Potential Antimicrobial Activities of Belimbing Buluh (*Averrhoa bilimbi*) Leaves Extracts against Common Human Pathogens

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Abstract: From ancient times plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health. Traditional medicines by using herbs have been practiced by Malay communities from the past until now. The knowledge on the benefits of certain herbs has been passed from generation to generation. The primary health care of 80% of the world's population is based on the use of medicinal plants derived from traditional system of medicine and local health practices. *Averrhoa bilimbi* is a member of the family Oxalidaceae known as "bilimbi" and native in Malaysia and Indonesian Moluccas. The plant has an enormous fiscal value since most of the plant parts such as leaves, bark, flowers, fruits, seeds and roots which are used as an alternative medicine to treat a variety of diseases. Thus, this study was conducted to investigate the potential antibacterial and antifungal activities of *Averrhoa bilimbi* leaves extracts against selected human pathogens. *A. bilimbi* leaves were extracted by using two polar solvents i.e. water and methanol. Two strains of bacteria, *Staphylococcus aureus* and *Bacillus subtilis* and two species of human pathogenic fungi, *Candida albicans* and *Aspergillus niger* were used to screen the antimicrobial activity and antifungal activity of *A. bilimbi* by well diffusion method. The minimal inhibitory concentration were determined by using different concentrations of leaves extracts (50, 100, 200, 400, 600, 800 µg/ml) using the same method. The phytochemical analyses of the leaves extracts were also conducted to determine the secondary metabolites presented in the *A. bilimbi* leaves extracts. Ampicilin and Ketonozole were used for positive control of antifungal and antibacterial activities respectively. Findings showed that methanol leaves extract gave larger zone of inhibition compared to water leaves extracts against *S. aureus* and *E. coli* with 6.0 mm and 7.0 mm respectively compared to 1.4 mm and 1.6 mm zone of inhibitions respectively at the same concentration (600 ug/uL). Meanwhile, for antifungal activities, methanol leaves extract showed higher zone of inhibition against *C. albican* compared to water leaves extract with 1.9mm and 1.2mm zone of inhibition respectively at the same concentration (200 ug/uL). However, there was no inhibition showed by *A. niger* for both of the leaves extracts. The Minimal Inhibitory Concentration (MIC) of methanol leaves extracts for antibacterial activity against *S. aureus* was at 600 ug/uL (6.0 mm) and *E. coli* was at 800 ug/uL (9.2 mm) and for antifungal activity against *C. albican* was at 200 ug/uL (1.9 mm). Meanwhile the MIC for water leaves extract for antibacterial activity was at 800 ug/uL (*S. aureus*) and 200 ug/uL (*E. coli*) and for antifungal activity against *C. albican* was at 800 ug/uL. The phytochemical analysis found that the *A. bilimbi* leaves extracts to contain Alkaloid, Glycoside, Flavonoid, Saponin, Tannin, Triterpene and Phenol. The antimicrobial and antifungal activities of the *A. bilimbi* were due to the presence of various secondary metabolites. Therefore, these plants can be used to develop new pharmaceuticals products and other research activities.

Keywords: *A. bilimbi*, Antibacterial activity, Antifungal activity, Secondary metabolites, Zone of inhibition

1. Introduction

Pathogenic fungi and bacteria cause many human diseases. These diseases are typically treated are by using synthetic antibiotics. However, some of these pathogenic microorganisms become resistant to modern synthetic antibiotic. Moreover, the price of the treatment using antibiotics keep on increasing over the years, thus medicinal plants extracts could become an

alternative treatment. The World Health Organization has identified the uses of plant extracts and with known antimicrobial properties as alternative sources of therapeutic treatments (World Health Organization – WHO, 2001). Medicinal plants have many medicinal properties which are the sources for maintenance of human health (Das et al., 2011). More than 12000 chemical compounds are known to exist in about 20% of plant species and only few have been exploited for medicinal purposes (Saxena et al., 2013). The secondary metabolites found in medicinal plants materials make the plants able to inhibit many pathogenic fungi and bacteria.

