Antimicrobial properties of crude guava leaf extract (*Psidium guajava*) against food-borne pathogens

By

Ms. Thanyathorn Charoenpornpanich

ID. 481-8911

A special project submitted to the Faculty of Biotechnology,
Assumption University in part fulfillment of the requirements for
the degree of Bachelor of Science in Biotechnology

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Title	:	Antimicrobial properties of crude guava leaf extract (*Psidium guajava*) against food-borne pathogens
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Department	:	Agro-Industry
Faculty	:	Biotechnology
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(Asst. Prof. Dr. Wunwisa Krasaekoopt)

Advisor

Abstract

Guava (*Psidiu guajava)* is a famous fruit in Thailand, which its leave has been used as a traditional herb for long time. Guava leaf has been considered interesting since it is used in folk medicine as remedies for many diseases, especially diarrhea. The aim of this study was to examine the antibacterial activities of guava leaf extracts against 5 food spoilage bacteria and pathogens (*Bacillus cereus, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* and *Salmonella typhimurium).* Moreover, the influence of extraction was considered as types of solvents (ethanol and methanol), concentrations of solvent (50, 70 and 90%), ratio of guava leaf: solvent (1:4, 1:5 and 1:6) and concentration of guava leaf extract used (50, 75 and 100%). The antimicrobial activity was tested by using disc diffusion method. The inhibition zones were recorded in centimeters. The extracts of *P. guajava* leaves were highly inhibit against the gram-positive bacteria as *S. aureus, B. cereus* and *L.monocytogenes* and virtually inactive against the gram-negative bacteria as *E. coli* and *S. typhimurium*. The extraction with 70% ethanol at ratio of 1:4 showed higher antimicrobial activity, against these bacteria compared to methanol extracts and water boiling extract.

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Ms.Thanyathorn Charoenpornpanich

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Introduction

Even though industries have produced a number of synthetic antimicrobial compounds, resistance of microorganisms against these compounds has increased. The use of natural antimicrobial substances has been of interest. Natural antimicrobial compounds have been isolated from a large number of plants (Vlietinck et al., 1995). Various types of natural antimicrobial compounds have a major function on plant defense. The use of plant extracts and phytochemicals, such as plyhenol compounds and flavonoids, which are a large group of secondary plant metabolite that possesses antimicrobial activities, can be great significance in preservation of food (Rauha et al., 2000). The antimicrobial properties of plants have been investigated by a number of researchers in different countries to prove such efficiency (Shapoval et l., 1994; Artizzu et al., 1995; Izzo et al., 1995).

Guava (*Psidium guajava* L.), belonging to the family of Myrtaceae, is a native of tropical America and has long been naturalized in Southeast Asia. The positive effects of guava extracts on human ailments have been described (Lozoya, 1999). The pharmacological actions and the medicinal uses of aqueous extracts of guava leaves in folk medicine include the treatment of various types of gastrointestinal disturbances such as vomiting, diarrhea, inhibition of the peristaltic reflex, gastroenteritis, spasmolytic activity, dysentery, abdominal distention, flatulence and gastric pain (Lozoya et al., 1994). Bronchitis, asthma attacks, cough, pulmonary diseases could be also treated with guava teas (Khan and Ahmad, 1985) and could also be useful as anti-inflammatory and hemostatic agent (Liu, 1988). Phytochemical studies have identified more than 20 compounds in guava extracts (Osman et al., 1974; Begum et al., 2002). The major constituents of its leaves were identified to be tannins, β-sitosterol, maslinic acid, essential oils, triterpenoids and flavonoids (Osman et al., 1974; Arima and Danno, 2002; Begum et al., 2002; 2004).

Although many studies reported an effective antimicrobial properties of guava leaf extract against a number of microbial strains, such as *Aeromonas hydrophila*, *Shigella* spp. and *Vibrio* spp. (Chulasiri et al., 1986), *Staphylococus aureus* and β-streptococcus group A (Jaiarj et al., 1999), *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* (Abdelrahim et al., 2002), the effectiveness of this extract against

food poisoning bacteria, such as *Listeria monocytogenes* and *Salmonella typimurium*, for the use in fresh-cut industry has not been reported elsewhere. Therefore, this project aimed to study the antimicrobial of Thai guava leaf extract against food poisoning bacteria and other bacteria for the further use in fresh-cut produces.

Objectives

1. To study the influence of solvent types and concentration for guava leaf extraction on the antimicrobial properties of crude guava leaf extract.

2. To study the influence of the ratio between guava powder and solvent; concentration used on the antimicrobial properties of crude guava leaf extract.

Literature Review

I. Guava

1. Botanical Information

The Guava (*Psidium guajava* L.), belonging to the family of Myrtaceae, is the origin of a tropical America and South-East Asia which contains about 100 species of tropical shrubs and small trees, low evergreen tree or shrub six to twenty-five feet high, with wide spreading branches and downy twigs. The branches are very strong and highly tolerant to high wind. The leaves are oblong or oval and blunt, 3 to 6 inches long, and feather-veined. The flowers are an inch or more across the calyx bell-shaped and splitting irregularly, the four to six petals are white, and the stamens are white with yellow anthers (Figure 1). Guava trees grow rapidly and fruit in 2 to 4 years from seed. They live 30 to 40 years but productivity declines after the 15th year. Orchards may be rejuvenated by drastic pruning (Anonymous, 2009b,c).

