

Antibacterial Activity of Water and Methanol Extracts of Banana Pulps against *Vibrio cholerae*

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ABSTRACT: Diarrheal disease is one of the top leading causes of death in children under five years old. Approximately, more than 700 000 children died due to this disease every year. *Vibrio cholerae* is an important agent of diarrheal disease, especially in the developing countries. Oral rehydration therapy and antibiotics are the most common treatments to reduce the severity of *V. cholerae* infection. However, the emergence of antimicrobial resistance cases in many parts of the world has encouraged the search of new and effective antimicrobial compounds to overcome this issue, especially from natural products such as plants. Banana (*Musa* spp.) has been claimed to have antidiarrheal activity against several microorganisms, including enteric pathogens. However, there are not many studies on the antibacterial activity of banana flesh (pulp) against *V. cholerae*. Therefore, this study was conducted to determine the antibacterial activity of different banana pulp extracts against *V. cholerae*. The antibacterial activity of water and methanol extracts of banana pulps from two different species, pisang perak (*Musa acuminata* AA) and pisang gala (*Musa balbisiana* BB), against *V. cholerae* was evaluated using the disc diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) test. The effect of the extracts on the protein profile of the bacteria was also investigated using SDS-PAGE. MIC and MBC tests showed that the banana pulp extracts have inhibitory effects against *V. cholerae*, however, disc diffusion assay did not show the same result. SDS-PAGE analysis revealed that the extracts also influenced the expression of the bacterial proteins. In conclusion, the water and methanolic extracts of banana pulps of pisang perak and pisang gala are potential indirect antibacterial agents against *V. cholerae*. However, further study needs to be carried out to support this finding.

Keywords: Antibacterial activity, banana pulp, *Musa* sp., disc diffusion assay, MBC, MIC, protein profile

Introduction

Cholera is caused by the ingestion of food or water contaminated with *Vibrio cholerae* (Kitaoka *et al.*, 2011). The disease is characterized as an acute severe watery diarrhea with the presence or absence of vomiting symptoms (Sharifa Ezat *et al.*, 2013). Cholera is responsible for approximately 28 000 to 142 000 deaths per year out of the 1.4 to 4.3 million cases worldwide (Ali *et al.*, 2012). Although the trend of cholera cases is decreasing in many countries, including Malaysia, the number of reported cases may not represent the actual number of cholera cases, which are probably much higher (WHO, 2015).

The transmission of the disease is facilitated by several factors, such as a poor latrine system, sanitation problems and overcrowded places which are common in developing countries (Mandal *et al.*, 2011; WHO, 2014a). Rapid transmission and spread of cholera disease with a high mortality rate (up to 50%) among severe and untreated cholera patients adequately justify the need for public health concern (Bandyopadhaya *et al.*, 2008; Farmer *et al.*, 2011). In cholera patients, the outcome of the disease is often dehydration due to massive loss of fluid and electrolyte in diarrhea (Centers for Disease Control and Prevention, 2014). In severe cases, besides rehydration therapy, antibiotics such as doxycycline, tetracycline or erythromycin are given as an adjuvant to reduce the severity and duration of the disease (WHO, 2014b; Centers for Disease Control and Prevention, 2015). Therefore, antibiotics are necessary to control cholera disease. However, there are several findings from previous studies about the presence of antibiotic resistance of *V. cholerae* among the clinical isolates (Ang *et al.*, 2010; Das *et al.*, 2013). Moreover, resistance to tetracycline and other antimicrobial agents among *V. cholerae* has been shown in various endemic and epidemic settings (Centers for Disease Control and Prevention, 2015).