Staphylococcus aureus is common in skin infections, respiratory disease and food-poisoning agents. *S. aureus* is the cause for the serious antibiotic resistant hospital infections (Heikkila & Saris, 2003). Pathogenic *E. coli* are harmful to their host (Elsas et al., 2011) and can cause mild to bloody diarrhea. They colonized the mucosal surface and infect urinary tract and gastrointestinal tract (Saosa, 2006). *C. albicans* is a group of microorganisms that live in mouth and intestine. *C. albicans* cause candidiasis that can affect skin, genitals and throat. Vulvovaginal candidiasis (VVC) is one of mucosal infection caused by *C. albicans* (Barousse et al., 2001). *A. niger* is the common fungal species which are able to produce mycotoxin in food. Mycotoxins are potent hepatocarcinogen in animals and humans (Irkin & Korukluoglu, 2007). Hepatocarcinogen cause carcinogenesis in the liver.

A. bilimbi belongs to family Oxalidaceaeis is native to Southeast Asia and found throughout the tropics. Different parts of *A. bilimbi* have various uses. The fruits of *A. bilimbi* is sour and high in vitamin C and contain many medicinal properties which can be used to treat several human diseases such as fever, inflammation, beriberi, coughs and to stop the rectal bleeding (Roy et al., 2011). The decoction and paste of the leaves can be used to treat fever, mumps, pimples, inflammation, cough, syphilis, itches, rheumatism and venereal diseases (Tan et al., 2005). Siddique et al. (2013) stated that methanol extracts of *A. bilimbi* barks at a concentration of 400 µg/disc revealed antimicrobial activity against the growth of *S. aureus, E. coli, A. niger* and *C. albicans* by having the zone of inhibition with diameter between 8 and 9 mm. Das et al. (2011), stated that methanol extracts of *A. bilimbi* leaves at concentration 400 µg/disc showed antibacterial activity against the growth of *S. aureus* and *E. coli* by having the zone of inhibition with diameter of 6.0 and 6.5 mm respectively. Siddique et al. (2013) also reported that the barks extract of *A. bilimbi* contain alkaloids, saponins and flavonoid.

The aim of this study was to evaluate the antimicrobial and antifungal activities of *A. bilimbi* leaves extracts against *S. aureus, E. coli, A. niger* and *C. albicans.* The types of secondary metabolites present in the *A. bilimbi* leaves extract were also determined.

2. Materials and Methods

Plant material

About 2.5 kg of leaves of *A. bilimbi* was collected at Jengka, Pahang Darul Makmur. The leaves were rinsed well with distilled water. Then, the leaves were air dried for 7 days and grounded by using dry grinder to obtain fine powder (Alabri et al., 2014; El-Chaghaby et al., 2014).

Plant Extraction

Two types of solvents were used for extraction i. e. methanol and water. About 200 g of powdered samples were soaked in 1000 mL of distilled water and another set in 600 mL of 100% methanol. After 24 hours of soaking, the macerations were filtered using Whatman No.1 filter paper. Each of the filtrates were dried using rotary evaporator with the temperature not exceeding 40°C until the methanol and water crude extract were obtained (James et al., 2007).

Phytochemical screening

The phytochemicals screenings were conducted against each of the leaves crude extracts to detect the presence of alkaloids, glycosides, flavonoid, saponin, tannins, triterpens, phenol and anthraquinone.

Test for alkaloids

Presence of alkaloid in the *A. bilimbi* leaves extracts were detected by using Mayer's reagent test. Six drops of Mayer's reagent were added in 1 mL of extract. The formation of yellow precipitate indicated the presence of alkaloids (Firdouse and Alam, 2011; Kodangala et al., 2010).

Test for glycosides

1 mL of extract was dissolved in 2.0 ml of glacial acetic acid, which contained one drop of 5% solution $FeCl_3$. Then 3 to 4 drops of concentrated H_2SO_4 were added to the solution. Formation of a brown ring indicated the presence of glycosides (Iqbal 2012; Rathore et al., 2012).

Test for flavonoid

About 2-3 mL of extract and few drops of sodium hydroxide (NaOH) were added in a test tube. Formation of intense yellow color that became colorless in addition of few drops of dilute HCl indicated the presence of flavonoid.