Figure 1: Guava

Guava is a well known shrub and popular for its delicious fruit in Thailand. Its fruit is normally eaten fresh or pickled or sweetened. Moreover, it is processed to gain guava juice. India ranks third in production of guava fruit behind Brazil and the United States. Guava fruit is one of the richest sources of vitamin and contains four to ten times more of this vitamin than citrus fruits (Jamayet, 2009). The fruit of the common tropical guava (*P. guajava*) is shaped like an apple or a pear and has white, pink, or

red flesh (depending on the variety) with a sweet, musky flavor and, usually, a yellow rind. The fruit varies in size (generally 1-4 inches in diameter) and has a pronounced musky odor. The butter varieties are soft when ripe, creamy in texture with a rind that softens to be fully edible. The seeds are numerous but small and in good varieties, fully edible. Actual seed counts have ranged from 112 to 535 (Anonymous, 2009b).

The leaves and bark are rich in tannin (10% in the leaves on a dry weight basis, 11-30% in the bark). The bark is used in Central America for tanning hides. Malayans use the leaves with other plant materials to make a black dye for silk. In Southeast Asia, the leaves are employed to give a black color to cotton; and in Indonesia, they serve to dye matting. In Asia, a drink is made from an infusion of guava fruits and leaves. The infusion made with guava tree leaves is considered a medicine (Julia, 2009).

2. Medicinal Uses of guava leaves

Guava leaves are used in folk medicine as a remedy for diarrhea and, as well as the bark, for their supposed antimicrobial properties and as an astringent. In some country, a tea made from the young leaves is used for diarrhea, dysentery and fever. Indians also employ it for sore throats, vomiting; stomach upsets, for vertigo, and to regulate menstrual periods. It has also shown to have a tranquilizing effect on intestinal smooth muscle, inhibit chemical processes found in diarrhea and aid in the re-absorption of water in the intestines. In other research, an alcoholic leaf extract was reported to have a morphine-like effect, by inhibiting the gastrointestinal release of chemicals in acute diarrheal disease. This morphine-like effect was thought to be related to the chemical quercetin. In addition, lectin chemicals in guava were shown to bind to *E. coli* (a common diarrhea-causing organism), preventing its adhesion to the intestinal wall and thus preventing infection (and resulting diarrhea). Tender leaves are chewed for bleeding gums and bad breath, and it is said to prevent hangovers Indians throughout the Amazon take a leaf decoction as a remedy for coughs, mouth sores, bleeding gums, or use it as a douche for vaginal discharge and to tighten and tone vaginal walls after childbirth. A decoction of the bark and/or leaves or a flower infusion is used topically for wounds, ulcers and skin sores. An extract is given in

epilepsy and chorea and a tincture is rubbed on the spine of children in convulsions. A combined decoction of leaves and bark is given to expel the placenta after childbirth. In a study with guinea pigs, Brazilian researchers reported that guava leaf extracts have numerous effects on the cardiovascular system which might be beneficial in treating irregular heart beat (arrhythmia). Previous research indicated guava leaf provided antioxidant effects beneficial to the heart, heart protective properties, and improved myocardial function (Anonymous, 2009a-c).

3. Plant Chemical

Phytochemical studies have identified more than 20 compounds in guava. The major constituents of its leaves were identified to be tannins, β-sitosterol,maslinic acid, essential oils, triterpenoids and flavonoids (Dweck, 2009). Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit) and contains appreciable amounts of vitamin A as well. Guava fruits are also a good source of pectin - a dietary fiber. The leaves of guava are rich in flavonoids, in particular, quercetin (Figure 2). Much of guava's therapeutic activity is attributed to these flavonoids. The flavonoids have demonstrated antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava; it is able to relax intestinal smooth muscle and inhibit bowel contractions. In addition, other flavonoids and triterpenes in guava leaves show antispasmodic activity. Guava also has antioxidant properties which are attributed to the polyphenols found in the leaves (Jaiarj, 1999).

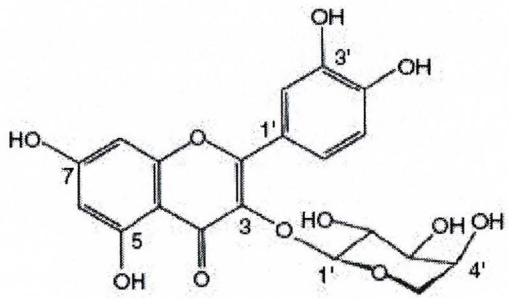

Figure 2: Structure of quercetin-3-O-a-L-arabinopyranoside (guaijaverin) (Prabu, 2006).

4. Antimicrobial activity

It has been reported that high level of antibacterial activity was detected in guava leaves. Leaf extracts have in vitro antimicrobial activity mostly associated with flavonoids, such as morin, glycosides, quercetin, and quercetin glycosides. In a study of 38 patients with various types of acne, the antimicrobial activity was compared with tea tree oil, doxycycline, and clindamycin (Qadan, 2005). Bark and leaf extracts have shown to have *in vitro* toxic action against numerous bacteria. In several studies guava showed significant antibacterial activity against such common diarrhea-causing bacteria as *Staphylococcus, Shigella, Salmonella, Bacillus, E. coli, Clostridium, and Pseudomonas* (Fathilah, 1998).

