A COMPARATIVE STUDY ON THE ANTIBACTERIAL ACTIVITY OF THE BANANA PULP EXTRACTS AGAINST SELECTED ORGANISMS

by

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CERTIFICATE

This was to certify that the dissertation entitled
"A comparative study on the antibacterial activity of the banana pulp extracts against selected organisms" was the bonafide record of research work done by
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during the period from July 2013 to May 2014
under my supervision.

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ABSTRAK

Pelbagai jenis bahagian pisang telah terbukti mempunyai kesan anti-mikrob berdasarkan kajian sebelumnya. Oleh itu, satu kajian perbandingan ke atas aktiviti antibakteria daripada ekstrak pulpa tiga spesis pisang yang berlainan iaitu Pisang Berangan (*Musa acuminate* AA / AAA), Pisang Mas (*Musa acuminate* AA) dan Pisang Nipah (*Musa balbisiana* BBB) telah dijalankan terhadap organisma tertentu. Data pengekstrakan menggunakan pelarut menunjukkan bahawa aseton mempunyai min hasil ekstrak yang paling tinggi (15.16) diikuti dengan metanol (13.73) dan larutan akueus (5.403). Aseton, metanol dan larutan akueus ekstrak pisang telah diuji dengan menggunakan kaedah agar resapan cakera untuk ujian sensitiviti antimikrobial. Bakteria yang terlibat adalah *Staphylococcus aureus, Streptococcus mutans, Pseudomonas aeruginosa* dan *Escherichia coli*. Ekstrak aseton dan metanol daripada semua jenis pisang menunjukkan purata zon diameter pemencilan yang hampir sama pada kepekatan 10 mg/disk terhadap bakteria gram negatif (*P. aeruginosa* dan *E. coli*) iaitu antara 7 mm kepada 8.5 mm, manakala ekstrak akueus bagi semua jenis pisang tidak mempunyai sebarang aktiviti pemencilan terhadap organisma yang diuji. Kesimpulannya, keputusan menunjukkan bahawa tiga jenis ekstrak pulpa pisang berpotensi menjadi sumber antimikrob, tetapi kajian lanjut perlu dijalankan untuk mengenal pasti komponen bioaktif yang bertanggungjawab untuk aktiviti antimikrob tersebut.

A COMPARATIVE STUDY ON THE ANTIBACTERIAL ACTIVITY OF THE BANANA PULP EXTRACTS AGAINST SELECTED ORGANISMS

ABSTRACT

Various parts of banana have shown to have antimicrobial effect based on previous study. Therefore, a comparative study on the antibacterial activity of the pulp extract of three different banana species, namely Pisang Berangan (*Musa acuminate* AA/AAA), Pisang Mas (*Musa acuminate* AA) and Pisang Nipah (*Musa balbisiana* BBB) was conducted against selected organisms. The acetone, methanol and aqueous solution of the banana extract were tested using agar disc diffusion method for antimicrobial sensitivity testing. The solvent extraction data showed that acetone had the highest mean of banana pulp extract yield (15.16), followed by methanol (13.73) and aqueous solution (5.403). The bacteria isolates were *Staphylococcus aureus, Streptococcus mutans, Pseudomonas aeruginosa* and *Escherichia coli*. The acetone and methanol extracts of all banana types showed an average with almost similar zone of inhibition activity at 10 mg/disc concentration against gram negative bacteria (*P. aeruginosa* and *E. coli*) ranging between 7 mm to 8.5 mm, whereas aqueous extract of all banana types did not show inhibitory action against tested organisms. In conclusion, the results implied that the pulp extract of three different banana species could be potential source of antimicrobial agents but further studies must be undertaken to identify the bioactive components responsible for their antimicrobial activity.

CHAPTER I

INTRODUCTION

1.1 Research question

Which species and which extraction method of banana pulp extract has greater antimicrobial activity against selected organisms?

1.2 Research hypothesis

A. Working hypothesis
The pulp extract obtained from three different banana species and extraction methods have comparable antimicrobial activity against selected organisms

B. Null hypothesis
The pulp extract obtained from three different banana species and extraction methods do not have comparable antimicrobial activity against selected organism.

