การตรวจสารพฤกษเคมีเบื้องต้นของสารสกัดถั่วขาว และฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคสัตว์น้ำ

Phytochemical Screening of *Bruguiera cylindrica* Extracts and Pathogenic Antibacterial Activities

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บทคัดย่อ

ถั่วขาว (*Bruguiera cylindrica*) เป็นพืชป่าชายเลนวงศ์ Rhizophoraceae พบได้ในพื้นที่เขตร้อน เก็บรวบรวมตัวอย่างแห้งของเปลือก ใบ ฝักและกิ่งของถั่วขาวมาสกัดด้วยตัวทำละลายเมทานอล นำสารสกัดหยาบตรวจสอบพฤกษเคมีเบื้องต้นและฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคสัตว์น้ำ 4 ชนิด คือ *Streptococcus agalactiae* SAAQ001, *Aeromonas hydrophila* AHAQ001, *Vibrio harveyi* VHAQ001 และ*V. parahemolyticus* โดยวิธี hole-plate diffusion พบว่า สารสกัดส่วนใหญ่ออกฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคทั้งแกรมบวกและแกรมลบ ตรวจสอบความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อแบคทีเรีย (Minimal Inhibitory Concentration, MIC) และความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อแบคทีเรีย (Minimal Bactericidal Concentration, MBC) พบว่าสารสกัดจากใบและกิ่งของถั่วขาวให้ค่าการยับยั้งและฆ่าแบคทีเรียต่ำสุดกับเชื้อ *S. agalactiae* การตรวจสารพฤกษเคมีเบื้องต้น พบว่าสารสกัดถั่วขาวมีสารพฤกษเคมีหลายกลุ่ม คือ แอนทราควิโนน เทอร์พีนอย ฟลาวานอยด์ ซาโปนิน ฟีนอลิก และ อัลคาลอยด์ โดยเฉพาะอย่างยิ่งเทอร์พีนอย ซึ่งพบเฉพาะในใบและกิ่งสนับสนุนการออกฤทธิ์ยับยั้งเชื้อแบคทีเรียก่อโรค ผลการทดลองดังกล่าวชี้ให้เห็นว่าสารสกัดถั่วขาวมีสารกลุ่มออกฤทธิ์ที่สำคัญ ช่วยเสริมการออกฤทธิ์ต้านแบคทีเรียก่อโรค ซึ่งสามารถนำแหล่งสารออกฤทธิ์ทางธรรมชาตินี้ไปพัฒนาเป็นผลิตภัณฑ์ต้านเชื้อก่อโรคสัตว์น้ำได้

คำสำคัญ: ถั่วขาว, ป่าชายเลน, ฤทธิ์ต้านแบคทีเรีย, การตรวจสารพฤกษเคมี

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ABSTRACT

Bruguiera cylindrica is a mangrove plant in the Rhizophoraceae family which founded in tropical regions. The dried samples of bark, leaf, pod and twig of *B. cylindrica* were collected and were macerated with methanol. Crude extract of each parts was determined the phytochemical screening and antibacterial activity with the aquatic pathogenic bacteria, *Streptococcus agalactiae* SAAQ001, *Aeromonas hydrophila* AHAQ001, *Vibrio harveyi* VHAQ001 and *V. parahemolyticus* by hole-plate diffusion method. Most of the plant extracts showed a wide range of antibacterial activity against gram-positive and gram-negative bacteria. In addition, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were investigated. The results showed that the leaf and twig extracts of *B. cylindrica* had the lowest MIC/MBC to *S. agalactiae*. Phytochemical screening revealed that the *B. cylindrica* extracts contained a rich source of various phytochemical such as anthraquinone, terpenoids, flavonoids, saponins, phenolics and alkaloids. Terpenoid especially founded in leaf and twig supported antibacterial activity. The results presented here suggested that the presence of bioactive substances from *B. cylindrica* extracts was the factors responsible for bioactivity against the chosen bacterial pathogens. Also, *B. cylindrica* was a potential source of biologically active compounds that could be used to develop natural and bioactive pharmaceutical products against bacterial pathogens in aquatic animals.

