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Research article

Effect of extraction solvents on phenolic compounds and flavonoids from Pongame oiltree (*Derris indica* [Lamk.] Bennet) aerial parts and their growth inhibition of aquatic pathogenic bacteria

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Abstract

Importance of the work: Various parts of *Derris indica* have been used in the Ayurvedic and Unani systems of medicine with antiinflammatory, antiplasmodial, antinociceptive, antihyperglycaemic, antilipid oxidative, antidiarrheal, antihyperammonemia and antioxidant properties, and for the treatment of liver disorders. However, there are no scientific reports regarding their use against aquatic pathogenic bacteria in the modern literature.

Objectives: To investigate the potential of phenolic compounds and flavonoids from *D. indica* against aquatic pathogenic bacteria.

Materials & Methods: The total phenolic and total flavonoid contents were analyzed of the aerial parts of *D. indica* extracted using hexane, ethyl acetate and methanol according to the polarity of solvents, respectively. The antibacterial activity levels of 15 extracts were evaluated against selected strains of bacteria using the hole-plate diffusion method and determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results: Quantitative analysis showed that the ethyl acetate extract had high levels of phenolic compounds and flavonoids contents (0.428 ± 0.022 mg gallic acid equivalents/g crude extract and 2.251 ± 0.102 mg rutin/g crude extract, respectively). Antibacterial screening showed that the maximum zone of inhibition was with ethyl acetate extract against *Streptococcus agalactiae* (zone diameter = 18.06 mm). The lowest MIC and MBC values were obtained with hexane and ethyl acetate extracts of the flower against *S. agalactiae* and *Aeromonas hydrophila* (MIC and MBC both 0.048 mg/mL) that were significantly lower than those of oxolinic acid (MIC = 0.19 mg/mL and MBC = 3.12 mg/mL). Two-way analysis of variance confirmed the effect of the solvents used for extracting each part of *D. indica* ($p < 0.001$).

Main finding: *D. indica* could be a potential source of natural bioactive compounds to develop pharmaceutical products against bacterial pathogens in aquatic animals.

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Introduction

Aquaculture plays an important role in many people's livelihoods and economic activity and is one of the fastest developing food production industries globally (Kobayashi et al., 2015). The development of aquaculture is hindered by disease outbreaks caused by bacteria and viruses; among the important pathogens, *Streptococcus agalactia* and *Aeromonas hydrophila* are well-known for causing serious outbreaks in many fish species, including Nile tilapia (*Oreochromis niloticus*; Syahidah et al., 2015). *Vibrio harveyi* is another species of bacteria that has damaged Thailand's shrimp industry, especially through luminous disease (Paranjpye et al., 2015). To cope with the problems, antibiotics have been heavily used and this has raised concerns about antibiotic resistance (Cabello, 2006). Therefore, one solution to reduce antibiotic use is their substitution with natural products, such as medicinal plants, herbs and extracts. Attempts have been made to explore the potential use of plants to prevent and suppress pathogens. For example, mangrove leaf extract of the Red mangrove (*Rhizophora macronata*) was used to control *A. hydrophila* (Panjaitan and Suprajitno, 2018). Medicinal mangrove plants, such as the Grey mangrove (*Avicennia marina*), Indian mangrove (*Avicennia officinalis*), Upriver orange mangrove (*Bruguiera sexangula*), Tall-still mangrove (*Rhizophora apiculata*) and White-flowered mangrove (*Lumnitzera racemosa*) were used against antibiotic-resistant *Staphylococcus aureus* and *Proteus* sp. (Abeysinghe, 2010). More evidence was reported on the methanolic extracts of *R. macronata* against *A. hydrophila* and *Escherichia coli* (Mahmiah et al., 2018) and the methanolic extracts of the Black mangrove (*Lumnitzera littorea*) on *S. agalactiae*, *A. hydrophila*, *V. harveyi* and *V. parahemolyticus* (Vittaya et al., 2020). Despite these previous attempts, there is still a need to explore new herbs as alternatives to replace antibiotic uses in aquaculture.

Derris indica [synonyms: *Pongamia pinnata*, *Pongamia glabra*] is a mangrove plant in the Leguminosae-Papilionoideae family that can survive the constantly changing tide and plays an essential role in the habitats of aquatic animals (Koysomboon et al., 2006; Decharchoochart et al., 2014). Extracts from various plant parts could inhibit free radicals and showed antibacterial, antioxidant and anti-inflammatory activities (Kumar et al., 2018; Su et al., 2020). For example, a secondary metabolite in the bark extract of this plant consists of phenolics and flavonoids that act against *Bacillus subtilis*, *E. coli* and *Aspergillus niger* (Niharika et al., 2021), and in leaf extract against *S. aureus* (Panigrahi and Mahapatra., 2016). Phenolic compounds and flavonoids are major antibacterial and antioxidant phytochemical substances (Mahmoudi et al., 2016). These substances could help stimulate immune systems, eliminate free radicals, reduce stress and prevent disease (Syahidah et al., 2015).

Several parts of *D. indica* have been used in folk medicine as antiseptics, liniment for rheumatism and diabetes, skin ailments or even to cure tumors (Chopade et al., 2008). The bark, leaf and seeds of the plant showed antioxidant and antibacterial activities (Sajid et al., 2012). Ethanolic extracts of the flowers and bark were used for their anti-lipid peroxidation and antihyperglycemic effects and to enhance antioxidant activity in alloxan-induced diabetic rats (Punitha and Manoharan, 2006). The extracts exhibited anti-inflammatory activity in a rat model (Badole et al., 2012). In addition, bioactive compounds from the hexane extracts of the fruit showed cytotoxicity against cholangiocarcinoma and HepG2 cell lines (Decharchoochart et al., 2014). The aqueous and organic solvent extracts of the bark, leaves and root contained alkaloids, steroids, triterpenoids, flavonoids, saponins and tannins that exhibited antimicrobial activity, in particular, the organic extracts of the plant showed good pharmacological and antibacterial properties (Khan et al., 2006; Mondal et al., 2019). In addition, isolation and purification of the plant yielded several bioactive compounds, such as flavonoids, flavone and chalcone derivatives, and furanoflavonoid. For example, candidone, a flavone derivative isolated from the hexane extract of *D. indica* fruit, exhibited antitumor activity on the model cell line and could be a treatment for cholangiocarcinoma (Kurasug et al., 2018). Derrivanone and derrischalcone showed potent cytotoxic activity against human hepatoma HepG2 cells (Decharchoochart et al., 2014). Pongamol, Karanjin, pongagalabrone, pinnatin, pongapin, kanjone, isopongachromene and glabrachalcone were also isolated from the seeds of *D. indica* (Chopade et al., 2008). The stem and leaves of the plant yielded several flavones and chalcone derivatives, such as pongone, galbone, pongalabol and pongagallone A and B (Li et al., 2006). In addition, flavonoids were isolated from the root bark (Tanaka et al., 1992). *D. indica* fruit yielded pongamoside A–C, furanoflavonoid glycosides and pongamoside D, a flavonol glycoside (Ahmad et al., 2004).