1.3 Background of study

Plant extracts derived from their parts (roots, stems, leaves, fruits) are now increasingly used in research due to their widespread, immediate availability, cheaper cost, besides having potential medicinal properties and ability to manage certain health conditions which have been growing in recognition. The banana fruit has been commonly known for their nutritional values, however very little study done in their medicinal properties. In Malaysia, the banana fruit is mostly considered as one of the major agriculture products grown for domestic purposes. In this research, banana pulp has been selected as the part to be studied for their antimicrobial effects. There have been many studies reported on the potential prospect of banana as a therapeutic agent (Venkatesh *et al.,* 2013). Banana has long been used as medicinal agent due to its properties that rich in nutrition. Banana is thought to have antimicrobial activity, antioxidant activity and other biological activity such as antidiabetic, antidiarrheic, antitumoral, antimutagenic, antihelminthic and antiulcerogenic (Kumar *et al*., 2012).

In the past studies, various parts of banana have been shown to have an inhibitory effect on pathogens making them as excellent candidates for the antimicrobial as well as antioxidant sources. The phytochemical components of banana include tannins, eugenol and tyramine have been proved to have antimicrobial effects (Zainab *et al.,* 2013). Other active compounds present in banana such as alkaloids, glycosides, flavonoids, saponins, steroids, serotonin and dopamine, also contribute to pharmacological effects (Subrata *et al*., 2011).

Subrata *et al.* (2011) has reported that the root extract of banana have antimicrobial properties. Traditionally, stem juice of banana plants has been used for dysentery and as an antidote for the snakebite (Venkatesh *et al.,* 2013). There is also evidence of antimicrobial activity of *Musa sapientum* leaves extract *in-vitro* (Repon *et al.,* 2013). Another study on the extracts of pulp of *Musa sapientum* var. *paradisiaca* shows a significant healing effect due to their antioxidant activities (Agarwal *et al.,* 2009). A study by Gurumaa revealed that peel and pulp of fully ripe banana have potential antifungal and antibiotics properties (Gurumaa, 2008).

In this research, the banana pulp (Figure 1.1) of three different banana types, namely Pisang Berangan (*Musa Acuminata* **AA/AAA**), Pisang Nipah *(Musa Balbisiana* **BBB**) and Pisang Mas *(Musa Acuminata* **AA**) were tested against selected gram positive bacteria (*Staphylococcus aureus* and *Streptococcus mutans)* and gram negative bacteria (*Pseudomonas aeruginosa,* and *Escherichia coli*) to determine their antimicrobial activity.

Figure 1.1: Pulp of banana (*Musa*) plant

Antimicrobial assay is conducted through agar disc diffusion technique. Agar disc diffusion technique serves as a primary screening to measure the overall sensitivity of antimicrobial agent against various organisms. Antimicrobial agent can be antibiotics, drugs or extract from various plant. This test uses banana pulp extract–impregnated disc which was put onto specific agar containing streaked bacterial culture to test whether the organisms are susceptible or resistance towards the plant extracts.

1.4 Problem statement

The increasing trend of microbial resistance towards antimicrobial agents and other chemotherapeutics drugs has led to the seeking of newly potential antimicrobial activity on several medicinal plants. In addition, the emergence of many cases related to various pathogenic microbial infections has led to the urgency in findings other effective treatment that can resolve the crisis.

Infectious disease is known to be one of the leading causes of morbidity and mortality worldwide. Based on World Health Organization, almost 15 million of the 57 million annual deaths worldwide are caused by infectious disease which account for more than 25% (WHO, 2004). Centre for Disease Control (CDC) reported that at least 23000 people die each year due to antimicrobial-resistance infections and it is estimated that the number of illnesses caused by antimicrobial resistance was 2049442 cases (CDC, 2013).

Recently, there had been a massive interest shown in studying the antimicrobial activities in some flowering plants due to their potential health promoting effect towards community. Odugbemi has shown that more than 400,000 spp. of tropical plants have medicinal properties which can be used as an alternative to the modern medicine (Odugbemi, 2006). Many studies have reported the potential application of different parts of banana as antimicrobial agent (Gurumaa, 2008; Subrata *et al.*, 2011; Repon *et al.,* 2013; Mokbe and Hashinaga, 2005). However, there are only a several studies that compared the antimicrobial activities of different types of banana species extracted using different type of solvents which was conducted in this research.

