Journal of **BIOLOGICAL RESEARCHES**

ISSN: 08526834 | E-ISSN:2337-389X

Volume 21| No. 1| December | 2015

Original Article

ANTIBACTERIAL ACTIVITY OF BELILIK (*Brucea javanica* (L). MERR) AND BENTA (*Wikstroemia androsaemofolia* DECNE) TO INHIBIT THE GROWTH OF ENTEROPATHOGENIC BACTERIA

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ABSTRACT

Several native Indonesia plants have been used to prepare traditional medicine since long time ago. One of common diseases in tropical country is diarrhea, it caused by the infection of enteropathogenic bacteria such as Enteropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Shigella* sp. Belilik (*Brucea javanica* (L.) Merr) and Benta (*Wikstroemia androsaemofolia* Decne) are herbals that utilized as medicine for diarrhea in Bangka Belitung, Indonesia. Parts of these plants are mostly can be utilized as medicine, such as leaf, root, and fruit. The aims of this study were to investigate the antibacterial activity of ethanol crude extract of *B. javanica* (root and fruit) and *W. androsaemofolia* (leaf and fruit) against enteropathogenic bacteria (EPEC, *P. aeruginoa, S. aureus*, *Shigella* sp.). Method that used was papper disc diffusion. The results showed that at concentration 10 mg/mL, 20 mg/mL, and 30 mg/mL of *B. javanica* ethanol extract of both root and fruit could not inhibit the enteropathogenic bacteria, while the ethanol extract of leaf and fruit of *W. androsaemofolia* were shown inhibition activity on the growth of enteropathogenic bacteria. *W. androsaemofolia* leaf extract performed the best inhibition activity to the growth of EPEC (20.55±1.5mm) and *S. aureus* (22.14±4.5mm), it was better than kanamycin performance at the same concentration (30 mg/mL). In addition, ethanol extract of *W. androsaemofolia* fruit showed the best inhibition activity against *Shigella sp*. (19.64±1.8mm).

Keywords: *antibacterial, Brucea javanica (L.) Merr, enteropathogenic bacteria, Wikstroemia androsaemofolia Decne*

INTRODUCTION

Antibiotic resistance has become a global problem in the world recently. One of the cases is the resistance of some infectious pathogenic bacteria that caused enteropathogenic disease, such as EPEC (Enteropathogenic *E. coli*), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Shigella* sp. Kusmala (2012). Noveria (2012) stated that those bacteria are resist to kanamycin, amoxillin, chloramphenicol, and tetracycline. The cases of resistance case encourage researchers to find alternative drugs to treat various diseases caused by microbial infection such as medicinal plants exploration. On the contrary to synthetic drugs, natural antimicrobials derived from plant are not associated with negative effects. Furthermore, it has therapeutic potential to treat other diseases (Anand *et al*, 2011).

The number of researchers that explore natural products in order to develop better drugs against cancer as well as viral and microbial infections is increase dramatically (Srinivasan *et al*, 2001). Total 80% of world's population relies on traditional medicines for primary healthcare and most of it involves the utilization of plant extracts (Sandhya *et al*, 2006). Various plants possess medicinal properties, such as antibacterial properties. Several medicinal plants that have been used today were known by the ancient cultures throughout the world (Zalika 1975 in Dipankar *et al*, 2011). Traditional usages of in indigenous society can provide information as the foundation of modern pharmaceutical development (Philip *et al*, 2009).

Indonesia is known as country with the second highest plant diversity in the world after Brazil (Sampurno 2007). Supported by indigenous cultures that have wide knowledge of traditional medicine, Indonesia posseses high diversity of medicinal plants. Bangka Belitung Islands is one of provinces in Indonesia which rich of folk medicines. Bangka Belitung community commonly utilize local plants such as belilik (*B. javanica* (L) Merr) and benta (*W. androsaemofolia* Decne) to overcome diarrhea. *B. javanica* is commonly used to treat malaria, amoebic dysentery, vaginal candidiasis, hemorrhoids, intestinal worms, papilloma, and nasopharyngeal cancer. In addition, its root has benefit in malaria treatment, fever, and food poisonous (Adelia, 2010; Dalimartha, 2001). Root and stem mixture is effective for fever and indigestion treatment (Hendrian & Haidah 1999). *B. javanica* fruit contains saponins and tannins that potential as antibacterial agents, it can be assumed that these compounds also can be found in root (Rahayu *et al*, 2009). Another plant that Bangka Belitung community use for medicine is *W. androsaemofolia*. It commonly used as anti-malaria, moreover people also used this plant as cure for diarrhea. *W. indica* shares the same genus with *W. androsaemo-*

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folia, because of that they contain similar active compound such as flavonoid, biflavonoid, coumarins, lignans, essential oils, and polysaccharides (Li *et al*, 2009). Likewise, this plant allegedly also has antibacterial properties.