Key words: *Bruguiera cylindrica*, mangrove, antibacterial activity, phytochemical screening

INTRODUCTION

Several parts of plants, such as bark, leaf, fruit and flower, may contain phytochemical constituents with a variety of pharmacological properties which are potential sources of therapeutic drugs. These are natural bioactive compounds that are formed during a plant's normal metabolic processes and are often referred as secondary metabolites. Commonly these chemicals include flavonoids, alkaloids, terpenoids, tannins, saponins and so on (Santhi and Sengottuve, 2016). Many secondary metabolites from plants, especially mangrove, are being widely used as antioxidants and antibacterial agents (Shelar *et al.*, 2012). It is a common practice to use plants in the form of crude extracts or decoctions to treat infections. Therefore, it is important to determine the phytochemical constituents in order to know the type of biological activity that may be exhibited by the plant (Vasanthi *et al*., 2014).

Bruguiera cylindrica is an evergreen mangrove tree belonging to the family Rhizophoraceae (Figure 1). It is a small to medium tree growing up to 15 meters tall. The bark is smooth and grey or brown with few lenticels and the trunk is buttressed by its roots. The leaves are opposite and elliptical with pointed ends. The flowers are in small bunches in the axils of the leaves (Krishnamoorthy *et al*., 2011). Previous reports indicated the use of *B. cylindrica* leaf, bark, root, stilt root, hypocotyl and flower as candidate insecticidal agents for mosquito control (Ali *et al*., 2012; 2014). Also, the antibacterial activity of this plant has been tested against antibiotic resistant bacteria and eye pathogens. It has also showed promising antibacterial activity against both the bacterial groups (Ravikumar *et al*., 2011). Methanolic leaf extract of *B. cylindrica* was tested for antibacterial and cytotoxic properties against pathogenic strains found in aquaculture and the results showed that it was effective against *Vibrio* pathogens (Shamsuddin *et al*., 2013). Krishnamoorthy *et al.* (2011), Vadlapudi and Naidu (2009) and Agoramoorthy *et al.* (2008) reported that *B. cylindrica* presented a good source of natural antioxidants by scavenging free radicals because of its rich phenolic and flavonoid

concents. Manilal *et al*. (2009) supported the compounds such as diterpenoid and diterpene have been isolated from this plant (Chantrapromma *et al*., 2003; Chantrapromma *et al*., 2007; Salae *et al*., 2007) and may be liable for various potential medicinal properties attributed to it. In addition, mangroves have been found to exercise strong antioxidant, antibacterial and antifungal activities (Shamsuddin *et al*., 2013). Thus, studying antibacterial activity from another parts of this plant is an interesting issue which may be used as cures for animal or human diseases. Bacteria that cause disease in aquatic animals are another problem that directly affects consumers. Therefore, finding bioactive substance from plants to prevent pathogenic bacteria will be useful information to replace chemicals and antibiotics as well as to also avoid chemical residues in aquatic animals. Thus, the aim of this work is to carry out preliminary phytochemical screening of bark, leaf, pod and twig of *B. cylindrica* and to determine their activity against aquatic pathogenic bacteria. Since there is no previous report on this activity of various part like bark, pod twig and leaf with four aquatic pathogenic bacteria (*S. agalactiae*, *A. hydrophila*, *V. harveyi* and *V. parahemolyticus*), to the best our knowledge, we are attempting the first study of the phytochemical and antibacterial activity of this species.

Figure 1 The various parts of *Bruguiera cylindrica*

MATERIALS AND METHODS

Plant material and extraction procedure

Fresh bark, leaf, pod and twig of *B. cylindrica* were collected from Rajamangala mangrove forest, Rajamangala University of Technology Srivijaya, Trang province (December 2015) and identified by Assistant Professor Sittichoke Junyong (The Forest Herbarium, Bangkok, voucher specimen number BKF.194807). The dried powder (1,000 g) of each parts (bark, leaf, pod, twig) was extracted with methanol in the ratio of 1:5 (w/v) for a week. The resulting extracts were evaporated to dry residue using a rotary evaporator with low temperature at 45 ^{o}C and refrigerated until used. The yield of bark, leaf, pod and twig extracts was 21.54 %, 53.76 %, 29.22 % and 15.00 %, respectively. All extracts were then determined for both phytochemical composition and antibacterial activity.