1.5 Research objectives

a) General objectives

To compare antimicrobial activities of three banana species against selected organism and to compare three different extraction method of banana pulp.

b) Specific objectives

i. To extract the crude of banana pulp using aqueous, acetone and methanol extraction solvents and to compare the yield of banana pulp extract of different extraction solvents

ii. To assess and compare the antibacterial effect of three types species of banana pulp extract against selected organisms using agar disc diffusion method

1.6 Research scope

- Three different types of banana namely Pisang Berangan (*Musa acuminate* **AA/AAA**, Pisang Nipah (*Musa balbisiana* **BBB**) and Pisang Mas (*Musa acuminate* **AA**) were selected to compare their antimicrobial activity.
- A part of banana namely the pulp was chosen from each banana types.
- The drying temperature was 50°C for 3 days.
- Three different types of extraction solvents namely acetone, methanol and distilled water were used to extract the crude of banana pulp. The extraction was done at room temperature.
- Antimicrobial activity was analysed using Kirby Bauer agar disc-diffusion test against selected organisms (*S. aureus, S.mutans, E.coli* and *P.aeruginosa*).

1.7 Rationale of study

Banana accounts for one of the major food crops worldwide especially in tropical and subtropical areas. According to the study by Chai and his colleague, banana is the second ranked cultivated fruit in Malaysia with total production of 530000 metric tons (Chai *et al.,* 2004). The by-products of banana are estimated to be 220 tones plant mass per hectare, and they could be useful source for natural products (Padam *et al.,* 2012).

Although previous studies has reported the *in-vitro* activity of banana extract against various organism (Akter *et al.,* 2011; Gurumaa, 2008; Subrata *et al.,* 2011 ; Mokbe & Hashinaga, 2005) , however, there are very few data published on the comparison of yield between different types of extracting solvent on the pulp of three different types of banana. Therefore, this study was conducted to know the percentage yield of the pulp of three types of banana extract using three types of extracting solvents namely acetone, methanol and distilled water. This study also focused on the effect of the banana extract on the commonly clinical isolated organisms such as *S. aureus, S. mutans, E. coli* and *P. aeruginosa.* This study may provide additional information regarding the antimicrobial activity of the common variants of banana in the region, and thus, provide possible plants leads that can be used as an alternative and less expensive source of treatment for the benefit of the local residents.

The variation of the banana and extraction solvent selection was made to enhance the most favorable condition that can lead to an optimize settings of antimicrobial determinations on natural-based products. The antimicrobial activity profile was then compared and analysed to uncover the most effective condition that yield the best effect based on their antimicrobial consistency and sensitivity.

CHAPTER II

LITERATURE REVIEW

2.1 Banana (*Musa* sp.)

2.1.1 Classification of banana, *Musa* sp.

Banana (*Musa*) is a monocotyledonous flowering plant belongs to the family *Musaceae* and has been assigned to the order Zingiberals. There are currently three genera belong to *Musaceae* family including *Musa, Musella* and *Ensete*. Most of the genera have been cultivated for a long time and can be found nearly around the world. The species of the genus *Musa* contains about 42-60 varieties while the genus *Ensete* have 6-8 species. The genus *Musella* contains only one. However, genus *Musa* has contributed to the most groups of edible banana which are of major interest in this study. The genus *Musa* is further divided into four different sections namely *Eumusa, Rhodochlamys, Australimusa* and *Calimusa*. Of all the *Musa* sections, the most cultivated banana is derived from *Eumusa* comprising two wild banana species namely *Musa acuminate* colla and *Musa balbisiana* colla. *Australimusa* also yields an edible banana but it is not widely distributed. *The Rhodochlamys* and *Calimusa* are probably known for their ornamental value. Banana is more commonly found in the tropics of Africa and Asia and well known as a tropical fruit (Krishnamurthy, 2002). Previous information indicated that banana is recognised as the major fruit crop in the world and it has been extensively cultivated to serve a variety of purpose ranged from a food source and ornamentals value.

