchers indicating that methanol extracts from grape seeds are potent antimicrobial agents (Shoko 1999; Baydar and others 2004).

50% Acetone

a. Measurement of Bioactive compounds effective against Gram negative pathogens using *Escherichia coli* 0157:H7 as a model

As shown in **Fig. 3c**, 4 h (2:1 and 4:1) extracts and 8 h 4:1 were most effective in inhibiting microbial growth. Further examination at 3 h of growth (**Table 2**) demonstrated that the 1 h 2:1, 2 h 4:1, 4 h 4:1 and 8 h 4:1 were significantly different from the control. Though the 8 h 4:1 extract had the most inhibition numerically, it was

not statistically significant ($p>0.05$) from the 1 h 2:1 extract. At 3 h of growth, results indicated that lower interaction times and ratios were sufficient to achieve inhibition of *E. coli* 0157:H7. At 4 h of microbial growth (**Table 3**), all the extracts except the 2 h 2:1 extract were significantly different from the control at a 95% confidence level. However, the extracts were not statistically significant from each other ($p>0.05$). The most economical extract should be the 1 h 2:1 extract based on growth levels at 3 h and 4 h, and absorbance at 0.5U because of lower interaction time and solvent utilizations.

b. Measurement of Bioactive compounds effective against Gram positive pathogens using *Staphylococcus aureus* as a model

Based on the time taken to reach microbial concentration of 0.5 absorbance units (**Fig. 4c**), all the extracts contained antimicrobial bioactive compounds. Further comparison at 3 h of growth (**Table 4**) demonstrates that all the extracts except the 2 h 2:1($p>0.05$) extract show significant antimicrobial activity. Though the 4 h 4:1 extract showed the most inhibition at 3 h of growth, it is not statistically significant from the 1 h 4:1 extract. Effects of the extracts at higher interaction times were not statistically different from those at lower extraction times ($p>0.05$). At 4 h of microbial growth (**Table 5**), all the extracts are statistically significant from the control. Also, evaluation of extracts at both 3 h and 4 h of growth correlates well with the conclusions from time taken to increase absorbance by 0.5 U. Based on the above results, it appears that the best extraction condition for the recovery of bioactive components with antimicrobial activity is that chosen on economical factors, namely 1 h 2:1.

In conclusion, our findings show that the extracts contain bioactive components in sufficient quantities to effectively inhibit *E. coli* 0157:H7 and *S. aureus* using 50%

acetone as a solvent. However, the most economical extracts would be those with lower interaction times and solvent utilizations. Hence, the1 h 2:1 extract appears to be the most suitable. Jayaprakasha and others (2003), Baydar and others (2004, 2006), Ozkan G and others (2004) have shown inhibitory effect of grape seed extract against *E. coli* 0157:H7 and *S. aureus* by using acetone: water: acetic acid (90: 9.5: 0.5) as an extraction solvent.

0.01% Pectinase

a. Measurement of Bioactive compounds effective against Gram negative pathogens using *Escherichia coli* 0157:H7 as a model

From **Fig. 3d,** it is observed that *E.coli* 0157:H7 was inhibited by all the extracts except 1 h 2:1 and 8 h 4:1 extracts. Further comparison of microbial growth at 3 h(**Table 2**) shows that inhibition of the organism by the aqueous extracts was significant ($p<0.05$) at 1 h 2:1 and 8 h 4:1. The comparison of the antimicrobial activity of the extracts by measuring microbial concentrations after 3 h of growth does not correlate well with the deductions from time taken to increased absorbance of microbial concentrations by 0.5 U. Hence, measuring growth rate after 3 h does not appear to be a good indicator for this extraction condition. At 4 h of growth (**Table 3**), all the extracts contained bioactive components in sufficient quantities to inhibit the growth of the organism. However, except for 8 h 4:1, they were not significantly different from each other ($p>0.05$). Thus, based on economical factors, it appears that the most suitable screening procedure is at 1 h 4:1.

b. Measurement of Bioactive compounds effective against *E. coli* pathogens using *Staphylococcus aureus* as a model

Estimation of bioactivity of the extracts based on antimicrobial studies against *S. aureus* shows that all the extracts have inhibitory action against the pathogen. Those with higher antimicrobial activity (longer time to reach an absorbance of 0.5 U) are 1 h 4:1 and 2 h 2:1 (**Fig. 4d**). Further examination at a microbial growth level at 3 h (**Table 4**) indicates that all the extracts are significantly different from the control except the 1 h 2:1 and 4 h 2:1 extracts. The 1 h 4:1 extract exhibits the most inhibition numerically and is statistically significant ($p<0.05$) from the remaining treatments. At a 4 h growth level (**Table 5**) for the pathogen, all the extracts are significantly different ($p<0.05$) from the control except the 1 h 2:1 and 4 h 2:1 extracts ($p>0.05$). Numerically, though the 2 h 2:1 extract shows the most inhibition, it is not significant ($p>0.05$) from the remaining extracts (including the 1 h 4:1 extract). The results based on microbial concentrations at 0.5 U and also on observations at 3 h and 4 h of microbial growth indicate that the 1 h 4:1 extract is the most suitable.

In conclusion, our findings reveal that 1 h 4:1 extract contains bioactive components in sufficient quantities to be effective against both the pathogens, *E. coli* 0157:H7 and *S. aureus*. Studies measuring antibacterial activity on grape pomace by using aqueous solvents are limited.

DISCUSSION

From the above data it can be concluded that all the extracts contain bioactive components. However, extracts that illustrated antimicrobial activity, contained active compounds in sufficient quantities to inhibit the pathogens. The extent of antimicrobial activity exhibited by the extracts was different. This implies that the extracts contain varying quantities of bioactive compounds. These bioactive compounds have been quantified and characterized as phenolic compounds and their derivatives by some researchers (Ozkan and others 2004; Pinelo and others 2005; Schieber and others 2001). Shoko and others (1999) also suggested that food borne pathogens can be inhibited by phenolic compounds like gallic acid.

Of the four solvents evaluated, it appears that 50% acetone contains higher amounts of bioactive components. This was evident in the ability of the extract to inhibit the pathogens to a greater extent. The time taken to increase microbial concentrations by 0.5 absorbance units was well beyond 4.2 h for *E.coli* (**Fig. 3c**) and 3.9h for *S. aureus* (**Fig. 4c**). These are much higher than the times of inhibition for the other solvents. Researchers have reported that acetone: water: acetic acid extracts inhibit bacteria at lower concentrations than methanol: water: acetic acid extracts (Baydar and others 2004; Jayaprakasha and others 2003). Jayaprakasha and others (2003) also have confirmed that the acetone extracts selectively extract more phenolics than the methanol extracts. Higher activity of 50% acetone can be explained on the basis of its ability to extract both the lipid soluble as well as aqueous components of the pomace.

The amount of solvent used also played a role in the extraction process. The principle of mass transfer in this case is the concentration gradient between the two phases, waste and solvent. Thus, higher amounts of solvent equate to greater extraction yields. But, the drawback would be that the extract is diluted. Based on our findings, greater inhibition of bacteria generally occurs at lower solvent: solid ratios. This is understandable, as lesser the amount of solvent used, more concentrated is the extract and hence a higher antimicrobial activity is demonstrated. From the standpoint of the processor, economical factors play an important role in selection of extraction solvents. It is the prerogative of the processor to choose between organic and aqueous solvents. Both aqueous as well as organic solvents contain bioactive components and show antimicrobial activity against food borne pathogens but at different levels.

Among petroleum ether, 70% methanol, 50% acetone and 0.01% pectinase extracts tested against *Staphylococcus aureus*, it appears that shorter interaction times were sufficient to achieve inhibition in all cases except petroleum ether. This may imply that the components leading to inhibition of the pathogen are more extractable under partial or complete aqueous conditions. For both the organisms, significant inhibition was achieved at lower interaction times and hence it appears that longer interaction times (8 h) are not significant. In case of *Escherichia coli* 0157:H7, significant inhibition was achieved within the 1 h and 2 h extraction times for all the solvents. It was found that extraction times of 4 h and 8 h had no significant effect on inhibition ($p>0.05$) of *E.coli* 0157:H7.