As the matter of facts, those medicinal plants are undoubtedly have important value to treatment of several diseases. The antibacterial activity of these plants against enteropathogenic bacteria through in vitro have not clearly revealed yet. Therefore, this study investigated the antibacterial activity of *B. javanica* and *W. androsaemofolia* against enteropathogenic bacteria. The aims of this study were to investigate the antibacterial activity of these plants and compare their effectivity to commercial antibiotic. This study will contribute in new approach of diarrhea treatment.

METHODS

Plant material

Herbals that used in this study were belilik (*B. javanica* (L). Merr) and Benta (*W. androsaemofolia* Decne). *B. javanica* were obtained from Selindung, Pangkalpinang, Bangka Island, while *W. androsaemofolia* were from Tanjungpandan, Belitung Island Indonesia.

Extraction

Extraction was done by soxhlet method. Each 100 g sample of *B. javanica* fruit, *B. javanica* root, *W. androsaemofolia* leaves, and *W. androsaemofolia* fruit were crushed separately then soaked in 96% of ethanol and heated to get the perfect extract, at the end of the process, solvent became clear. Solvent then were dried using vaccum rotary evaporator. Total yields of extraction were then measured.

Phytochemical Analysis

Phytochemical components were analyzed qualitatively. Purpose of this analysis was use to investigate active compound contains in fruit and root of *Belilik* (*Brucea javanica* (L.) Merr) and *W. androsaemofolia* leaves and fruit. There are some active compound that have been analyzed in both plants such as alkaloids, flavonoids, phenols, saponins, steroids, tannins, and triterpenoids.

Alkaloids

30 mg of extract were diluted in 10 mL of $CHCl_3$ – NH_3 and filtered, filtrate was accumulated into test tube. 3-5 drops of 2M sulfuric acid was added into the filtrate then shook until formed two layers. Acid layer (top layer) was pipetted into another test tube, then Dragendorff reagent was added. Alkaloids will be detected by the formation of orange to reddish brown sediment (Robinson 1995).

Flavonoids

30 mg of extract were added into 100 mL of hot water, boiled for 5 minutes, then filtered. Filtrate was added into 5 mL of 0.05 mg Mg powder and concentrated HCl then shaken vigorously. Positive test was indicated by formation of red, yellow, or orange colour in the (Harborne 1996).

Phenols

10 drops of 1% $FeCl_3$ were added into 30 mg of extract. Phenols were represented by the production of green, red, purple or dark black colour (Harborne 1996).

Saponin

30 mg of extract was re-extracted with 5 mL of diethyl ether to divide it into two fractions (soluble and insoluble). Insoluble fraction of diethyl ether then added into 5 mL of water in a test tube then they were shaken. Positive test result indicated by for about 1-3 cm foam production in 15 minutes (Harborne, 1996).

Tannins

30 mg of the extract was added into hot water and boiled for 5 minutes. The mixture then filtered and the filtrate divided into 2 parts and each of it was added 1% of $FeCl_3$. The presence of tannins and polyphenol were characterized by the formation of blue-green colour. Besides of that, the presence of tannins will characterized by the formation of a white precipitate after gelation addition (Harborne 1996).

Triterpenoids and Steroids

Saponin soluble fraction in diethyl ether separated and added with 10 drops of glacial CH_3COOH and 2 drops of concentrated H_2SO_4. The solution was shaken slowly and was left for few minutes. Steroids gave blue or green colour, while triterpenoids gave red or purple colour (Harborne 1996).

Tested Microorganism

Four enterophatogenic bacterial strains were used in this study: EPEC, *P. aerugionosa*, *S. aureus*, *and Shigella* sp. These bacteria were obtained from Microbiology Laboratory, Bangka Belitung University, Indonesia. Bacterial strains were cultivated at 37°C and maintained on Nutrient Agar (NA) slant (Oxoid, USA) at 4°C.