Flavonoids (guaijaverin) have also been reported to have potential antiplaque agent by inhibiting the growth of *S. mutans*, a plaque-forming bacterium (Prabu, 2006). Inhibitory activities against the growth of *Neisseria* spp, *Staphylococcus* spp. and *Streptococcus viridans*, which are oral flora in oral cavity, were also reorted (Abdelrahim, 2002). It has also demonstrated antifungal, anti-yeast (candida), anti-amebic, and antimalarial actions.

5. Toxicology

Guava has a long history of traditional use, much of which is being validated by scientific research. It is a wonderful natural remedy for diarrhea - safe enough even for young children. For infants and children under the age of 2, just a cup daily of guava fruit juice is helpful for diarrhea. For older children and adults, a cup once or twice daily of a leaf decoction is the tropical herbal medicine standard. Toxicity studies with rats and mice, as well as controlled human studies show both the leaf and fruit to be safe and without side effects. Acute toxicity tests in rats and mice have proven the LD_{50} of guava leaf extracts to be more than 5 g/kg. In vitro genotoxicity and mutagenicity tests on *P. guajava* in human peripheral blood lymphocytes found no disturbances in cell division. (Kanta, 2008; Roncada, 2004)

II. Microorganism Background

1. Characteristic of *Bacillus cereus*

Bacillus cereus is an endemic, soil-dwelling, facultative aerobes gram-positive rod-shaped beta hemolytic bacterium belonging to the genus *Bacillus*. *Bacillus* can produce protective endospores. Some harmless strains of *B. cereus* are used as a probiotic feed additive to reduce *Salmonella* in the intestines and cecum. This improves the animals' growth as well as food safety for humans who eat their meat. Some strains are harmful to humans and cause foodborne illness, *B. cereus* is responsible for a minority of foodborne illnesses (2–5%). It is a spore forming bacterium capable of facultative aerobic metabolism. *Bacillus* foodborne illnesses occur due to survival of the bacterial endospores when food is improperly cooked and refrigerated. There are two principal type of food poisoning caused by *B. cereus*:

1) An emetic (vomiting) intoxication due to the ingestion of a toxin preformed in the food. This toxin known as cereulide may be formed by certain strains of *B. cereus* if the vegetative cell count exceeds 10^5 cfu/g. The toxin is extremely stable and cannot be inactivated by reheating the food. The cereulide toxin is extremely heat resistant and can survive at 126°C for 90 minutes. Symptoms can appear within 5 hours and include nausea and vomiting. The duration of illness is usually 6 to 24 hours.

2) A diarrhoeal infection due to the ingestion of cells which produce enterotoxins in the small intestine and ingestion. This infection occurs when *B. cereus* levels exceed 10^6cfu/g in the food and sufficient amounts of the enterotoxin are formed in the small intestine of the host. The diarrheal toxin is heat sensitive and can be inactivated by heating at 56°C for 5 minutes. Symptoms usually appear within 24 hours and include abdominal pain, watery diarrhea and nausea. The duration of illness is usually 12 to 24 hours.

B. cereus spores are variable in their resistance to heat. Most are moderately heat resistant but some are extremely heat resistant. Low levels of *B. cereus* cells or spores are found on virtually every raw agricultural commodity (e.g. herbs, spices, vegetables, milk, meat etc). These levels are generally too low to cause foodborne poisoning; however, the ability of *B. cereus* to form spores ensures its survival through all stages of food processing and subsequent time/temperature abuse can result in

spore germination and multiplication low level of the vegetative cells to dangerous levels of cells or toxins in the food at the time of consumption. Hence, foods should be cooked to a core temperature of 75°C instantaneous or 70°C for 2 minutes or equivalent. After cooking, foods should be cooled as quickly as possible to ensure they can be refrigerated within 2 hours (Anonymous, 2009f-g).

2. Characteristic of *Escherichia coli*

Escherichia coli is a common gram-negative non-sporing rod-shaped bacterium that ferments lactose. They are catalase positive, oxidase negative and indole positive. It lives in the intestinal tracts of animals in health and disease. Most types of *E. coli* are harmless. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 or by preventing the establishment of pathogenic bacteria within the intestine. However, some types cause travelers' diarrhea. The worst type of *E. coli* causes bloody diarrhea, and can sometimes cause kidney failure and even death. These problems are most likely to occur in children and in adults with weak immune systems.

Human and animal can get *E. coli* infections by eating foods containing the bacteria or swallowing water in a swimming pool contaminated with human waste. However, the regular presence of *E. coli* in the human intestine and feces has led to tracking the bacterium in nature as an indicator of fecal pollution and water contamination. As such, it is taken to mean that, wherever *E. coli* is found, there may be fecal contamination by intestinal parasites of humans. Most cases of E. coli infection get better without treatment in 5 to 10 days (Anonymous, 2009h-i; Todar, 2009a).