Overall, all the extracts showed greater inhibition against *E. coli* 0157:H7 than against *S. aureus* (**Fig. 3 and Fig. 4**). On the contrary, Jayaprakasha and others (2003)

and Baydar and others (2004) showed that Gram positive bacteria are inhibited more than the Gram negative bacteria. Possible differences could be that the base substrate used in their study was grape seeds while we incorporated the skin, seeds and stems. Also, the method of extraction plays an important role in the mass transfer during extraction. Grape cultivars vary by region of growth and climatic conditions. These factors may be responsible for the quantities of individual bioactive components of the extracts. Studies on antimicrobial activities against extracts obtained from grape pomace are limited and comparisons are therefore made with grape seed extracts. Lack of a standardized method of extraction of bioactive components from grape seed extract leads to variations in extraction procedures from lab to lab (Veluri and others 2006). This explains the variation observed in the action of bioactive properties from varying systems.

CHAPTER IV

CONCLUSION

In conclusion, value of bioactive compounds in grape pomace can be extracted due to its potent antimicrobial properties. These compounds have potential applications in the food industry to enhance the safety of the foods. Both *Escherichia coli* 0157:H7 and *Staphylococcus aureus* were inhibited by the grape pomace. This study was conducted on a lab scale and can be considered as a preliminary step for further scale up of the process. For commercial production of extracts from grape byproducts, it is vital to determine optimum extraction conditions that produce economical bioactive components. Thus, grape pomace extracts possess bioactive components that exhibit antimicrobial properties against food borne pathogens *Escherichia coli* 0157:H7 and *Staphylococcus aureus*. Further research needs to be conducted to characterize and identify the components of the extracts obtained.

Figure 1: Structure of some basic phenolics (Goldberg 2003)

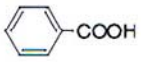
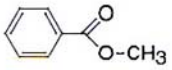
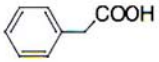
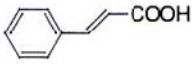
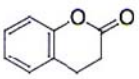
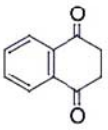
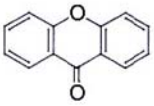
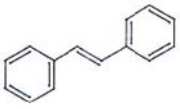
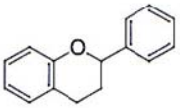
Carbon No.	Skeleton	Classification	Example	Basic structure
7	C_6–C_1	Phenolic acids	Gallic acid	
8	C_6–C_2	Acetophenones	Xanthoxylin	
8	C_6–C_2	Phenylacetic acid	*p*-Hydroxyphenyl-acetic acid	
9	C_6–C_3	Hydroxycinnamic acids	Caffeic acid	
9	C_6–C_3	Coumarins	Esculetin	
10	C_6–C_4	Naphthoquinones	Juglone	
13	C_6–C_1–C_6	Xanthones	Gentisin	
14	C_6–C_2–C_6	Stilbenes	Resveratrol	
15	C_6–C_3–C_3	Flavonoids	Quercetin	