Hence, to overcome the emergence of antibiotic resistance issue, the search for alternative treatment is indeed necessary. Interestingly, Newman and Cragg (2007) stated that natural products serve as important components of drug development. Plants and plant derived compounds have been traditionally used to treat various complications and human diseases

(cited in Yamasaki *et al.*, 2011; Ghonime *et al.*, 2015). *Musa* spp. or banana is one of the plants which have been screened for several medicinal effects such as antidiarrheal activity (Alvarez-Acosta *et al.*, 2009; Rabbani *et al.*, 2010) and antimicrobial activity (Jawla *et al.*, 2012; Okorondu *et al.*, 2012; Venkatesh *et al.*, 2013; Rattanavichai and Cheng, 2014). These effects may correspond to the active compounds present in the banana plant such as flavonoid, glycosides, terpenoids, tannins, alkaloids phenolic compounds, starch and carbohydrate binding protein or lectin (Rabbani *et al.*, 2010; Okorondu *et al.*, 2012; Venkatesh *et al.*, 2013; Singh *et al.*, 2014; Pereira and Maraschin, 2015). However, the effects of banana extract as antibacterial agents against gastrointestinal infections especially *V. cholerae* are still unclear. Hence, this study was carried out to determine the effect of different banana pulp extracts against *V. cholerae*.

In this study, crude extracts of different banana pulps, namely pisang perak (*Musa acuminata* AA) and pisang gala (*Musa balbisiana* BB), were evaluated for antibacterial activities using the disk diffusion test, minimum inhibitory concentration (MIC) test and minimum bactericidal concentration (MBC) test. The effects of the plant extracts on protein profile of the bacteria were also evaluated using SDS-PAGE. The results of the study could potentially be used to understand the inhibitory activity of the plant extracts as an antibacterial agent against *V. cholerae* infection.

Material and Methods

Plant Collection

Banana pulp (fruit) of two different banana species, pisang perak (*Musa acuminata* AA) and pisang gala (*Musa balbisiana* BB), were collected from Kampung Sireh, Kota Bharu Kelantan. The collected plants were identified taxonomically and authenticated by the botanist of the School of Biological Sciences, Universiti Sains Malaysia (USM11621 for *M. acuminata* and USM11622 for *M. balbisiana*). The bananas were washed with tap water and wiped dry with a clean cloth. The peels and seeds were manually separated from the pulps. The banana pulps were cut into smaller pieces and allowed to dry in an incubator at 50°C for four to five days. The dried pulps were ground into the powder form using an electric blending machine and kept at -20°C prior to its use.

Plant Extraction

Water extraction of the samples was carried out by boiling 50 g of dried banana pulp powder in a 1000 ml conical flask containing 600 ml of distilled water for approximately 60 minutes on a stirring hot plate. The extract was then filtered through a clean cloth and centrifuged at 3000 rpm for 15 minutes. The supernatant was stored at -20°C overnight and subjected to freeze-drying. As for methanol extract, 50 g of banana pulp powder was soaked in 600 ml of 95% methanol solution in a 1000 ml conical flask for 3 days and stirred continuously using a magnetic stirrer. The extraction process was carried out in the fumehood at room temperature. The methanolic extract was then filtered through a clean cloth and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected, filtered using Whatman filter paper, and then concentrated using a rotary evaporator. The extract was allowed to air dry for approximately 5 to 7 days in a fume hood and stored in a sterile container at -20°C until further use.

V. cholerae Strain

Vibrio cholerae strain was collected from the Medical Microbiology and Parasitology Laboratories, Department of Medical Microbiology and Parasitology, Universiti Sains Malaysia. Gram stain and several biochemical tests were performed to confirm the authenticity of *V. cholerae* isolate.

Disc Diffusion Test

The disc diffusion method was carried out according to the Clinical and Laboratory Standards Institute (2007) with some modifications. Basically, 50 µl of each extract concentration (20, 40, 60, 80, 100 mg/ml) were impregnated onto 6 mm, sterile blank discs to give a final concentration of 1, 2, 3, 4 and 5 mg/disc. The discs were left to dry in the fumehood overnight. Colonies from nutrient agar were suspended in sterile saline water to form a turbidity of 0.5 McFarland standards using nephelometry. The bacterial suspensions were streaked onto Mueller-Hinton agar plates using sterile cotton swabs. The extract-impregnated disc, negative and positive control discs were placed on the agar within 15 minutes duration following the adjustment of bacterial suspension to 0.5 McFarland standard. Tetracycline (30 µg/ disc) served as a positive control while the impregnated disc with sterile distilled water

served as a negative control. The culture plates were incubated at 37°C for 18 hours. The diameter of inhibitory or clearing zone around each of the discs was measured in millimeter (mm) to determine the antimicrobial activity. The test was conducted in triplicate measurements and the reliability of the result was also compared with *E. coli* ATCC 25922.

Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration (MIC) of both water and methanolic extracts was determined using the microbroth dilution method based on the Clinical and Laboratory Standard Institute (2012). In sterile 96-well plates, two-fold serial dilution of each extract was performed, with the final concentration ranging from 0.625 mg/ml to 320 mg/ml. These assays were repeated three times. Wells, which contained Mueller Hinton broth with bacterial suspension, were used as the positive control while wells which contained extracts without bacteria were used as the negative control. The microtiter plate was incubated at 37°C for 24 hours. The MIC values were taken as the lowest concentration of each extract that completely inhibit the bacterial growth after the period of incubation by comparing the turbidity of the tests with both controls.

Minimum Bactericidal Concentration (MBC) Assay

The minimum bactericidal concentration (MBC) values were determined by sub culturing the wells which show growth inhibition on the Mueller-Hinton agar. The growth inhibition was determined by comparing the turbidity of the tests in each well with both positive and negative control. The plates were incubated at 37°C for 24 hours. The MBC values were defined as the lowest concentration of extract which shows no bacterial colony growth on the agar plates during the incubation time. The assays were repeated three times.

Bacterial Protein Isolation for SDS-PAGE Analysis

V. cholerae was cultured overnight in 10 ml Luria-bertani (LB) broth using conical flasks in an orbital shaker at 37°C and 200 rpm. The colonies were diluted (1:50) into LB broth and incubated on a shaker for 2 hours at 37°C. The bacterial cultures were adjusted to 0.5-1.0 McFarland and used in subsequent experiments. The extracts were added to the 50 ml of bacterial culture to achieve a final concentration of 0.2 x MIC [modified from Ismail *et al.*

(2013)]. Untreated bacterial culture was set as the control. All flasks were incubated at 37°C for 18 hours with constant shaking (200 rpm). Then, the treated and untreated bacterial cultures were collected and centrifuged at 3000 rpm for 30 minutes at 4°C to separate the bacterial cell pellet from the supernatant. The supernatant was collected and filtered through a 0.2 µm filter and mixed with absolute ethanol until the final concentration of the ethanol reached up to 70%. The mixture was mixed properly and kept at -20°C overnight for precipitation of exoproteins. The precipitated proteins were collected by centrifugation at 3000 rpm for 30 minutes. The pellets were dissolved in RIPA buffer at 4°C, overnight and centrifuged at 12 000 rpm for 20 minutes at 4°C. The supernatant was collected and stored at -80°C. Prior to conducting the SDS-PAGE, the protein concentrations were measured using spectrophotometer NanoDrop 2000 (Thermo Scientific™).

SDS-PAGE Gel Analysis

SDS-PAGE gel electrophoresis was carried out using 10% resolving gel [30% acrylamide mix (Amresco, USA), 1.5 M resolving buffer, 10% SDS (Amresco,USA), 10% APS], and 5% stacking gel [30% acrylamide mix, 1.0 M stacking buffer, 10% APS, 10% SDS]. The bacterial proteins were treated with the sample buffer and β-mercaptoethanol (Biorad, USA) in 1:1 ratio, heated at 95°C for 5 minutes, before being loaded into the wells. Gel electrophoresis was run at 100V, until the bromophenol blue (BPB) reached the bottom of the gel plate. The protein gel was then stained with Coomassie Brilliant Blue R-250, and the molecular weights of the bacterial proteins were determined using commercial protein markers (Fermentas, USA).

Results

Effect of Banana Pulp Extracts on the Growth of V. cholerae

Both water and methanol extracts of the banana pulps showed negative inhibitory activity against *V. cholerae* when tested using the disk diffusion assay (Table 1). This data indicated that the plant extracts are unable to inhibit the growth of *V. cholerae*. However, further analysis using MIC and MBC assays were carried out to confirm the result.