3. Characteristic of *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive rod-shaped bacterium, motile via flagella at 30°C and below but usually not at 37°C, grows from below 1°C up to 44°C. It grows at pH values of between 4.4 and 9.6. Flagella are produced at room temperature but not at 37°C. Hemolytic activity on blood agar has been used as a marker to distinguish *Listeria monocytogenes* among other *Listeria* species, but it is not an absolutely definitive criterion. It is the agent of listeriosis, a serious infection

caused by eating food contaminated with the bacteria. It is one of the most virulent foodborne pathogens with 20 to 30% of clinical infections resulting in death. Studies suggest that up to 10% of human gastrointestinal tracts may be colonized by *L. monocytogenes*. It is the third most common cause of meningitis in newborns. *L. monocytogenes* is easily destroyed by heat

Responsible for approximately 2,500 illnesses and 500 deaths in the United States annually, Listeriosis is the leading cause of death among foodborne bacterial pathogens with fatality rates exceeding even *Salmonell*a and *Clostridium botulinum*. The disease affects primarily pregnant women, newborns and adults with weakened immune systems. An outbreak of listeriosis in Halifax, Nova Scotia involving 41 cases and 18 deaths, mostly in pregnant women and neonates, was epidemiologically linked to the consumption of coleslaw containing cabbage that had been treated with *L. monocytogenes* contaminated raw sheep manure (Anonymous, 2009j-k; Todar, 2009b).

4. Characteristic of *Salmonella typhimurium*

Salmonella is a cylindrical rod of size about 2 microns by 0.5 microns, ram-negative, facultative anaerobic bacteria of the family Enterobacteriaceae, catalase positive and oxidase negative. The organism can grow at temperatures ranging from 7 to 47°, and at pH values of between 4.0 and 9.5. The minimum water activity value for growth is 0.96, and the organism can survive for long periods of time in foods containing fats and at low water activities. They are easily destroyed by heating (Anonymous, 2009l-m).

There are over 2,500 different serotypes know. Most but not all are human pathogens, *Salmonella typhimurium* multiplies in the gastrointestinal tract of many animal species after consumption of contaminated food or water but it usually causes no disease, but in humans *S. typhimurium* cause "Salmonellosis", which is the most food borne disease caused by ingestion of contaminated water or food (usually poultry or beef). The micro-organism will adhere to the walls of the small intestine and grow and release enterotoxin. Invasion of salmonellae results in varying degrees of damage to the mucous membrane of the small intestine and colon. In addition to enterotoxin,

cytotoxin is also produced by most strains of *Salmonella*. The disease is often referred to as salmonellosis.

Frequently, the source of *Salmonella* sp. is originated in the poultry and swine. Also it lives in the intestinal tracts of warm and cold blooded animals. The area that often detected salmonella sp. includes water, soil, insects, factory surfaces, kitchen surface, animal feces, raw material, raw poultry and raw seafood. In human, *Salmonell*a sp. are the cause of two disease called salmonellosis and enteric fever (typhoid), resulting from bacteria invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection or intoxication (Walderhaug, 1992).

The number of *Salmonella* generally around 10^5 *Salmonella* per gram of food is enough to cause illness, although outbreaks have occurred where between 1 to 10 cells per gram of food was detected. The illness usually occurs 8 to 72 hours after the ingestion of the contaminated food. Illness may begin with nausea and vomiting, often followed by diarrhea. In healthy adults the disease is usually self-limiting with good medical care, but it is more serious in the young, the old and those with weakened immune systems. Duration of symptom is usually last for 2 to 5 days. The preventing or minimizing the risk of *Salmonella* sp. are to store perishable foods chilled or frozen, wash vegetables and fruit well if these are to be eaten raw, cook food to at least an internal temperature of 75°C and if not eaten immediately cool rapidly and refrigerate at 5^0C or less (Anonymous, 2009l-m).

5. Characteristic of *Staphylococcus aureus*

Staphylococci are Gram-positive facultative anaerobes that yield principally lactic acid when fermented. Staphylococci have perfectly spherical shape approximately 1 micrometer in diameter. *S. aureus* forms a fairly large yellow colony on rich medium, it always occurs in microscopic clusters resembling grapes. *S. aureus* can grow at a temperature range of 7 to 48° C but grow poorly when they are in the presence of other microorganisms. It often hemolysis when grow on blood agar plates

S. aureus was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses. *S. aureus* can be differentiated from most other staphylococci by the coagulase test. *S. aureus* is primarily coagulase-

positive, that causes clot formation while most other *Staphylococcus* species are coagulase-negative. (Stoppler, 2009) The treatment for *S. aureus* infection is penicillin; but in 1943, *S. aureus* resistant to penicillin was discover by Alexander Fleming.

S. aureus is always considered as a potential pathogen. Over 30 different types of Staphylococci can infect humans, but most infections are caused by *Staphylococcus aureus*. Staphylococci can be found in the air, dust, water, human feces, nose, on the skin and utensil handled by man. In the majority of cases, the bacteria do not cause disease. However, damage to the skin or other injury may allow the bacteria to overcome.