In earlier time, the classification of banana was according to Karl Linnaeus nomenclature system including plantain, *Musa paradisiaca* and dessert banana, *Musa sapientum*. Banana was classified according to their different properties and characteristics in terms of fruit texture and anatomical aspect of the flowers. The higher diversity of banana cultivars makes the classification even more difficult and added more confusion to the banana nomenclature. In 1955, new nomenclature system based on the origin of edible banana was introduced, and according to Simmonds and Shephard (1955), the edible bananas were derived from *Musa acuminate* colla and *Musa balbisiana* colla wild species which are common in Southeast Asia. They claimed that the *Musa paradisiaca* and *Musa sapientum* were hybrid cultivars originated from a combination of two wild banana species. The criteria that are used to grouped and differentiate between *Musa acuminate* from *Musa balbisiana* and their hybrids were shown in Table 2.1. The banana was further grouped according to their genomic background of diploids, triploid or tetraploid of natural or hybrid wild types. *Musa acuminate* cultivars account for the most numerous clones as compared to *Musa balbisiana* and their hybrids (Valmayor *et al.*, 2000). The nomenclature system of the banana species was created based on their haploid number stated as letter A and B for *Musa acuminate* and *Musa balbisiana* respectively. Of all the banana cultivars, only three types of banana were used in this study, namely Pisang Berangan (*Musa acuminate* **AA/AAA**), Pisang Mas (*Musa acuminate* **AA**) and Pisang Nipah (*Musa Balbisiana* **BBB)** due to their availability and the higher consumption among the local population.

Table 2.1: Criteria for the differentiation of bananas (Simmons & Shepherd, 1995)

Characters	*Musa acuminata*	*Musa balbisiana*
Pseudostem color	More or less heavily marked with brown or black blotches	Blotches slightly or absent
Peduncle	Usually downy or hairy	Glabrous
Petiolar canal	Margin erect or spreading with scarious wings below, not clasping pseudostem	Margin inclosed , not winged below, clasping pseudostem
Pedicels	Short	Long
Ovules	Two regular rows in each loculus	Four irregular rows in each loculus
Bract shoulder	Usually high	Usually low
Bract curling	Bract reflex and roll back after opening	Bract lift but do not roll
Bract shape	Lanceolate or narrowly ovate, tapering sharply from the shoulder	Broadly ovate, not tapering sharply
Bract apex	Acute	Obtuse
Bract color	Red, dull purple or yellow outside; pink, dull purple or yellow inside	Distinctive brownish-purple outside; bright crimson inside
Color fading	Inside bract color fades to yellow towards the base	Inside bract color continues to base
Bract scars	Prominent	Scarcely prominent
Free tepal of male flowers	Variably corrugated below tip	Rarely corrugated
Male flower color	Creamy white	Variably flushed with pink
Stigma color	Orange or rich yellow	Cream, pale yellow or pale pink

2.1.2 Anatomy and growth of banana, *Musa* sp.

The general characteristics that distinguished *Musaceae* from other Zingiberals are their helical-like leaves, male and female flowers are separated as well as the pulp on its fruit. Genus *Musa* is one of the important banana that is popular in most countries due to their edible properties (Orhan, 2001), and it is native to India, Malaysia, New Guinea, Sri Lanka, Vietnam, Myanmar, Thailand, Java, Philippines, China and Queensland. Most of the time, banana can be eaten freshly or cooked as well as manufactured as other food products.

Banana is a monocotyledonous having a pseudostem with size approximately 2-9 meters tall that propagated from corms (Nelson *et al.,* 2006). They are widely adapted and usually grow in a well-drained soils associated with other tropical vegetative forest plants. Banana is a non-woody, perennial with no true stems. The trunk is known as pseudostem in which they consist of leaf petioles bound together that vegetatively propagated form underground rhizome and grow vertically above the ground. The juicy succulent of the pseudostem can support the banana trees of up to 25 meters. The leaves extended form the underground rhizome having a size of 150-400 cm long and 70-100 cm wide. The individual leaves are supported by 30-90 cm petiole and characterized by their notable midrib and pinnated vein (Nor Adlin, 2008). The inflorescence later emerges at the top of the banana tree through the center of the pseudostem consisting of female flowers, neutral flowers and male bud which arranged radially on the peduncle and is wrapped by a bract. Later in time, the bract falls off whereas the remaining female flowers ovaries produced edible seedless fruits under favorable conditions. Pedicles attached the fruits to their respective peduncles.