Although several reports have revealed the medicinal uses of *D. indica* as mentioned above, no detailed work has been presented on the anti-bacterial properties of aquatic pathogens of the leaf, flower, pod, twig, and bark of *D. indica*. Therefore, the main objective of the present study was to screen phenolic

compounds and flavonoids using standard phytochemical methods and to quantitatively analyze the total phenolic and flavonoid contents of the hexane, ethyl acetate and methanol extracts obtained from five different parts of the plant. Antibacterial activity was investigated against four aquatic pathogenic bacteria: *S. agalactiae* SAAQ001, *A. hydrophila* AHAQ001, *V. harveyi* VHAQ001 and *V. parahaemolyticus*. The correlations between the chemical compositions of the plant and its antibacterial property were determined and analyzed. The knowledge gained from this study would benefit the efficient utilization of *D. indica* for aquatic animal disease control.

Materials and Methods

Ethics statements

This study was approved by the Ethics Committee of Rajamangala University of Technology Srivijaya (Approval no. D 02-01-62).

Reagents

Gallic acid and rutin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Folin-Ciocalteu reagent and aluminum chloride, were purchased from Loba Chemie (Mumbai, India). All chemicals used were of analytical grade and were used without further purification.

Plant collection

Plants were collected from Trang province, Thailand at 7°31'32.8"N 99°19'34.2"E and identified by comparing with specimens of plants of the same species. A voucher specimen (BKF. 194786) was deposited at the Forest Herbarium Bangkok, Department of National Parks, Wildlife and Plant Conservation Thailand. During 2017, fresh leaves and flowers were collected in June. Fruit and twigs were collected during July and August. Bark was collected last in September 2017.

Preparation of extracts

All parts were cut into small pieces, air-dried for 1 wk at room temperature (28–30 ℃) and coarsely ground. The dried samples (1,000 g each) of each part were sequentially extracted with hexane, ethyl acetate and methanol using a ratio of 1:5 (plant dry weight-to-solvent volume) for 1 wk. The resulting extracts were evaporated to dryness using a rotary evaporator at 45℃. The concentrated extracts were stored in a refrigerator until analyzed. The yields of leaf, flower, pod, twig and bark extracts were reported.

Determination of phenolic compounds and flavonoids

Qualitative analysis

The leaf, flower, pod, twig and bark extracts of *D. indica* were screened for phenolic compounds and flavonoids using a standard procedure (Galina et al., 2009). Briefly, 0.1 g of plant materials was added to 5 mL of water and heated to 50 ℃ for 5 min. A few drops of 1% $FeCl_3$ solution were added to the filtrate. Brownish-green or blackish-blue precipitates indicated the presence of phenolic compounds. In the case of flavonoids, 50% ethanol was used instead of water. Two small pieces of magnesium were placed in the mixture. Then, the solution was filtered before adding 2–3 drops of concentrated HCl. The presence of flavonoids was confirmed by yellowish-orange precipitates.

Quantitative analysis

Determination of total phenolic content

The total phenolic content (TPC) of each extract was determined spectroscopically using the Folin-Ciocalteu colorimetric method (Vittaya et al., 2019) with gallic acid as a standard. The absorbance of the mixture was recorded at 765 nm using an ultraviolet (UV)-visible spectrophotometer. The TPC of each sample was calculated alongside the standard curve of gallic acid at concentrations in the range 50–250 μg/mL and expressed in terms of gallic acid equivalents (mg GAE/g CE). The data were calculated according to the equation $y = 0.0041x - 0.0011$ (coefficient of determination (R^2) = 0.9992).

Determination of total flavonoid content

The extracts' total flavonoid content (TFC) was determined using the aluminum chloride method (Vittaya et al., 2019) with rutin as a standard. The absorbance of the reaction mixture was measured against a blank sample at 510 nm using UV-visible spectrophotometry. The total flavonoid content of each sample was calibrated alongside the standard curve of rutin at concentrations of 100–500 µg/mL and expressed in terms of rutin equivalents (mg RU/g CE). The data were calculated according to the equation $y = 0.0015x - 0.0075$ (R^2 = 0.9996).

Antibacterial activity

Preliminary screening of antibacterial activity

In vitro antibacterial screening of *D. indica* extracts was carried out using the hole-plate diffusion method (Brantner et al., 1994) against four pathogenic strains: *S. agalactiae* SAAQ001, *A. hydrophila* AHAQ001, *V. harveyi* VHAQ001 (derived from Aquatic Animals Health Management Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University) and *V. parahemolyticus* (derived from Songkhla Aquatic Animal Health Center, Department of Fisheries, Songkhla, Thailand). To obtain the inoculum density for a susceptibility test, the NaCl turbidity standard was adjusted to 1×10^8 colony forming units (CFU)/mL to test the antibacterial activity. In addition, the culture media of *Vibrio* sp. subculture (Sritunyalucksana et al., 2005) were supplemented with 1.5% NaCl. The dried surface of a Mueller Hinton agar plate was inoculated by streaking the swab over the sterile agar surface with 1×10^8 CFU/mL of each inoculum. After 5 min drying time, a 6 mm hole was made in the agar and aseptically filled with 40 μL of the extract dissolved in dimethyl sulfoxide (DMSO; 50 mg/mL) to yield a final concentration of 4 mg/hole. Oxolinic acid (Oxo) and DMSO were used as positive and negative controls, respectively. Inoculated agar plates were incubated at 35℃ for 18–24 hr. At the end of the incubation period, the antibacterial activity of each extract was determined by measuring the diameter of the inhibition zone (in millimeters). Final values were calculated from quadruplicate sets.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

