

Antibacterial Assays of Malaysian Medicinal Plant *Polygonum minus* Using Different Extraction Methods

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Abstract: Medicinal plants and herbal preparations are gaining renowned interest in scientific communities nowadays due to their reliable pharmacological actions and affordability to common people which makes them effective in control of various diseases. The present study was aimed to evaluate the antibacterial activities of aqueous, methanol and ethanol extracts against gram positive and gram negative microorganism. The combination effects of *Polygonum minus* methanol extracts with streptomycin sulfate against gram positive *Bacillus subtilis* which showed 16.7% were synergy (2 out of 12 combinations), 16.7% were additive and (2 out of 12 combinations), 66.7% were indifferent (8 out of 12 combinations). While, combination effects between *P. minus* ethanol extract with streptomycin sulfate against *Bacillus subtilis* indicated that about 50% (6 out of 12 combinations) were antagonistic, 16.67% (2 out of 12 combinations) were indifferent and 33.33% (4 out of 12 combinations) were additive. The finding of this study concluded that gram-positive bacteria were more susceptible than the gram-negative and the combination effects between *P. minus* ethanol extract with streptomycin sulfate may lead to the development of a new and vital antimicrobial drugs against simultaneous infections of gram positive and gram negative microorganism.

Key words: *Polygonum minus*, antibacterial, ethanol extract, methanol extract, *Bacillus subtilis*, against

INTRODUCTION

In recent years, the prevention of many disorders like cardiovascular diseases and cancer has been found to be associated with the consumption of vegetables, fresh fruits, plant beverages and tea that are rich in natural antioxidants. The antioxidant and antimicrobial potentials of these plants are in turn attributed to the several compounds present in them. These compounds have unique mechanisms of action, some are proteins and enzymes while others exist as low molecular weight compounds such as vitamins, carotenoids, flavonoids, anthocyanins and other phenolic compounds (Khan *et al.*, 2003). Plants that possess therapeutic properties or exert beneficial pharmacological effects on

the human body are generally known as medicinal plants. The rate at which bacteria resist antibiotics has increased alarmingly and the aftermaths of antibiotics usage are a major problem in the treatment of infectious diseases. New antimicrobial agents are needed to treat diseases in humans and animals that are caused by drug resistant microorganisms (Zink, 1997). Hence, the search for novel substances with antimicrobial properties is important. Medicinal plants, however have been utilized in the development of drugs from long time and compounds with antimicrobial activity from plant origin are the possible alternatives to the challenges of using synthetic antimicrobial compound (Vikram *et al.*, 2014). Antimicrobial compounds of plant origin may occur in stems, roots, leaves, bark, flowers and fruits of

plants. Plants derived phytoalexin, sothiocynates, allcins, anthocyanins and essential oils, tannins and polyphenols and terpenoids have demonstrated antibacterial and/or antifungal activities. These compounds are bactericidal and/or bacteriostatic inducing lag time, growth rate and maximum growth of microorganisms (Borchardt *et al.*, 2008). Hence, the aim of this study was to study the antimicrobial effect of the leaves extract of *P. minus*. The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effects they produced. These limitations demand for improved pharmacokinetic properties which necessitate continued research for new antimicrobial compounds for the development of drugs (Al-Haj *et al.*, 2009). There have been a number of reports of antibacterial activity from natural resources (Al-Haj *et al.*, 2010) and special attention has been reported for antibacterial and/or antifungal activities related to natural product against several pathogens. On the other hands various studies have reported that *P. minus* possess antioxidant activity (Maizura *et al.*, 2010; Huda-Faujan *et al.*, 2013), antimicrobial activity (Jamal *et al.*, 2011; Imelda *et al.*, 2014), antiulcer activity and cytotoxicity (Wasman *et al.*, 1996), anti-inflammatory activity (George *et al.*, 2014) and antiviral activity (Wasman *et al.*, 1996; Qader *et al.*, 2011). *P. minus* was chosen for the purpose of this study because of its enormous use in traditional medicine. It has also been reported exhibiting antibacterial activity against *Helicobacter pylori* (Uyub *et al.*, 2010), *Bacillus subtilis* (Jamal *et al.*, 2011) and *Escherichia coli* (Imelda *et al.*, 2014).