The growth cycle of banana involved two important cycles including vegetative phase followed by reproductive phase. In vegetative phase, the sucker that arises from corms begins to produce leaves which go all the way above the ground and stopped growing when the inflorescence appears. Reproductive phase takes place where the flower bud shoots from the vegetative meristems and begins the vertical growth of the pseudostem until the inflorescence develops. Therefore, the important stages of banana growth cycle consist the synthesis of three essential part namely an underground corm consisting of suckers and roots, a pseudostem comprise a leaves and their combined petiole network and an inflorescence with female flower that further develop into fruits (Swennen & Oritz, 1997).

2.1.3 Antimicrobial activity of banana, *Musa* Sp.

Previous study has discovered the medicinal properties of tropical flowering plant which hold a great promise for the current and future treatment of disease (Odugbemi, 2006). Based on early human culture, banana has long been used as an agent to treat diarrhea, dysentery, menorrhagia, hypoglycemic, diabetes and hypolipidemic. Beside of its nutritional value, bananas also shows high antioxidant activity (Someya *et al.,* 2002) and possesses potential antimicrobial activity. According to Kanazawa & Sakakibara banana pulp found to have variety of antioxidants such as beta-carotene and vitamins A (Kanazawa & Sakakibara, 2000). Study have found that the peel and the pulp of ripe banana have potential antifungal and antibiotic activities (Kumar *et al.,* 2012). Ono *et al.* (1998) in Japan performed antibacterial activity using banana fruit against *E. coli* and *S. aureus,* and found that the growth of the bacteria is inhibited.

According to Biswas *et al.* (2011), the ethanol extract of *Musa paradisiaca* root showed an *in-vitro* antibacterial activity against *S. aureus, E. coli, P. aeruginosa* and *Vibrio cholerae.* Other study also reported the antibacterial activity of banana against *E. coli* and *S. aureus* (Ono *et al.*, 1998). Fagbemi and his colleagues conducted a study to investigate the antimicrobial activity of unripe banana (*Musa sapientum L.*) using ethanol and water as a solvent to compare their efficacy. It is found that *S. aureus, E. coli* and *P. aeruginosa* were susceptible to ethanolic extract of unripe banana, but *E. coli* and *P. aeruginosa* are not susceptible to aqueous extracts of the unripe banana (Fagbemi *et al.,* 2009). Another study revealed that the seed extract of the *Musa sapientum* showed significant activities against *E. coli* and moderate activity against *P. aeruginosa* followed by the weakest activity against *S. aureus* (Hossain *et al.*, 2011).

2.2 Extraction solvents

To assess the antimicrobial activity of three types of banana species, high yield of banana pulp crude need to be extracted using extraction solvent. Extraction solvent is a process whereby the substance of interest is separated from one another depending on the various solubility of the compound inside the plant. Extraction solvent must be properly chosen according to the type of substances to be extracted. According to the findings by many researchers, different extraction solvents and technique of extraction could largely affect the natural product (Michiels *et al.,* 2012) and the solvent selection is mainly dependent on the plant material used (Zhou & Yu, 2004).

Lolita *et al*. (2012) has reported that the solvent can significantly affected the total phenolic content and antioxidant in various fruit and vegetables. The most important criteria to look upon is the solvent polarity, where it has been reported that higher polarity will yield better solubility in phenolic compound hence high quality of extract can be isolated (Naczk & Shahidi, 2006). Water can be added to the ethanol as an alternative to increase the polarity however, too high water content can cause impurities as it allow the extraction of other compound and subsequently lower the concentration of compound of interest (Spigno *et al.,* 2007). Literature data also show that ethanol and the mixture of ethanol/water solutions can be regarded as the best solvents. Study by Fagbemi and his colleagues show that the potency of antimicrobial activity of unripe banana is enhanced by the type of solvents used where they found that some of the active components in the banana dissolved well in ethanol than in water (Fagbemi *et al*., 2009). Numerous parts of banana such as seed, peel, pulp or stem can be extracted to seek for their antioxidant activities, antimicrobial activities and medicinal purpose. In addition, the decision to use whether solvent or aqueous solution must be appropriate to the respective substance in terms of their solubility and purification ability as it can affect the purity and validity of the outcome.

