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CHEMICAL COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF ACETONE *Musa acuminate AA/AAA* LEAF STALK EXTRACTS ON SELECTIVE GRAM-NEGATIVE BACTERIA

(Sebatian Kimia dan Aktiviti Antimikrob dari Ekstrak Aseton Pelepah Daun *Musa acuminate AA/AAA* pada Bakteria Jenis Gram Negatif Terpilih)

Tuan Nadrah Ismail, Raja Azman Awang, Suharni Mohamad, Wan Nazatul Shima Shahidan*

School of Dental Sciences, Health Campus,
Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

**Corresponding author: shima@usm.my*

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Abstract

Banana is a familiar tropical fruit and is acclaimed for its therapeutic uses. A previous study has found that acetone banana leaf stalk extract of the Cavendish group (*Musa acuminate AA/AAA*) have antibacterial properties on selected Gram-negative bacteria using the agar disc diffusion method. Hence, this study is aimed to evaluate the phytochemical analysis and antibacterial activities *Musa acuminate AA/AAA*. The chemical composition of *Musa acuminate AA/AAA* leaf stalk extract was analysed using gas chromatography–mass spectrometry (GC-MS) and the mass spectra library was used to identify an unknown chemical in the sample mixture. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Musa acuminate AA/AAA* leaf stalk extract was investigated using macrobroth dilution on *Pseudomonas aeruginosa* and *Escherichia coli*. The GC/MS analysis revealed the presence of more than forty individual compounds and a significant difference in the percentage composition of leaf stalk parts was observed. The extract inhibited bacterial growth at an MIC of 125 mg/ml and 250 mg/ml against *Pseudomonas aeruginosa* and *Escherichia coli* respectively, while the MBC was 500 mg/ml against both organisms with comparable effects to chlorhexidine. *Musa acuminate AA/AAA* leaf stalk extract possesses good antibacterial activities against the tested bacteria. The results indicate that *Musa acuminate AA/AAA* leaf stalk can be used against other Gram-negative bacteria.

Keywords: phytochemical, *Musa acuminate AA/AAA* leaf stalk, acetone extract, Gram-negative bacteria

Abstrak

Pisang adalah buah tropika yang biasa dan telah diiktiraf untuk kegunaan terapeutiknya. Kajian kami sebelum ini telah mendapati bahawa ekstrak tangkai daun pisang berangan (*Musa acuminate AA/AAA*) menggunakan aseton mempunyai ciri-ciri antibakteria terhadap bakteria gram-negatif terpilih dengan menggunakan kaedah penyebaran cakera agar. Oleh itu, objektif utama kajian ini adalah untuk menganalisa fitokimia dan aktiviti antibakterianya. Komposisi kimia ekstrak tangkai daun *Musa acuminate AA/AAA* dianalisa menggunakan GC/MS dan perpustakaan spektrum jisim telah digunakan untuk mengenal pasti bahan kimia yang tidak diketahui dalam campuran sampel. Aktiviti antibakteria ekstrak telah disiasat oleh MIC dan MBC menggunakan pencairan macrobroth terhadap strain patogen *Pseudomonas aeruginosa* dan *Escherichia coli*. GC/MS analisis menunjukkan kehadiran lebih daripada empat puluh sebatian individu dan perbezaan yang signifikan dalam komposisi peratusan bahagian tangkai daun dapat dilihat. Ekstrak menghalang pertumbuhan bakteria pada MIC 125 mg/ml terhadap *Pseudomonas aeruginosa* dan 250 mg/ml terhadap *Escherichia coli*, manakala MBC adalah 500 mg/ml terhadap kedua-dua organisma dengan kesan setanding klorheksidina. Perbezaan ketara dalam komposisi peratusan bahagian tangkai daun dilihat. Keseluruhan ekstrak dalam kajian ini memiliki aktiviti antibakteria yang baik terhadap bakteria yang diuji. Keputusan menunjukkan bahawa kajian terhadap tangkai daun pisang boleh diteruskan terhadap beberapa bakteria Gram-negatif.

Kata kunci: fitokimia, pelepah daun pisang *Musa acuminate AA/AAA*, ekstrak aseton, bakteria gram-negatif