MATERIALS AND METHODS

Plant collection: Fresh healthy leaves of *Polygonum minus* (huds) samples were obtained from Banji local market, Selangor, Malaysia. The fresh samples were cleaned and washed using running tap water to remove sand and dust particles and then divided into two parts, leaf and whole plant. The samples were dried using an oven (Sheldon Manufacturing, Inc., FX2-2, USA) at 40°C and then ground for approximately 2-3 min using a grinder (Multifunction disintegrator BR-15, Brun) and kept in airtight bottle before any further processing (Fig. 1)

Bacteria: Two bacteria which consisted of one gram positive bacteria *Bacillus subtilis* and one gram-negative *Escherichia coli* were used for the purpose of carrying out this research. These bacteria were obtained from



Fig. 1: *Polygonum minus*

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Extraction of plant material: The extraction of plant material and extracts was done adopting the method of (Al-Alhaj *et al.*, 2010; El-Mahmood, 2009) with slight modifications. The aqueous, methanolic and ethanolic extracts were prepared by dissolving 100 g of fine powder extract of *P. minus* separately in 1000 mL of distilled water, methanol and ethanol, respectively. The contents were kept in rotary shaker for 72 h at room temperature with speed around 100 rpm. Then the extracts were filtered through filter study No. 1. The solvent residue was further evaporated using rotary evaporator at 45°C and speed of 65 rpm to obtain crude extracts. The crude extracts were preserved in airtight bottle at 4°C. For antimicrobial assay, the aqueous, methanol and ethanol extracts were diluted in Mueller-Hinton Broth (MHB, Oxoid.UK) to give a range concentration between 31.25-500 mg/mL. The reconstituted extracts were maintained at a temperature of about 2-8°C.

Assay of antibacterial activity on agar well diffusion: Stock cultures were maintained at 4°C on nutrient agar slants for bacteria prior to assay. Agar-well diffusion assay was carried out using the method described by Bbosa *et al.* (2007) with modifications. Wells of 6 mm

diameter and 5 mm depth was made in solidified Mueller Hinton Agar (MHA) using a sterile borer. Cultures of the microorganisms at the concentration of 10^7 cell/mL were then inoculated separately on the solidified agar on each petri dish by streaking using sterilize cotton swabs. About 10 μ L of for each extracts at the concentration of 31.25, 62.5, 125, 250 and 500 mg/mL was dispensed into the respective wells. Each extracts of ethanol, methanol and distilled water were used as negative control while an aqueous solution of 10 mg/mL of streptomycin sulfate was used as positive control. The plates were allowed to stand for 1 h for pre-diffusion of the extract to occur and then incubated at 37°C for 24 h and the zones of inhibition were measured to the nearest mm. All the tests were carried out in triplicates.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

Microdilution method was done as described by Adegoke *et al.* (2010) with modifications. A 100 μ L of overnight bacterial inoculum was added into each wells of 96-well microtiter plate containing 100 μ L of the *P. minus* extracts and controls. The microtiter plates were further incubated for an overnight at 37°C. The wells were then observed for visible growth based on turbidity. The lowest concentration that showed no bacterial growth (non-turbid) was reported as Minimum Inhibition Concentration (MIC). For determining MBC values, the bacterial suspension from the MIC wells that did not show any growth were sub-cultured into MHA plates by streaking and further growth overnight at 37°C. The lowest concentration that showed no bacterial growth was recorded as MBC value.

The agar diffusion method, the inhibition zone relate to the susceptibility of the tested bacteria to the plant extracts. According by Nascimento *et al.* (2000), microorganism can be characterized as being susceptible if it produces inhibition zone of equal to or >7 mm in diameter or resistant if the inhibition zone is <7 mm in treatments with extracts. All the extracts of *P. minus* were effective except for some concentrations of aqueous extract which recorded <7 mm.