Test for saponin

About 1 mL of the extract was dissolved in 9 mL of distilled water and shook in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponin (Iqbal 2012; Tiwari et al., 2011).

Test for tannins

Few drops of 10% lead acetate solution were added to 1 mL of extract. Formation of yellow or red precipitate indicated the presence of tannins (Rathore et al., 2012).

Test for triterpenes

Libermann-Burchard test was used to detect the presence of triterpenes. About 1 to 2 mL of acetic acid anhydrate was added to 2 mL of extract together with 2 drops of H_2SO_4 concentrate in a test tube. Reddish violet color formed at the junction of the two layers bluish green color in the acetic acid layer which indicated the presence of unsaturated sterols (triterpenes).

Test for phenol

Few drops of 5% ferric chloride (FeCl) solution were added to 1.0 mL of extract. Formation of intense color indicated presence of phenols (Iqbal, 2012).

Tested microorganism

Two strains of bacteria used were *S. aureus* and *E. coli* and the two species of human pathogenic fungal used were *C. albicans* and *A. niger*. The cultures were obtained from stock culture that were maintained in nutrient agar.

Antibacterial activity

The antibacterial activities of *A. bilimbi* leaves extracts were conducted using standard disc diffusion method. About 10 mL of Nutrient Agar (NA) (40°C) was poured into the petri plate and allowed to solidify. Then, 50 µL of bacterial suspension was poured uniformly by using hockey stick on the solidified NA (Datta et al., 2011). The bacterial agar plate was divided into three compartments. Sterile blank paper disc was impregnated with 15 µL extract of different concentrations (50, 100, 200, 400, 600 and 800 µg/µL) were placed on the agar plate. Ampicillin acts as positive control while dimethyl sulfoxide (DMSO) was used as negative control (Chakraborthy and Aeri, 2009; Costa et al., 2010). The plates were incubated at 37°C for 24 hours to allow maximum growth of the organisms. The antibacterial activities of the extracts were determined by measuring the diameter of zone of inhibitions expressed in millimeter (mm). The experiment was carried out in five replicates and mean values were taken.

Antifungal activity

The antifungal activities of *A. bilimbi* against *A. niger* and *C. albicans* were conducted by using well diffusion method. Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were used as media growth for *A. niger a*nd *C. albicans* respectively. A 5 mm diameter of fungal pathogens were placed at the center of the respective plates and three wells were bored in each plate at the periphery side by using aseptic cork borer (Costa et al., 2010). About 15 µL extract of different concentrations (50, 100, 200, 400, 600 and 800 µg/µL) were filled into the wells by using Pasteur pipette. Ketoconazole was used as positive control and NaCl was used as negative control (Timothy, 2012). The plates were incubated at 28 °C for 72 hours (Chakraborthy et al., 2009; Costa et al., 2010; Manimegalai et al., 2012). The antifungal activities of the extracts were determined by measuring the diameter of zone of inhibitions which were expressed in millimeter (mm). The experiment was carried out in five replicates and mean values were taken.

3. Result and Discussion

Antibacterial Activity of *Averrhoa bilimbi*

The findings showed that methanol leaves extract of *A. bilimbi* gave larger zone of inhibition against *S. aureus* and *E. coli* compared to water leaves extracts that gave 6.0 mm and 7.0 mm respectively compared to 1.4 mm and 1.6 mm zone of inhibitions respectively at the same concentration (600 ug/uL). The antibacterial of *A. bilimbi* methanol and water leaves extracts were shown in Table 1 and Figure 1. This results contradicted with Zakaria et al, (2007) which stated that *S. aureus* were more susceptible to water and chloroform leaves and fruits extracts of *A. bilimbi* compared to *E. coli.*

Table 1. Antibacterial activity of methanol and water extract of *A. bilimbi* leaves

SOLVENT EXTRACT	CONCENTRATION OF EXTRACT (µg/µL)	ZONE OF INHIBITION (mm)	
		Staphylococcus aureus	*Escherichia coli*
Methanol	50	2.60 ± 0.89	1.40 ± 0.89

