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Bactericidal Efficacy of Selected Medicinal Plant Crude Extracts and their Fractions against Common Fish Pathogens

(Keberkesanan Bakterisid ialah Ekstrak Mentah Tumbuhan Ubatan Terpilih dan Fraksinya terhadap Patogen Biasa Ikan)

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ABSTRACT

The emergence of new diseases and the increased use of antibiotics have led to the development of resistant bacterial strains. Thus, there is greater attention to seek new antibacterial agents from the natural sources for combating fish diseases in the aquaculture industry. The present study evaluated the bactericidal efficacy of crude methanolic and aqueous extracts from Polygonum chinense, Syzygium polyanthum, Premna foetida, Pimenta dioica, Brucea javanica, Vitex negundo, Alpinia conchigera *and* Clinacanthus nutans *against* Vibrio harveyi, Vibrio alginolyticus, Vibrio parahaemolyticus *and* Aeromonas hydrophila *using disc diffusion method. The results showed that methanolic extracts of* P. dioica, P. foetida *and* P. chinense, *and aqueous extracts of* P. dioica *and* S. polyanthum *showed moderate to strong activity (10.8 to 17.2 mm) against all the tested bacteria. These five potential crude extracts were fractionated using liquid-liquid extraction method to obtain the methanol, dichloromethane and ethyl acetate fractions. Among the fractions, ethyl acetate fraction showed the highest activity against all tested bacteria, with minimum inhibition concentration (MIC) values between 0.625 and 10.000 mg/mL. In addition, the five potential crude extracts had low to moderate toxicity with* LC_{50} *>100 µg/mL using brine shrimp cytotoxicity assays. The results of this study indicated that methanolic extracts of* P. chinense *and* P. foetida *that showed high bactericidal activity and low toxicity could be good potentials for use in fish culture.*

Keywords: Bactericidal activity; brine shrimp toxicity; disc diffusion; medicinal plants; minimum inhibition concentration

ABSTRAK

Kemunculan penyakit baharu dan peningkatan penggunaan antibiotik telah membawa kepada penghasilan strain bakteria yang rintang. Oleh itu, terdapat banyak perhatian untuk mencari agen antibakteria baru daripada sumber semula jadi bagi memerangi penyakit ikan dalam industri akuakultur. Kajian ini telah menguji keberkesanan bakterisid bagi ekstrak mentah metanolik dan akueus daripada Polygonum chinense, Syzygium polyanthum, Premna foetida, Pimenta dioica, Brucea javanica, Vitex negundo, Alphinia conchigera *dan* Clinacanthus nutans *terhadap* Vibrio harveyi, Vibrio alginolyticus, Vibrio parahaemolyticus *dan* Aeromonas hydrophila *menggunakan kaedah penyebaran cakera. Hasil kajian telah menunjukkan ekstrak metanolik* P. dioica, P. foetida *dan* P. chinense, *dan ekstrak akueus* P. dioica *serta* S. polyanthum *mempunyai aktiviti antimikrob sederhana hingga kuat (10.8 hingga 17.2 mm) terhadap semua bakteria yang diuji. Kesemua lima ekstrak mentah yang berpotensi ini seterusnya difraksikan menggunakan kaedah pengekstrakan cecair-cecair untuk mendapatkan fraksi metanol, diklorometana dan etil asetat. Antara fraksi ini, fraksi etil asetat menunjukkan aktiviti tertinggi terhadap semua bakteria yang diuji dengan nilai kepekatan perencatan minimum (MIC) antara 0.625 hingga 10.000 mg/mL. Di samping itu, lima ekstrak mentah yang berpotensi ini mempunyai ketoksikan yang rendah hingga sederhana dengan* LC_{50}*> 100 µg/mL menggunakan pengasaian kesitotoksikan udang brin. Hasil kajian ini menunjukkan bahawa ekstrak metanolik* P. chinense *dan* P. foetida *yang mempunyai aktiviti bakterisid tinggi dan ketoksikan rendah mempunyai potensi yang baik untuk digunakan dalam kultur ikan.*

Kata kunci: Aktiviti bakterisid; kepekatan perencatan minimum; kesitotoksikan udang brin; penyebaran cakera; tumbuhan ubatan

INTRODUCTION

Aquaculture is currently the fastest growing food producing sector in the world. The total aquaculture production has almost doubled from 61 million metric tonnes (mt) in 2006 to over 110 million mt in 2016 (FAO 2017). Most productions were from Asian countries where China played a major role in the aquaculture growth as it contributed almost 60% of the global production (FAO 2017). The intense growth of aquaculture industry provides half of all the fish for human consumption thus increasing the world per capita fish supply up to 20 kg in 2014 (SOFIA 2016).