Combination effects of *P. minus* with streptomycin sulfate:

The combination effects between each *P. minus* methanol and ethanol extracts with streptomycin sulfate on *B. subtilis* and *E. coli* were used. Combination test was done based on method by Bergeret and Raymond (1999) and Palmer and Rybak (1997). About 50 μ L of each Streptomycin sulfate solution with different concentration (including 2.5, 0.25, 0.025 and 0.0025 mg/mL) was mixed separately with 50 μ L of each *P. minus* extracts with range of MIC value (2.0, 1.0 and 0.5 MIC). The combination

mixtures were then added with 100 μ L of bacterial culture (10^7 cells/mL). As a control, 100 μ L of the bacterial culture (10^7 cells/mL) were mixed with either 100 μ L of streptomycin sulfate (with different concentration ranging from 2.5-0.0025) or 100 μ L of *P. minus* extracts (2.0, 1.0 and 0.5 MIC) separately. After an overnight incubation at 37°C, 20 μ L of each mixture was added with 180 μ L of Mueller-Hinton Broth (MHB). Then, 100 μ L of each dilution mixtures was applied onto each Mueller Hinton Agar (MHA) and was further spread by using hockey stick. Colony counting was done after the agar plates were incubated for an overnight at 37°C.

RESULTS AND DISCUSSION

Antibacterial activity of aqueous extract of *P. minus*:

Antibacterial activity of five different concentrations of aqueous extracts of *P. minus* assayed against two bacteria strains is shown in Fig. 2. The range of inhibition zone for the aqueous extract was between 6-13 mm in diameter, while the highest inhibition zones were obtained at concentration of 500 mg/mL against all tested bacteria. Concentration of 125 mg/mL with maximum inhibition zones were obtained only against *E. coli* (6 mm). No inhibition zone were shown with any concentration against *B. subtilis*.

Antibacterial activity of methanol and ethanol extract of *P. minus* against *B. Subtilis* and *E. coli*:

The antibacterial activity of methanol extract of *P. minus* showed maximum inhibition of 11.5 mm and minimum inhibition of 10 mm in diameter only on *B. subtilis* (Fig. 3a) and the zone of inhibition of the ethanol extract against *E. coli* at concentration of 250 mg/mL was 13 mm (Fig. 3b), the maximum zone of inhibition was against *B. subtilis* (11.5 mm) at concentration of 500 mg/mL and minimum at 250 mg/mL but no zone of inhibition was observed at concentration of 31.25 mg/mL. Concentration of 500 mg/mL while the maximum zone of inhibition (13 mm) against *E. coli*.

MIC and MBC evaluation: The MIC and MBC values of methanol extract of the plant material is shown in Table 1. Lowest MIC and MBC value of the extract was shown against *B. subtilis*. Whereas, lowest MIC value of the ethanol extract of *P. minus* (250 mg/mL) was shown against *B. subtilis* and *E. coli*. Lowest MBC of ethanol extract (250 mg/mL) was found against *B. subtilis* and *E. coli*.

Combination effects of *P. minus* extracts with streptomycin sulfate:

The result of combination effects between *P. minus* methanol extracts with streptomycin sulfate against *B. subtilis* as shown in Fig. 4-6.