Stock solutions of the four pathogenic bacteria (*S. agalactiae* SAAQ001, *A. hydrophila* AHAQ001, *V. harveyi* VHAQ001 and *V. parahemolyticus*) were used, and all tested inoculums were produced as 1×10^6 CFU/mL for Mueller Hinton broth (MHB) using the modified method of Sritunyalucksana et al. (2005). All extracts and control stock solutions were prepared at an initial 100 mg/mL concentration and serially diluted two-fold with MHB to obtain a concentration in the range 0.01–50,000 μg/mL. In 96-well plates, each concentration of 50 µL was mixed with 50 µL of MHB. Then, the standardized inocula at 1×10^6 CFU/mL were filled in each well and gently mixed using a multichannel auto-pipette to produce a final 5×10^5 CFU/mL concentration. Then, the plates were covered with sterile plate sealers. The turbidity of the solutions was measured after incubation at 35 ℃ for 24 hr. Bacterial growth was confirmed by discoloration of *p*-iodonitrotetrazolium chloride dye from yellow to pink when added to each well. The MIC was defined as the lowest concentration of the extract, inhibiting bacterial growth completely by preventing this color change, including a negative growth control of DMSO in a quadruplicate test (Eloff, 1998). The MBC was determined after a broth microdilution test using the streaking plate technique. Briefly, the presence of one loopful of the clear solution in the broth microdilution test was streaked on MHB plates. After the agar plates were incubated at 35 ℃ for 24 hr, the MBC was estimated from the appearance of bacterial colonies for which antibacterial agent concentrations were specified. The lowest concentration presenting no bacterial colony was considered the MBC (Eloff, 1998).

Statistical analysis

The data were expressed as mean ± SD values of triplicate determination. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Two-way and three-way ANOVA were used to investigate the interaction between plant parts (leaf, flower, pod, twig and bark) with the three organic solvents (hexane, ethyl acetate and methanol) at 24 hr and 48 hr incubation times. Correlations between the values for TPC and TFC in each part of the plant and evaluation of phytochemical composition (TPC and TFC) with four pathogenic bacteria were performed using Pearson's correlation coefficient.

Results and Discussion

Yield of plant extracts

In this work, *D. indica* leaf, flower, pod, twig and bark samples were extracted using hexane, ethyl acetate and methanol. The extraction yields from the different parts of *D. indica* were in the range 0.70–1.45% for hexane extracts, 1.28–5.63% for ethyl acetate extracts and 4.91–27.25% for methanol extracts (Table 1). Notably, the extraction yields from methanol were relatively high compared to the other two solvents. The extraction yields increased with the polarity of the extracting solvent. Nonetheless, the obtained yields included components other than phenolic compounds, such as carbohydrates and proteins, contributing to the higher yield (Sata et al., 2013; Do et al., 2014; Rajasree et al., 2021). The results of this study were in agreement with the reported extraction yields from Wild grape (*Ampelocissus martini*) (Vittaya et al., 2019) and another medicinal plant (Desert cauliflower, *Anabasis aretioïdes*) (Senhaji et al., 2020).

Table 1 *Derris indica* extract yields

Plant part	Solvent	Yield (% weight per weight)
Leaf	Hexane	1.04
	Ethyl acetate	1.28
	Methanol	10.46
Flower	Hexane	1.45
	Ethyl acetate	2.72
	Methanol	27.25
Pod	Hexane	1.38
	Ethyl acetate	3.85
	Methanol	12.10
Twig	Hexane	0.70
	Ethyl acetate	2.32
	Methanol	4.91
Bark	Hexane	1.08
	Ethyl acetate	5.63
	Methanol	10.16

Phenolic and flavonoid compounds of D. indica extracts

The TPC was in the range 0.021–0.428 mg GAE/g CE while the TFC was 0.145–2.251 mg RU/g CE as shown in Table 2. Two-way ANOVA revealed that the plant parts and solvents exerted a significant effect on the TPC ($F = 57.018$, $p < 0.001$ for plant parts; $F = 325.471$, $p < 0.001$ for solvents). Interestingly, interaction between plant parts and solvents also had a significant effect ($F = 34.025$, $p < 0.001$), as shown in Fig.1. Similarly, the plant parts and solvents also showed highly significant effects on the TFC ($F = 63.818$, $p < 0.001$ for plant parts; $F = 339.037$, $p < 0.001$ for solvents) as well as the interaction between plant parts and solvents ($F = 45.194$, $p < 0.001$), as shown in Fig. 2. Among plant parts (averaged across solvents), the TPC was predominantly found in the pod (0.324 ± 0.018 mg GAE/g CE), whereas TFC was highest in the leaf extract (1.396 ± 0.170 mg RU/g CE) as shown in Table 3. When the effects of solvents were considered, TPC was mostly found in both ethyl acetate (0.297 ± 0.023 mg GAE/g CE) and methanol (0.293 ± 0.013 mg GAE/g CE), whereas TFC was highest in both hexane (1.403 ± 0.087 RU/g CE) and ethyl acetate (1.340 ± 0.144 RU/g CE), as shown in Tables 4.

Table 2 Total phenolic and total flavonoid contents of *Derris indica* extracts from different plant parts and solvents and the two-way analysis of variance table showing effects of plant parts (PP), the solvent used (S) and their interaction

Plant part	Solvent	Total phenolic content (mg GAE)/g CE	Total flavonoid content (mg RU/g CE)
Leaf	Hexane	0.051 ± 0.011^{j}	1.894 ± 0.185^{b}
	Ethyl acetate	0.258 ± 0.015^{fg}	1.534 ± 0.016^{c}
	Methanol	0.322 ± 0.032^{cd}	0.759 ± 0.059^{hi}
Flower	Hexane	0.021 ± 0.004^{j}	0.938 ± 0.150^{gh}
	Ethyl acetate	0.428 ± 0.022^{a}	0.693 ± 0.033^{i}
	Methanol	0.307 ± 0.022^{de}	0.429 ± 0.024^{j}
Pod	Hexane	0.263 ± 0.040^{fg}	1.405 ± 0.146^{cd}
	Ethyl acetate	0.364 ± 0.045^{b}	1.056 ± 0.071^{fg}
	Methanol	0.346 ± 0.045^{bc}	0.774 ± 0.013^{hi}
Twig	Hexane	0.124 ± 0.015^{i}	1.303 ± 0.017^{de}
	Ethyl acetate	0.233 ± 0.014^{gh}	1.165 ± 0.232^{ef}
	Methanol	0.280 ± 0.026^{ef}	0.523 ± 0.024^{j}
Bark	Hexane	0.126 ± 0.006^{i}	1.474 ± 0.035^{cd}
	Ethyl acetate	0.203 ± 0.025^{h}	2.251 ± 0.102^{a}
	Methanol	0.213 ± 0.010^{h}	0.145 ± 0.015^{k}

Two-way ANOVA (*p*-values of the factors)

Variable	df	F	*p*	F	*p*
PP	4	57.018	< 0.001	63.818	< 0.001
S	2	325.471	< 0.001	339.037	< 0.001
PPS	8	34.025	< 0.001	45.194	< 0.001
Error	30				

GAE = gallic acid equivalents; CE = crude extract; RU = rutin

Data shown as mean ± SD values from analysis of triplicate analysis.