Although the aquaculture production is growing rapidly, the intensification of the culture systems has led to many issues and challenges. Infectious diseases are among the major concern in aquaculture industry as it has caused high fish mortality and severe economic losses

(Lafferty et al. 2015). To mitigate the losses due to disease outbreaks, farmers save their crops by indiscriminate use of antibiotics, disinfectants, hormones and other chemicals in fish feed and culture water (Rico et al. 2012). Regardless of some positive effects of these chemicals on fish, they have attracted controversy because of their potential side and residual effects to the fish and consumers (Rico & Van den Brink 2014). The prolonged use of antibiotics/chemicals has led to the development of resistant strains of bacteria (Watts et al. 2017). In the efforts to overcome this problem, medicinal plants have attracted the attention of scientists and researchers as alternatives in preventing disease occurrence in aquaculture.

Medicinal plants have been globally known and used for thousands of years as traditional medicine (Bulfon et al. 2015). Consequently, medicinal plants have been tested in fish and shellfish, and successfully being proven as growth promoter, immunostimulant, agent for antibacterial, antiviral, antifungal and anti-parasitic activities and appetite stimulators (Syahidah et al. 2015). These biological activities were contributed by the secondary metabolites, either in pure or mixture compounds, derived from medicinal plants (Radulović et al. 2013) and can be potential source for new drugs in order to control diseases (Savoia 2012).

The present study evaluated the *in vitro* bactericidal efficacy of methanolic and aqueous extracts of leaves of *Polygonum chinense*, *Syzygium polyanthum*, *Premna foetida*, *Pimenta dioica*, *Brucea javanica*, *Vitex negundo*, *Alphinia conchigera* and *Clinacanthus nutans*, and their selected potential fractions against common fish bacterial pathogens. The potential crude extracts from plants were further subjected to toxicity test in brine shrimp to determine the safety levels of the extracts.

Materials and Methods

Plant Collection

The leaves of eight selected medicinal plants as shown in Table 1 were collected from University Agriculture Park, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (2°59'20.3"N 101°42'29.6"E). Plant identification was done by University Agriculture Park. The plant material was cleaned using tap water three times and twice more with sterile distilled water. The samples were dried under shades for a few days and turned over every day to avoid rotting and to ensure complete drying. Then, the samples were coarsely powdered using electric household blender. The samples were kept at 4°C until further use (Ngugi et al. 2015).

Extraction of Plant Materials

Crude extract The plant extracts were exhaustively extracted using maceration technique (Jovanović et al. 2017) with two solvents, 80% methanol (Merck, Darmstadt, Germany) and sterile double distilled water (ddH_2O). The samples were homogenised with the respective solvent at a ratio of 1:10 (w/v) for 48 h at room temperature with regular shaking (150 rpm) (Eloff 1998). The mixture was centrifuged at 2000 × g for 20 min and filtered through a Whatman No. 1 filter paper (Whatman, Maidstone, England). The filtrate was kept at 4°C and the marc was macerated again in the respective solvent two times in order to obtain the maximum extracts (Eloff 1998). The filtrates were combined and solvents were evaporated using a rotary vacuum evaporator (Rotavapor R-215, Buchi, Switzerland) at 30 to 35°C and the residues obtained were freeze-dried (FreeZone Freeze Dryer, Labconco, USA) to powder form. The extracts were stored at -20°C until further use. These procedure was carried out similarly for the eight plant materials.

Fractionation

Fractionation was carried out using the liquid-liquid extraction method (Blum et al. 2019) with some modifications on the solvents used. The potential dried crude extracts were fractionated using three solvents which were methanol (MeOH), dichloromethane (DCM) and ethyl acetate (EtOAc). All the pooled MeOH, DCM and EtOAc fractions of each plant extract were evaporated using a rotary vacuum evaporator (Rotavapor R-215, Buchi, Switzerland) and freeze-dried. All extracts were stored at -20°C until further analysis.