Figure 2: Basic Steps involved in red wine making

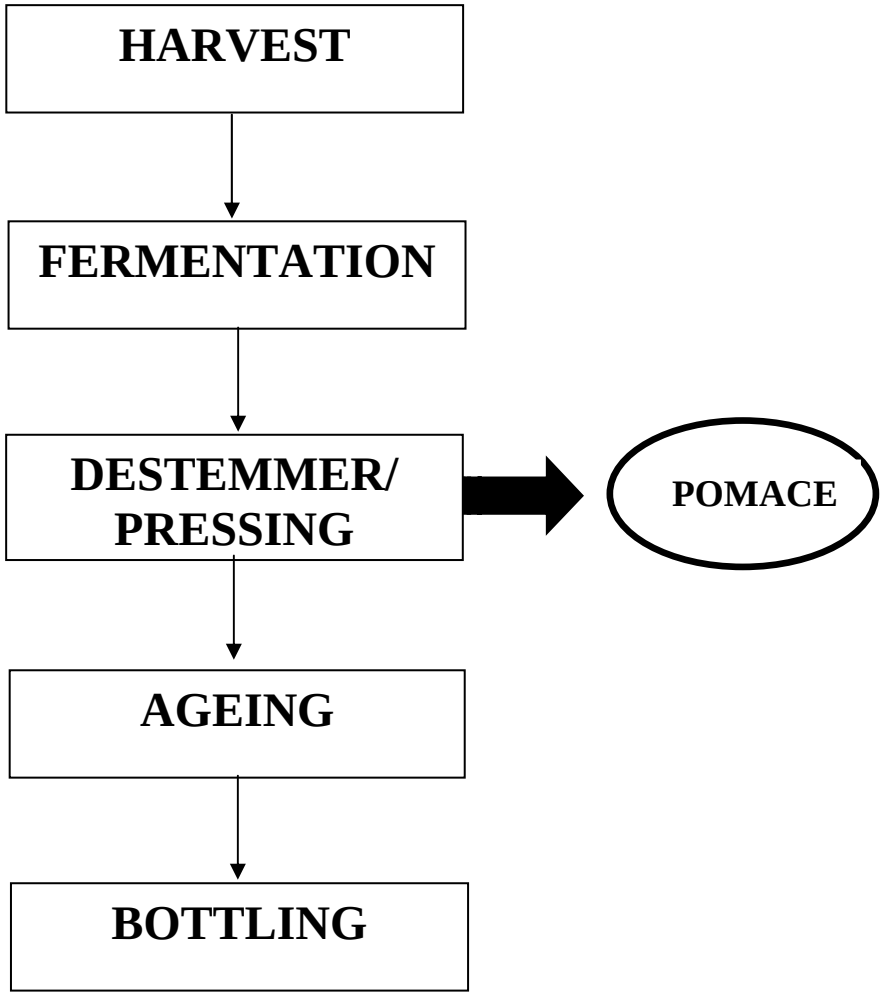
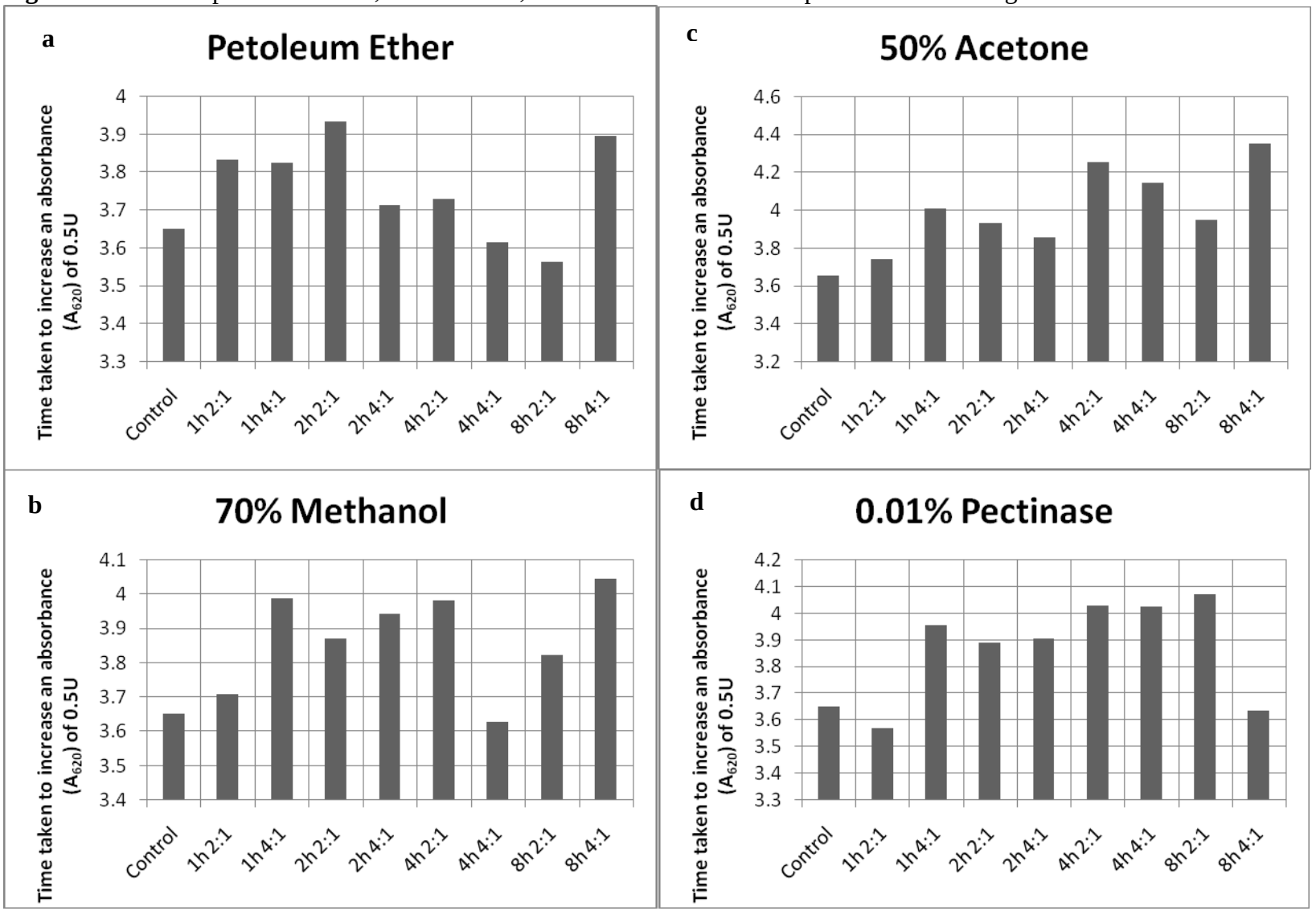
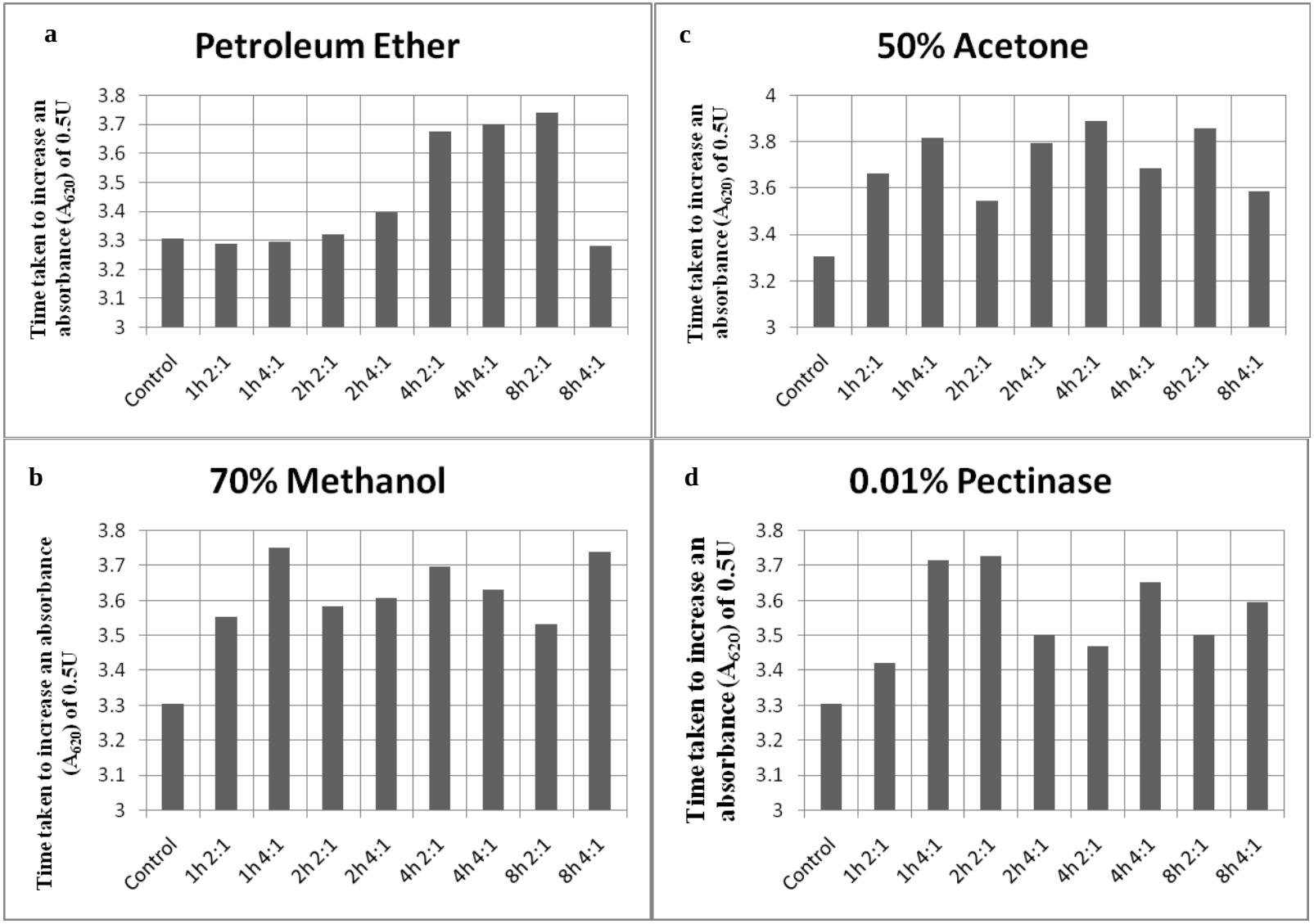


Figure 3: Action of petroleum ether, 50% acetone, 70% methanol and 0.01% pectinase extracts against *Escherichia coli* 0157:H7



Each treatment is representative of values from 3 replicates analyzed in duplicate.

Fig 4: Action of petroleum ether, 50% acetone, 70% methanol and 0.01% pectinase extracts against *Staphylococcus aureus*.



Each treatment is representative of values from 3 replicates analyzed in duplicate.

Table 1: Approximate acreage, percentage, and number of vines breakdown of wine grape types in Oklahoma (Stafne 2006)

Grape Color			
Color	**Acres**	**%**	**# of vines**
Red	137.5	59.2	85101
White	94.8	40.8	57201
Grape Type			
American	17.7	7.3	9661
Hybrid	34.5	14.3	19793
Vinifera	188.8	78	117971
Muscadine	1	0.4	242

Table 2: Inhibition of *Escherichia coli* 0157:H7 at a 3 h growth level[1]

Extraction time/ Solvent: waste ratios	**Petroleum Ether**	**70% Methanol**	**50% Acetone**	**0.01% Pectinase**
Control	0.3278 ± 0.0583^{a}	0.3278 ± 0.0583^{a}	0.3278 ± 0.0583^{a}	0.3278 ± 0.0583^{a}
1 h 2:1	0.2002 ± 0.0543^{b}	0.1600 ± 0.0883^{ab}	0.2329 ± 0.0544^{bc}	0.1901 ± 0.0489^{bc}
1 h 4:1	0.1923 ± 0.0908^{ab}	0.2455 ± 0.0699^{ab}	0.2701 ± 0.0659^{acd}	0.2785 ± 0.0639^{ac}
2 h 2:1	0.3369 ± 0.0692^{a}	0.3314 ± 0.0714^{ab}	0.3077 ± 0.0659^{ac}	0.2616 ± 0.0736^{ac}
2 h 4:1	0.3650 ± 0.0733^{a}	0.3601 ± 0.0726^{a}	0.1729 ± 0.0482^{bd}	0.3055 ± 0.0670^{a}
4 h 2:1	0.3493 ± 0.0716^{ac}	0.2824 ± 0.0639^{ab}	0.2787 ± 0.0673^{ac}	0.2706 ± 0.0647^{ac}
4 h 4:1	0.2508 ± 0.0577^{bc}	0.1708 ± 0.0704^{b}	0.2397 ± 0.0586^{bcd}	0.2545 ± 0.0605^{ac}
8 h 2:1	0.2881 ± 0.0634^{ab}	0.3254 ± 0.0874^{ab}	0.2724 ± 0.0666^{ac}	0.2529 ± 0.0606^{ac}
8 h 4:1	0.1610 ± 0.0871^{ab}	0.3253 ± 0.0696^{ab}	0.1669 ± 0.04610^{b}	0.1776 ± 0.0513^{b}

Numerical values in the above table are representative of microbial growth at an absorbance of 620 nm (A_{620}). Also, each treatment is representative of values from 3 replicates analyzed in duplicate. The values are corrected for time 0 readings.