Mean ± SD values in a column with different lowercase superscripts denote significant ($p < 0.05$) differences.

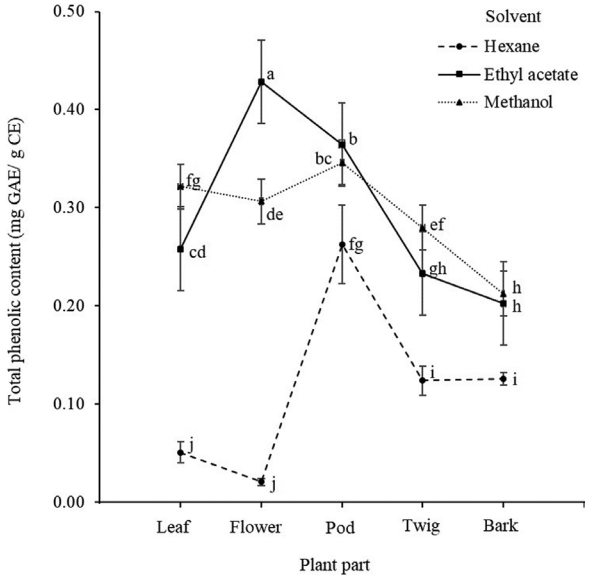

Fig. 1 Interaction effect of plant parts and solvents on total phenolic content (TPC) in *Derris indica* extracts, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

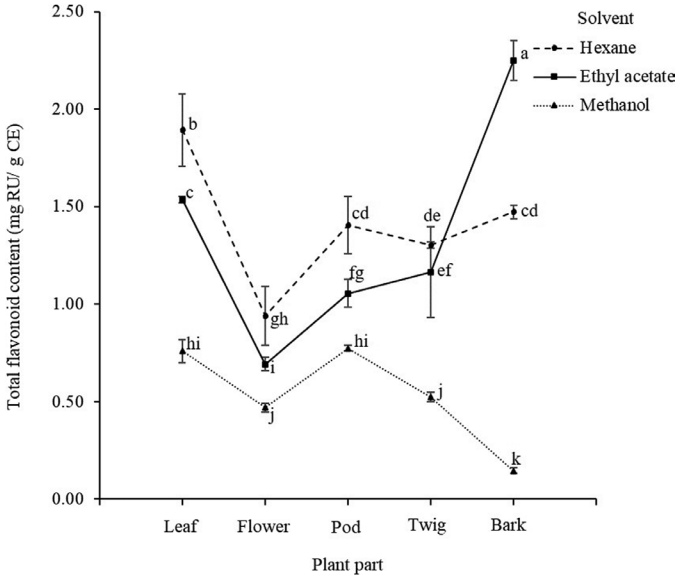

Fig. 2 Interaction effect of plant parts and solvents on the quantity of TFC in *Derris indica* extracts, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

Table 3 Total phenolic and total flavonoid contents in each plant part averaged across solvents

Plant parts	Total phenolic content (mg GAE/g CE)	Total flavonoid content (mg RU/g CE)
Leaf	0.210±0.040^{c}	1.396±0.170^{a}
Flower	0.252±0.060^{b}	0.700±0.072^{d}
Pod	0.324±0.018^{a}	1.078±0.095^{c}
Twig	0.212±0.024^{c}	0.997±0.126^{c}
Bark	0.180±0.014^{d}	1.290±0.308^{b}

GAE = gallic acid equivalents; CE = crude extract; RU = rutin
Data shown as mean ± SD values from analysis of triplicate analysis.
Mean ± SD values in a column with different lowercase superscripts denote significant ($p < 0.05$) differences.

Table 4 Total phenolic and total flavonoid contents obtained from the extraction using different solvents averaged across plant parts

Solvent	Total phenolic content (mg GAE/g CE)	Total flavonoid content (mg RU/g CE)
Hexane	0.117±0.023^{b}	1.403±0.087^{a}
Ethyl acetate	0.297±0.023^{a}	1.340±0.144^{a}
Methanol	0.293±0.013^{a}	0.534±0.062^{b}

GAE = gallic acid equivalents; CE = crude extract; RU = rutin
Data shown as mean ± SD values from analysis of triplicate analysis.
Mean ± SD values in a column with different lowercase superscripts denote significant ($p < 0.05$) differences.

Figs. 1 and 2 show the interactions between plant parts and solvents, indicating that the highest quantities of TPC or TFC in different plant parts were found in different solvents. For example, the highest amount of TPC in the leaf was found in the methanol extract, while the highest amount of TPC in flowers was in ethyl acetate. A weaker degree of interaction was seen for TFC, whereby all but one plant part (except for bark) produced the highest TFC in hexane extract, followed by ethyl acetate and methanol. Ethyl acetate extract of the bark showed the highest TFC, followed by hexane and methanol (Fig. 1).

Discrepancies in the phenolic and flavonoid contents in each part with different solvent extractions have been reported (Abbaszadeh et al., 2013; Wakeel et al., 2019; Elshaafi et al., 2020), due to the solubility principle of chemical substances whereby a solvent is efficient in extracting natural compounds with similar polarity (Do et al., 2014). For example, bark may contain high molecular weight substances, which were less soluble in low polarity solvents, such as hexane, but more soluble in medium to high polarity solvents, such as ethyl acetate and methanol. However, the solubility of phenolic and flavonoid compounds depends not only on the

extracting solvent but also the degree of polymerization and interaction of phenols with other phytochemicals, minerals and vitamins (Naczk and Shahidi, 2004), as well as other primary metabolites, such as the sugar, starch, protein, lipids, vitamins and amino acids in several parts of *D. indica* (Sagwan et al., 2010; Sajid et al., 2012). Furthermore, the variations in the phenolic compounds and flavonoids in extracts from different solvents might be related to environmental factors, such as other natural chemical ingredients of the dissolved plant material, the soil state, maturity at harvest and post-harvest storage conditions (Huang et al., 2005).