Introduction

Banana plant extracts derived from their parts (roots, stem, leave, and fruits) are now increasingly used in research due to their availability and cheap cost. They also have potential medicinal properties as a therapeutic agent and the ability to manage certain health conditions [1]. In past studies, various parts of banana have been shown to have an inhibitory effect on pathogens, making them excellent candidates for antimicrobial as well as being a good antitumoral, antimutagenic, antihelminthic, and antiulcerogenic source [2-4]. The phytochemical compounds present in banana include sterol, terpenoids, fatty acids, phenolic compound, and tocopherol [5-7]. A recent study done by Ehiowemwenguan [8] has reported that ethanol extract of *Musa sapientum* peels had significant *in-vitro* broad-spectrum antimicrobial activity. Thus, they concluded that the extract from banana peel could be used to control infections caused by both Gram-positive and Gram-negative bacteria. ~~A~~ study conducted by Fairus Fadhilah [9] has reported that acetone banana leaf stalk extracted from three different banana species, including Cavendish group (*Musa acuminate AA/AAA*), has antibacterial properties on selected Gram-negative bacteria when tested using the agar diffusion method. However, the phytochemical analysis and antimicrobial activity of this banana leaf stalk extract against these selected Gram-negative bacteria has not been investigated yet. Hence, this study aims to screen its phytochemical compound. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of acetone extract of *Musa acuminate AA/AAA* against pathogenic strains *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) were also determined to maximise its therapeutic effects.

Materials and Methods

Collection and preparation of plant extracts

Banana leaf stalks of *Musa acuminate AA/AAA*) were obtained from the district of Kubang Kerian, Kelantan, Malaysia (Figure 1). The banana leaf stalks were washed thoroughly using tap water and wiped using a clean cloth. The succulent parts of the banana leaf stalk were cut into two-centimetre thickness. Next, the cut leaf stalk was allowed to dry in the oven at 50 °C for three days. The dried leaf stalk was powdered using an electric blending machine and kept at 4 °C in a tight-capped bottle.

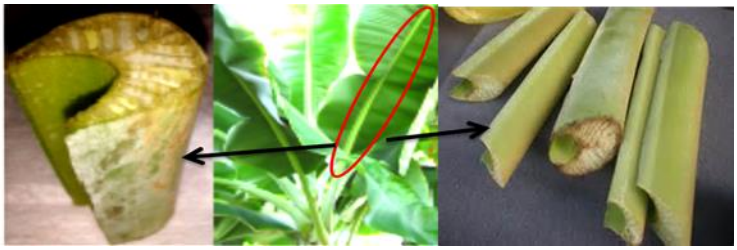

Figure 1. Leaf stalk of *Musa acuminate AA/AAA* which was used for extraction

Preparation of extracts

Soxhlet apparatus technique was used to extract the crude compound from the banana leaf stalk using acetone as its extraction solvent. Twenty grams 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 millilitres of extraction solvent were added into a distillation flask and the rest of the Soxhlet apparatus consisting of a condenser and a middle chamber, with thimble, was attached 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 No 1 (11 µm) Whatman filter paper and transferred into a 50 ml falcon tube. The solvents were removed using a Concentrator Plus machine yielding the extracted compound.

GC-MS analysis

The analysis was carried out using a Hewlett Packard 789A Gas Chromatograph with 5975C Mass Selective Detector. The column was a fused silica capillary, HP-5 column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Agilent Technologies, USA). The carrier gas was helium with a flow rate of 1.0 ml/min with oven temperature programmed at 50 °C (held for 5 minutes) to 300 °C (held for 10 minutes) at a rate of 25 °C/min. The injection and interface temperatures were set at 250 °C and 280 °C, respectively. One microlitre of the sample was injected *via* GC auto sampler (7693 Auto sampler, Agilent Technologies, USA) in split-less mode and was analysed in MS full scan mode (m/z 40-650). The electron ionisation was fixed at 70 eV. The mass spectra library was used to identify an unknown chemical in the sample mixture. The banana leaf stalk compounds were identified by matching their mass spectra with NIST02 and WILEY275 libraries.

Bacterial strains

The bacterial species used in this study were obtained from the Medical Microbiology and Parasitology Laboratory, School of Medical Sciences, Universiti Sains Malaysia. Malaysia comprises of two types of Gram-negative bacteria, P. aeruginosa and E. coli. All bacterial strains were grown and maintained by sub-culturing on MacConkey agar (Oxoid, UK). All agar plates were incubated for 24 hours at 37 °C and maintained at 4 °C.

Determination of minimum inhibitory concentration (MIC)

The determination MIC of the extract was carried out using the macro-dilution technique as described by a double-fold serial dilution using Muller Hinton broth (Oxoid, UK). The following concentrations were used: 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5mg/ml. Concentration was based on a previous study's results [9]. Colonies from the plates were suspended into sterile Mueller Hinton broth to form turbidity of 0.5 McFarland standards using a densitometer (DEN-1, Grant Instruments, Cambridge) and further diluted into 1x106 CFU/ml. Then, equal volumes of extract and bacterial suspension were dispensed into sterilised test tubes, which were incubated aerobically at 37 ^{o}C for 24 hours. A tube containing broth and inoculums without extract served as organism control. The tube with broth and extract without inoculums served as extract control. Chlorhexidine digluconate (C-9394, Sigma Chemical Co., St. Louis, MO, USA) diluted to 0.12% was used as a positive control. The experiment was performed in triplicates for each concentration and organism. The lowest concentration of the extracts which inhibited microbial growth (no turbidity) was recorded as the MIC [10].

