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In-vitro screening of antioxidant, antibacterial and antifungal properties of herbs for aquaculture

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Abstract
The use of herbs in treating diseases is getting attention in aquaculture practices. Methanolic extracts from three fresh local herbs were screened for the antioxidant, antibacterial and antifungal activities *in-vitro*. The three selected herbs were *Piper betle* (betel), *Curcuma longa* (turmeric) and *Etlingera coccinea* ("tuhau"). The antioxidant activity analysis was done using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity. Antibacterial activity was screened using the disc-diffusion method using four types of marine pathogens which is the *Vibrio* species; *V. alginolyticus*, *V. anguillarum*, *V. harveyi* and *Vibrio parahaemolyticus*. The antifungal activity of the herbs was tested on a marine fungus, *Fusarium moniliforme* by dipping the fungus block into herbs' extract and observed after 24 to 48h. Concentrations of 0, 20, 50 and 100 mg/mL were used for testing both antibacterial and antifungal activities. Highest antioxidant activity was found in betel (more than 50% of activity), followed by turmeric (20 – 40%), and tuhau (20 – 30%). Apart from betel, the other herbs have less than 50% of activity. Betel also exhibited strong antibacterial activity (>14.0 mm), followed by a moderate activity of tuhau (10 – 14mm), and a weak activity of turmeric (<10mm). Meanwhile, for the antifungal activity, all herbs extract showed 100% inhibition where no mycelial growth was observed after dipping the fungus block into the extract. Screening of these herbs *in-vitro* showed that betel leaves gave the best results for the antioxidant, antibacterial and antifungal activity.

Keywords: herbs, local, betel, turmeric, tuhau, antioxidant, antibacterial, antifungal, aquaculture

1. Introduction
Medicinal plants have been used in mankind as a source of medicine centuries ago [1] The use of these medicinal plants as a basis for maintaining good health has been discovered in most developing countries [2]. Treatments of bacterial diseases with various medicinal plants have been safely used widely in organic agriculture, veterinary and human medicine [3] World aquaculture has grown rapidly in terms of production over the recent years and the intensive and stressful rearing conditions always result in the farmed fish being highly susceptible to different infectious diseases which is now the major problem in the aquaculture industry as it causes heavy economic losses [4] Due to the development of bacterial resistance to antibiotics and increasing popularity of medicinal plants has led researchers to investigate further the properties in medicinal plants [5].

Betel (*Piper betle*) is a perennial dioecious plant. Its leaves possess a strong and pungent aromatic smell and used widely especially in Asia as masticators [6]. Betel is commonly used in India and China as a traditional medicine and it treats bronchitis, asthma, cough and leprosy. This plant has digestive stimulant, carminative and aphrodicac properties [6].

Turmeric (*Curcuma longa*) is a perennial plant and is widely cultivated in Asia.[7] In Indian traditional medicine, they practice the use of turmeric in curing biliary disorders, anorexia, coryza cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis [8].

Tuhau, *Etlingera coccinea* and other members of the same genus are widely utilized as salad or used in traditional medicine in Borneo. Tuhau is normally consumed as pickles and as traditional remedy to cure stomach related pains such as stomach ache, food poisoning and gastric problems [9]. So far, less studies had been done on this plant.

These three plants have similar compounds however the main and most active components of betel leaves are its oil; chavibetol and chavicol [10]. For turmeric, it is mostly dominated by curcuminoids and essential oils [11]. Meanwhile for Tuhau, the most abundant and volatile essential oil is borneol [12].

Vibrios are Gram-negative bacteria where it takes form in a curved, rod-shaped. They are natural inhabitants in the marine environment. The transmission of this bacterial infections is commonly through the consumption of raw or undercooked shellfish or exposure of wounds to warm seawater [13]. In tropical marine environment, *Vibrio harveyi* is rampant and widely reported to cause vibriosis to marine fishes and other aquatic organisms [14].

Fusarium moniliforme is the most common fungi associated with humans and animal. It causes ear rot and stalk rot of corn and widespread of this fungus causes infection of corn kernels [15].

The local medicinal plants that are used in this present study are those that can be commonly found in Sabah, Malaysia. Among them are betel (*Piper betle*), turmeric (*Curcuma longa*) and tuhau (*Etlingera coccinea*).