[1]Values with different superscripts within the same column are significantly different ($p<0.05$). All values are mean ± standard error of the mean.

Table 3: Inhibition of *Escherichia coli* 0157:H7 at a 4 h growth level[1]

Extraction time/ Solvent: waste ratios	**Petroleum Ether**	**70% Methanol**	**50% Acetone**	**0.01% Pectinase**
Control	0.5537 ± 0.0643^{a}	0.5537 ± 0.0643^{a}	0.5537 ± 0.0643^{a}	0.5537 ± 0.0643^{a}
1 h 2:1	0.5026 ± 0.0701^{a}	0.4794 ± 0.0945^{ab}	0.4342 ± 0.0669^{b}	0.4973 ± 0.0691^{bc}
1 h 4:1	0.4971 ± 0.0851^{a}	0.5541 ± 0.0708^{abc}	0.4347 ± 0.0683^{b}	0.4498 ± 0.0678^{bc}
2 h 2:1	0.4821 ± 0.0683^{b}	0.4339 ± 0.0688^{b}	0.5057 ± 0.0688^{a}	0.4719 ± 0.0730^{bc}
2 h 4:1	0.5363 ± 0.0692^{a}	0.4765 ± 0.0690^{b}	0.4117 ± 0.0671^{b}	0.4297 ± 0.0673^{c}
4 h 2:1	0.5374 ± 0.0684^{a}	0.5053 ± 0.0683^{bc}	0.3766 ± 0.0654^{b}	0.4271 ± 0.0679^{c}
4 h 4:1	0.5080 ± 0.0685^{a}	0.4800 ± 0.0824^{abc}	0.4395 ± 0.0674^{b}	0.3926 ± 0.0666^{c}
8 h 2:1	0.5264 ± 0.0689^{a}	0.5039 ± 0.0774^{abc}	0.3895 ± 0.0656^{b}	0.4184 ± 0.0671^{c}
8 h 4:1	0.4673 ± 0.0866^{a}	0.4947 ± 0.0687^{bc}	0.4003 ± 0.0667^{b}	0.4844 ± 0.0710^{b}

Numerical values in the above table are representative of microbial growth at an absorbance of 620 nm (A_{620}). Also, each treatment is representative of values from 3 replicates analyzed in duplicate. The values are corrected for time 0 readings.

[1]Values with different superscripts within the same column are significantly different ($p<0.05$). All values are mean ± standard error of the mean.

Table 4: Inhibition of *Staphylococcus aureus* at a 3 h growth level[1]

Extraction time/ Solvent: waste ratios	**Petroleum Ether**	**70% Methanol**	**50% Acetone**	**0.01% Pectinase**
Control	0.4304 ± 0.0115 [a]	0.4304 ± 0.0091 [a]	0.4597 ± 0.0195 [a]	0.4588 ± 0.0163 [a]
1 h 2:1	0.4726 ± 0.0296 [a]	0.3477 ± 0.0272 [b]	0.2898 ± 0.0207 [c]	0.4027 ± 0.0714 [abc]
1 h 4:1	0.4581 ± 0.0293 [a]	0.1680 ± 0.0186 [d]	0.2210 ± 0.0160 [d]	0.2356 ± 0.0465 [d]
2 h 2:1	0.4624 ± 0.0314 [a]	0.3795 ± 0.0185 [b]	0.4065 ± 0.0354 [ab]	0.2384 ± 0.0116 [d]
2 h 4:1	0.4229 ± 0.0278 [ab]	0.3708 ±0.0302 [ab]	0.2704 ± 0.0183 [c]	0.3397 ± 0.0493 [bc]
4 h 2:1	0.2358 ± 0.0753 [bc]	0.2346 ± 0.0288 [c]	0.2772 ± 0.0191 [c]	0.4922 ± 0.1312 [abc]
4 h 4:1	0.2370 ± 0.0231 [c]	0.3459 ± 0.0353 [b]	0.1995 ± 0.0145 [d]	0.3197 ± 0.0267 [c]
8 h 2:1	0.2366 ± 0.0185 [c]	0.3850± 0.0378 [ab]	0.3262 ± 0.0335 [c]	0.3197 ± 0.0266 [b]
8 h 4:1	0.4926 ± 0.0321 [a]	0.2401 ± 0.0137 [c]	0.3862 ± 0.0258 [b]	0.3576 ± 0.0176 [bc]

Numerical values in the above table are representative of microbial growth at an absorbance of 620 nm (A_{620}). Also, each treatment is representative of values from 3 replicates analyzed in duplicate. The values are corrected for time 0 readings.

[1]Values with different superscripts within the same column are significantly different ($p<0.05$). All values are mean ± standard error of the mean.

Table 5: Inhibition of *Staphylococcus aureus* at a 4 h growth level[1]

Extraction time/ Solvent: waste ratios	**Petroleum Ether**	**70% Methanol**	**50% Acetone**	**0.01% Pectinase**
Control	0.7305 ± 0.0092 [a]	0.7305 ± 0.0099[a]	0.7449 ± 0.0196[a]	0.7430 ± 0.0215 [a]
1 h 2:1	0.7138 ± 0.0210 [ab]	0.5571 ±0.0277 [bcd]	0.5702 ± 0.0247 [b]	0.6250 ± 0.0886 [a]
1 h 4:1	0.7263 ± 0.0219 [a]	0.5484 ± 0.0385 [bcd]	0.5786 ± 0.0255 [b]	0.5933 ± 0.0344 [b]
2 h 2:1	0.7220 ± 0.0231 [ab]	0.6011 ± 0.0185 [bc]	0.5827 ± 0.0302 [b]	0.5665 ± 0.0227 [b]
2 h 4:1	0.6662 ± 0.0205 [bc]	0.5103 ± 0.0264 [bd]	0.5111 ± 0.0211 [cd]	0.5491 ± 0.0643 [b]
4 h 2:1	0.5887 ± 0.0886 [ac]	0.5241 ± 0.0408 [bc]	0.4997 ± 0.0210 [d]	0.6279± 0.1342 [a]
4 h 4:1	0.6400 ± 0.0294 [c]	0.6180 ± 0.0398[b]	0.5733 ± 0.0252 [bc]	0.5884 ± 0.0396 [b]
8 h 2:1	0.4874± 0.0255 [d]	0.5912 ± 0.0367 [b]	0.5122 ± 0.0307 [bc]	0.6106 ± 0.0287 [b]
8 h 4:1	0.7260 ± 0.0222 [a]	0.5466 ± 0.0197 [b]	0.5620 ± 0.0235 [bc]	0.5850 ± 0.0236 [b]

Numerical values in the above table are representative of microbial growth at an absorbance of 620 nm (A_{620}). Also, each treatment is representative of values from 3 replicates analyzed in duplicate. The values are corrected for time 0 readings.

[1]Values with different superscripts within the same column are significantly different ($p<0.05$). All values are mean ± standard error of the mean.