Determination of minimum bactericidal concentration (MBC)

Sterile Mueller Hinton agar plates were inoculated with samples from each of the test tubes that showed no visible growth from the MIC test. The plates were then incubated at 37 ^{o}C for 24 hours. Chlorhexidine digluconate (C-9394, Sigma Chemical Co., St. Louis, MO, USA) diluted to 0.12% was used as a positive control. The experiment was performed in triplicates for each concentrations and organisms. The highest dilution that yielded no single bacterial colony was taken as the MBC [11]

Data analysis

The MIC and MBC were expressed as means ± SE.

Results and Discussion

The GC-MS analysis shows that the classes of compounds identified in *Musa acuminate AA/AAA* leaf stalk were terpenoids, phytosterol, phenolic compounds, fatty acids, hydrocarbon, and tocopherol. More than forty individual compounds were identified as shown in Table 1. The main classes of compounds identified were phytosterol (18.53%), terpenoids (15.86%), fatty acids (6.61%) and tocopherol (5.54%). The major identified phytosterol was gamma-sitosterol (8.26%) followed by stigmasterol (6.3%). Seven terpenoids compounds were identified and squalene (12.02%) was found most abundant in banana leaf stalk. Palmitic acid was the highest fatty acid (4.38%) and alpha-tocopherol was the highest tocopherol found in banana leaf stalk.

Table 1. Chemical compound banana leaf stalk extract from *Musa acuminate AA/AAA*

Compounds	**% of Total Ion Chromatogram**
Terpenoids	(15.86)
(-)-Loliolide	0.15
Phytol	0.78
Farnesol	0.19
Cycloeucanol	0.97
Cycloartenol	1.19
3-o-acetyl-delta24-cycloartenol	0.56
Squalene	12.02
Phytosterols	(18.53)
Campesterol	3.97
Stigmasterol	6.30
Gamma-sitosterol	8.26
Phenolic	(0.33)
2-methoxy-4vinylphenol	0.10
Pradox 146	0.23
Fatty acids	(6.61)
Lauric acid	0.30
Pentadecanoic acid	0.29
Palmitic acid	4.38
Margaric acid	0.61
Stearic acid	0.86
Nonadecanoic acid	0.17
Hydrocarbon	(3.33)
Tridecane	0.29
2-methyl-z-4-tetradecane	0.61
Cyclopentadecane	0.13
Eicosane	0.16
Cyclotetracosane	0.17
Heptacosane	0.89
Cyclooctasane	0.56
Tetratetracontane	0.52
Tocopherol	(5.54)
Alpha-tocopherol	5.08
Gamma-tocopherol	0.46

Table 1 (cont'd). Chemical compound banana leaf stalk extract from *Musa acuminate AA/AAA*

Compounds	**% of Total Ion Chromatogram**
Others	(5.01)
2-Pentanone,4-hydroxy-4-methyl	0.13
6-Methyl-5-Hepten-2-One	0.06
4-Methyl-1,5-Heptadiene	0.04
Decanoic acid, methyl ester	0.07
Palmitic acid, (2,2-dimethyl-1,3-dioxolan-4-yl) methyl ester	0.23
Tricosanoic acid, methyl ester	0.12
6,11-Dimethyl-2,6,10-dodecatrien-1-ol	0.22
9,12,15-octadecatrienoic acid, methyl ester, (Z, Z, Z)	0.67
9,12,15-Octadecatrien-1-ol, (Z, Z, Z)	0.45
9,12,15-Octadecatrienoic acid, (Z, Z, Z)	2.99
Trans-Geranyl acetone	0.11
Cis-Geranylacetone	0.11
Cyclopentane, -1-ethyl-2-methyl-cis	0.04

The MIC and MBC of the acetone extract of *Musa acuminate AA/AAA* leaf stalk are shown in Table 2. The acetone extract of the pulp had MIC values ranged from 125 mg/ml to 250 mg/ml. The acetone extract of the leaf stalk had MIC values of 125 mg/ml against *P. aeruginosa* and 250 mg/ml against *E. coli*, respectively. The MBC of acetone extract of *Musa acuminate AA/AAA* leaf stalk for both organisms was 500 mg/ml.