Antimicrobial activity assay

Antimicrobial activity of extracts was determined by observation of their ability against four pathogenic bacteria using disc diffusion method. Crude ethanol extract and antibiotic (kanamycin as control) were dissolved in aquadest. Extract and antibiotic were tested in three different concentrations (10mg/mL, 20 mg/mL, and 30 mg/mL). 20 ml of Nutrient Agar and 100 µL of bacteria suspension at log phase (10^6-10^8 cell/mL) were poured into petri dishes (90 mm each side), mixed, and homogenized. After agar medium solidified and innoculated, 6 mm diameter of antibiotic disc that had been soaked in extracts and antibiotic for 5 minutes then placed on the surface of the medium. Petri dishes were incubated at 37°C for 24 hour. Growth inhibition zone sorround the disc represents the antimicrobial activity of extract. Negative control were paper disc soaked in aquadest. The diameter of inhibition zone measured using digital Vernier

Caliper. Inhibition zone was calculated with reduce the total of inhibition with paper disc diameter (6 mm).

RESULTS

Medicinal plant extracts

The extraction using ethanol yielded different extract mass for each part of plants. Yield of *B. javanica* fruits extraction resulted 2.9% of extract, while root resulted 8.39% of extract. *W. androsaemofolia* fruits extraction resulted 11.21% of extract and 4.4% for leave.

The results of phytochemical test

Phytochemical analysis through qualitative method indicated that *B. javanica* fruit contain alkaloids, flavonoids, and phenols. In addition, *B. javanica* root contain alkaloids, phenols, and saponins. Phytochemical test of *W. androsaemofolia* showed that phenols, saponins and steroids are contained in fruit, while leaves contained phenols and steroids (Table 1).

Table 1. Phytochemical component of each part of the plants

Compounds	*B. javanica* fruits	*B.javanica* roots	*W. androsaemofolia* fruits	*W. androsaemofolia* leaves
Alkaloids	+	+	-	-
Flavonoids	+	-	-	-
Phenols	+	+	+	+
Saponins	-	+	+	-
Steroids	-	-	+	+
Tanins and Poliphenols	-	-	-	-
Triterpenoids	-	-	-	-

Note: (+) =present, (-)= no

Table 2. Inhibition Zone of Extracts investigated in diffusion assay

Extract/ antibiotic	Concentration ($mgmL^{-1}$)	Means of Inhibition zone (mm) against			
		EPEC	P.a	S.a	Sh
***B. javanica* fruits**	10	0.0	0.0	0.0	0.0
	20	0.0	0.0	0.0	0.0
	30	0.0	0.0	0.0	0.0
***B. javanica* leaves**	10	0.0	0.0	0.0	0.0
	20	0.0	0.0	0.0	0.0
	30	0.0	0.0	0.0	0.0
***W.androsaemofolia* fruits**	10	11.13±1.3	13.47±2.5	16.59±3.0	17.13±1.7
	20	15.39±1.0	14.54±0.8	15.88±3.0	18.58±2.3
	30	12.63±1.1	14.17±1.7	16.48±3.0	19.64±1.8
***W.androsaemofolia* leaves**	10	17.33±2.5	12.24±0.7	16.09±4.5	12.89±2.1
	20	19.36±0.9	13.07±0.4	17.49±4.3	11.06±1.4
	30	20.55±1.5*	14.74±2.2	22.14±4.5*	14.74±1.0
Kanamycin	30	15.3±6.6	23.58±5.6*	19.61±6.0	22.21±0.3*

note:*=the higest inhibition zone against tested bacteria

Antibacterial Test Result

Antibacterial activity test results show that ethanol extract of *W. androsaemofolia* could inhibit the growth of enteropathogenic bacteria effectively. At the concentration of 10, 20, 30 mg/mL *B. javanica* extract could not inhibit the growth of enteropathogenic bacteria (Table 2). *W. androsaemofolia* leaves performed the widest inhibition zone against EPEC (20.55±1.5 mm) and *S. aureus* (22.14±4.5 mm) even when it compared to kanamycin (15.3±6.6 mm for EPEC and 19.61±6.0 mm for *S. aureus*) at concentration 30 mg/mL.

Although, *W. androsaemofolia* fruit extract could not perform better inhibition zone compared to kanamycin, but Duncan Multiple Range Test (DMRT) result showed that *W. androsaemofolia* fruit against *Shigella* sp. delivered similar wide of inhibition zones as *W. androsaemofolia* leaf against EPEC and *S. aureus* (Table 3).

DISCUSSION

Both ethanol crude extract of *B. javanica* fruit and leaf could not inhibit the growth of EPEC, *P. aeruginosa*, *S.aureus*, and *Shigella* sp. *B. javanica* is called as belilik in Bangka, it is called makassar fruit in other province in Indonesia, and Ya- and -Zi in China. It is widely used to treat cancer. It also can perform as anti-pyretic, detoxification, anti-inflammatory, and anti-virus with low level of toxicity (Chen *et al*, 2013).