Table 1 : Diameter of inhibition zones of pisang perak and pisang gala extracts against *V. cholerae*

Extraction solvent	Sample type	Diameter of inhibition zone (mm)					Positive control tetracycline (30 µg/disc) (mm)
		Concentration of extracts (mg/ml)					
		20	40	60	80	100	
Water	Pisang Perak (<i>Musa acuminata</i> AA)	-	-	-	-	-	23
	Pisang Gala (<i>Musa balbisiana</i> BB)	-	-	-	-	-	25
Methanol	Pisang Perak (<i>Musa acuminata</i> AA)	-	-	-	-	-	24
	Pisang Gala (<i>Musa balbisiana</i> BB)	-	-	-	-	-	24
- No inhibition zone							

The zone of inhibition for *E. coli* ATCC 25922 against standard antibiotic was 21-23 mm, which is within the acceptable QC ranges (18-25 mm), as stated in guideline provided by Clinical and Laboratory Standards Institute (2007).

In contrast to the disc diffusion test, all banana pulp extracts demonstrated inhibition activity against *V. cholerae* in MIC and MBC assays. The methanolic extract of pisang perak successfully inhibited *V. cholerae* with a MIC of 20 mg/ml while water extract of pisang perak successfully inhibited *V. cholerae* with MIC of 80 mg/ml. Similar to methanolic extract of pisang perak, water extract of pisang gala also inhibited the growth of the bacteria at MIC of 20 mg/ml while methanolic extract of pisang gala successfully inhibited the bacteria at MIC of 80 mg/ml. For MBC assay, The methanolic extract of pisang perak completely killed *V. cholerae* at MBC of 40 mg/ml while water extract of pisang perak successfully killed *V. cholerae* at MBC of 80 mg/ml. Water extract of pisang gala completely killed the bacteria at MBC of 40 mg/ml while methanolic extract of pisang gala successfully killed the bacteria at MBC of 160 mg/ml. Table 2 summarizes both MIC and MBC assay results.

Table 2 : MIC and MBC assay results

Extraction solvent	Sample type	Minimum inhibitory concentration (MIC) (mg/ml)	Minimum bactericidal concentration (MBC) (mg/ml)
Water	Pisang Perak (<i>Musa acuminata</i> AA)	80	80
	Pisang Gala (<i>Musa balbisiana</i> BB)	20	40
Methanol	Pisang Perak (<i>Musa acuminata</i> AA)	20	40
	Pisang Gala (<i>Musa balbisiana</i> BB)	80	160

Effect of Banana Pulp Extracts on Protein Profile of V. cholerae

In order to determine the effect of banana pulp extracts on protein profile of *V. cholerae*, 0.2 x MIC was the concentration of the extracts used to treat the protein. Therefore, based on the MIC results, the concentration of the methanolic extract of pisang perak and water extract of pisang gala used in this study was 4 mg/ml while the concentration of water extract of pisang perak and the methanolic extract of pisang gala used was 16 mg/ml. As shown in Figure 1, treatment of the bacteria with 4 mg/ml methanolic extract of pisang perak and water extract of pisang gala showed the expression of *V. cholerae* protein at approximately 40 kDa (**d**).

For bacteria treated with 16 mg/ml of water extract of pisang perak and methanolic extract of pisang gala, only proteins of more than 40 kDa were expressed. Four protein at approximately 66 kDa (**a**), 65 kDa (**b**), 47 kDa (**c**) and 40 kDa (**d**) were detected in cells treated with 16 mg/ml of the water extract of pisang perak while three proteins at approximately 66 kDa (**a**), 65 kDa (**b**) and 40 kDa (**d**) were detected in cells treated with 16 mg/ml methanolic extract of pisang gala. However, the expression of proteins lower than 40 kDa were reduced or inhibited in these cells.

Interestingly, the data also showed that the expression of the approximately 40 kDa protein was much higher in the treated bacteria compared to the untreated bacteria. Similar finding was also observed for the 47 kDa protein in cell treated with 16 mg/ml of water extract of pisang perak.