Staphylococcal food poisoning is an illness of the bowels that causes nausea, vomiting, diarrhea, abdominal pain and dehydration. It is caused by eating foods contaminated with toxins produced by *Staphylococcus aureus*. Symptoms usually develop within one to six hours after eating contaminated food. The gastroenteritis is self-limiting with the person getting better in 8–24 hours. Patients with this illness are not contagious, since toxins are not transmitted from one person to another. The prevention is, to minimize the time food is stored in the temperature danger zone (5^0C to 60°C).

Some strains of *S. aureus,* growing under conditions in which there is little or no oxygen and temperature range between 10-48°C, will produce the exotoxin TSST-1 cause of bacteria toxic shock syndrome. It is characterized by the sudden onset of high fever, vomiting, diarrhea, and muscle aches, followed by low blood pressure (hypotension), which can lead to shock and death. There may be a rash resembling sunburn, with peeling of skin (Stoppler, 2009; Anonymous, 2009n-o; Todar, 2009c).

Materials and Methods

1. To study the influence of solvent types and concentration for guava leaf extraction on the antimicrobial properties of crude guava leaf extract.

1.1 Guava leaf preparation

Guava leaves were collected from Nakornpathom Province. The leave were dried at 40°C and triturated in a mechanical mill. The guava leaf powder was kept in the airtight container at room temperature.

1. 2. Preparation of crude guava leaf extracts

The extraction of guava leave was modified from Betoni et al. (2006). Dried guava leaf powder was extracted with 50, 70 and 90% methanol and ethanol using the ratio of dried leave to alcohol as 1:4. The mixtures were then filtered after 48 h. The plant residue was re-extracted with addition of solvent, and after 24 h it was filtered again. Combined filtrates were concentrated on a water bath at 45°C for solvent elimination, and the extracts were kept in sterile bottles under refrigerated conditions until use.

1.3 Culture preparation

There were five cultures used in this experiment, namely *Escherichia coli, Listeria monocytogenes*, *Salmonella typhyimurium, Bacillus cereus* and *Staphylococcus aureus.* A loop of culture was activated into 5 ml nutrient broth (or BHI for *L. monocytogenes*) and the tubes were incubated at 37°C for 24 h. Then, one milliliter of each active culture was transferred into 10 ml of broth media and incubated at 37°C for 24 h.

1.4 Antimicrobial activity testing

The extracts were dissolved in sterile water and filtered through 0.22 μm Millipore. Agar plates were inoculated with these cultures; *B. cereus, E. coli, L. monocytogenes, S. typhimurium and S. aurues* at visible growth's concentration by spread plate technique (Table 1). Subsequently, filter paper discs (6 mm in diameter),

saturated with extracts (25 μL), and were placed on surface of each inoculated plate. The controls were the solvents used for each extract. The plates were then incubated at 37°C for 24 h. Diameters of the resulting zone of inhibition formed in the medium were measured to determine the antibacterial effectiveness of each testing solution, and a diameter of 7 mm or more was considered a positive result. All tests were performed in triplicate.

Table 1: Visible growth's concentration of each microorganism

Culture	Concentration (CFU/ml)
Bacillus cereus	10^8
Escherichia coli	10^{10}
Listeria monocytogenes	10^{11}
Salmonella typhimurium	10^8
Staphylococcus aureus	10^{11}

2. To study the appropriate ratio between guava leaf powder to solvent and concentration of crude guava leaf extract used

2.1 Preparation of guava leaf extraction

Crude guava leaf extracts were prepared by using the ratio of guava leaf powder to 70% ethanol as 1:4, 1:5 and 1:6. The mixtures were then filtered after 48 h. The plant residue was re-extracted with addition of solvent, and after 24 h it was filtered again. Combined filtrates were concentrated on a water bath at 45°C for ethanol elimination, and the extracts were kept in sterile bottles under refrigerated conditions until use.

2.2 Culture preparation

The procedure is the same as mention in section 1.3.

2.3 Antimicrobial activity testing

The extracts were diluted to 50, 75 and 100% in sterile water and filtered through 0.22 μm Millipore. Agar plates were inoculated with these cultures; *B. cereus, E. coli, L. monocytogenes, S. typhimurium and S. aurues* at visible growth's concentration by spread plate technique (Table 1). Subsequently, filter paper discs (6 mm in diameter), saturated with extracts (25 μL), and were placed on surface of each inoculated plate. The controls were the solvents used for each extract. The plates were then incubated at 37°C for 24 h. Diameters of the resulting zone of inhibition formed in the medium were measured to determine the antibacterial effectiveness of each testing solution, and a diameter of 7 mm or more was considered a positive result. All tests were performed in triplicate.

3. To study the antimicrobial properties of crude guava leaf extracts produced by using solvent extraction in comparison with boiling water extraction

3.1 Solvent extraction

Crude guava leaf extracts were produced by using ethanol extraction with the ratio of powder to ethanol as 1:4. The mixture was then filtered after 48 h. The plant residue was re-extracted with addition of solvent, and after 24 h it was filtered again. Combined filtrates were concentrated on a water bath at 45°C for solvent elimination, and the extracts were kept in sterile bottles under refrigerated conditions until use.

3.2 Boiling water extraction

One part of guava leaf powder was boiled in four parts of boiling water. The solution was boiled for 15 min. This process was used to produce traditional tea from guava leaf (Kanta, 2008). The mixture was then cooled and kept in sterile bottle under refrigerated conditions until use.