Table 3. DMRT interaction between *W. androsaemofolia* extract and bacteria

Extract andbacteria	Inhibition zone (mm)
***W.androsaemofolia* leaves to EPEC**	19.078[a]
***W.androsaemofolia* fruits to *Shigella* sp.**	18.901[a]
W.androsaemofolia* leaves to *S.aureus	18.568[a]
W.androsaemofolia* fruits to *S.aureus	16.322[b]
W.androsaemofolia* fruits to *P.aeruginosa	14.060[c]
W.androsaemofolia* leaves to *P.aeruginosa	13.348[c]
***W.androsaemofolia* fruits to EPEC**	13.120[c]
***W.androsaemofolia* leaves to *Shigella* sp.**	12.790[c]

* same letter show the same effect of extract

It is noticeable in this study that *B. javanica* fruit contain alkaloids, flavonoids, and phenols, whereas *B. javanica* root contain alkaloids, phenols, and saponin (Table 1). Several studies stated that *B. javanica* produces various active compounds, including alkaloids (brucamarine, yatanine), glucoside (brucealin and yatanoside), and phenol (bruceno and bruceolic acid). *B. javanica* seeds

contain brusatol and bruceine (A, B, C, E, F, G, H). Flesh of *B. javanica* fruit contains fatty oil, oleic acid, linoleic acid, stearic acid, and palmitic acid. Moreover, the most abundance active compound found in its fruit was brucein (Wijayakusuma, 2008; Chen *et al*, 2013).

Although, *B. javanica* contain antimicrobial compound, as the matter of fact it could not inhibit bacterial growth that tested in this study. It can be expected that the increasing of extract concentration and usage of another solvent for both fruits and leaves of *B. javanica* might be able to inhibit the growth of tested bacteria. It supported by Senthilnath *et al*, (2013) study that stated plant extracts contain antibacterial substances in sufficient concentrations that will kill bacteria effectively. It also possible that active compounds contained in extracts are not soluble in solvent that used in the study.

Sornwatana *et al*, (2013) stated that brucin, an antibacterial peptide derived from fruit of *B. javanica* showed antibacterial activity potential against *S. pyogenes*. Rahayu *et al* (2009) stated that extract of *B. javanica* through soxhletation is more effective than extract derived from maceration at 30%, 60%, 90% concentration against *Shigella dysentriae* ATCC 9361 through in vitro method. *W. androsaemofolia* performed inhibition activity to the growth of all tested bacteria, both gram-positive and gram-negative. The inhibition zone of plant extracts against both gram-positive and gram-negative bacteria may correlate to the presence of broad antibiotic compounds (Lans *et al*, 2001). *W. androsaemofolia* leaf extract showed the best inhibition against EPEC and *S. aureus* at concentration of 30mg/mL, it remains the same when it was compared to kanamycin at the same concentration. *W. androsaemofolia* fruit extract performed good inhibition to *Shigella* sp. DMRT showed that the ability of *W. androsaemofolia* fruit extract to inhibit *Shigella* sp. is similar to *W. androsaemofolia* leaf extract in inhibition of EPEC and *S.aureus* growth (Table 3 and Figure 1). The effect of both leaf and fruit extract of *W. androsaemofolia* against four tested bacteria were different (Table 2.), but *W. androsaemofolia* generally can inhibit growth of both gram-positive and gram-negative bacteria.

Gywali *et al*, (2013) stated that the activity of antimicrobial agents depend on microorganism types. Gram-positive bacterial cell walls contain peptidoglycan and teichoic or teichuronic acid, also this bacteria may or may not be surrounded by protein or polysaccharide envelope. Gram-negative bacterial cell walls contain peptidoglycan, lipopolysaccharide, lipoprotein, phospholipid, and protein. The critical attack site of antimicrobial agents is peptidoglycan layer. This layer is essential for bacterial survival in hypotonic environments. As the matter of this fact, loss or damage of this layer will alter bacterial cell wall rigid that lead to cell death. Outer layer of gram-positive and gram-negative bacteria cell wall and porin channels of Gram-negative bacteria allow antimicrobial agents diffuse easily through them. Gram-positive bacteria outer wall channels are loose, whereas gram-negative bacteria outer wall channels are narrow (Harold *et al*, 1996). It can be assumed that both leaf and fruit extract of *W. androsaemofolia* cause cell wall damage of gram-positive and gram-negative bacteria, due to the extracts ability to inhibit the growth of both gram-positive bacteria (*S. aureus*) and gram-negative bacteria (EPEC, *Shigella* sp., and *P. aeruginosa*).

Figure 1. Inhibition zone caused by *W. androsaemofolia* a. leaves against EPEC b. fruits against *Shigella* sp.