Preliminary screening of phytochemical test

The different qualitative chemical tests were carried out using standard procedures to identify the constituents of various extracts of *B. cylindrica* which were described by (Tukiran, 2013; Bhatt and Dhyani, 2012; Mouafi *et al*., 2014 and Vittaya and Chalad, 2016) as follows:

Anthraquinone test

Two hundred and fifty of each extract was boiled with10% sulfuric acid for a 2-3 minutes in a water bath, filtered, and allowed to cool. The filtrate was then extracted with chloroform and 3% ammonia solution was added. The formation of a rose pink color in the ammonia layer indicated the presence of anthraquinone (Tukiran, 2013)

Terpenoid test

Two hundred and fifty of each extract of bark, leaf, pod and twig were extracted with petroleum ether. To the filtrate, chloroform was added and then concentrated sulfuric acid. A reddish brown ring at the junction of the two layers indicated the presence of terpenoid (Tukiran, 2013).

Flavonoid test

Three mL of 95% ethanol was added to small amounts of each extract (0.250 g.), which were later treated with a few fragments of metallic magnesium. After filtration, concentrated hydrochloric acid was added. The formation of a cherry color indicated the presence of flavonoids (Bhatt and Dhyani, 2012).

Saponin test

About 0.250 g of each extract was mixed with water and heated in a water bath. After filtration, 5 mL of distilled water was added and shaken well. The formation of froth showed the presence of saponin (Vittaya and Chalad, 2016).

Phenolic test

Two hundred and fifty of each extract of bark, leaf, pod and twig were shaken in water separately and warmed. The mixture was filtered and ferric chloride was added to the filtrate. A dark green, dark blue or black color indicated the presence of phenolic compounds (Mouafi *et al*., 2014).

Alkaloid test

Two hundred and fifty of each extract was dissolved in dilute sulfuric acid and warmed before being filtered. A few drops of Dragendroff's reagent were added to the filtrate. The formation of an orange-yellow precipitate indicated the presence of alkaloids (Mouafi *et al*., 2014).

Antibacterial properties investigation

Inoculums preparation

Four bacterial strains causing severe disease in aquatic animals including *Streptococcus agalactiae* SAAQ001, *Aeromonas hydrophila* AHAQ001, *Vibrio harveyi* VHAQ001 (derived from Kasetsart University) and *Vibrio parahemolyticus* (derived from Songkhla Aquatic Animal Health Center, Thailand) were used for producing the inoculums tested with *B. cylindrica* extracts. All tested inoculums were produced through the modified method of Sritunyalucksana *et al*. (2005). The concentrations were adjusted at 1×10^8 CFU ml^{-1} in NaCl solution and 1×10^6 CFU ml^{-1} in Mueller Hinton broth (MHB), used for testing antibacterial activity and MIC/MBC determination. In addition, the 1.5% NaCl was supplemented in the cultured media for *Vibrio* spp. subculture.

Screening of antibacterial activity

The preliminary screening of antibacterial properties existing in the extract samples, derived from bark, leaf, pod and twig of *B. cylindrica*, was done in quadruplicate with hole-plate diffusion method (Brantner *et al*., 1994 Modified method). Firstly, Petri disc containing Muller Hinton agar (MHA) was evenly swabbed with cotton swab soaked with 1×10^8 CFU ml^{-1} of each tested inoculum, dried for 5 mins, and then made the hole, having the standard size as 6 mm in diameter. Secondly, 40 µl of each extract sample dissolved in DMSO with the concentration level of 100 $mg.ml^{-1}$. The mixture was filled into the created hole with aseptic technique. Also, Oxytetracycline

and DMSO were introduced into the other holes as positive and negative controls, respectively. Finally, the agar plates were incubated at 35 ^{o}C for 18-24 hrs, then the antibacterial activity of each extract sample was evaluated by measuring zone of inhibition (clear zone, diameter in mm.) surrounding the hole.