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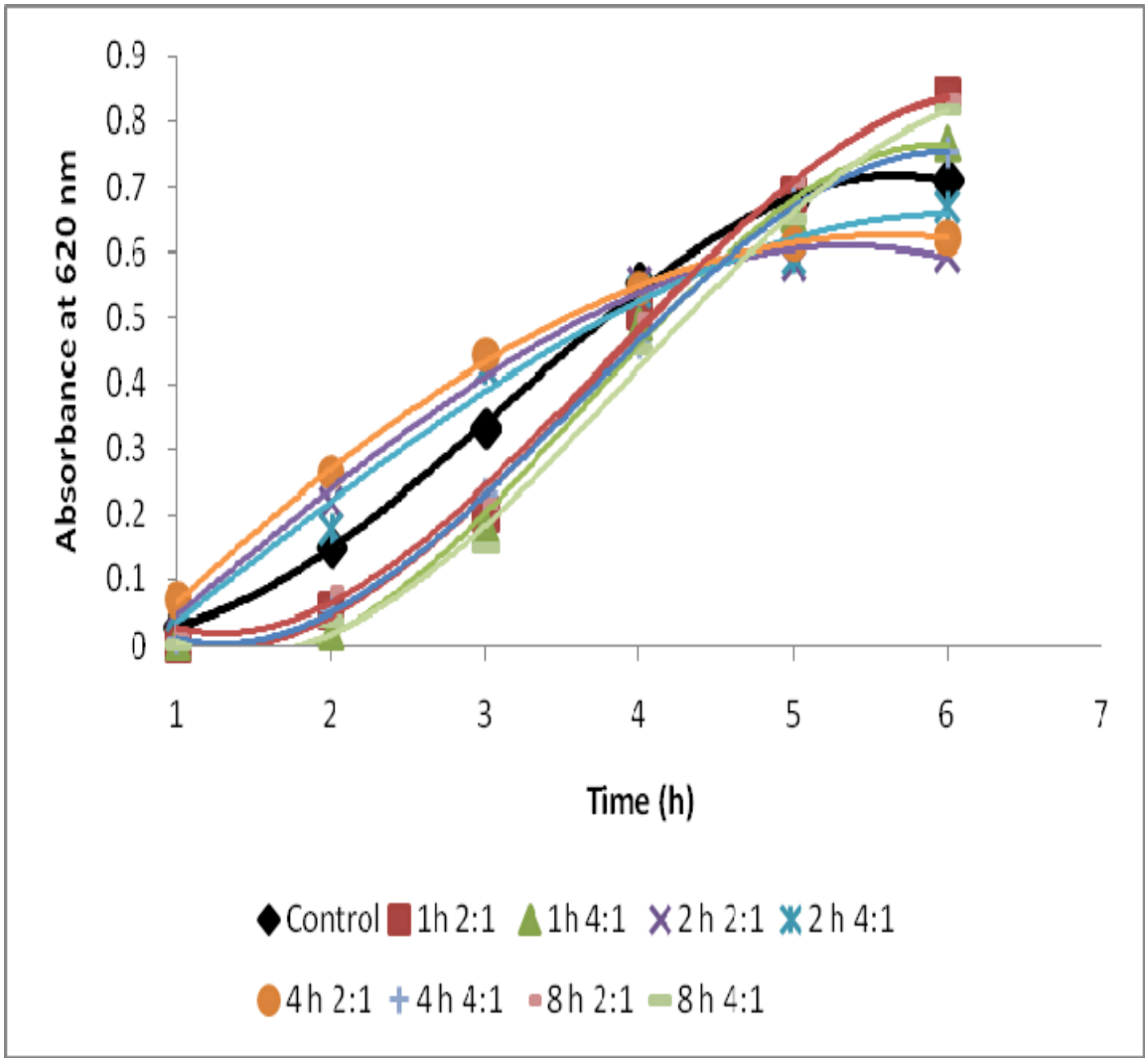
APPENDIX 1

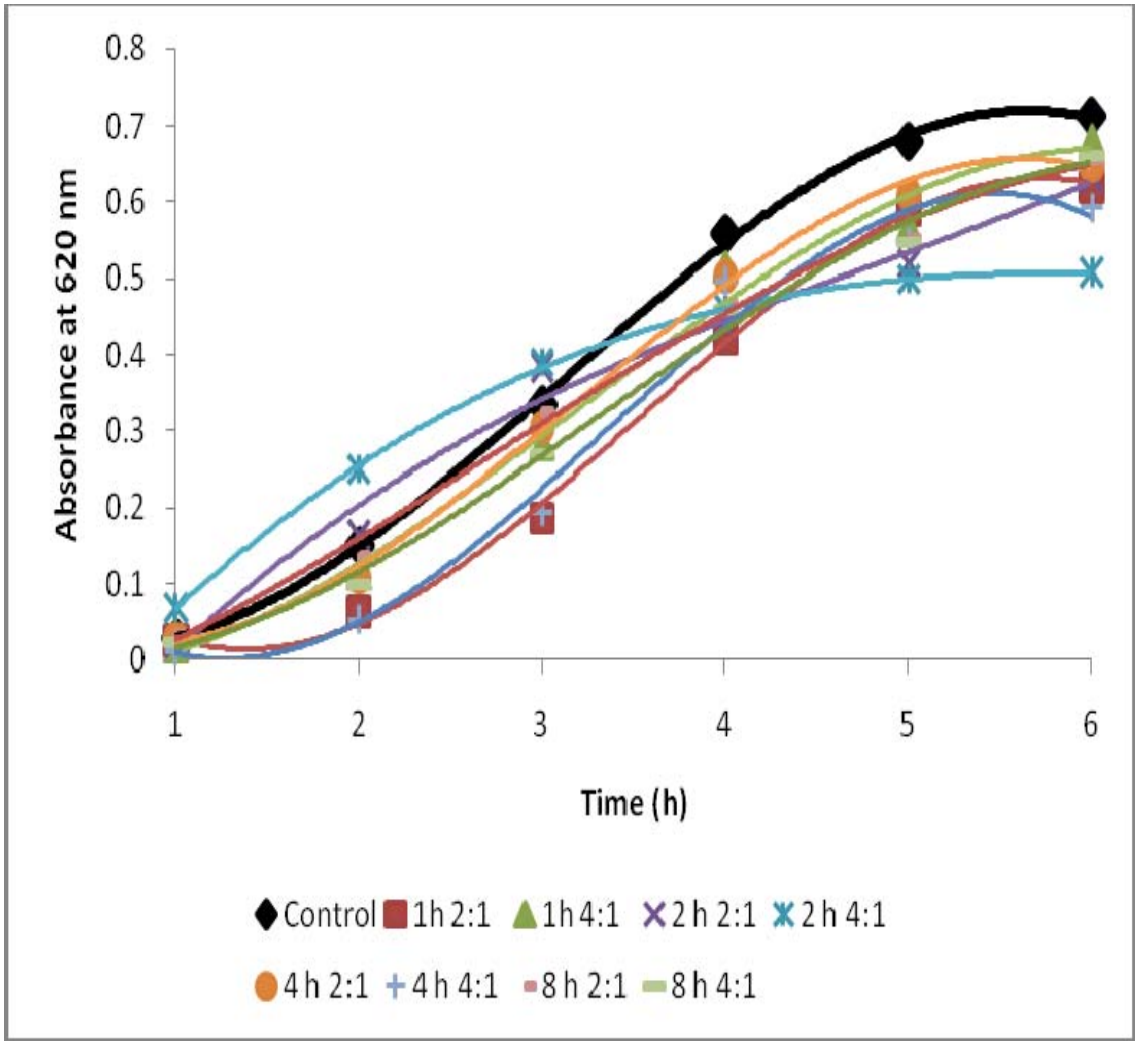
Growth of *Escherichia coli* 0157:H7 with Petroleum Ether as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 2