Test Microorganisms

The four fish bacteria pathogens used in the study were obtained from the bacterial collection of the Aquatic Animal Health Unit, Universiti Putra Malaysia, Selangor, Malaysia. These strains were isolated from diseased grouper (*Epinephelus* sp.) and identified using 16S rRNA sequencing which yielded fragments showing high similarities of >90% using nBLAST analysis. The bacteria were *Vibrio alginolyticus* (JN188406.1), *V. harveyi* (AB793708.1), *Aeromonas hydrophila* (EU696781.1) and *V. parahaemolyticus* (JN188418.1). Stock cultures of the bacterial isolates were stored in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) containing sterile 20% glycerol at -80°C.

Antimicrobial Assay

The effects of the various crude extracts and fractions on the four selected bacterial strains were assayed using disc diffusion method (CLSI 2013; Su et al. 2015). The extracts and fractions were diluted in 2% dimethyl sulfoxide (DMSO) (Nacalal Tesque, Japan) at a final concentration of 300 mg/mL. The bacterial cultures were adjusted to 10^8 CFU/mL (equivalent to a MacFarland No. 0.5 standard solution) and 100 µL of bacterial suspension were spread with a sterile swab on the Muller-Hinton agar (MHA) plates. Sterile blank susceptibility discs (Thermo Scientific, United Kingdom), 6 mm in diameter, were soaked in 15 µL of the plant extracts (4.5 mg/disc) and placed on the

agar plates. Reference antibiotics namely oxytetracycline, deoxytetracyline and chloramphenicol discs (Thermo Scientific, United Kingdom) containing 30 μg of drug per disc were used as positive controls. All the plates were then incubated at 30°C for 24 h. After 24 h incubation, each plate was examined and the diameter of the zone of inhibition was measured (Gupta et al. 2016). The assay was performed three times.

MINIMUM INHIBITION CONCENTRATION

Minimum inhibition concentrations (MIC) of all extracts were determined using broth microdilution method (Tittikpina et al. 2018). The four bacterial inocula (*V. harveyi, V. alginolyticus, V. parahaemolyticus* and *A. hydrophila*) were prepared from 24 h broth cultures and the bacterial suspensions were adjusted to 0.5 MacFarland standard turbidity (10^8 CFU/mL). All extracts were dissolved in 2% DMSO at the highest concentration (40 mg/mL) according to Cacciatore et al. (2015) and Mudzengi et al. (2017), and then were serially diluted two-fold into concentrations ranging from 0.3125 to 40 mg/mL in 5 mL sterile glass vials containing nutrient broth (NB) (Becton, Dickinson and Company, France).

Ninety-five millilitres of NB and 5 µL of bacterial inoculum were dispensed into each of the 96-well plates. From the stock solutions of each extract (40 mg/mL), 100 µL of aliquot was added into the first well of the respective plate. Then, 100 µL of the serial dilution solutions were added into the 10 consecutive wells. The last well represented as negative control which contained 195 µL of NB and 5 µL of bacterial inoculum, without extract. All the wells had 200 µL of final volume and all assays were performed in triplicate. Finally, the plates were covered with sterile plate sealer and incubated at 30°C for 24 h. The bacterial growths were determined by observing the absorbance reading at 600 nm using universal microplate reader (Tecan, Austria) and confirmed by plating 10 µL of samples from clear wells on nutrient agar (Becton, Dickinson and Company, France) plates to observe any visible bacterial growth. The MIC was defined as the lowest concentration of the extract which inhibited the bacterial growth.

BRINE SHRIMP CYTOTOXICITY TEST

Cytotoxicity of selected potential crude plant extracts were evaluated using brine shrimp, *Artemia salina* (O.S.I., Pro 80™, Ocean Star International, USA) (Meyer et al. 1982). The brine shrimp eggs (1 g/L) were hatched in seawater provided with continuous aeration and light for 48 h. The hatched nauplii were harvested and transferred into new chamber. Ten healthy nauplii were introduced into vials containing 5 mL of 10, 100, 500 and 1000 µg/mL of each crude extracts. The vials were incubated under light for 24 h. After 24 h, the dead nauplii were counted under light microscope and recorded. The lethal concentration (LC_{50}) of each extract were determined using Probit analysis (Finney 1971).