Table 1: Antioxidant, antibacterial and antifungal activities of selected medicinal plants

Plant	Extract	Activity	Result
Betel	Hot water, Cold ethanolic	Antioxidant activity	Extracts obtained had good antioxidant activities [16].
	Aqueous, Ethanol	Antibacterial activity	Both types of extracts showed promising antibacterial activities towards tested pathogens [17].
	Ethanol	Antifungal activity	Extracts showed antifungal activity against fish water molds with increased concentration [18].
Turmeric	Methanol	Antioxidant activity	Extracts showed a dose dependant antioxidant activity [19].
	Methanol	Antibacterial activity	Extracts showed antibacterial activity against various gram positive and gram-negative bacteria [20].
	Aqueous, Ethanolic	Antifungal activity	Both types of extracts showed potent antifungal activity however aqueous extract showed better results [21].
Tuhau		Antibacterial activity	Antibacterial activity of the essential oils inhibited all tested bacteria with MIC values less than 10µg/mL [12].

*MIC – Minimum Inhibitory Concentration

Malaysia is among the countries that is blessed with the abundance of medical benefit plants [22]. Hence the present study was done to investigate the potential of local medicinal plants on its antioxidant, antibacterial and antifungal activity.

Materials and Methods

Preparation and Extraction

Three different species of herbs were obtained freshly from Kota Kinabalu's local market. For betel, the leaves were used while for turmeric, its rhizomes and for tuhau, its stems. The herbs were washed with sterilized distilled water and oven-dried in the oven for 24 hours at 50 °C, shredded into smaller pieces and grinded into powder form using and electric blender (Panasonic MX-337, Malaysia) [23]. The powder form of herbs was stored in a zip-lock bag until further analysis was conducted.

The extraction of herb was done by soaking 100g of the powdered herbs in 1000mL methanol solvent and stored in dark condition for 3 days at room temperature. After that, the extracts were filtered out using a sterile Whatman No. 1 filter paper and evaporated to dry using a rotary vacuum evaporator (Eyela, Japan). Its residue was collected, weighed and stored in sterile amber bottles at 4 °C until further analysis [24].

Antioxidant assay

The total antioxidant activity of the test herbs was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical test.[25] Butylated hydroxyl toluene (BHT) was used as the standard and distilled water as control. The herbs' extracts were prepared to different concentrations ranging from 0.02-0.10 mg/mL from the stock solution of 100 mg/mL. 4 ml of DPPH in methanol solution was added to the herb. Mixture was shaken thoroughly and let to stand in dark condition at standard room temperature for 30 minutes before it was inserted into the UV-Vis spectrophotometer for its absorbance reading. Methanol was used as blank. For standard, it was prepared exactly like the samples except without the extract. The absorbance of the resulted reaction was read at 517nm using a UV-Vis Spectrophotometer (HACH DR 5000 UV-Vis Spectrophotometer).

Activity of DPPH radicals were determined in inhibition (I%) and calculated using the equation by Hassim *et al*. [26].

$$\text{Inhibition, \%} = \frac{[\text{Absorbance (control)} - \text{Absorbance (sample)}]}{\text{Absorbance (control)}} \times 100$$

Absorbance (control) = Distilled water

Absorbance (sample) = Sample absorption

Table 2: The antioxidant activity was determined using the scale by Chua and Aminah [27].

Antioxidant activity range (%)	Activity Status
0	Nil
1 – 39	Low
40 – 69	Moderate
70 – 100	High

Antibacterial assay

Four marine bacterial strains of *Vibrio* species; *Vibrio alginolyticus* (ATCC 17749), *Vibrio anguillarum* (ATCC 19264), *Vibrio harveyi* (ATCC 35084), *Vibrio parahaemolyticus* (ATCC 17802) obtained from American Type Culture Collection (ATCC) were used in the antibacterial assay. This assay was conducted using the disc-diffusion method [28]. In this assay, Whatman No. 3 filter paper discs were impregnated with different concentrations of herb extracts at 0, 20, 50 and 100 mg/mL. Muller-Hinton Agar (MHA) was used for the disc-diffusion testing. A sterile cotton bud was used to streak the bacteria evenly on the medium to make a bacterial lawn for the assay. The impregnated paper discs (6mm in diameter) were then placed on the surface of the bacterial lawn. This assay was done in triplicates for all tested concentrations of herbs and bacteria. Kanamycin discs (Oxoid™) were used as the positive control meanwhile methanol as the negative control. The agar plates were then incubated at 28 °C for 24 hours and inhibition zones were measured to grade the results [28, 29].