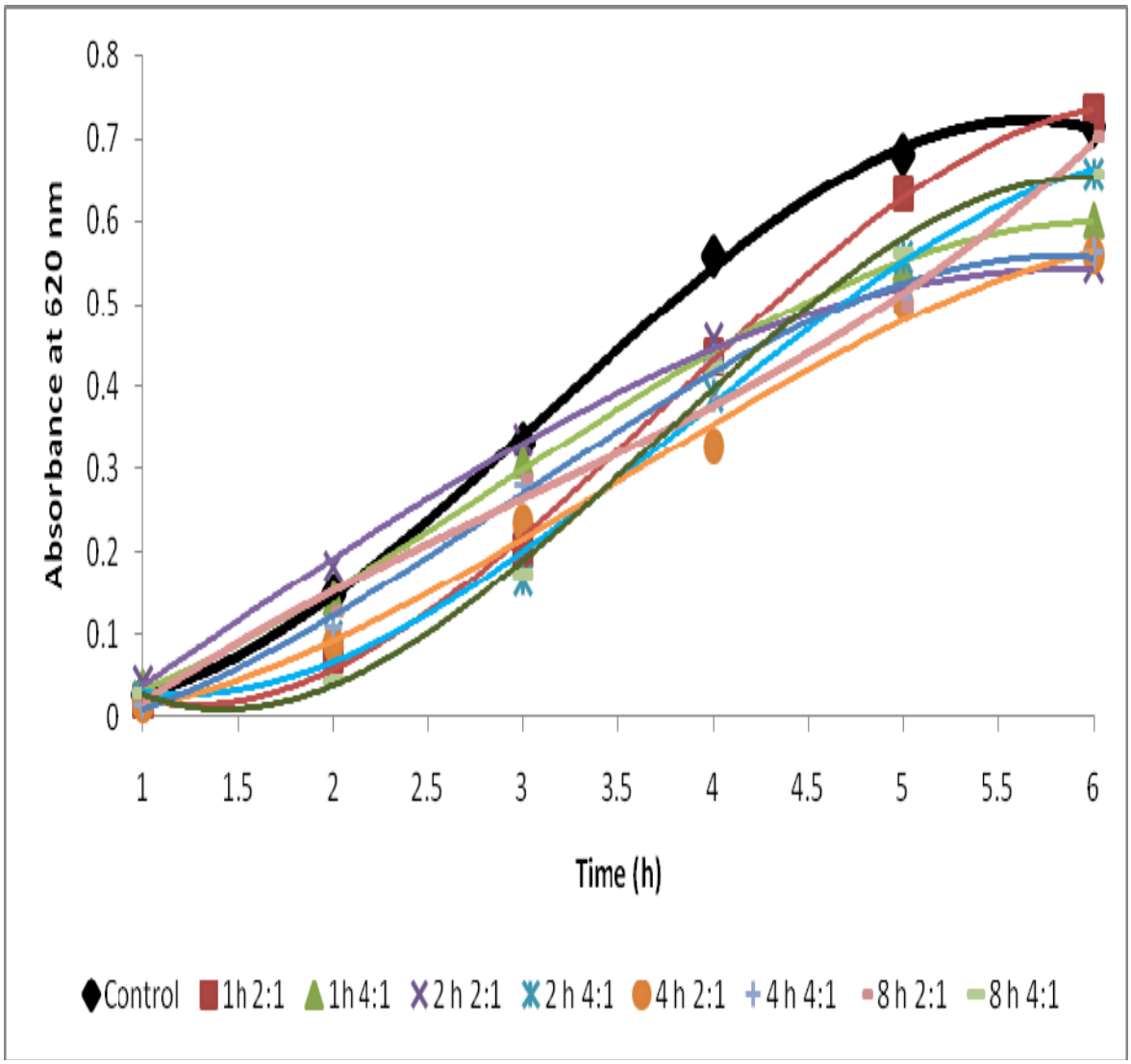
Growth of *Escherichia coli* 0157:H7 with 70% Methanol as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 3

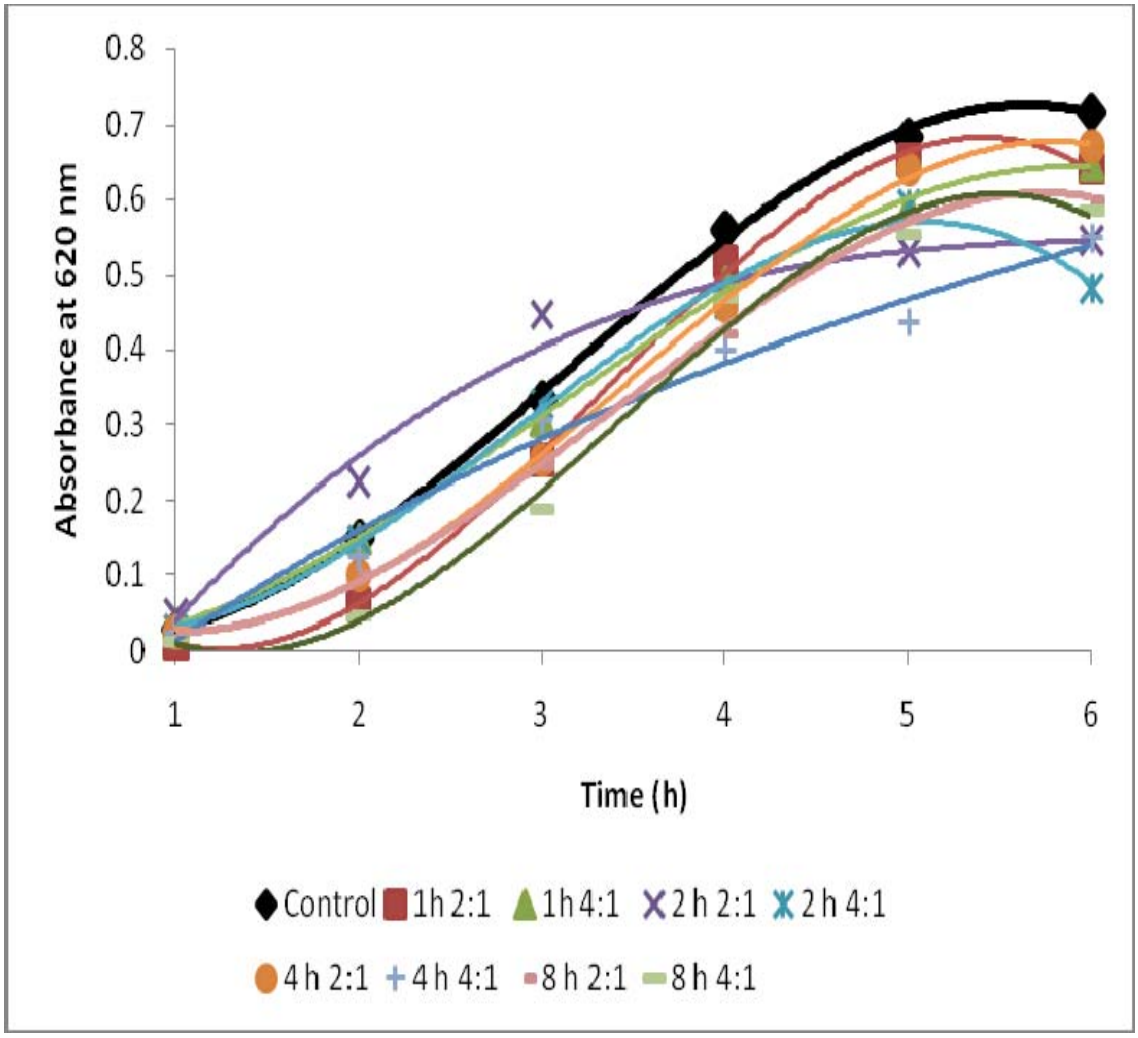
Growth of *Escherichia coli* 0157:H7 with 50% Acetone as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 4