Solvent with different polarity is readily available to be used in the extraction process. The commonly used extraction solvent is water, ethanol, methanol, acetone and ethyl acetate (Montelongo *et al.,* 2010). Phytochemical analysis of the *Musa sapientum* pulp using methanol as a solvent shows the presence of chemical compound such as carbohydrates, alkaloids, steroids, glucosides and flavonoids (Imam *et al.,* 2011). Apart from the solvent selection, the extraction technique also plays an important part in the total isolation of the active ingredient of banana. Previously, sonication, stirring, percolation and shaking have been extensively used for extraction purpose especially in the experimental research. Recently, the emergence of new, automated technologies such as soxhlet method, microwave, superficial fluid extraction, Ankum batch extraction and pressurized liquid extraction have enable the optimisation of sample preparation (Luthria, 2006).

2.3 Mechanism of actions of antimicrobial agent

The action of antimicrobial agent commonly focused on the inhibition of the synthesis of bacterial part that is crucial for their growth and function which involved inhibiting their cell wall synthesis, suppressing their protein synthesis, blocking their nucleic acid synthesis or disrupting their cell membrane activity (Jawetz, 1989). Antimicrobial agent can be bacteriostatic or bactericidal which solely depending on their mechanism of action towards the bacteria. Bacteriostatic simply means the drug or antimicrobial agents that are capable of inhibiting the bacterial growth temporarily. The bacterial continue growing once the antimicrobial agent is stopped. Bactericidal is defined as the drug or antimicrobial agent that causes cell death. Bactericidal drug action is required for some cases such as in a patient whose immune system is compromised (Woods & Washington, 1995).

In general, plant has a capacity to synthesize aromatic substances (Geissman, 1963). Most of these substances played an important role for the defense action against pests or microorganism. Previous findings of preliminary phytochemical study using ethanol extract has reported that the roots of *Musa paradisiaca* Lam. contain several compound such as alkaloids, glycosides, steroids, flavonoids, saponins, reducing sugars and tannins (Biswas *et al.*, 2011).

Phenolic compounds are one of the simple examples of bioactive phytochemicals (Cowan, 1999). In the previous study, it is found that the inhibitory effect towards microorganism is found higher in oxidized phenol (Scalbert, 1991). The phenolic toxicity toward organism is mediated by oxidized substances which are responsible for the inhibition of the enzyme activity in the organisms through protein interaction (Mason & Wasserman, 1987). Other examples of common phenolic compounds are quinones, flavonoids, tannins and coumarins.

Quinones caused the changes of color in injured fruit and vegetables. Quinones usually present in the human skin and responsible for melanin production (Schmidt,1988). The antimicrobial potential of quinones is thought to be caused from its action where it can bind irreversibly with nucleophilic amino acid in proteins of the organism causing the loss of protein function (Stern *et al.,* 1996). The most likely component that would be targeted by quinones is the surface-exposed adhesins, membrane-bound enzyme and polypeptide cell wall (Cowan, 1999). As for the flavonoids, these substances are known to be specifically synthesised by plants for the defense against microbial infections (Dixon *et al.,* 1983). The action of flavonoids is most likely due to the ability to bind with soluble proteins and bacterial cell walls which can destroy the microbial membranes (Cowan, 1999). Tannins are commonly found in plant parts such as leaves, fruit and roots (Scalbert, 1991). The antimicrobial action of tannins involved their capacity to inactivate enzyme, microbial adhesins and block cell envelope transport proteins. A previous study has reported that tannins can be virulent to fungi, yeast and bacteria (Cowan, 1999). Coumarins are highly known for their antithrombotic, anti-inflammatory and vasodilator activities. Some types of coumarins possess antimicrobial activity where *in-vitro* researches have proved that these substances can inhibit *Candida albicans* (Cowan, 1999).