STATISTICAL ANALYSIS

The collected data were analysed using one-way and two-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using IBM SPSS Statistics 21.0 (IBM Corporation 2012) computer software. The results were expressed as the mean value (n=3) ± standard error.

Results and Discussion

BACTERICIDAL ACTIVITY OF METHANOLIC AND AQUEOUS EXTRACTS

The bactericidal activities of all the tested plant extracts which were assessed against four Gram-negative bacteria (*V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila*) presented in Table 1 shows that methanolic extracts of *P. dioica* and *P. chinense* significantly (p<0.05) inhibited the growth of *V. harveyi* with inhibition zones of 17.2 ± 0.1 and 16.9 ± 0.2 mm, respectively, compared to the aqueous extracts. These two methanolic extracts also exhibited moderate to strong bactericidal activities against the three other tested bacteria. The result of the present study was in accordance with the findings of Maharajan et al. (2012) whereby ethanolic extract of *P. chinense* had strong antibacterial and antifungal activities against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Aspergilus niger*. Likewise, Asha et al. (2013) and Manasa et al. (2013) discovered that the bark and leaf extracts of *P. dioica* showed high inhibition against clinical isolates of bacteria with inhibition zone of 1.0 to 2.0 cm. They suggested that the high inhibitory potential of these extracts could be attributed by the presence of diverse secondary compounds in these plants. The genus *Polygonum* and *Premna* have been reported to contain antimicrobial compounds (Compean & Ynalvez 2014) such as flavonoids (De Soysa et al. 2016; Loke et al. 2016), alkaloids (George & Joseph 2013), quinones (Sindhu & Manorama 2014), coumaric acid, quercetin (Shukor et al. 2013), glycosides, tannin (Kalaichelvi & Dhiyva 2016), terpenoids (Loke et al. 2016), phenolic acids (Huang et al. 2017), lignan (Wang et al. 2013) and anthraquinones (Wang et al. 2019). Moreover, Ezhilan and Neelamegam (2012) identified five major compounds in the ethanolic extract of *P. chinense* using GC-MS chromatogram analysis, where four of them (squalene, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, 1,2-benzenedicarboxylic acid, diheptylester and 8-, methyloctahydrocoumarin) had been reported to possess antimicrobial activity.

Meanwhile, only the aqueous extracts of *S. polyanthum* and *P. dioica* showed moderate to strong bactericidal activities against all tested bacteria. *S. polyanthum* showed significantly higher (p<0.05) inhibition zone of 16.0 ± 0.0 mm against *V. harveyi*, and *P. dioica* showed strong bactericidal activity against *V. parahemolyticus* and *A. hydrophila* compared with their methanolic extracts. According to Esimone et al. (2012), aqueous extract showed higher activity as it contained more active compounds than the methanolic extract. Furthermore, the use of plant

TABLE 1. Bactericidal activities of the methanolic and aqueous extracts of selected medicinal plants against four fish pathogenic bacteria