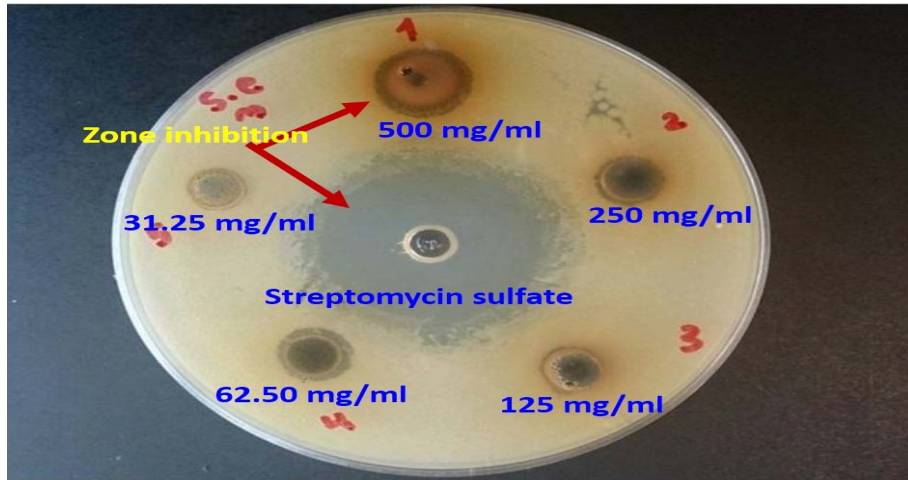


Fig. 2: Antibacterial activity of aqueous extract of *P. minus*

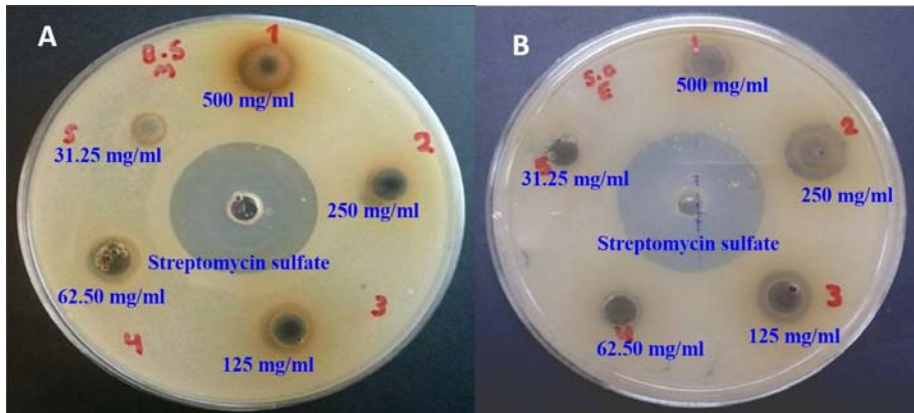


Fig. 3: A, B) Antibacterial activity of methanol and ethanol extract of *P. minus* against *B. subtilis* and *E. coli*

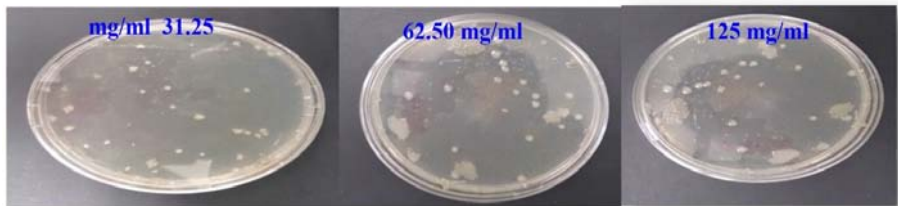


Fig. 4: Combination effect between *P. minus* methanol extracts with streptomycin sulfate against *B. subtilis*

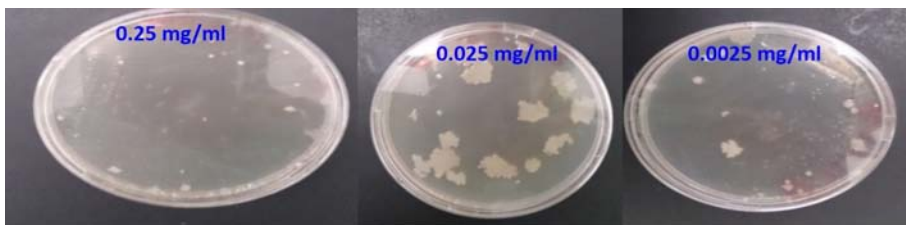


Fig. 5: Low concentration of streptomycin sulfate indicate more than 20 CFU/mL

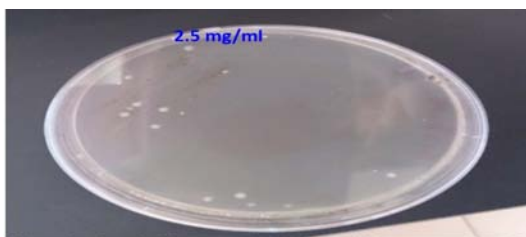


Fig. 6: Control of streptomycin sulfate with high concentration 2.5 mg/mL indicate low colony count