Determination of MIC and MBC

Stock solutions of four pathogenic bacteria, *S. agalactiae* SAAQ001, *A. hydrophila* AHAQ001, *V. harveyi* VHAQ001 and *V. parahemolyticus* were used and all tested inoculums had been produced as1×10^{6} CFU ml^{-1} through the modified method of Sritunyalucksana *et al*. (2005). The stock solutions of each extract and control were prepared with an initial concentration of 100 mg ml^{-1}, and serially diluted two-fold with Mueller Hinton broth (MHB) to obtain a concentration range from 0.01-50000 $\mu g.ml^{-1}$. Fifty μl of each concentration was added in 96 well plates containing 50 μl of MHB. The inoculums standardized at 1×10^{6} CFU ml^{-1} were filled in each well and gently mixed by multichannel auto-pipette in order to produce the final concentration of 5×10^{5} CFU ml^{-1} in each well. After mixing, the plates were covered with a sterile plate sealer and incubated at 35 ^{o}C for 24 hrs. The turbidity of the solutions was then checked, and *p*-Iodonitrotetrazolium chloride (INT) was added to each well to confirm bacterial growth from discoloration of the mixture. The presence of microorganisms reduced the yellow dye to a pink color. MIC was defined as the lowest concentration of the extract that prevented this change and exhibited complete inhibition of bacterial growth. In addition, a negative growth control of DMSO was included in every test in quadruplicate following the aforementioned method and four wells of inoculums were set up as a negative control (Eloff, 1998 modified method). Minimal bactericidal concentration (MBC) was streak plate technique performed after a broth microdilution test. Briefly, one loopful of the clear solution presenting in the broth microdilution test was streaked onto agar plates, Mueller Hinton agar (MHB). The agar plates were incubated at 35 ^{o}C for 24 hrs. The MBC value was estimated from the appearance of bacterial colonies on the agar plates, on which antibacterial agent concentrations were specified. The lowest concentration presenting no bacterial colony was the MBC value (Eloff, 1998; National Committee for Clinical Laboratory Standards (NCCLS), 2000).

Statistical analysis

Data obtained were expressed as four times the mean ± standard deviation. The data were subjected to ANOVA test to measure whether there was significant difference in zones of inhibition between extract and antibiotic used, and finally, the Duncan's Multiple Range Test (DMRT) with $p < 0.05$ as a limit of significant.

RESULTS AND DISCUSSIONS

Phytochemical screening

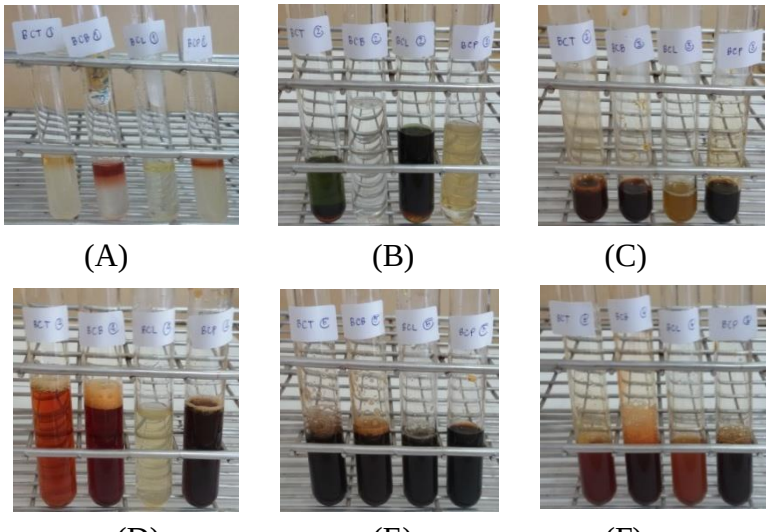
The phytochemical screenings of crude methanolic extracts of bark, leaf, pod and twig samples of *B. cylindrica* revealed the presence of some secondary metabolites or bioactive compounds such as anthraquinone, terpenoid, flavonoid, tannin, saponin and alkaloid as shown in Table 1 and Figure 2. These phytochemical compounds are known to have medicinal and pharmacological importance (Adebayo and Ishola, 2009), giving reason for their study as potential pharmaceuticals. In this work, all parts contained flavonoids and phenolic compounds, which are one of the most unique groups of plant metabolites (Singh *et al*., 2007). These compounds have biological properties, such as anticandidal activity (Hong *et al*., 2011) and antioxidant activity (Vijayavel *et al*., 2006; Loo *et al*., 2007; Loo *et al*., 2008; Rahim *et al*., 2008; Gao and Xiao, 2012). Terpenoid was observed only in leaf and twig. Its therapeutic properties have been generally reported of use against bacteria, fungi and cancers (Premanathan *et al*., 1999; Hong *et al*., 2011; Sulaiman *et al*.,