Plant	Solvent	Inhibition zone diameter (mm)			
		Vibrio harveyi	*Vibrio alginolyticus*	*Vibrio parahaemolyticus*	*Aeromonas hydrophila*
Clinacanthus nutans	Methanol	6.0 ± 0.0a	6.0 ± 0.0a	6.8 ± 0.1b	6.0 ± 0.0a
	Water	6.0 ± 0.0a	6.0 ± 0.0a	6.0 ± 0.0a	6.0 ± 0.0a
Vitex negundo	Methanol	11.5 ± 0.3	7.7 ± 0.1c	10.8 ± 0.2e	8.3 ± 0.2b
	Water	8.8 ± 0.1c	6.0 ± 0.0a	8.9 ± 0.2d	8.9 ± 0.1
Alphinia conchigera	Methanol	9.6 ± 0.1	8.2 ± 0.2	9.0 ± 0.1d	7.7 ± 0.1
	Water	6.0 ± 0.0a	6.0 ± 0.0a	6.0 ± 0.0a	6.0 ± 0.0a
Brucea javanica	Methanol	8.6 ± 0.1bc	6.0 ± 0.0a	10.3 ± 0.1	6.0 ± 0.0a
	Water	6.3 ± 0.1a	7.0 ± 0.1b	6.3 ± 0.1a	6.0 ± 0.0a
Polygonum chinense	Methanol	16.9 ± 0.2d	11.8 ± 0.0f	16.5 ± 0.2f	15.5 ± 0.2c
	Water	6.0 ± 0.0a	9.7 ± 0.1d	6.0 ± 0.0a	6.0 ± 0.0a
Syzygium polyanthum	Methanol	8.3 ± 0.0b	7.1 ± 0.1b	7.3 ± 0.1c	7.0 ± 0.0
	Water	13.5 ± 0.1	14.0 ± 0.0	11.5 ± 0.1	16.0 ± 0.0
Pimenta dioica	Methanol	17.2 ± 0.1d	11.5 ± 0.2ef	16.5 ± 0.2f	15.5 ± 0.2c
	Water	15.3 ± 0.1	10.0 ± 0.2d	16.0 ± 0.1	10.0 ± 0.2
Premna foetida	Methanol	14.8 ± 0.2	11.2 ± 0.1e	10.8 ± 0.1e	14.2 ± 0.2
	Water	7.1 ± 0.1	7.3 ± 0.1bc	7.0 ± 0.1bc	8.5 ± 0.2b
Deoxytetracyline*		23.2 ± 0.2	21.6 ± 0.2	23.9 ± 0.2	25.3 ± 0.1
Oxytetracyline*		18.1 ± 0.1	22.6 ± 0.2	22.6 ± 0.2	22.3 ± 0.1
Chloramphenicol*		37.6 ± 0.2	28.6 ± 0.2	31.9 ± 0.3	31.6 ± 0.1

Similar superscripts within columns indicate no significant differences between means among plant extracts ($p<0.05$). Means that have no superscript in common are significantly different from each other ($p<0.05$). *Positive control (antibiotic)

infusion and decoction methods are commonly practised in the traditional medicine preparation. In the study by Daood (2011), the aqueous extract of *Mentha aquatica* and *Thymus vulgaris* showed significantly higher inhibitory effect against three fish pathogens, *A. hydrophila*, *A. caviae* and *A. sobria* than the methanolic extract. The result of the present study was similar to Turker and Yildirim (2015), where the growth of *V. anguillarum*, a common fish pathogen was also inhibited by the aqueous extracts of *Alchemilla mollis*, *Eryngium campestre* and *Mentha longifolia* but their methanolic extracts did not show any inhibition effect. Moreover, the dietary intake of aqueous extracts of *Ocimum sanctum* (Das et al. 2015), *Psidium guajava* (Gobi et al. 2016) and *Musa acuminata* (Rattanavichai & Cheng 2015) also increased the resistance against bacterial infection in *Labeo rohita*, *Oreochromis mossambicus* and *Macrobrachium rosenbergii*, respectively.

Based on the results of the 16 extracts, five extracts showed moderate to strong bactericidal activity against all the tested bacteria at 300 mg/mL. These five crude extracts were further partitioned with MeOH, DCM and EA to separate the polar and non-polar compounds, and also subjected to bactericidal testing.

BACTERICIDAL ACTIVITY OF FRACTIONS OF POTENTIAL PLANT EXTRACTS

A. hydrophila was the most susceptible to all the tested fractions. Bactericidal activity results showed that all fractions of extracts gave highly significant ($p<0.001$) inhibitory effects against all the tested bacteria, and there was significant correlation ($p<0.001$) between them. From the three fractions of all extracts, EtOAc fractions showed moderate, strong to very strong bactericidal activity against all the tested bacteria except for *P. foetida* extract against *V. parahaemolyticus*. The strongest bactericidal activity (25.5 ± 0.2 mm) was exhibited by EtOAc fractions of *S. polyanthum*, followed by EtOAc fraction of *P. foetida* against *A. hydrophila* (Table 2). This indicated that the EtOAc with low polarity had higher bactericidal potential than the higher polarity solvents (DCM and MeOH). Su et al. (2015) reported similar results where both ethyl ether and EtOAc extracts with medium polarity showed higher antibacterial activities against six clinical drug resistant and four standard bacterial strains compared with hexane, chloroform (non-polar) and aqueous (polar) extracts of *P. cuspidatum*. Lopes et al. (2015) stated that the polarity of the solvent affect the constituents that will be present in the extract and the interaction between the bacterial cell wall components. The EtOAc fraction usually contained higher phenolic compounds and flavonoids (Assefa et al. 2016; Sundowo et al. 2017) which are known to possess antimicrobial properties (Compean & Ynalvez 2014). Moreover, four fractions obtained from *P. capitatum* also showed antibacterial activity against five standard bacterial strains (Liao et al. 2011) and Zhang et al. (2012) reported that the active fraction of *P. capitatum* comprises of 14 compounds with flavonols and glycosides as its major components which might be responsible for its biological effects.