2.4 Antimicrobial susceptibility test (AST)

Based on Bauer *et al.,* (1966), the standard disc test includes the measurement of diameter of zone of inhibition followed by the set-up of minimum inhibitory concentration (MIC) through agar or broth dilution susceptibility tests to see their comparisons (Schwalbe *et al.,* 2007). In the earlier time, well diffusion instead of drug-impregnated disc bacteria is used to assess the effect of drug/test extract on the bacteria. It is Foster and Woodruff who introduced the used of disc strips incorporated with antibiotics as an alternative source of the diffusion test (Foster & Woodruff, 1943).

The result interpretation of disc diffusion test is initially regarded as susceptible if there is zone of inhibition or resistance if there is no zone of inhibition. However, it is found that many factors can influenced the size of the inhibition zone. Disc diffusion test is a qualitative test which is not benefited for further interpretation. Therefore, multiple discs with different concentrations of the tested extract/drug were setup (Bauer *et al.,* 1959). The categorical interpretations of the result depend upon the zone of inhibition, whereby the appearance of zone around the disc indicates susceptibility, and the presence of inhibition zone around the high potency disc indicates intermediate susceptibility while no zone of inhibition indicates resistance (Schwalbe *et al.,* 2007).

The principle behind the agar disc diffusion test relied on the tested extract or antibiotics-impregnated disc that comes in contact with an agar surface containing bacteria inoculation with which the diffusion and growth inhibition begin. Once the impregnated disc comes in contact with the inoculated agar, the molecules of extract/antibiotics diffuse out of the disc into the surrounding agar, forming a gradient of extract/antibiotic concentration. At the same time, the test organism starts to divide until at some point it achieved the critical mass and stopped growing. The outermost edge zone of inhibition shows that the concentration of extract/antibiotic has reaches a sufficiently higher cell mass and unable to further inhibit the growth.

CHAPTER III

MATERIALS AND METHODS

3.1 Study design

This was an *in-vitro* experimental study to assess the diameter of zone of inhibition of the selected bacterial strain. The experiment was conducted in School of Health Sciences and School of Dental Sciences, Universiti Sains Malaysia, Kubang Kerian from July 2013 to Mei 2014.

3.2 Materials

Table 3.1 show the list of materials used in this project.

Table 3.1 List of materials

No.	Materials
1	Aqueous extract of Pisang Berangan, Pisang Nipah and Pisang Mas
2	Methanol extract of Pisang Berangan, Pisang Nipah and Pisang Mas
3	Acetone extract of Pisang Berangan, Pisang Nipah and Pisang Mas
4	Mac Conkey agar powder (Oxoid, Thermo Scientific)
5	Columbia Sheep blood agar (commercially prepared; Oxoid, Thermo Scientific)
6	Mueller Hinton agar (commercially prepared; Oxoid, Thermo Scientific)
7	Mueller Hinton broth powder (Oxoid, Thermo Scientific)
8	Imipenem antibiotics disc (10 µg/disc) (Oxoid, Thermo Scientific)
9	Tetracycline antibiotics disc (30 µg/disc) (Oxoid, Thermo Scientific)
10	Ampicillin clauvinic antibiotics disc (30 µg/disc) (Oxoid, Themo Scientific)
11	Sterile filter paper disc (Whatman No. 1, 6 mm)
12	Sterile distilled water

3.3 Instrument and disposable items

Table 3.2 show the lists of instrument and disposable items used in this project

Table 3.2 List of instrument and disposable item

No.	Item
1.	Incubator (Sanyo, Japan)
2.	Refrigerator (4°C)
3.	Freezer (-81°C) (Ilshin, Korea)
4.	Biological Safety Cabinet (BSC) Level II (Heraeus, Germany)
5.	Autoclave machine (HVE-25, Japan)
6.	Micropipette (Eppendorf, Germany)
7.	Scott Duran bottle
8.	Centrifuge machine (Eppendorf, Germany)
9.	Conical flask
10.	Filter Funnel
11.	Nephelometry
12.	Freeze dry machine (Scanvac, Germany)
13.	Concentrator Plus machine (Eppendorf, Germany)
14.	Filter paper (Whatman No, 150 mm)
15.	Eppendorf tube
16.	Falcon tube
17.	Sterile cotton swab
18.	Pipette tips
19.	Parafilm seal
20.	Electrical grinding machine (Pensonic, Malaysia)
21.	Soxhlet apparatus

3.4 Plant collection

Three types of banana pulp (*Musa*) were obtained from Kubang Kerian, Kelantan and were identified based on the physical characteristics of the fruit as shown in Figure 3.1, 3.2 and 3.3.