Interestingly, TPC and TFC in each plant part showed a negative trend with a correlation coefficient ranging between -0.225 (in bark) and -0.851 (in leaves). However, the Pearson's correlation coefficient (r) was correlated only for the leaf (r = -0.851) and twig (r = -0.761) extracts (Table 5). Negative correlations between the phenolic and flavonoid contents have been reported elsewhere, indicating that there were other secondary metabolites in the extract of each plant part (Senhaji et al., 2020; Boulfia et al., 2021). Thus, various parts of *D. indica* are good sources of bioactive substances that could be used in pharmaceuticals or food products as well as in aquaculture as an alternative to currently used antibiotics.

Table 5 Pearson's correlation coefficient (r) values of total phenolic (TPC) and total flavonoid (TFC) contents in the extracts from each plant parts of *Derris indica*

Assay	TFC (mg GAE/g CE)				
	Leaf	Flower	Pod	Twig	Bark
TPC (mg RU/g CE)	-0.851 ($p = 0.004$)	-0.649 ($p = 0.058$)	-0.608 ($p = 0.083$)	-0.761 ($p = 0.017$)	-0.225 ($p = 0.553$)

GAE = gallic acid equivalents; CE = crude extract; RU = rutin

Antibacterial activity

All the extracts of *D. indica* showed antibacterial activity against the tested four bacteria, with the diameters of the inhibition zones in the ranges 8.56–18.06 mm against *S. agalactiae*, 8.38–12.38 mm against *A. hydrophila*, 10.06–12.94 mm against *V. harveyi* and 6.00–11.68 mm against *V. parahaemolyticus*. Furthermore, the influences from plant parts, extracting solvents and extraction time and their combinations were different in each bacterium, as shown in Table 6. For *S. agalactiae*, plant parts and the interaction between plant parts and solvents were highly significant ($p < 0.001$), as shown in Fig. 3. The best antibacterial activities were found in the following experiments: flower extracted with ethyl acetate, pod extracted with each of the three solvents and twig extracted with hexane or methanol.

For *A. hydrophila*, plant parts, solvent, time and the interaction between plant parts and solvents were highly significant ($p < 0.001$) and showed the highest antibacterial activity for flowers extracted using ethyl acetate, as shown in Fig. 4. In addition, treating this bacterium for 24 hr resulted in a larger inhibition zone than those treated for 48 hr.

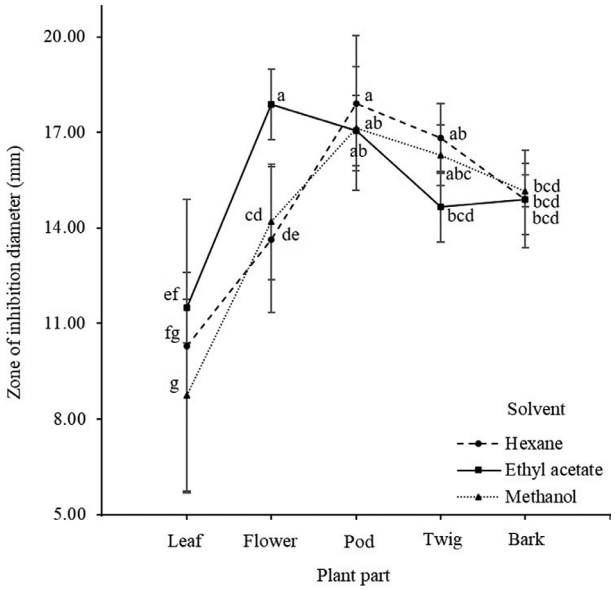

Fig. 3 Interaction of plant parts and solvents on antibacterial activity of *Derris indica* extracts against *Streptococcus agalactiae*, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

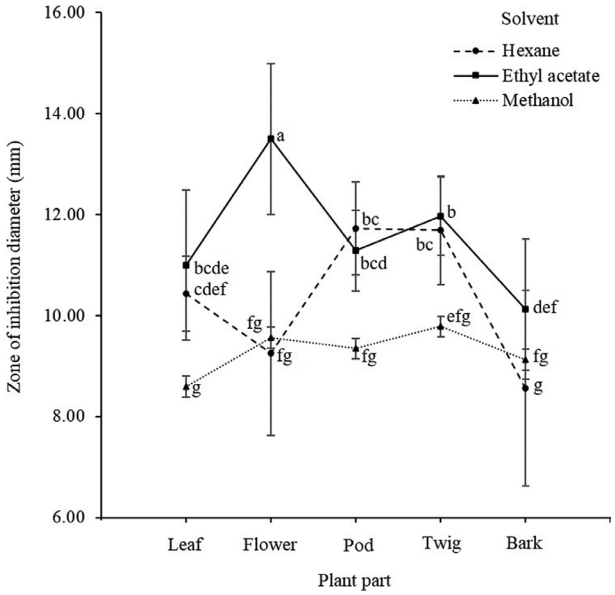

Fig. 4 Interaction of plant parts with solvents on antibacterial activity of *Derris indica* extracts against *Aeromonas hydrophia*, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

Table 6 Zones of inhibition from various extracts of *Derris indica* against four pathogenic bacteria (*Streptococcus agalactiae*, *Aeromonas hydrophila*, *Vibrio harveyi* and *V.parahaemolyticus*) at different times (24 hr and 48 hr) and the three-way analysis of variance table showing the effects of plant parts (PP), the solvent used (S) and time (T) and their interactions