Table 2. The minimum inhibitory and bactericidal concentrations of acetone extract of *Musa acuminate AA/AAA* leaf stalk

Bacterial Strain	**MIC[a] (mg/ml)**	**MBC[b] (mg/ml)**
Pseudomonas aeruginosa	125 ± 1.0	500 ± 1.0
Escherichia coli	250 ± 1.0	500 ± 1.0

[a]MIC and [b]MBC are presented as the mean value of triplicate values ± SE in mg/ml; all tested bacteria showed inhibitory and bactericidal effects against chlorhexidine digluconate (0.12%)

The search for antimicrobial compounds for the benefit of humanity is necessitated by the inherent ability of pathogens to develop and adopt mechanisms of resistance against antibiotics. The present work demonstrates that the composition of volatile leaf stalk extract of *Musa acuminate AA/AAA* is a complex mixture of several classes of components, mainly terpenoids, phytosterol, phenolic compounds, fatty acids, hydrocarbon, tocopherol and other minor compounds, which is in agreement with other banana parts (peel, pulp, flowers, petioles/midrib, leaf blades/sheaths, floral stalk and rachis) and species reported [5, 12]. Phytosterol was the major class of compounds identified and this finding was similar with previous studies of other parts and varieties of banana [5, 7]. Dikshit [7] found that methanol extract of a banana's stem from *Musa sapientum* is rich in sterol and has antihypercholesterolemic and antioxidant effects in cholesterol-fed wistar rats. Other studies found that phytosterol has antibacterial [13], anticancer [14], and antidiabetic [15] properties. Tocopherol was also identified in this banana leaf stalk and concordant with previous findings of another Musa spp. [6].

Tocopherol is a potent antioxidant and recent findings showed that it reduces inflammatory responses in type 2 diabetes patients [16]. The terpenoids were found abundantly in this banana leaf stalk compared to the previous study of the unripe peel [5] and ripe pulp of *Musa acuminate AA/AAA* [17]. Squalene (12.02%) is the major identified compound in the terpenoids class and recent studies found that it has antibacterial [18], anticancer [19], and anti-inflammatory [20] activities. Palmitic acid (hexadecanoic acid) is the most abundant fatty acid (4.38%) identified and this finding is similar with previous studies of other Musa varieties [5]. Interestingly, a recent study by Nagata [21] showed that palmitic acid has anti-multiple myeloma activity. Low phenolic compounds in this banana leaf extract were in agreement with previously published results obtained from other Musa varieties [5].

The present work demonstrates that leaf stalk extract of *Musa acuminate AA/AAA* possessed antibacterial properties against two strains of Gram-negative bacteria (*E. coli* and *P. aeruginosa*) which is in agreement with the previous study [9] which used agar disc-diffusion method. To date, only Gram-negative bacteria (*E. coli* and *P. aeruginosa*) are susceptible to this stalk extract of *Musa acuminate AA/AAA* compared to previous studies using Gram-positive bacteria namely *Staphylococcus aureus* and *Streptococcus mutans* [9]. In addition, there is no other similar antimicrobial study reported on a specific leaf stalk extract of *Musa acuminate AA/AAA* on a specific Gram-negative bacterium. This finding could be correlated with identified compounds found in this study that has antibacterial property. The antimicrobial effects of acetone extract against these organisms may be due to the ability of the acetone to extract some of the active properties of these plants such as phenolic compounds, saponin, bryophyllin and other secondary metabolites which are reported to be antimicrobial [22].

Microorganisms vary widely in their degree of susceptibility to antimicrobial agents [3]. Based on the results, it is suggested that both bacteria are susceptible to the extract of the leaf stalk. *Musa acuminate AA/AAA* leaf stalk acetone extract showed MIC at 125 mg/ml for *P. aeruginosa* and 250 mg/mL for *E. coli*. The relatively higher MIC and MBC values against *P. aeruginosa* and *E. coli* might be contributed to their cell wall properties. Gram-negative bacteria are protected by their lipopolysaccharide layer (LPS), hindering the direct exposure of the inner membrane layer to the natural antibacterial activities [23, 24]. It has been reported that Gram-negative bacteria are usually more resistant to the plant-origin antimicrobials and even show no effects, compared to gram-positive bacteria [25]. Moreover, it was also suggested that the crude extract might contain compounds that inhibited the antibacterial activity of the effective compounds.

Conclusion

Based on the results obtained in this investigation, it can be concluded that acetone extract of *Musa acuminate AA/AAA* leaf stalk possesses antimicrobial activities against *P. aeruginosa* and *E. coli*. Thus, extracts from this plant possess the capabilities of being a candidate to control infections and/or diseases caused by these Gram-negative bacteria. However, the *in-vivo* experiment and pharmacological research of the identified compounds are very limited. Therefore, future work should be focused on *in-vivo* and pharmacological assays of known compounds, especially phytosterol and squalene that have antimicrobial properties. A better understanding of the antimicrobial compounds from *Musa acuminate AA/AAA* leaf stalk is crucial to identify the potential side effects and trace out the new host target and molecular mechanisms, which will provide evidence to further clinical applications of these compounds.

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