TABLE 2. Bactericidal activities of the fractions of potential plant extracts against four fish pathogenic bacteria

Plant	Fraction	Inhibition zone diameter (mm)			
		Vibrio harveyi	*Vibrio alginolyticus*	*Vibrio parahaemolyticus*	*Aeromonas hydrophila*
Syzygium polyanthum	MeOH	14.3 ± 0.1	11.5 ± 0.1^{f}	9.8 ± 0.2^{c}	11.5 ± 0.2^{a}
(aqueous)	DCM	9.0 ± 0.1	9.0 ± 0.1^{b}	7.0 ± 0.1^{a}	15.5 ± 0.2^{c}
	EtOAc	18.3 ± 0.2^{e}	16.5 ± 0.2	14.3 ± 0.2	25.5 ± 0.2
Premna foetida	MeOH	8.0 ± 0.2^{a}	7.3 ± 0.1	7.3 ± 0.1^{a}	12.5 ± 0.2
(methanolic)	DCM	12.8 ± 0.2	10.3 ± 0.2^{cd}	10.0 ± 0.2^{c}	14.8 ± 0.2^{b}
	EtOAc	10.3 ± 0.1^{b}	10.3 ± 0.3^{cd}	8.8 ± 0.2^{b}	18.5 ± 0.3
Pimenta dioica	MeOH	13.5 ± 0.3	10.8 ± 0.1^{de}	16.5 ± 0.3^{d}	13.5 ± 0.3
(methanolic)	DCM	10.6 ± 0.2^{b}	11.5 ± 0.2^{f}	11.3 ± 0.2	16.5 ± 0.2^{de}
	EtOAc	16.0 ± 0.2^{c}	11.3 ± 0.2^{ef}	18.0 ± 0.2	16.0 ± 0.3^{cd}
Pimenta dioica	MeOH	15.7 ± 0.2^{c}	10.0 ± 0.2^{c}	13.6 ± 0.2	9.6 ± 0.2
(aqueous)	DCM	10.3 ± 0.2^{b}	8.6 ± 0.2^{ab}	8.5 ± 0.2^{b}	10.6 ± 0.2
	EtOAc	17.8 ± 0.3^{de}	10.8 ± 0.2^{de}	17.0 ± 0.2^{d}	14.3 ± 0.2^{b}
Polygonum chinense	MeOH	8.3 ± 0.1^{a}	8.0 ± 0.2^{a}	9.0 ± 0.2^{b}	11.6 ± 0.2^{a}
(methanolic)	DCM	10.3 ± 0.2^{b}	10.5 ± 0.2^{cd}	10.3 ± 0.2^{c}	14.5 ± 0.2^{b}
	EtOAc	17.5 ± 0.2^{d}	10.5 ± 0.3^{cd}	17.0 ± 0.2^{d}	16.8 ± 0.2^{e}

Similar superscripts within columns indicate no significant differences between means among fractions (p<0.05). Means that have no superscript in common are significantly different from each other (p<0.05).
Note: MeOH – methanol; DCM – dichloromethane; EtOAc – ethyl acetate

MINIMUM INHIBITION CONCENTRATION OF SELECTED CRUDE EXTRACTS AND THEIR FRACTIONS

Minimum inhibition concentration is used to determine the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the bacteria under defined test conditions (Mudzengi et al. 2017). In the present study, the MIC of the extracts and fractions ranged from 0.625 to 20.000 mg/mL (Table 3). The highest MIC value (20 mg/mL) was demonstrated by MeOH fraction of *P. foetida* against *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila*. On the contrary, EtOAc fraction of *S. polyanthum* against *A. hydrophila*, and EtOAc fraction of

TABLE 3. The minimum inhibition concentration (MIC) of the crude extracts and their fractions against four fish pathogenic bacteria