2011). Anthraquinone and saponin were detected in the bark, pod and twig extract but not in the leaf extract. Saponin has been found to exercise antimicrobial activity against a wide range of microorganisms in vitro (Saad *et al*., 2011). This activity is probably due to the polarity of these compounds across the microorganisms' cell membranes. However, alkaloid was found in all extracts except pod extract and several works have reported its biological activity as anti-inflammatory (Augusto *et al*., 2011), antimicrobial (Benbott *et al*., 2012) and antimalarial (Dua *et al*., 2013).

Table 1 Qualitative phytochemical analysis in the extract of *B. cylindrical*

Plant constituents	**Verification method**	**observations**	***B. cylindrica* extracts**			
			Bark	**Leaf**	**Pod**	**Twig**
Anthraquinone	Borntrager's test	Formation of a rose pink color	+	-	+	+
Terpenoid	Salkowski's test	Reddish brown ring	-	+	-	+
Flavonoid	Reduction of metal	Formation of a cherry color	+	+	+	+
Saponin	Forth test	Formation of a stable form	+	-	+	+
Phenolic	Ferric chloride test	Green-bluish, dark green	+	+	+	+
Alkaloid	Dragendroff's test	Formation of an orange-yellow precipitate	+	+	-	+

Note: "+" means present and "-" means absent



(A) (B) (C)

(D) (E) (F)

Figure 2 Qualitative phytochemical analysis in extracts of *B. cylindrica* bark (BCB), leaf (BCL), twig (BCT) and pod (BCP): (A) Anthraquinone; (B) Terpenoid; (C) Flavonoid; (D) Saponin; (E) phenolic; (F) Alkaloid

Antibacterial screening

The antibacterial activity of phytochemical compounds like flavonoids and phenolic compounds has been variously reported (Ravikumar *et al*., 2011) and in this work, we report the antibacterial screening of bark, leaf, pod and twig extracts of *B. cylindrica*

compared with oxotetracycline as antibiotic agent. The results were listed in Table 2 and presented in Figure 3. The antibacterial activity of all the extracts was slightly different in the inhibition zone producing a range from 7.44-12.38 mm against the four pathogenic bacterial strains (*S. agalactiae*: gram positive and *V. harveyi*, *A. hydrophila* and *V. parahaemolyticus*: gram negative). The effective growth inhibition of the bioactive component present in these extracts showed as clear areas surrounding the hole. This antibacterial activity may be due to active compounds like anthroquinone, terpenoids, flavonoids, saponin, phenolics and alkaloids which are present in this plant (Table 1). Some of these phytochemical compounds have already been reported to have antibacterial properties (Santhi and Sengottuve, 2016). In addition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined and the results presented in Table 3 and showed in Figure 4 and Figure 5, respectively. The results of antibacterial activity assay showed that all extracts of *B. cylindrica* were not different in MIC (6250-25000 $ug.ml^{-1}$) and MBC (12500-25000 $ug.ml^{-1}$) for *V. harveyi* and *V. parahaemolyticus*, respectively. However, it showed slightly different in *A. hydrophila* with MIC (3125-12500 $ug.ml^{-1}$) and MBC (3125-12500 $ug.ml^{-1}$). For *S. agalactiae*, the result showed remarkable different MIC (1562.5-3125 $ug.ml^{-1}$) and MBC (1562.5-12500 $ug.ml^{-1}$). From above data, it was founded that the extracts of *B. cylindrica* were more against the gram positive bacterial strain of *S. agalactiae* than the gram negative strains of *A. hydrophila*, *V. harveyi* and *V. parahaemolyticus* with lower MIC/MBC values, especially leaf and twig extracts. From above phytochemical screening (Table 1), terpenoid substance was only founded in leaf and twig. This may be play an important role to antibacterial activity. This data was supported by the discovery of terpenoid from *B. cylindrica* by Chantrapromma *et al*. (2003). In addition, the phenolic and flavonoid compounds presented by the phytochemical analysis of the extracts could also be inhibitory to the cell protein synthesis of the bacteria (Ravikumar and Kathiresan, 1993; Scalbert, 1991). The phenolic structure of flavonoids contains one carbonyl group in its molecule which permeates cell walls and soluble proteins (Ravikumar *et al*., 2011), thus exhibiting its antibacterial activity. Generally, gram positive bacteria are believed to be weaker having only an outer peptidogly can layer which is not an effective impermeable barrier, whereas gram negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide compound. The presence of a peptidoglycan and phospholipidic bilayer makes the cell membranes of these bacteria impermeable to pharmaceutical constituents. In the face of this barrier the phytochemical constituents were effective in inhibiting the growth of these pathogenic strains (Ravikumar *et al*., 2011). Based on the results, it is possible to conclude that methanolic extracts of various parts of *B. cylindrica* have potential as a source of antibacterial agents against pathogenic aquatic bacteria. Furthermore, the quantitative determination of its phytochemical constituents is of importance for further study.