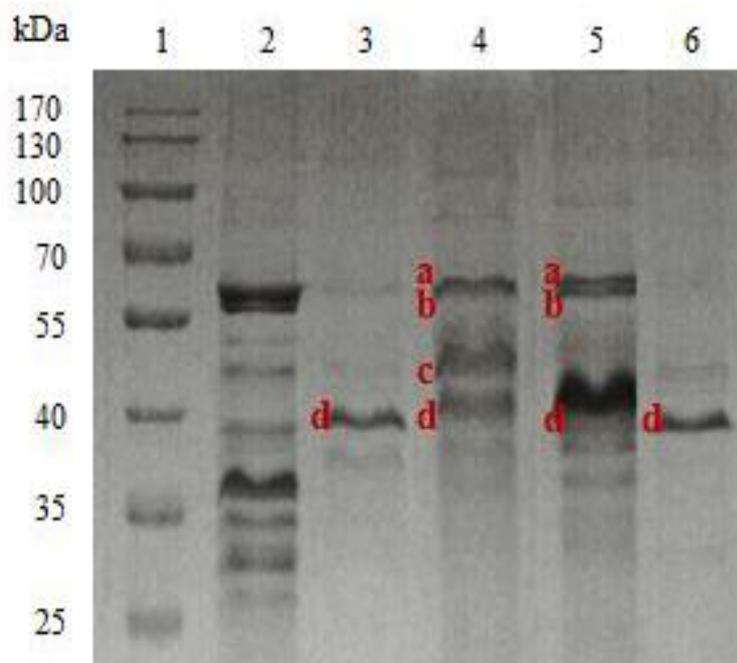


Figure 1: Protein expression profile of *V. cholerae* cell treatment. Lane 1: PageRuler Prestained Protein Ladder; Lane 2: Untreated *V. cholerae*; Lane 3: *V.cholerae* treated with 4 mg/ml of the methanolic extract of pisang perak; Lane 4: *V.cholerae* treated with 16 mg/ml of the water extract of pisang perak; Lane 5: *V.cholerae* treated with 16 mg/ml of the methanolic extract of pisang gala; Lane 6: *V.cholerae* treated with 4 mg/ml of the water extract of pisang gala. a - d: expressed proteins.

Discussion

Plants and plant-derived compounds have been proven to have medicinal values for the treatment of various human diseases including diarrhea. However, the inhibitory mechanisms of these plants are still unclear. Therefore, this study was conducted to determine the effect of banana pulp extracts from two different species, namely pisang perak and pisang gala against *V. cholerae*, an important pathogen in diarrhoeal disease using three different methods: disc diffusion, MIC and MBC assays. The effects of the extracts on the protein profile of the bacteria were also evaluated using a proteomic approach. This study demonstrated that the plant extracts only inhibited the growth of *V. cholerae* in broth dilution techniques but not in disc diffusion test. The result probably indicated that disc diffusion test was not sufficiently

sensitive to detect antibacterial properties of the banana pulp extracts compared to both microbroth dilution tests. *V. cholerae*, like the other Gram negative bacteria, is less susceptible to the potential antibacterial compounds present in the extracts due to its outer lipopolysaccharide membranes, which protect the inner membrane layer from direct exposure to the compounds (Ahmad and Beg, 2001; Alzoreky and Nakahara, 2003; Negi and Jayaprakasha, 2003; Naz *et al.*, 2007; Munyendo *et al.*, 2011). Therefore a higher concentration of banana pulp extract should be used.

This data also demonstrated that inhibition zone in disc diffusion assay did not totally indicate the presence or absence of any potential antibacterial compounds present in the extracts. Factors such as polarity, solubility and volatility of the extracts, as well as the size of the active compounds influence the rate of extract diffusion on the agar media (Scorzoni *et al.*, 2007). In addition, the amount of compound added to the disk, adsorption by the disc and microbial strains used in the study may also influence the size of the inhibition (Pauli, 2006). Therefore, we assumed that the potential antibacterial compounds in our samples are likely consisted of high molecular weight compounds based on the poor diffusion rate in the media.

The MIC and MBC results also suggested that the methanolic extract of pisang perak has better antibacterial activity against *V. cholerae* (MIC of 20 mg/ml and MBC of 40 mg/ml) than the water extract (MIC of 80 mg/ml and MBC of 80 mg/ml). The data is in agreement with a study done by Padam *et al.* (2012) which suggested that a low polarity solvent such as methanol is the best extracting solvent to extract potential antibacterial compounds from banana inflorescence. A study by Negi and Jayaprakasha (2003) also demonstrated a similar finding in which methanol extract of a pomegranate peel has higher activity than the water extract against all the bacteria tested such as *B. cereus* and *S. aureus*. In contrast, the results also showed that water extract of pisang gala had better antibacterial activity against *V. cholerae* (MIC of 20 mg/ml and MBC of 40 mg/ml) than the methanolic extract (MIC of 80 mg/ml and MBC of 160 mg/ml) which contradicted previous findings suggesting that methanol extract is more efficient than the water extract in extracting the antibacterial compounds of the plant. These data indicate that both polar and non-polar solvents can be used to extract the antibacterial compounds presence in the banana pulp extracts, but the efficiency depends on the species of the plant. Different solvents are capable to extract and dissolve different compounds present in the plant extract (Nur Syukriah *et al.*, 2014), and therefore, exhibit different antibacterial activity. We assumed that different antibacterial