3.3 Antimicrobial activity testing

The ethanol and water extracts were filtered through 0.22 μm Millipore. Agar plates were inoculated with these cultures; *B. cereus, S. aurues* and *L. monocytogene* at visible growth's concentration by spread plate technique (Table 1). Subsequently, filter paper discs (6 mm in diameter), saturated with extracts (25 μL), and were placed

on surface of each inoculated plate. The controls were the solvents used for each extract. The plates were then incubated at 37°C for 24 h. Diameters of the resulting zone of inhibition formed in the medium were measured to determine the antibacterial effectiveness of each testing solution, and a diameter of 7 mm or more was considered a positive result. All tests were performed in triplicate.

4. Statistical analysis

A completely randomized 2x3 and 3x3 factorial designs with 3 replications and analysis of variance (ANOVA) were used for sections 1 and 2, respectively. Comparisons of means were carried out by using Duncan's multiple range tests.

Result and Discussion

The Guava leaf extracts were tested for antimicrobial activity against five microorganisms namely *Bacillus cereus, Escheriachia coli, Listeria monocytogenes, Salmpnella typhimurium* and *Staphylococcus aureus* at visible range at 10^8, 10^{10}, 10^{11},10^8 and 10^{10}, respectively. Agar disc diffusion method was used to determine the inhibitory effect of guava leaf extracted with different conditions, i.e. type of solvent, concentration of solvent, ratio of guava leaf: solvent and concentration of extract.

I. The influence of solvent types and concentration for guava leaf extraction on the antimicrobial properties of crude guava leaf extract.

Guava leaves were extracted by using 2 types of alcoholic solvent, which were ethanol and methanol. The concentration of alcoholic solvent was also considered to affect on antimicrobial activity. Three levels of alcohol as at 50, 70 and 90% were used in this part. The ratio between guava leaf powder and solvent used was 1 to 4 and 100% crude extracts were used for microbiological testing. The results are shown in Table 2. It was noticed that crude guava leaf extracts, produced by using either ethanol or methanol at different concentrations, could not inhibit gram negative like *E. coli* and *S. typhimurium*. These organisms are the index organisms used as safety and quality indicators in the food industry, whereas, all treatments were able to inhibited gram positive like *B. cerues*, *L. monocytogenes* and *S. aureus* , which are food poisoning bacteria. This may be caused by the difference in structure of their cell walls that posses an outer membrane consisting of various lipid complexes while gram positive bacteria does not possess a lipid outer membrane (Anonymous, 2009d).

Moreover, the ethanol extraction demonstrated the higher antimicrobial effectiveness of crude guava leaf extract than methanol extraction for all concentration levels against *B. cereus*, *S. aureus* and *L. monocytogenes*. For *B. cereus*, the highest inhibitory effect was obtained when 70 and 90 % ethanol were used. The inhibitory zones were 1.57 and 1.52 mm for 70 and 90%, respectively, which was no significant

different ($p<0.05$). For the economical reason, 70% ethanol was considered as more worthy.

Table 2: The antimicrobial activity (cm of inhibition zone) of 100% crude guava leaf extract using different types and concentrations of solvents at the ratio of guava leaf powder to solvent as 1:4 against various types of microorganisms

Microorganisms	Type of solvent	Concentration of solvent (%)		
		50	70	90
Bacillus cereus	EtOH	1.4^{b}	**1.57^{a}**	1.52^{a}
	MetOH	1.04^{d}	1.23^{c}	1.2^{c}
Staphylococcus aureus	EtOH	1.8^{b}	**2.03^{a}**	1.48^{cde}
	MetOH	1.6^{cde}	1.68^{bc}	1.63^{bcd}
Escherichia coli	EtOH	N	N	N
	MetOH	N	N	N
Listeria monocytogenes	EtOH	0.93^{bc}	0.88^{bcde}	**1.07^{a}**
	MetOH	0.92^{bcde}	0.92^{bcd}	0.98^{ab}
Salmonella typhimurium	EtOH	N	N	N
	MetOH	N	N	N

* The same letters mean there are no significant different at 95% confidential level and the statistical analysis were done separately for each bacterium.

**N refers to not detectable

For *S. aureus*, crude guava leaf extract produced by using 70% ethanol provided the highest inhibition (2.03 mm). It was recognized that extraction with ethanol was better than methanol except for 90%. On the other hand, the highest inhibitory effect against *L. monocytogenes* was obtained at 90% for ethanol and methanol, which were 1.07 and 0.98 mm, respectively.

A better inhibitory effect of crude guava leaf extract produced by using ethanol extraction may be due to a higher content of inhibitory compounds found in the crude guava leaf extract (Table 3). It is implied that the inhibitory compounds in guava leaf may be dissolve well in ethanol rather than in methanol. The solid content of crude guava leaf extracts produced by using ethanol was approximately 0.07 mg/ml, which were higher than those of methanol (0.05 mg/ml).

Table 3: Concentration of inhibitory compounds (mg/ml) extracted from guava leaf ratio of 1:4 by using different types of solvents and concentration.