Chinese traditional medicine apply *Wikstroemia* as antioxidant, antimicrobial, anti-inflammation, antivirus, antitumor, anticancer, and antibrowning (Huang *et al*, 2010; Lu *et a*,. 2011; Li *et al*, 2012; Lu *et al*, 2012; Ko *et al*, 2013). Some studies reported that *Wikstroemia* contain flavonoids, biflavanoids, coumarin, aetheric oil, lignan, polysacharide, and other important compounds (Huang *et al*, 2010; Li *et al*, 2010; Li *et al*, 2012; Ko *et al*, 2013; Lu *et al*, 2011; Lu *et al*, 2012). *Wikstroemia* contain flavonoid/biflavonoid, such as Naringin, 5,6,7-Trihydroxy-4'-methoxy-dihydroflavonol,kaempferol-3-O-b-D-ucopyra noside, kaempferol-3-robinoside-7-rhamnoside, wikstrol

A, wikstrol B, chamaejasmin, neochamaejasmin, isochamaejasmin, chamaechromone, genkwanin, quercetin, quercitrin, sikokianin B, sikokianin C, sikokianin D, stelleranol, genkwanol C, genkwanol B, tricin, and 4'-methoxydaphnodorin (Li *et al*, 2005; Huang *et al*, 2010; Chen *et al*, 2012; Li *et al*, 2012; Wei *et al*, 2012; Yongqin *et al*, 2012; Ko *et al*, 2013). Yang & Zu (2006) reported that *Wikstroemia indica* extract through decoction showed antibacterial activity against *Bacillus coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Sarcina lutea* using the doubling dilution method. The minimum inhibitory concentration (MIC) of *Bacillus coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Sarcina lutea* were 156mg/mL, 78mg/mL, 78mg/mL and 39mg/mL, respectively. Furthermore, the minimum bactericidal concentration (MBC) of these four species of bacteria were 312mg/mL, 156mg/mL, 156mg/mL and 78mg/mL, respectively. In conclusion, antibacterial activity performance can be classified from strong to weak as follow *Sarcina lutea*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus coli*.

Both leaf and fruit extracts of *W. androsaemofolia* showed difference ability against tested bacteria, which probably influenced by different compound contained in each extract. Arulmozhi *et al* (2007) stated that the antibacterial properties of medicinal plants correlated to the presence of chemical agents contain which commonly called as bioactive antimicrobial compounds. Different concentration of extract showed different ability to form inhibition zone. Yang & Zu (2006) stated that antibacterial activity depend on the concentration of substances.

The effectiveness of medicinal plant in curing disease is noticeably due to the combination of different compounds activities originally in the plant (Bhandarkar *et al.*, 2003). Phenol compounds have capacity to link with proteins and bacterial cell membrane then form complexes (Zongo *et al*, 2011). Steroids have been reported to have antibacterial properties by link to the cell membrane lipids, for the consequences it will alter membrane sensitivity for steroidal compound associated with liposomes leakages (Raquel *et al.*, 2007). Antimicrobial property of saponin is related to its ability to stimulate leakage of several proteins and certain enzymes from the cell (Zablotowicz *et al.*, 1996).

The best concentration to inhibit the growth enteropathogenic bacteria was 30 mg/mL after compared to commercial antibiotic aminoglycoside kanamycin at the same concentration. Aminoglycosides are complex sugars connected to glycosidic linkage. Antimicrobial activity of these agents are depend on the free NH_4 and OH groups, which aminoglycosides bind to specific ribosomal proteins (Harold *et al*, 1996). Bactericidal effect of the aminoglycoside kanamycin has been investigated extensively, it irreversibly binds to 16S of 30S ribosomal subunit (Franklin & Snow 2005). In addition, in order to targeting the protein synthesis machinery, kanamycin inhibit DNA synthesis and cellular membrane composition. The increase of glucose incorporation lipids and hydrophobic into the membrane fraction showed that cellular membrane was also damaged by kanamycin treatment (Faraji *et al*, 2006).

As has been noted, *W. androsaemofolia* can be used as alternative antimicrobial agents in new drugs development for infectious diseases caused by enteropathogenic bacteria especially EPEC, *S. aureus*, and *Shigella* sp. Therefore, the enhancement of plant extracts activity, in particular by using another solvent, extraction techniques, or purification are needed for further investigation. It is also necessary to ensure the safety of plant extracts before widely used.

ACKNOWLEDGEMENT

The author would like to thank to Umajaya and Yuliana who assisted in *Brucea javanica* antibacterial activity test in Microbiology Laboratory, Bangka Belitung University.

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