activity of our banana pulp extracts observed in this study may correlate with the efficiency of both water and methanol to dissolve the compounds present in the banana pulp such as phenolic and flavonoid compounds which exhibit antimicrobial effects (Alam *et al.*, 2005; Pereira and Maraschin, 2015).

Further investigation was done to determine the effects of the plant extracts on the proteins profile of the bacteria. As shown in Figure 1, differentially expressed proteins of approximately 28-66 kDa were identified from *V. cholerae* cell free supernatant. These proteins are believed to have contributed to the *V. cholerae* virulence: the outer membrane proteins (23-49 kDa), flagellar protein (47 kDa), mature vibriolysin (35 kDa), hemagglutinin/protease; HA/P (32 kDa), active cytolysin/hemolysin (65 kDa), variant of cytolysin (50 kDa), and pro-vibriolysin (66 kDa) (Yamamoto *et al.*, 1990; Zitzer *et al.*, 1995; Nagamune *et al.*, 1996; Wu *et al.*, 1996; Olson and Gouaux, 2005; Xicohtencatl-Cortes *et al.*, 2006; Iqbal *et al.*, 2011).

As shown in Figure 1, cell treated with 4 mg/ml methanolic extract of pisang perak and water extract of pisang gala expressed protein at approximately 40 kDa. The reduced expression of several proteins probably indicates a disintegration of the respective proteins or an inhibition of protein synthesis (Yong *et al.*, 2015). Similar finding was also observed for the expression of proteins lower than 40 kDa in cells treated with 16 mg/ml of pisang perak and methanolic extract of pisang gala. This finding is in agreement with a previous study by Wong *et al.* (2014) which showed reduced expression levels of bacterial proteins upon treatment with *Melastoma candidum* extract. This phenomenon not only affects the structure of the proteins, but may also affect their function.

In the presence of 16 mg/ml water extract of pisang perak and methanolic extract of pisang gala, some of the bacterial proteins showed higher expression level compared to the untreated *V. cholerae*. We assumed that treatment with these extracts affected the integrity of the bacterial membrane and caused the release of some periplasmic proteins into the culture medium. Therefore, the amount of proteins in the culture supernatant increased significantly than that of the untreated *V. cholerae* and resulted in higher protein expression shown on the SDS gel. In addition, a study conducted by Arokiyaraj *et al.* (2014) also demonstrated that there was an increase in the protein concentration of *E. coli* and *P. aeruginosa* compared to the control upon treatment with an oil extract from the ambrette seed, which proving that

there was an intracellular protein leakage due to the disruption of the bacterial membrane. A study by Henie *et al.* (2009) also found that the amount of protein detected in several microorganisms such as *S. aureus*, *B. cereus*, *S. enteritidis* and *V.cholerae* following treatment with guava leaves extract increased gradually over time due to protein leakage. This finding suggested that treatment with the extracts may inhibit the growth of the bacteria by interrupting the bacterial protein translation pathway (Wong *et al.*, 2014), thus decreases or increases the expression of their proteins. However, further study should be carried out to determine whether the treatment can also affect the mRNA transcription activity.

Conclusion

Both banana pulp extracts of pisang gala and pisang perak have potential antibacterial activity against *V. cholerae*. However, further study is required to support this finding.

Acknowledgement

We extend our heartfelt gratitude to the School of Health Sciences, USM for the facilities and all the staff members of Biomedicine and Culture Laboratories, School of Health Sciences USM, especially Ms Wan Razlin Wan Zaabar and Ms Siti Kurunisa, for their assistance throughout this project.

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