Type of solvent	Concentration of solvent (%)		
	50	70	90
EtOH	0.07^{a*}	0.07^{a}	0.06^{ab}
MetOH	0.05^{b}	0.05^{b}	0.05^{b}

* The same letters mean there are no significant different at 95% confidential level

Finally, the 70% ethanol extraction was used for antimicrobial testing of crude guave leaf extracts against *B. cereus* and *S. aureus*; and 90% ethanol extraction for *L. monocytogenes.*

2. The influence of ratio between guava leaf powder to solvent and concentration of crude guava leaf extract used

From the section (1), crude guava leaf extracts were produced by using 70 and 90% ethanol for the antimicrobial testing against *B. cerues, S. aureus* and *L. monocytogenes*, respectively. In this part, the various ratios between guava leaf powders to ethanol were used as 1:4, 1:5 and 1:6. After extraction, the crude guava leaf extract were diluted to 50, 75 and 100% for the antimicrobial testing against these microorganisms. The results were shown in Table 4.

It was noticed that the crude guava leaf extract produced by using the ratio of powder to ethanol as 1:4 had the highest inhibitory effect, which may possibly due to higher amount of inhibitory compounds in the crude extracts (Table 4). The solid contents of crude guava leaf extracts of the ratio 1:4 were higher (0.07 mg/ml) than

those of other ratios (0.05-0.6 mg/ml) for all concentration of ethanol. Moreover, the undiluted guava leaf extracts (100%) also gave the maximum inhibition zone. The maximum inhibition zones were 1.57, 2.03 and 1.18 cm for *B. cereus*, *S. aureus* and *L. monocytogenes*, respectively.

Table 4: The antimicrobial activity (cm of inhibition zone) of crude guava leaf extract produced by using ethanol extraction at different ratios of guava leaf powder to ethanol and different concentration of extracts used

Microorganisms	Ratio	Concentration of extract (%)		
		50	75	100
Bacillus cereus	1:4	1.22^{cde}	1.28^{cd}	**1.57^{a}**
	1:5	1.08^{g}	1.2^{def}	1.47^{b}
	1:6	1.02^{h}	1.17^{ef}	1.3^{c}
Staphylococcus aureus	1:4	1.38^{ef}	1.68^{c}	**2.03^{a}**
	1:5	1.35^{efg}	1.57^{d}	1.78^{b}
	1:6	1.22^{h}	1.35^{efg}	1.43^{e}
Listeria monocytogenes	1:4	0.88^{defg}	0.98^{bcd}	**1.18^{a}**
	1:5	0.8^{fgh}	0.93^{de}	1.1^{ab}
	1:6	0.82^{efgh}	0.88^{def}	1.07^{abc}

* The same letters mean there are no significant different at 95% confidential level and the statistical analysis were done separately for each bacterium.

Table 5: Concentration of inhibitory compounds (mg/ml) extracted from guava leaf by using different ration of guava leaf power to ethanol and different concentrations of ethanol.

Ratio of guava powder to ethanol	Concentration of ethanol (%)		
	50	70	90
1:4	0.07^{a}	0.07^{a}	0.06^{b}
1:5	0.06^{b}	0.06^{b}	0.06^{b}
1:6	0.06^{c}	0.05^{c}	0.05^{c}

* The same letters mean there are no significant different at 95% confidential level

3. The antimicrobial properties of crude guava leaf extracts produced by using solvent extraction in comparison with boiling water extraction

Guava leaf extract produced by boiling water for 15 minutes could also inhibit theses three cultures but the level of efficiency was lesser than extraction with ethanol (Table 4). It was implied that the some antimicrobial agents in guava leaves were not water-soluble compounds; therefore, organic solvent such as ethanol was required. Another reason was that alcoholic extraction was applied as a cold extract method, no heat treatment was used. The cold extract process is usually more expensive than the normal heat extraction because heat speeds up, and simplifies the extraction process. Conversely, some antimicrobial compounds may be destroyed by heat treatment (Anonymous, 2009e). The inhibition zones of ethanol extraction were 1.57, 2.03 and 1.18 mm for *B. cereus*, *S. aureus* and *L. monocytogenes*, respectively.

Table 6: Antimicrobial properties (cm of inhibition zone) of crude guava leaf extracts produced by ethanol and boiling water extractions.

Microorganisms	Extraction method	Inhibition zone (cm)
Bacillus cereus	ethanol	**1.57**[a]
	Boiling water	1.33[b]
Staphylococcus aureus	ethanol	**2.03**[a]
	Boiling water	1.40[b]
Listeria monocytogenes	ethanol	**1.18**[a]
	Boiling water	1.00[a]

* The same letters mean there are no significant different at 95% confidential level and the statistical analysis were done separately for each bacterium.

Conclusion and Recommendation

A hundred percentage of guava leaf extract produced by using 70% ethanol with the ratio of guava leaf powder to ethanol as 1:4 had the highest antimicrobial activity against only gram positive bacteria as *B. cereus*, *S. aureus* and *L. monocytogenes*. Moreover, the inhibition effect of water boiling extraction, which was the traditional method for making guava leaf tea, against these three cultures had lesser than ethanol extraction. Antimicrobial activities were 1.57, 2.03 and 1.18 cm for *B. cereus*, *S. aureus* and *L. monocytogenes*, respectively. The application of crude guava leaf extract in fresh-cut produces should be further examined.