Table 1: MIC and MBC values of aqueous, methanol and ethanol extract of *P. minus*

Extracts/Test bacteria	MIC value (mg/mL)	MBC value (mg/mL)	MBC/MIC ratio
Aqueous			
<i>B. subtilis</i>	62.50	250.00	4
<i>E. coli</i>	250.00	250.00	1
Methanol			
<i>B. subtilis</i>	62.50	62.50	1
<i>E. coli</i>	250.00	- ^a	-
Ethanol			
<i>B. subtilis</i>	250.00	250.00	1
<i>E. coli</i>	250.00	250.00	1

The combination effects between *P. minus* methanol extracts with Streptomycin sulfate indicated moderate inhibition activity against *B. subtilis*. The synergistic effects were clarified by the combination between *P. minus* methanol extracts at high concentration (2.0 MIC) with 2.5 and 0.025 mg/mL of streptomycin sulfate respectively. Likewise, the additive effects were determined for the combination of *P. minus* methanol extracts at concentration of 1.0 MIC with 2.5 and 0.25 mg/mL of streptomycin sulfate subsequently.

Generally, each single treatment with 0.5 MIC of ethanol extracts and 0.0025 mg/mL of streptomycin sulfate showed low inhibition activity as there are Too Numerous To Count (TNTC) colonies was recorded. Moderate inhibition effects were shown by single treatment of 1.0 MIC ethanol extract and 0.025 mg/mL of streptomycin sulfate. Meanwhile, each single treatment with high concentration of streptomycin sulfate (2.5 mg/mL) and ethanol extract (2.0 MIC) indicated high inhibition activity as there are no bacterial growth and 6 colonies of bacterial produced, respectively. The additive effects could be observed for each following combinations, streptomycin sulfate at low concentration (0.0025 mg/mL) with either 2.0 or 1.0 MIC of ethanol extracts, streptomycin sulfate at concentration of 0.025 mg/mL with either 1.0 or 0.5 MIC of ethanol extracts. Besides, indifferent effects showed by two combinations as follow, high concentration of ethanol extracts (2.0 MIC) with high concentration of

streptomycin sulfate (2.5 mg/mL), low concentration of ethanol extracts (0.5 MIC) with low concentration of streptomycin sulfate (0.0025 mg/mL). Meanwhile, the other combinations exhibited antagonism effects. In general, combination effects between *P. minus* ethanol extract with streptomycin sulfate against *B. subtilis* showed that about 50% (6 out of 12 combinations) were antagonistic, 16.67% (2 out of 12 combinations) were indifferent and 33.33% (4 out of 12 combinations) were additive.