	100	3.60 ± 1.82	4.00 ± 2.35
	200	5.00 ± 1.73	5.20 ± 3.03
	400	4.60 ± 0.89	4.40 ± 2.88
	600	6.00 ± 2.83	7.00 ± 4.30
	800	4.60 ± 1.95	9.20 ± 3.63
	DMSO	0.00	0.00
Water	50	0.60 ± 0.42	0.60 ± 0.42
	100	1.60 ± 0.55	1.70 ± 1.40
	200	0.70 ± 0.45	2.40 ± 2.07
	400	0.90 ± 0.22	1.80 ± 1.64
	600	1.40 ± 0.55	1.60 ± 1.14
	800	1.80 ± 1.10	1.60 ± 1.14
	Distilled water	0.00	0.00

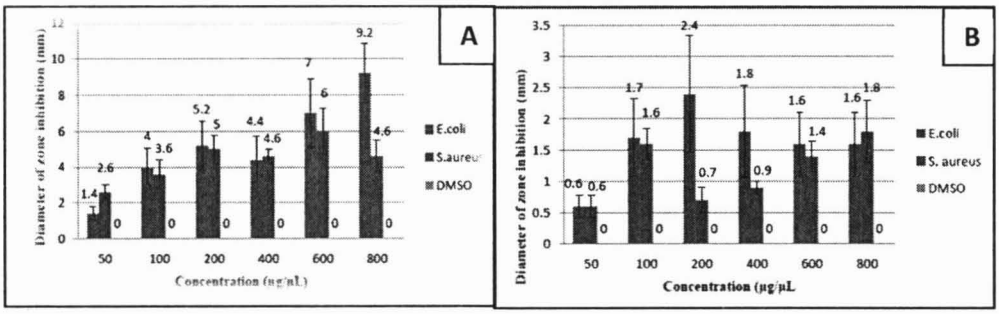

Fig. 1 Antibacterial activity methanol (A) and water (B) leaves extracts of *A. bilimbi* at different concentrations against *E. coli* and *S. aureus*

The findings also indicated that the Minimal Inhibitory Concentration (MIC) of methanol leaves extracts against *S. aureus* was at 600 ug/uL (6.0 mm) and against *E. coli* was at 800 ug/uL (9.2 mm). Meanwhile the MIC for water leaves extract for against *S. aureus* was at 800 ug/uL and against *E. coli* was at 200 ug/uL. This indicated that *S. aureus* and *E. coli* were more susceptible to methanol leaves extract compared to water leaves extract where the zone of inhibition given by both bacterial against methanol leaves extracts were larger than the water leaves extracts. This indicated that methanol leaves extract contained more phytochemical substances that were stronger antibacterial activity against both of pathogenic bacteria.

Antifungal Activity of *Averrhoa bilimbi*

Antifungal activity of *A. bilimbi* methanol and water leaves extract showed that only *C. albicans* showed the antifungal activity against the extracts, meanwhile *A. niger* did not showed any antifungal activity. Results found that the methanol leaves extract gave the higher zone of inhibition against *C. albicans* compared to water leaves extract at same concentration of 200 ug/uL with 1.9 mm and 1.2 mm zone of inhibition respectively as showed in Table 2 and Figure 2.

Table 2. Antifungal activity of methanol and water extract of *Averrhoa bilimbi* leaves

SOLVENT EXTRACT	CONCENTRATION OF EXTRACT (µg/µL)	ZONE OF INHIBITION (mm)	
		Aspergillus niger	*Candida albicans*
Methanol	50	0.00	1.60 ± 0.65

	100	0.00	1.60 ± 1.08
	200	0.00	1.90 ± 0.74
	400	0.00	0.80 ± 0.67
	600	0.00	0.20 ± 0.45
	800	0.00	0.00
	DMSO	0	0.00
Water	50	0.00	1.20 ± 0.76
	100	0.00	1.60 ± 0.96
	200	0.00	1.20 ± 0.67
	400	0.00	1.80 ± 0.84
	600	0.00	1.80 ± 1.44
	800	0.00	2.80 ± 1.15
	Distilled water	0	0.00