Part used	Solvent	Zone of inhibition diameter (mm)							
		S. agalactiae		*A. hydrophila*		*V. harveyi*		*V. parahaemolyticus*	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Leaf	H	$10.63\pm5.34^{e-f}$	$9.94\pm4.55^{g-h}$	$10.63\pm1.03^{c-e}$	$10.25\pm0.35^{c-e}$	$12.31\pm0.47^{b,c}$	$11.69\pm0.47^{b-d}$	$7.50\pm1.73^{e-g}$	$7.00\pm1.41^{d,e}$
	E	$11.94\pm4.83^{c-f}$	$11.06\pm3.93^{f-h}$	$11.13\pm1.81^{c-e}$	$10.88\pm1.36^{c,d}$	$11.31\pm0.94^{c,d}$	$11.06\pm1.30^{b-d}$	$8.19\pm1.60^{d-f}$	$7.44\pm1.20^{c-e}$
	M	8.94 ± 3.39^{f}	8.56 ± 3.10^{h}	8.75 ± 0.98^{f}	$8.44\pm1.09^{e,f}$	$11.50\pm0.46^{c,d}$	$10.75\pm0.35^{c,d}$	$6.94\pm1.09^{f,g}$	6.00 ± 0.00^{e}
Flower	H	$13.81\pm2.33^{b-e}$	$13.44\pm2.59^{c-g}$	$10.00\pm1.27^{e,f}$	$8.50\pm1.73^{e,f}$	$12.63\pm0.92^{b,c}$	$12.63\pm0.75^{b,c}$	$8.75\pm0.93^{c-e,A}$	$6.25\pm0.50^{e,B}$
	E	18.06 ± 0.66^{a}	17.69 ± 0.24^{a}	13.88 ± 1.45^{b}	13.13 ± 1.65^{b}	15.19 ± 1.74^{a}	15.31 ± 1.70^{a}	12.56 ± 0.66^{a}	11.68 ± 0.38^{a}
	M	$15.44\pm0.55^{a-c,A}$	$12.94\pm1.81^{d-g,B}$	$10.50\pm0.94^{d-f}$	$8.63\pm1.80^{e,f}$	13.38 ± 2.03^{b}	12.94 ± 1.85^{b}	6.25 ± 0.50^{g}	6.00 ± 0.00^{e}
Pod	H	17.59 ± 1.36^{a}	$17.38\pm2.06^{a,b}$	$12.31\pm0.55^{b,c,A}$	$11.13\pm0.85^{c,d,B}$	$11.31\pm1.28^{c,d}$	$11.31\pm1.03^{b-d}$	$8.75\pm1.21^{c-e}$	$8.63\pm1.97^{b-d}$
	E	$16.88\pm1.09^{a,b}$	$17.25\pm1.24^{a,b}$	$11.94\pm0.31^{c,d,A}$	$10.62\pm0.48^{c,d,B}$	$11.19\pm0.90^{c,d}$	$10.75\pm1.06^{c,d}$	$8.88\pm0.14^{c-e}$	$8.19\pm0.85^{b-d}$
	M	$17.06\pm2.50^{a,b}$	$17.19\pm1.57^{a-c}$	$10.19\pm0.55^{d,f}$	$8.50\pm1.00^{e,f}$	$11.38\pm1.51^{c,d}$	$11.25\pm1.89^{b-d}$	$8.00\pm1.02^{d-g}$	$7.25\pm0.50^{c-e}$
Twig	H	$15.72\pm1.69^{a,b}$	$15.75\pm1.70^{a-d}$	$11.63\pm0.52^{c-e}$	$11.75\pm1.55^{b,c}$	10.06 ± 0.77^{d}	10.31 ± 0.77^{d}	$6.75\pm0.96^{f,g}$	$7.00\pm0.00^{d-e}$
	E	$14.50\pm1.59^{a-d}$	$14.81\pm1.36^{a-e}$	$12.38\pm0.52^{b,c}$	$11.56\pm0.83^{b,c}$	$11.19\pm0.75^{c,d}$	$10.44\pm0.59^{c,d}$	$10.12\pm0.92^{b,c}$	9.50 ± 1.24^{b}
	M	$16.32\pm0.62^{a,b}$	$16.25\pm1.31^{a-d}$	$9.94\pm0.83^{e,f}$	$9.63\pm0.48^{b,c}$	10.25 ± 0.54^{d}	$10.50\pm0.58^{c,d}$	$7.38\pm0.43^{e-g,A}$	$6.00\pm0.00^{e,B}$
Bark	H	$16.06\pm0.52^{a,b}$	$13.75\pm1.26^{b-f}$	$9.88\pm1.38^{e,f}$	$7.25\pm1.50^{d,e}$	$10.50\pm0.74^{c,d}$	$10.50\pm1.00^{c,d}$	$9.31\pm1.18^{c,d}$	$7.63\pm2.14^{c-e}$
	E	$14.94\pm1.16^{a-d}$	$14.88\pm1.49^{a-e}$	$10.81\pm1.75^{c,e}$	9.44 ± 0.38^{f}	10.25 ± 0.46^{d}	$10.63\pm0.95^{c,d}$	$9.75\pm1.08^{c,d}$	$8.40\pm0.71^{b,c}$
	M	$15.50\pm0.20^{a-c}$	$14.81\pm0.47^{a-e}$	$9.88\pm1.59^{e,f}$	$8.38\pm0.97^{d,e}$	$10.75\pm1.59^{c,d}$	$10.63\pm1.93^{c,d}$	$7.50\pm1.02^{e-g}$	$6.88\pm1.03^{d,e}$
Oxo	-	$11.63\pm1.59^{d-f}$	$12.25\pm2.35^{e-f}$	$29.50\pm0.68^{a,A}$	$28.13\pm0.78^{a,B}$	$11.63\pm1.59^{b,d}$	$12.25\pm2.35^{b-e}$	$11.63\pm1.59^{a,b}$	12.25 ± 2.35^{a}
Three-way analysis of variance									
Variable	df	F	*p*	F	*p*	F	*p*	F	*p*
PP	4	32.708	< 0.001	10.186	< 0.001	83.668	< 0.001	6.884	< 0.001
S	2	1.507	0.227	39.512	< 0.001	4.173	0.018	69.573	< 0.001
H	1	1.813	0.182	24.780	< 0.001	3.530	0.064	18.150	< 0.001
PP×S	8	2.862	0.007	6.358	< 0.001	3.680	0.001	9.999	< 0.001
PP×H	4	0.282	0.889	2.131	0.083	1.214	0.311	0.474	0.755
S×H	2	0.293	0.747	0.128	0.880	1.283	0.282	0.074	0.928
PP×S×H	8	0.241	0.982	0.378	0.930	1.101	0.370	1.134	0.349
Error	96								

Oxo = oxolinic acid; Sol = solvent; H = hexane; E = ethyl acetate; M = methanol; df = degrees of freedom

Values for zones of inhibition are expressed as means (mm) ± SD ($n = 4$);

Lowercase superscripts (a–h) in each column denote significant ($p < 0.05$) differences among means in each plant, while uppercase superscripts (A–B) in each row denote significant ($p < 0.05$) differences between incubation times (24 hr versus 48 hr).

For *V. harveyi*, the influences of plant parts, solvent and the interaction between plant parts and solvents were highly significant ($p < 0.001$), as shown in Fig. 5. Antibacterial activities of the flower extracted with ethyl acetate were significantly higher than those of other plant parts.

For *V. parahaemolyticus*, the influences of plant parts, solvent and the interaction between plant parts and solvents were highly significant ($p < 0.001$), as shown in Fig. 6. Within each plant part, ethyl acetate extracts showed higher antibacterial activities than the other solvents and the ethyl acetate extract of flowers produced the best results ($p < 0.05$). Notably, the influence of extraction time was highly significant, whereby the bacterium being treated for 24 hr showed a larger inhibition zone than those treated for 48 hr.