Table 2 Zone of inhibition from methanolic extracts of *B. cylindrica* against four pathogenic bacteria causing infectious disease in aquaculture

Part of plant	Zone of inhibition diameter (mm)			
	S. agalactiae	***A. hydrophila***	***V. harveyi***	***V. parahaemolyticus***
Bark	10.50 ± 1.70^{b}	9.00 ± 3.16^{bc}	11.00 ± 1.84^{b}	12.38 ± 1.80^{b}
Leaf	10.75 ± 1.74^{b}	11.38 ± 2.66^{b}	6.44 ± 0.55^{d}	10.25 ± 2.40^{b}
Pod	8.31 ± 1.61^{b}	7.50 ± 2.38^{c}	7.44 ± 1.09^{cd}	9.19 ± 0.63^{b}
Twig	9.25 ± 0.61^{b}	7.50 ± 1.91^{c}	8.63 ± 1.79^{c}	11.00 ± 2.04^{b}
Oxytetracycline	37.50 ± 2.27^{a}	34.88 ± 0.14^{a}	40.13 ± 1.16^{a}	33.50 ± 1.02^{a}

Note: Values are zone of inhibition and expressed as mean (mm) ± S.D (n = 4) (Conc. 4 mg/hole); superscripts a - d indicate the difference compared with the others (in the same column by the Duncan's Multiple Range Test: DMRT) is significant at 0.05 level zone of inhibition diameter from higher to lowest values

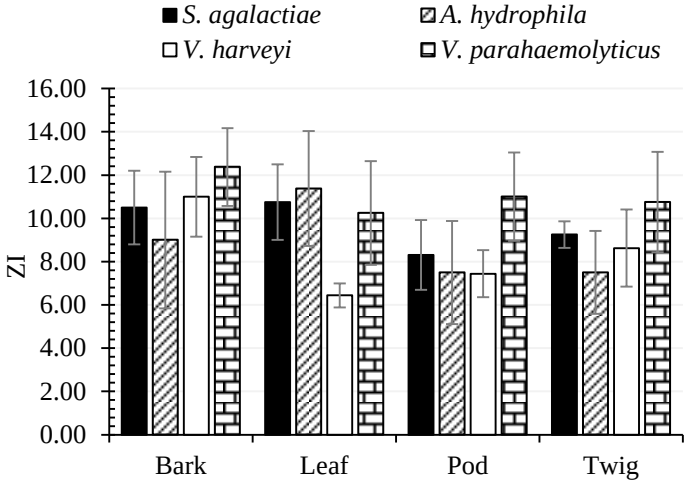


Figure 3 Zone of inhibition (ZI: mm) of *B. cylindrica* bark, leaf, pod and twig with *S. agalactiae*, *A. hydrophilic*, *V. harveyi* and *V. parahaemolyticus*

Table 3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (μg.ml^{-1}) of the extracts from *B. cylindrica* against four pathogenic bacteria causing infectious disease in aquaculture