Every plant on Earth is useful to mankind either directly or indirectly. Many plants have created history in the treatment of diseases. The classical examples include opium for pain management, digitalis for cardiac failure and cinchona for malaria. With the recognition of the role of antioxidants in many diseases there was a worldwide search for natural antioxidants. Plants containing these principles are nowadays screened for a variety of pharmacological properties. The plant *Polygonum minus* is not an exception in this context as it is reported to be antibacterial effect and hence has gained great attention. Therefore, in agar diffusion method, the inhibition zone relate to the susceptibility of the tested bacteria to plant *Polygonum minus* extracts. According to Nascimento *et al.* (2000), microorganism can be characterized as being susceptible if it produces inhibition zone of equal to or >7 mm in diameter or resistant if the inhibition zone is >7 mm in treatments with extracts. Overall, *P. minus* aqueous extracts was effective towards all the two bacteria strains with least activity on *B. subtilis*. The aqueous extract of *P. minus* was reported to have the highest total flavonoid content. Aqueous extract besides, this current research indicated that only the aqueous extract of *P. minus* was effective against *E. coli*. The methanol extracts of *P. minus* at varying concentrations also exhibited inhibition against *E. coli*. Meanwhile, the ethanol extract of *P. minus* was effective against *B. subtilis* and *E. coli*. The antimicrobial activity of *P. minus* methanolic and ethanolic extracts may be due to the present of bioactive compounds such as phenolics, flavonoids, alkaloids, terpenoids and steroids (Imelda, 2014). However, the formation of clear zone of inhibition from agar-well diffusion assay could not indicate the effectiveness of the antimicrobial activity. Also, the antimicrobial activity could be affected by certain factors such as culture media selected, the volume of bacteria inoculates, pH of the medium, growth phase, the incubation time and temperature (Friedman *et al.*, 2002; Rios *et al.*, 1988). Generally, the extracts were quite sticky in which could affect the diffusion of compounds into agar, resulting a smaller inhibition zone compared to the positive control (Streptomycin sulfate). Thus, this assay

only useful as preliminary screening tool to determine the antimicrobial potential of *P. minus* extracts and further experiment on MIC and MBC evaluation was carried out. Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that will inhibit the growth of a microorganism at the end of stipulated time of incubation period. Minimum Bactericidal Concentration (MBC) is that concentration of a microbial agent that will kill or eliminate a microorganism at the end of stipulated time of incubation period (Mehta *et al.*, 2013). MIC and MBC values of the plant extracts were determined towards *B. subtilis* and *E. coli*. For the aqueous extract, the lowest MIC was shown against *B. subtilis* (62.50 mg/mL) (Nurain *et al.*, 2012). Generally, the MIC value of *P. minus* extracts were lower (except in some cases which is equal) than the MBC values. It is showed that the plant extracts were bacteriostatic at lower concentration but bactericidal at higher concentration (Maji *et al.*, 2010). Al-Haj *et al.* (2010) stated that when MBC/MIC value is ≤ 4 the strains is considered to be susceptible while if the ratio is >4 then the strains is considered to be tolerant while (Canillac and Mourey, 2001) stated that when MBC/MIC value is ≤ 4 the strains is considered to be susceptible while if the ratio is >4 then the strains is considered to be tolerant. In general, all test bacteria were susceptible to the different extracts of *P. minus*. Generally, the combination effects of *P. minus* methanol extracts with streptomycin sulfate against *B. subtilis* showed that about 16.7% were synergy (two out of 12 combinations), 16.7% were additive (two out of 12 combinations) and 66.7% were antagonistic (eight out of 12 combinations). Meanwhile, the combination effects between *P. minus* ethanol extract with streptomycin sulfate against *B. subtilis* showed that about 50% (six out of 12 combinations) were antagonistic, 16.67% (two out of 12 combinations) were indifferent and 33.33% (four out of 12 combinations) were additive. The presence of abundant phytochemical might be the reason of the effective combination results among methanol and ethanol *P. minus* extracts with streptomycin sulfate. Priyavardhini *et al.* (2012) stated that the abundant and complexity of the compounds in plants were responsible for imparting them with the future of being therapeutically effective with the advantage of synergistic and additive effects. However, the antimicrobial effect of very highly active compounds could be suppressed or masked by the other low active compound resulting antagonism effect (Ejim *et al.*, 2011).

CONCLUSION

Plants containing antibacterial activities are nowadays, extensively screened for various pharmacological activities. *P. minus* abundantly available in Southeast Asia and possesses high antibacterial and antioxidant activity. This plant has been

long used in traditional medicine and Malay cuisine. The biological activity of this plant extracts depends on the type of solvent used and methanol was shown to be the best solvent to extract the phenolic compounds. There are four types of relation including additive, antagonism, indifferent and synergy effects could be observed in the combination method of this study. Thus, it may help in improving the treatment of antibiotic resistance microorganism in efficient and safe way.

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