Overall, these results demonstrated the superior bactericidal activities of ethyl acetate extracts from flowers against the four tested aquatic pathogens.

Comparing the TPC and TFC quantity profiles (Figs. 1–2) with the inhibition zones (Figs. 3–6), it was apparent that the antibacterial activity was consistent with the number of phenolic compounds but not the flavonoids found in the different plant parts extracted with each solvent. For example, the ethyl acetate extracts from flowers, which had the highest TPC values but low TFC values, showed the highest antibacterial activity against all bacteria examined. There was a similar trend for pods. In contrast, the hexane and ethyl acetate extracts of the leaves or bark, which had a high quantity of TFC, showed low antibacterial activity. Thus, the present study indicated that phenolic compounds might play an essential role in bacterial growth inhibition rather than flavonoid compounds. A similar study was conducted by Sajid et al. (2012) using *D. indica* bark, leaf and seed extracts and they reported that compounds, such as protocatechuic, ellagic, ferulic, gallic, gentisic, 4-hydroxybennzoic acid, 4-hydroxycinnamic acids, vanillic acid and tannic acid, possessed antibacterial properties against *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *E. coli*. Phenolic compounds contain hydroxyl groups in their molecules which facilitate cell membrane permeation of the pathogens, while flavonoids contain a more hydrophobic carbonyl group (Ravikumar et al., 2010). There have been several reports on the antibacterial properties of compounds isolated from the bark, root and stem of *D. indica* (Khan et al., 2006; Koysomboon et al., 2006; Rao et al., 2009; Badole et al., 2011). Another more recent report showed that the change in the chemical composition of each extract directly affected its bioactivity and characteristic of bioactive compounds are important in providing access across the cell walls of bacteria (Vittaya et al., 2020).

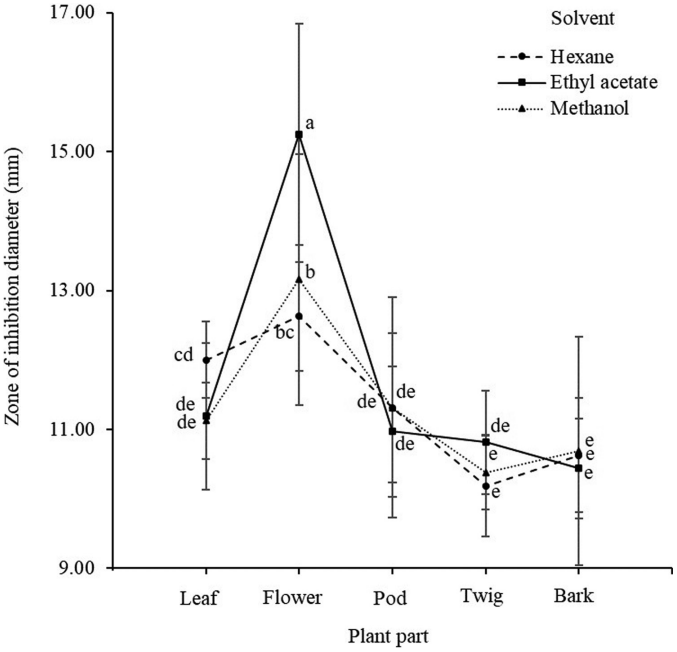

Fig. 5 Interaction of plant parts with solvents on antibacterial activity of *Derris indica* extracts against *Vibrio harveyi*, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

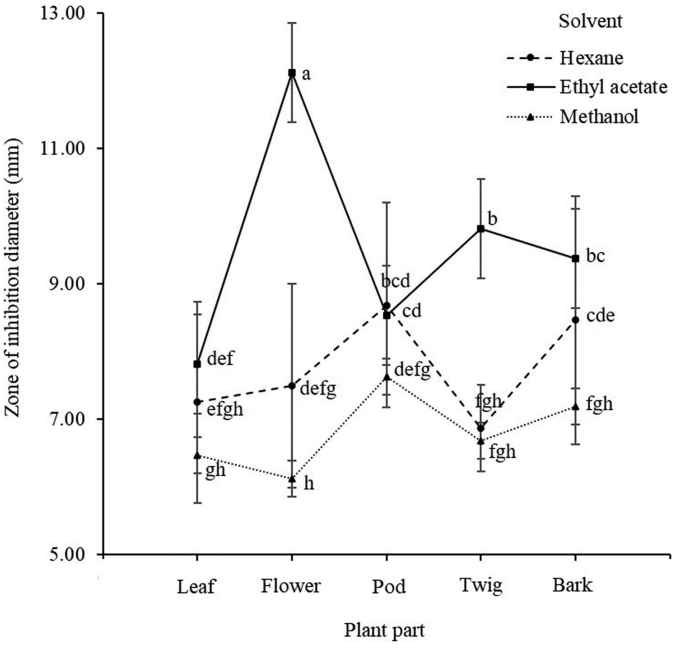

Fig. 6 Interaction of plant parts with solvents on antibacterial activity of *Derris indica* extracts against *Vibrio parahaemolyticus*, where different lowercase letters indicate significant ($p < 0.05$) differences among means and error bars represent ± SD

Table 7 Pearson's correlation coefficient (*r*) between phytochemical composition (phenolic and flavonoid) and their antibacterial properties against four pathogenic bacteria (*Streptococcus agalactiae*, *Aeromonas hydropilla*, *Vibrio harveyi* and *V. parahaemolyticus*) of *Derris indica*

Variable	TPC	TFC	*S. agalactiae*	*A. hydropilla*	*V. harveyi*	*V.parahaemolyticus*
TPC	1					
TFC	-0.414**	1				
S. agalactiae	0.213^{ns}	-0.054^{ns}	1			
A. hydropilla	0.313*	0.176^{ns}	0.308**	1		
V. harveyi	0.129^{ns}	-0.212^{ns}	0.001^{ns}	0.284**	1	
V.parahaemolyticus	0.195^{ns}	0.099^{ns}	0.211*	0.476**	0.277**	1

TPC = total phenolic content; TFC = total flavonoid content

*, ** = significant different from 0 at $p < 0.05$ and $p < 0.01$, respectively; ns = non-significant ($p > 0.05$)

Additionally, the Pearson correlation coefficient was used to establish a relationship between the TPC and TFC in the extracts and the antibacterial property (Table 7). The antibacterial activity against the four bacteria did not significantly correlate with the phenolic and flavonoid contents, except for the phenolic content against *A. hydrophila* (r = 0.313, ($p < 0.05$). However, a low correlation coefficient was observed between TPC and TFC in *D. indica* against the four bacteria. It was possible that the discrepancies in antibacterial activity involved other specific phenolic and flavonoid compounds in each extract or other primary metabolites, such as sugars, carbohydrates, proteins and amino acids (Santhi and Sengottuvel, 2016). Similarly, Lyu et al. (2020) reported no significant or a negative correlation between the phenolic and flavonoid contents and their antibacterial activity against Cranberry hibiscus (*Hibiscus acetosellsai*).