Part of plant	MIC/MBC (ppm, ugml^{-1})			
	S. agalactiae	***A. hydrophila***	***V. harveyi***	***V. parahaemolyticus***
Bark	3125/12500	6250/6250	6250/12500	6250/25000
Leaf	1562.5/1562.5	3125/3125	12500/12500	12500/25000
Pod	3125/12500	12500/12500	12500/12500	12500/25000
Twig	1562.5/6250	6250/12500	6250/25000	12500/25000
Oxytetracycline	0.05/0.05	0.01/0.19	0.19/0.38	0.01/0.01

Note: Values are Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) and expressed as mean (mm) ± S.D (n = 4)

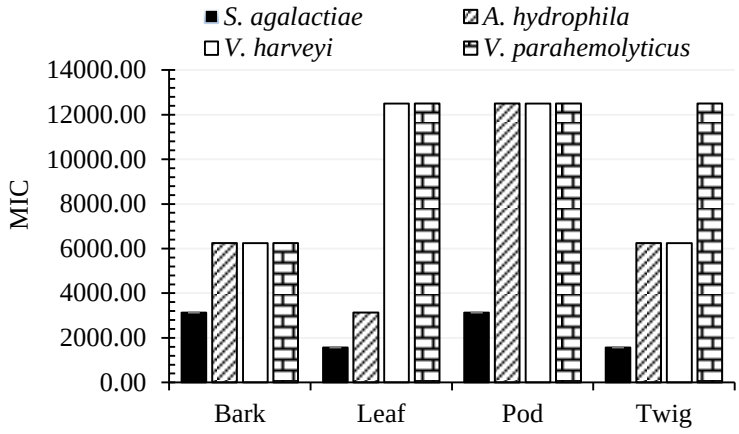


Figure 4 Minimum Inhibitory Concentration (MIC: ugml^{-1}) of *B. cylindrica* bark, leaf, pod and twig with *S. agalactiae, A. hydrophilic*, *V. harveyi* and *V. parahaemolyticus*

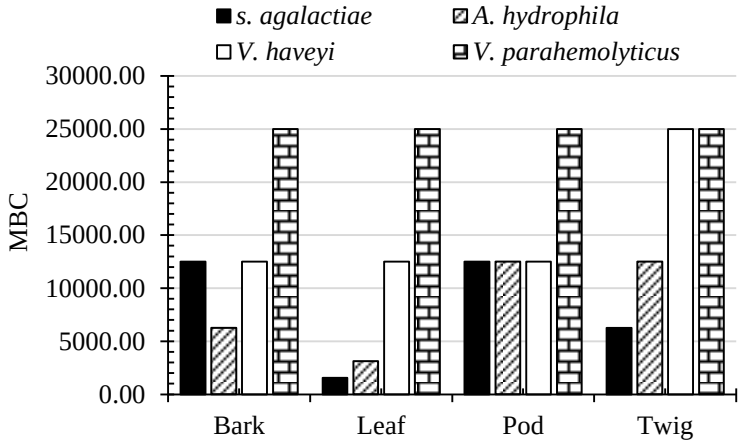


Figure 5 Minimum Bactericidal Concentration (MBC: ugml^{-1}) of *B. cylindrica* bark, leaf, pod and twig with *S. agalactiae, A. hydrophilic*, *V. harveyi* and *V. parahaemolyticus*

CONCLUSION

The results of the present in vitro antibacterial assay and MIC/MBC test revealed that the methanolic extracts of various parts of *B. cylindrica* were effective against pathogenic aquatic bacteria of both gram-negative and gram-positive strains, especially leaf and twig extracts with the lowest MIC/MBC to *S. agalactiae*. This activity was supported by bioactive components such as anthraquinone, flavonoids, saponins, phenolics, alkaloids, especially terpenoids which only founded in leaf and twig. These are well known to possess antibacterial and other therapeutic properties. The present study provides evidence of antimicrobial properties that correspond to the phytochemical study which showed the active ingredients in *B. cylindrica*. This species could probably provide alternative bioactive agents to mitigate the problems of diseases currently proliferating in aquaculture.

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