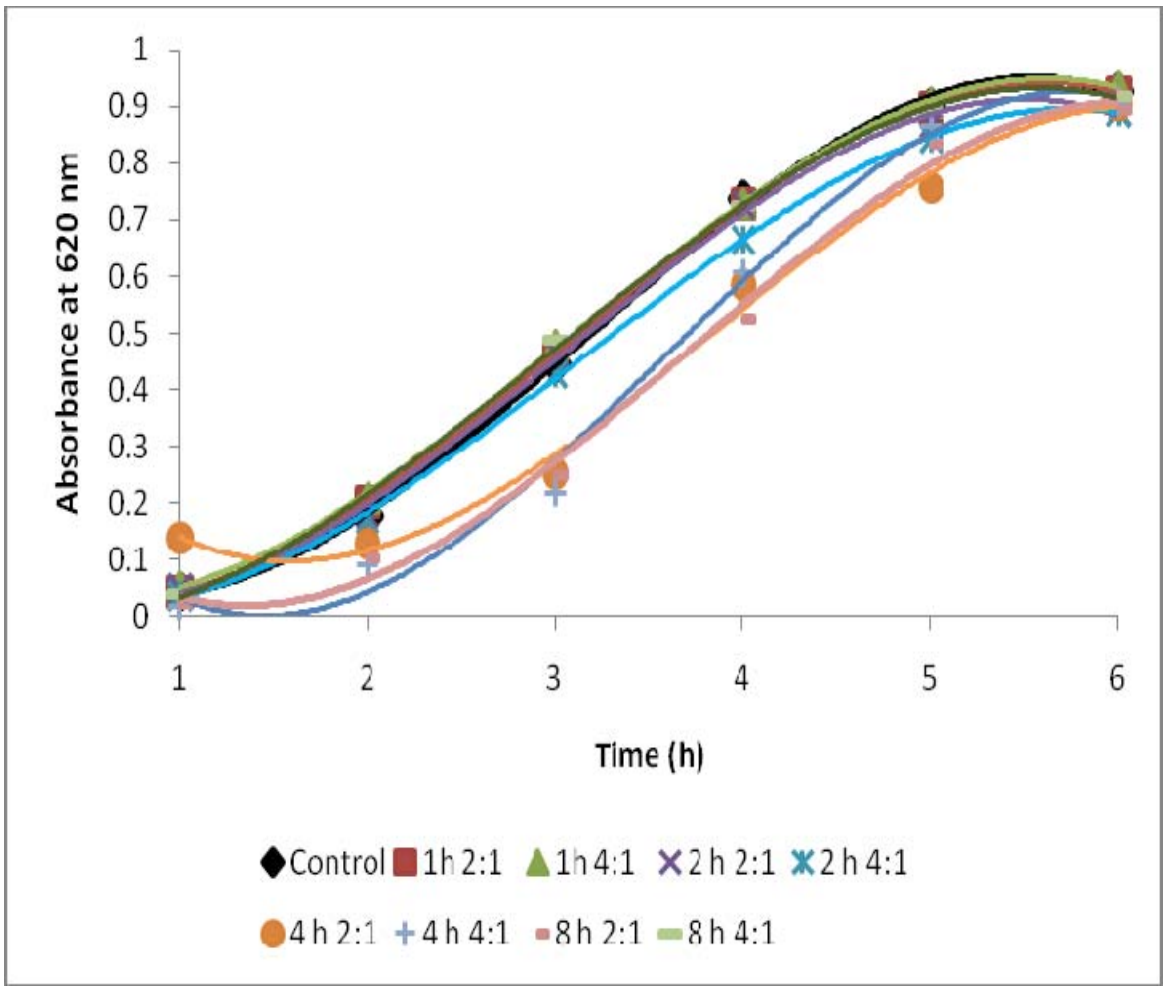
Growth of *Escherichia coli* 0157:H7 with 0.01% Pectinase as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 5

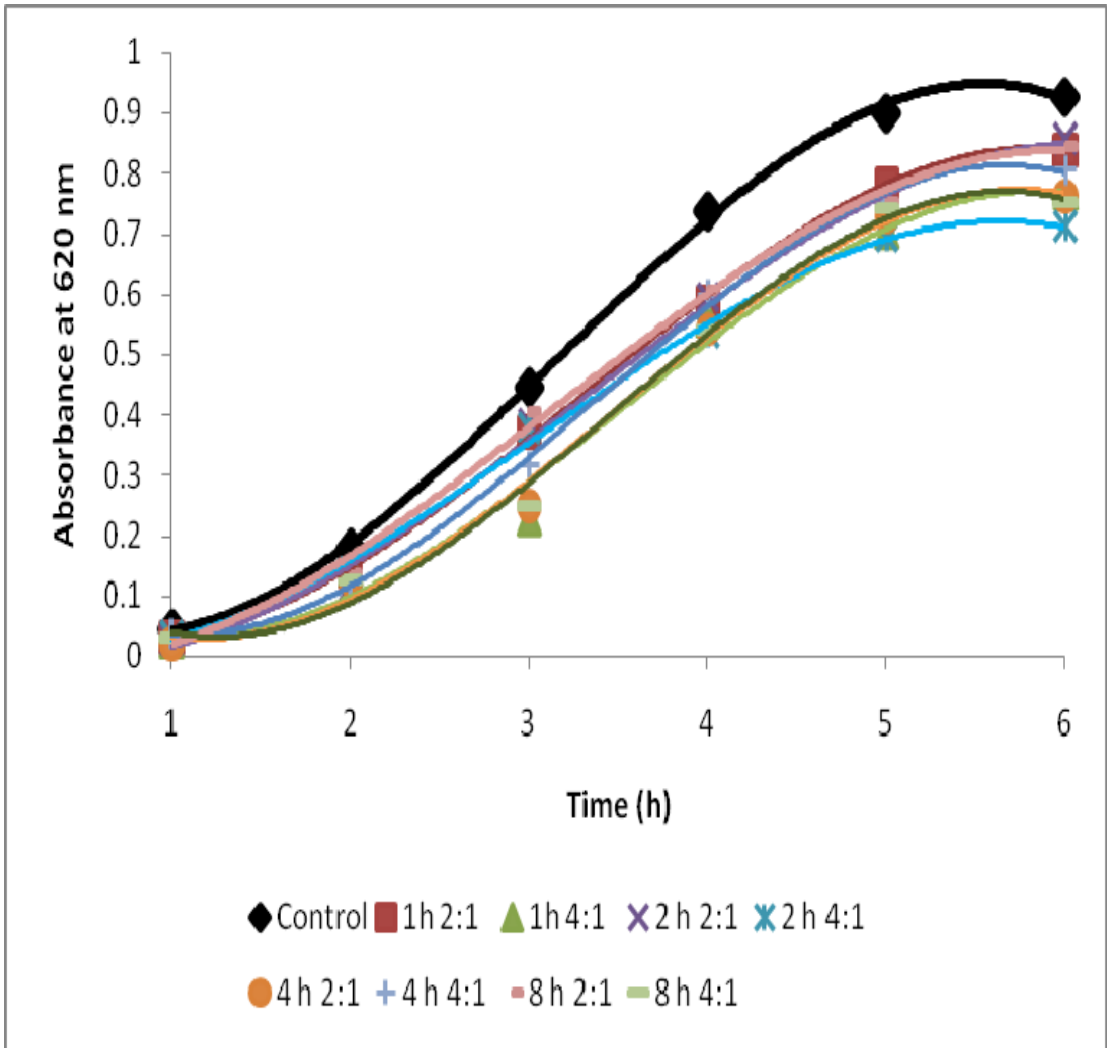
Growth of *Staphylococcus aureus* with Petroleum Ether as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 6