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Appendix

Statistically Analysis

Bacillus cereus

Table A.1: Influence analysis of solvent types and concentration for guava leaf extraction on inhibition against *B. cereus*

Source of Variation	SS	df	MS	F	P-value	F crit
Type of solvent	0.5083	1	0.5084	185.3291*	1.17E-08	4.7472
Conc.of solvent	0.1055	2	0.0528	19.2278*	0.0002	3.88529
Interaction	0.0013	2	0.0007	0.2405	0.7899	3.88529
Error	0.0329	12	0.0028			
Total	0.6481	17				

Table A.2: The influence analysis of the ratio for extraction and the concentration of crude guava leaf extract used on inhibition against *B.cereus*

Source of Variation	SS	df	MS	F	P-value	F crit
Ratio	0.1705	2	0.0853	12.1184*	0.0005	3.5545
Conc. of Extract	0.5372	2	0.2686	38.1710*	3.35E-07	3.5545
Interaction	0.0222	4	0.0055	0.7895	0.5470	2.9277
Error	0.1267	18	0.0070			
Total	0.8567	26				

Table A.3: Statistically analysis inhibition against *B.cerues* compare between ethanol solvents and water boiling extraction.

Source of Variation	SS	df	MS	F	P-value	F crit
70%EtOH 1:4 100% vs boiling extract	0.0817	1	0.0817	11.5294*	0.0274	7.7086
Error	0.0283	4	0.0071			
Total	0.11	5				

Listeria monocytogenes

Table A.4: Influence analysis of solvent types and concentration for guava leaf extraction on inhibition against *L.monocytogenes*

Source of Variation	SS	df	MS	F	P-value	F crit
Type of solvent	0.0022	1	0.0022	0.2963	0.5962	4.7472
Conc. of solvent	0.0525	2	0.0262	3.5	0.0635	3.88534
Interaction	0.0102	2	0.0051	0.6852	0.5227	3.8853
Error	0.09	12	0.0075			
Total	0.155	17				

Table A.5: Influence analysis of the ratio for extraction and the concentration of crude guava leaf extract used on inhibition against *L.monocytogenes*

Source of Variation	SS	df	MS	F	P-value	F crit
Ratio	0.0624	2	0.0312	2.2026	0.1394	3.5545
Conc. Of Extract	0.3124	2	0.1562	11.0261*	0.0007	3.5545
Interaction	0.0148	4	0.0037	0.2614	0.8988	2.9277
Error	0.255	18	0.0142			
Total	0.6447	26				

Table A.6: Statistically analysis inhibition against *L.monocytogenes* compare between ethanol solvents and water boiling extraction.

Source of Variation	SS	df	MS	F	P-value	F crit
90% Et 1:4 100% vs Water boiling	0.006667	1	0.006667	16*	0.01613	7.708647
Error	0.001667	4	0.000417			
Total	0.008333	5				

Staphylococcus aureus

Table A.7: Influence analysis of solvent types and concentration for guava leaf extraction on inhibition against *S.aureus*

Source of Variation	SS	df	MS	F	P-value	F crit
Type of solvent	0.08	1	0.08	2.9844	0.1097	4.7472
Conc. of solvent	0.2703	2	0.1351	5.0414*	0.02575	3.8852
Interaction	0.1975	2	0.0988	3.6839	0.0566	3.8852
Error	0.3217	12	0.0268			
Total	0.8694	17				

Table A.8: Influence analysis of the ratio for extraction and the concentration of crude guava leaf extract used on inhibition against *S.aureus*

Source of Variation	SS	df	MS	F	P-value	F crit
ratio	0.62	2	0.31	30.7156*	1.58E-06	3.5545
Conc. of extract	0.845	2	0.42	41.8624*	1.7E-07	3.5545
Interaction	0.1433	4	0.0358	3.5504*	0.0265	2.9277
Error	0.1817	18	0.01			
Total	1.79	26				

Table A.9: Statistically analysis inhibition against *S.aureus* compare between ethanol solvents and water boiling extraction.

Source of Variation	SS	df	MS	F	P-value	F crit
70% Et 1:4 100% vs Water boiling	0.601667	1	0.6016	206.2857*	0.0001	7.708647
Error	0.011667	4	0.0029			
Total	0.613333	5				

Table A.10: Statistically analysis of concentration of inhibitory compounds (mg/ml) extracted from guava leaf ratio of 1:4 by using different types of solvents and concentration.

Source of Variation	SS	df	MS	F	P-value	F crit
Type of Solvent	0.0004	1	0.000417	25	0.0377	18.5128
% Sol	3.33E-05	2	1.67E-05	1	0.5	19
Error	3.33E-05	2	1.67E-05			
Total	0.0005	5				

Table A.11: Statistically analysis of Concentration of inhibitory compounds (mg/ml) extracted from guava leaf by using different ration of guava leaf power to ethanol and different concentrations of ethanol.

Source of Variation	SS	df	MS	F	P-value	F crit
Type of Solvent	0.000267	2	0.0001	8	0.04	6.9442
% Sol	6.67E-05	2	3.33E-05	2	0.25	6.9442
Error	6.67E-05	4	1.67E-05			
Total	0.0004	8				