Minimum inhibitory concentrations and minimum bactericidal concentrations of D. indica extracts against aquatic bacterial pathogens

The MICs and MBCs of *D. indica* extracts were investigated; the ratios of MBCs to MICs was defined as R values of the bacteriostatic and bactericidal activities for extracts against aquatic bacterial pathogens comparing with those obtained from the standard antibiotic agent, oxolinic acid (Oxo) (Table 8). Generally, a value of R < 4 indicates that the extracts are bactericides (Boulfia et al., 2021). The extracts of *D. indica* leaf, flower, pod, twig and bark were active against all of the tested bacteria except for three occasions, namely, pod in hexane and twig in ethyl acetate were bacteriostatic (R = 4) against *V. harvei*, as was the flower in methanol against *V. parahaemolyticus* (R = 4). Based on the MIC and MBC values, the hexane extract of flowers was the most potent against *S. agalactiae* (MIC = MBC = 0.048 mg/mL). It should be noted that the hexane extracts of other plant parts were also potent against this bacterium, with slightly higher MICs and MBCs (MIC = MBC = 0.39–0.78 mg/mL). For *A. hydrophila*, good antibacterial activity was found with the ethyl acetate extracts of flowers (MIC = MBC = 0.048 mg/mL). The hexane extracts from all plant parts were also slightly potent against *A. hydrophila.*

All of the extracts seemed to be less effective when tested against *V. harveyi* and *V. parahaemolyticus* (Table 8). Only the ethyl acetate extract of flowers showed a comparable potency with the previous tests against *S. agalactiae* and *A. hydrophila,* with MIC = MBC = 0.097 against *V. harveyi* and MIC = MBC = 0.78 against *V. parahaemolyticus* (Table 8). The antibacterial activity of *D. indica* against other bacteria has been studied, with Sharma et al. (2021) reporting that extracts from flowers and bark were active against Gram-positive and Gram-negative bacteria (*Bacerus cereus*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Salmonella typhi*, *E. coli*). The antibacterial activity was proposed to result from compounds, such as alkaloids, flavonoids and tannins. Bodiba et al. (2018) using *D. indica* leaves reported antibacterial activity against *Streptococcus mutans*. In addition, Panigrahi and Mahapatra (2016) showed that the methanolic extracts of *D. indica* leaves were active against *S. aureus,* and the antibacterial properties were proposed to result from alkaloids, anthraquinone glycosides and flavonoids. Bark extracts of *D. indica* were active against *B. subtilis* and *E. coli* due to flavonoid and phenolic compounds, as reported by Niharika et al. (2021).

Table 8 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts of *D. indica* against four pathogenic bacteria that cause infectious diseases in aquaculture conditions

Species	Solvent	MIC and MBC (mg/mL)											
		S. agalactiae			*A. hydrophila*			*V. harveyi*			*V. parahaemolyticus*		
		MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
Leaf	Hexane	0.39	0.39	1	0.39	0.78	2	3.12	6.25	2	12.50	12.50	1
	Ethyl acetate	0.78	0.78	1	0.39	0.39	2	25.00	25.00	1	25.00	25.00	1
	Methanol	6.25	6.25	1	3.12	6.25	2	3.12	6.25	2	12.50	25.00	2
Flower	Hexane	0.048	0.048	1	0.39	0.39	1	3.12	3.12	1	12.50	12.50	1
	Ethyl acetate	3.12	3.12	1	0.048	0.048	1	0.097	0.097	1	0.78	0.78	1
	Methanol	1.56	3.12	2	1.56	1.56	1	3.12	3.12	1	6.25	25.00	4
Pod	Hexane	0.78	0.78	1	0.39	0.39	1	3.12	12.50	4	12.50	12.50	1
	Ethyl acetate	1.56	1.56	1	1.56	1.56	1	3.12	6.25	2	12.50	12.50	1
	Methanol	1.56	1.56	1	3.12	3.12	1	6.25	6.25	1	12.50	25.00	2
Twig	Hexane	0.39	0.39	1	0.39	0.39	1	3.12	6.25	2	12.50	12.50	1
	Ethyl acetate	3.12	3.12	1	1.56	1.56	1	3.12	12.50	4	6.25	6.25	1
	Methanol	3.12	3.12	1	1.56	1.56	1	6.25	6.25	1	12.50	25.00	2
Bark	Hexane	0.78	0.78	1	0.78	0.78	1	3.12	6.25	2	12.50	12.50	1
	Ethyl acetate	6.25	6.25	1	3.12	3.12	1	3.12	6.25	2	12.50	25.00	2
	Methanol	6.25	12.50	2	6.25	6.25	1	6.25	6.25	1	12.50	25.00	2
Oxolinic acid		0.19	3.12	16	0.19	3.12	16	0.19	3.12	16	0.19	3.12	16

Values are expressed as means from quadruplicate determination (n = 4).

R values are calculated from MBC/MIC (R < 4.00 indicates bactericidal extract, R > 4.00 indicates bacteriostatic extract)

For the first time, the present study presented the utilization of *D. indica* for controlling aquatic animal diseases. Various extracts of the plant exhibited antibacterial activity against the four bacteria, both Gram-positive (*S. agalactiae*) and Gram-negative (*A. hydrophila*, *V. hydrophila* and *V. parahaemolyticus*). Remarkably, the ethyl acetate extracts of flowers exhibited excellent *in vitro* bactericidal effect against these tested pathogenic bacteria with MIC/MBC = 1. Furthermore, based on the inhibition zone comparisons, the antibacterial activity of the extracts depended on the total phenolic content of the extracts. Based on the MIC/MBC values, most extracts had a bactericidal effect, particularly the flower extracts, which had the lowest MIC/MBC value and therefore, were considered an attractive antibacterial agent source. Although the extracts from flowers produced the highest antibacterial activity, leave extracts were recommended to be used in aquaculture due to their strong antibacterial activity (MIC/MBC = 1–2) and their availability throughout the year. Collectively, the results from the present study indicated that extracts of *D. indica* could be a promising source of antibacterial agents to be used in aquaculture.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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