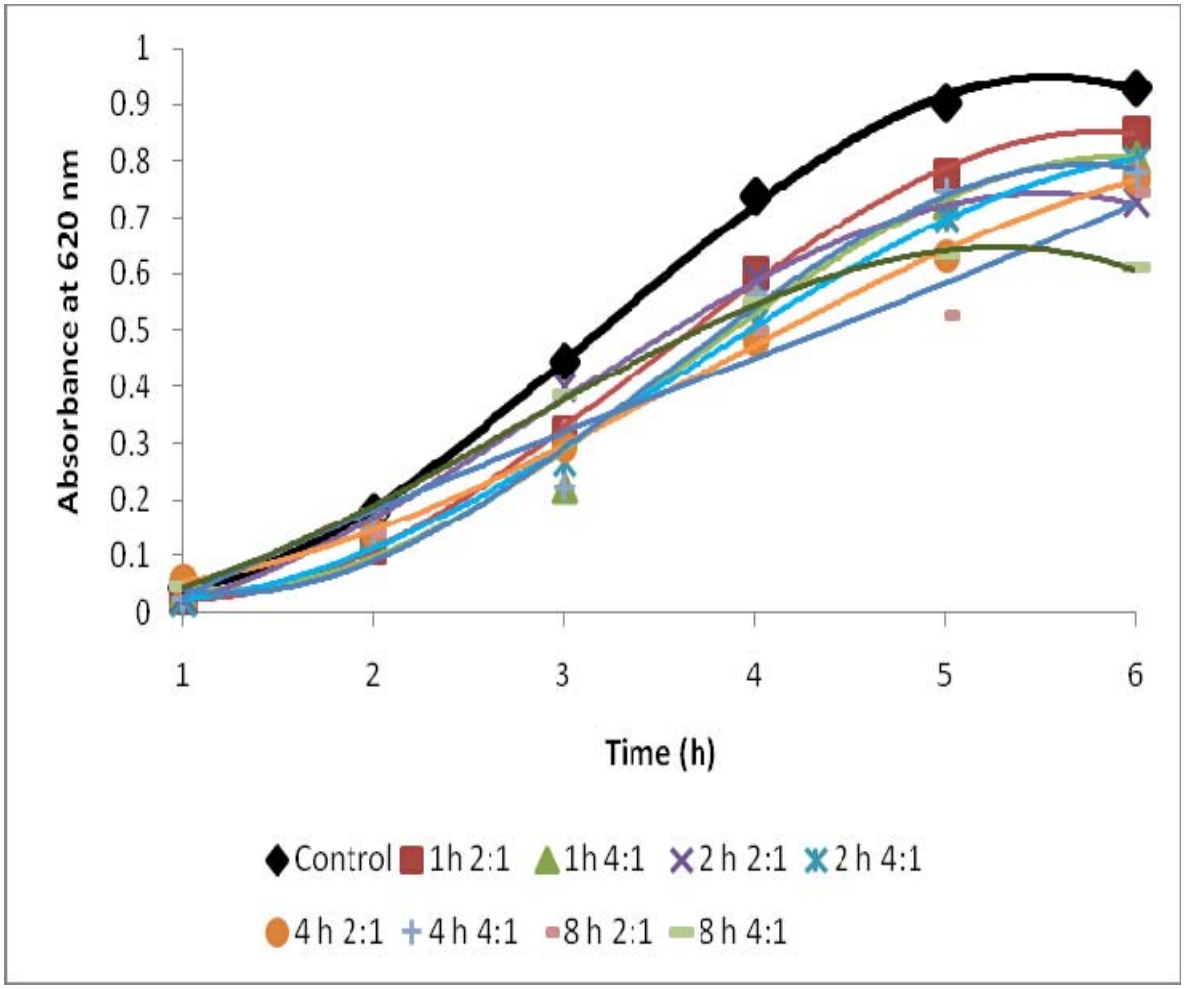
Growth of *Staphylococcus aureus* with 70% Methanol as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 7

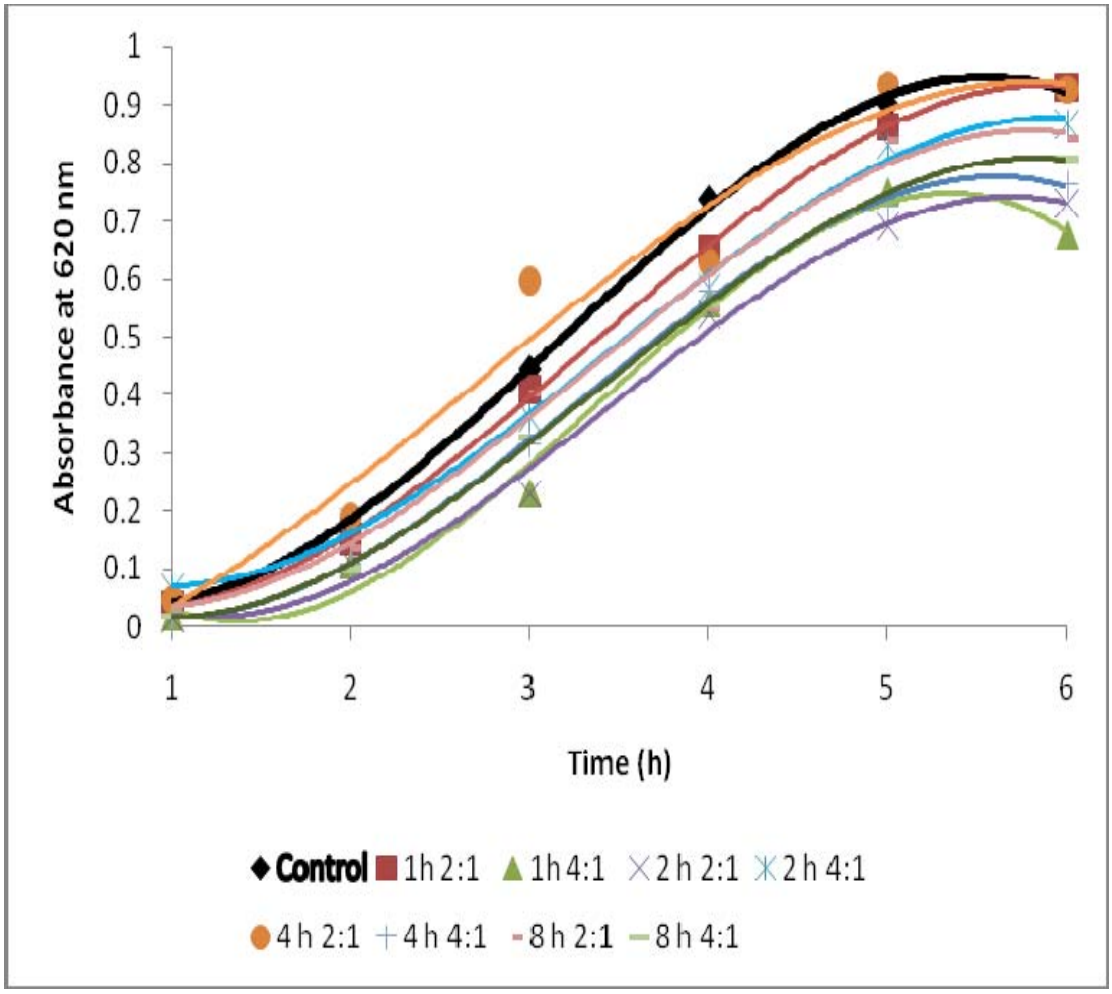
Growth of *Staphylococcus aureus* with 50% Acetone as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

APPENDIX 8

Growth of *Staphylococcus aureus* with 0.01% Pectinase as the extracting solvent



Each treatment is representative of values from 3 replicates analyzed in duplicate.

VITA

Akhila Vasan

Candidate for the Degree of

Master of Science

Thesis: EXTRACTION OF BIO-ACTIVE COMPONENTS FROM FRUIT AND VEGETABLE PROCESSING WASTES: USING GRAPE WASTE FROM THE WINE PROCESSING INDUSTRY AS A MODEL

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EXTRACTION OF BIO-ACTIVE COMPONENTS FROM FRUIT AND VEGETABLE PROCESSING WASTES: USING GRAPE WASTE FROM THE WINE PROCESSING INDUSTRY AS A MODEL

Pages in Study: 61 Candidate for the Degree of Master of Science

Major Field: Food Science

Scope and Method of Study: The aim of the study was to develop a screening procedure to extract valuable components from fruit and vegetable processing wastes which can be easily replicated on a large scale. Variety of extraction conditions such as solvent utilization, ratio of solvent: waste and interaction time were tested for their effect on the extraction procedure. The activity of the extracts was tested based on antimicrobial activity against common food pathogens: *Escherichia coli*0157:H7 and *Staphylococcus aureus*.

Findings and Conclusions: The results from the study concluded that the extracts contain bioactive components in sufficient quantities to inhibit the pathogens. Thus, value can be extracted in the form of bioactive antimicrobial compounds from grape waste. The most effective solvent was 50% acetone, which implies that this extract contains bioactive components in higher amounts than the other solvents to inhibit the pathogens. The experiment was conducted on a lab scale and further research needs to be conducted to scale up the process. Further research also needs to be conducted to identify and characterize the compounds.

ADVISER'S APPROVAL: Dr. Christina DeWitt