Plant	Crude/ Fraction	MIC (mg/mL)			
		Vibrio harveyi	*Vibrio alginolyticus*	*Vibrio parahaemolyticus*	*Aeromonas hydrophila*
Syzygium polyanthum	Crude	1.250	2.500	5.000	2.500
(aqueous)	MeOH	5.000	5.000	2.500	5.000
	DCM	2.500	5.000	5.000	20.000
	EtOAc	1.250	1.250	2.500	0.625
Premna foetida	Crude	1.250	5.000	2.500	2.500
(methanolic)	MeOH	2.500	20.000	20.000	20.000
	DCM	2.500	5.000	2.500	10.000
	EtOAc	1.250	5.000	5.000	10.000
Pimenta dioica	Crude	1.250	2.500	1.250	1.250
(methanolic)	MeOH	2.500	5.000	2.500	1.250
	DCM	1.250	5.000	5.000	5.000
	EtOAc	0.625	5.000	0.625	0.625
Pimenta dioica	Crude	10.000	10.000	2.500	2.500
(aqueous)	MeOH	10.000	5.000	2.500	2.500
	DCM	5.000	20.000	10.000	20.000
	EtOAc	2.500	5.000	1.250	1.250
Polygonum chinense	Crude	1.250	5.000	1.250	1.250
(methanolic)	MeOH	5.000	5.000	10.000	10.000
	DCM	2.500	5.000	10.000	10.000
	EtOAc	1.250	10.000	1.250	1.250

Note: MeOH – methanol; DCM – dichloromethane; EtOAc – ethyl acetate

TABLE 4. Cytotoxicity of potential plant extracts on brine shrimp, *Artemia salina*

Plant extract	LC_{50} (µg/mL)	95% Confidence intervals
Syzygium polyanthum (aqueous)	267.65 ± 11.46[c]	244.74 – 290.56
Premna foetida (methanolic)	756.41 ± 12.83[a]	730.76 – 782.06
Pimenta dioica (methanolic)	102.80 ± 6.25[d]	90.31 – 115.29
Pimenta dioica (aqueous)	461.52 ± 17.70[b]	426.13 – 496.91
Polygonum chinense (methanolic)	707.09 ± 5.46[a]	696.17 – 718.01

Similar superscripts within columns indicate no significant differences between means among plant extracts ($p<0.05$

P. dioica against *V. harveyi*, *V. parahaemolyticus* and *A. hydrophila* showed the lowest MIC.

BRINE SHRIMP CYTOTOXICITY TEST

Brine shrimp is usually tested as the first line in evaluating the cytotoxicity effect and the presence of the bioactive compounds in the plant extracts (Geethaa et al. 2013; Meyer et al. 1982; Moshi et al. 2010). In the present study, all the crude extracts were virtually non-toxic to the brine shrimps (Table 4). They exhibited low to moderate toxicity since the LC_{50} values obtained were greater than 100 µg/mL. The methanolic extract of *P. dioica* was the most significantly toxic with LC_{50} value of 102.8 µg/mL. Meanwhile, two of the crude extracts with strong bactericidal activities, viz, methanolic extracts of *P. foetida* and *P. chinense*, showed significantly lower toxicity with LC_{50} of >700 µg/mL. However, Das (2015) reported moderate toxicity with LC_{50} of 140 µg/mL when using ethanolic extract of *P. chinense*. The variation of toxicity in the two studies might be attributed by the different solvents used in the extraction (Adeogun et al. 2016). Adeogun et al. (2016) also found that when the ethanol and aqueous extracts of *Thaumatococcus daniellii* were used they were non-toxic but the hexane and acetone extracts were toxic to brine shrimp.

CONCLUSION

The findings of the present study demonstrated the effectiveness of the crude extracts and fractions of *P. dioica*, *P. chinense*, *P. foetida* and *S. polyanthum*, as bactericidal agents. Furthermore, the methanolic crude extracts of *P. chinense* and *P. foetida* showed promising results against fish bacterial pathogens and had the lowest toxicity level when tested against brine shrimp. Thus, the extracts of these plants have potentials and could be further explored for their antibacterial properties. Further pharmacological and chemical analysis studies will be useful to confirm the effectiveness of these extracts and identify the phytochemicals responsible for the biological activity.

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