Figure 3.1: Pisang Berangan (*Musa acuminate* **AA/AAA**)


Figure 3.2: Pisang Mas (*Musa acuminate* **AA**)


Figure 3.3: Pisang Nipah (*Musa balbisiana* **BBB**)

The banana pulps were washed thoroughly using tap water and wiped using a clean cloth. The succulent parts of the banana pulp were cut into about two centimeter thickness. Next, the cut pulp was allowed to dry in the oven at 50°C for three days. The dried pulp was powdered using electric blending machine and kept at 4°C in a tight-capped bottle.

3.5 Preparation of extracts

The extract preparation used several type of solvents namely acetone, methanol and distilled water. Soxhlet apparatus technique was used to extract the crude compound from the banana pulp using methanol and acetone as its extraction solvent. Twenty gram of each banana pulp powder was placed inside a thimble made from thick filter paper. The thimble was loaded into the middle chamber of the soxhlet extractor. Three hundred milliliter of extraction solvent was added into a distillation flask and the rest of the soxhlet apparatus consisting of condenser and the middle chamber with thimble was attach to the flask. The solvent was heated to begin the distillation process and the cycle was allowed to stand for three days. Next, the extract was filtered using Whatman filter paper and transferred into a falcon tube. The solvents were removed by means of Concentrator Plus machine yielding the extracted compound.

As for the aqueous extract, distilled water was used for the solvent where 20 gram of pulp powder was dissolved into 300 ml of distilled water. The mixture was boiled for about 45 minutes. Next, the extract was filtered using Whatman filter paper and was subjected to freeze-dry.

The extracts were stored in a sterile container at 4°C until further use. The extracts were weighed and freshly dissolved in sterile distilled water to a final concentration of 50, 100 and 500 mg/ml respectively for agar disc diffusion test.

3.6 Determination of extraction yield (%)

The yield (%, w/w) from all the dried extracts was calculated as:

$$\text{Yield (\%)} = \frac{W1}{W2} \text{ X } 100$$

Where W1 was the weight of the extract after lyophilization/evaporation of solvent and,W2 was the weight of the plant powder

3.7 Bacterial strains

The bacterial species used in this study were purchased from the Medical Microbiology and Parasitology Laboratory, School of Medical Sciences comprising of gram positive bacteria (*S. aureus, S. mutans*) and gram negative bacteria (*P. aeruginosa, E. coli)*. All bacterial strains were grown and maintained by subculturing on specific types of media including Mac Conkey agar and Sheep Blood agar for gram negative and gram positive bacteria, respectively. All agar plates were incubated for 24 hours at 37°C and maintained at 4°C.

3.8 Agar disc diffusion test

The antimicrobial screening using agar disc diffusion technique was carried out based on Clinical and Laboratory Standards Institute (CLSI, 2012) with some modifications. *In-vitro* antibacterial activity was examined for acetone, methanol and aqueous extract from banana pulp. Colonies from the plates were suspended into sterile Mueller Hinton broth to form a turbidity of 0.5 McFarland standards using nephelometry. Bacteria suspensions with approximately $1x10^8$ CFU/ml suspension were streak onto Mueller-Hinton agar plates using sterile swab stick. The test disc was prepared by incorporating 20 µL of each extract (50, 100 and 500 mg/ml) to 6 mm sterilized filter paper disc to give a final concentration of 1, 2 and 10 mg/disc. The discs were left to dry under the biosafety cabinet overnight. Then, the impregnated disc, blank disc (negative control) and positive control disc were placed gently into the respective location. Imipenem served as positive control against *P. aeruginosa* to confirm that their growth were inhibited by antibiotics; tetracyline was used as a positive control to inhibit the growth of *E. coli* and *S. aureus* and amoxicillin was used as positive control to inhibit the growth of *S. mutans*. The culture plates were incubated at 37°C for 24 hour. Microbial growth was determined by measuring the diameter of zone of inhibition in mm. Statistical analysis by ANOVA method with post-hoc analysis was used to compare the significant antimicrobial activity among three different extracts.