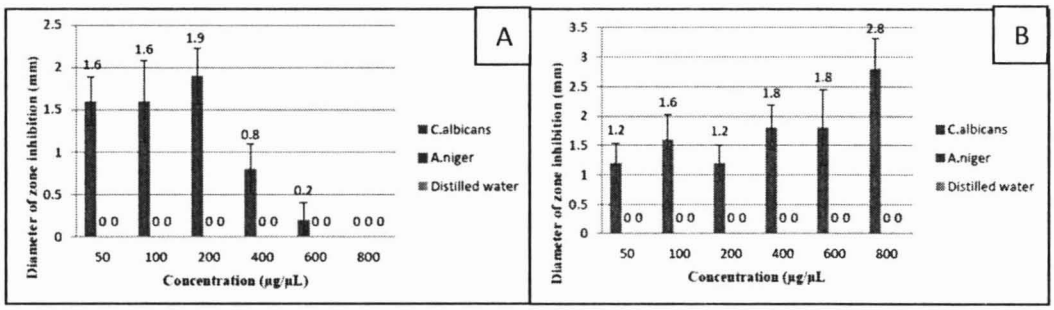

Fig. 2 Antifungal activity methanol (A) and water (B) leaves extracts of *A. belimbi* at different concentrations against *C. albicans* and *A. niger.*

Results also indicated that the Minimal Inhibitory Concentration (MIC) of antifungal activity of methanol leaves extracts for against *C. albicans* was at 200 ug/uL (1.9 mm) while, for water leaves extract was at 800 ug/uL (2.8 mm). This indicated that, *C. albicans* was more sensitive to water leaves extract compare to methanol leaves extract as it gave the larger zone of inhibition with 2.8 mm (water leaves extract) as compared to 1.9 mm (methanol leaves extract). This indicated that *A. bilimbi* water leaves extracts have stronger antifungal activity compared to methanol leaves extract.

Phytochemical Screening Test

Phytochemical analysis screening indicated that the leaves of *A. bilimbi* methanol extracts contained alkaloid, glycoside, flavonoid, saponin, tannin, triterpene and phenol, while water leaves extract contained only glycoside, flavonoid, tannin, triterpene and phenol as showed in Table 3

Table 3. Phytochemical analysis screening of *A. belimbi* leaves extracts

PHYTOCHEMICAL CONSTITUENTS	METHANOL	DISTILLED WATER
Alkaloid	+	-
Glycoside	+	+
Flavonoid	+	+
Saponin	+	-
Tannin	+	+
Triterpene	+	+

Phenol	+	+

The result of phytochemical analysis screening was consistent with the findings of Siddique et. al. (2013), where they reported that the barks extract of *A. bilimbi* contained alkaloids, saponins and flavonoid. The results from this study also indicated that the leaves of *A. bilimbi* contain more phytochemical constituent than the bark of *A. bilimbi*.

4. Conclusions and Recommendations

In this study, *A. bilimbi* leaves extract showed inhibitory activities towards both *E. coli* and *S. aureus* at every concentration but with different diameter of zone of inhibition. However, *A. bilimbi* only showed inhibitory activity towards *C. albicans* but not towards *A. niger*. Thus, this showed that *C. albicans* are more susceptible towards the leaves extracts. The minimum inhibitory concentration for methanol and distilled water leaves extract in inhibiting *S. aureus, E. coli* and *C. albicans* were at concentration 50 µg/µL.

It is also recommended to use methanol leaf extract rather than distilled water leaves extract in inhibiting the bacteria and fungi because it is a polar solvent and showed more antimicrobial activity compared to distilled water. The phytochemical screening analysis proved that solvent methanol is able to extract more chemical constituent from the leaf of *A. bilimbi* as compared to distilled water. The presence of all the phytochemical constituent in leaves extract of *A. bilimbi* proved that this plant has the potentials in treating various types of ailments. It is recommended to use leaves of medicinal plant *A. bilimbi* as alternatives to synthetic drugs because of its potentials.

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