Table 3: Grading of result was done based on MacKeen *et al.* [30]

-	No inhibition zones
+	Zone of inhibition < 10mm in diameter (Weak activity)
++	Zone of inhibition between 10 – 14mm in diameter (Moderate activity)
+++	Zone of inhibition between > 14mm in diameter (Strong activity)

Antifungal assay

The antifungal assay was done according to Borisutpeth *et al.* [31]. The fungal agar blocks were taken from vegetative colony and were submersed in petri dishes containing 20 mL of the different concentrations of herbs' extract. After 1, 2 and 24 hours, the fungal blocks were dipped in sterile seawater to rinse all the extracts out of the blocks. The fungal blocks were then inoculated onto a peptone-yeast-glucose-seawater (PYGS) agar and incubated for another 24 and 48 hours. Sterile seawater was used as a negative control where the fungal agar blocks were also submersed into it without the herbs' extract. Growth of hyphae was monitored and compared to the control. The fungistatic activity shown by percentage of hyphal growth inhibitory was calculated using this formula:

$$\text{Inhibition, \%} = \frac{[\text{Colonial radius (control)} - \text{Colonial radius (sample)}]}{\text{Colonial radius (control)}} \times 100$$

Results

Antioxidant activity

Antioxidant activity was tested at 5 concentrations which are 0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical test method. Figure 1 shows the percentage inhibition of DPPH of the three herbs at different concentrations and compared with different concentrations of the butylated hydroxytoluene (BHT). The highest percentage of inhibition was shown by betel where at 0.10 mg/mL it was 94.79%. This was followed by turmeric with 42.27% at the highest concentration tested which indicates a low to moderate activity. Meanwhile, Tuhau has the lowest antioxidant activity with only 32.12% at 0.10mg/mL. However, all three herbs have a pattern in which it the percentage of inhibition increases with the increase of concentration. Betel and turmeric has higher antioxidant activity compared to BHT while tuhau has lower antioxidant activity.

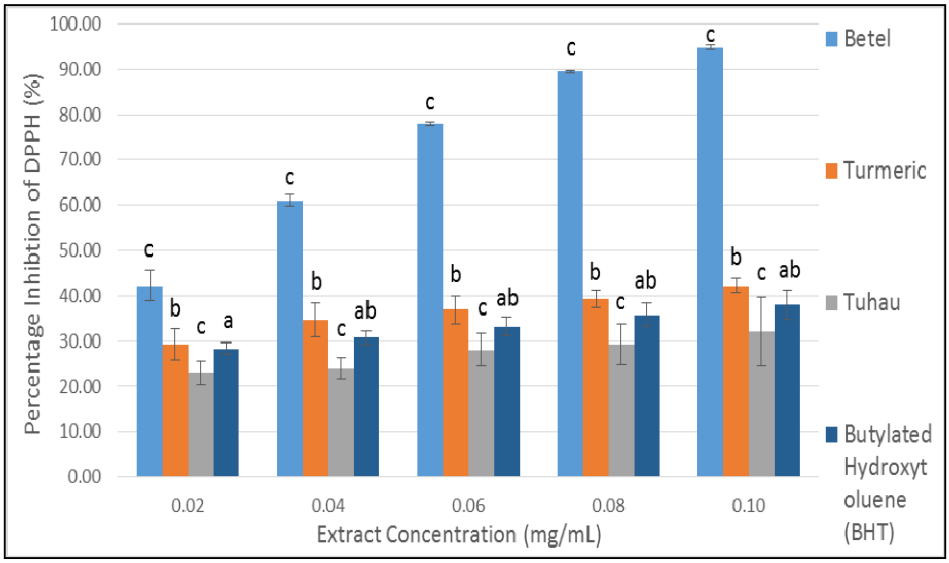

Fig 1: Percentage inhibition of DPPH *Superscripts a and b denote significant differences among the treatments ($P < 0.05$, Duncan test)

Antibacterial activity

Among the three herbs examined, betel exhibited the widest spectrum of antibacterial activities at all the tested concentrations of its extract against the four tested *Vibrio* species (Table 4). It is followed by tuhau, where at higher concentrations (100mg/mL) of its extract, it can inhibit the other varieties of *Vibrio*. Meanwhile for turmeric, it seems to be selective on the inhibition of bacteria where it only inhibited *Vibrio anguillarum* in all tested concentrations. When statistical analysis was run, it shows significant difference for the readings from different extract concentrations of all tested herbs, the readings of inhibition zones by different extract. Readings from extract concentration of 20mg/ml is significantly lower than 100mg/ml for all extracts. For the readings of inhibition zones by different extracts, betel is significantly higher than turmeric and tuhau.

Table 4: Antibacterial susceptibility of herb extracts against *Vibrio* species

Bacteria pathogen			Diameter of inhibition zone (mm)			
			***V. alginolyticus* ATCC 17749**	***V. anguillarum* ATCC 19264**	***V. harveyi* ATCC 35084**	***V. parahaemolyticus* ATCC 17802**
Extract Concentration (mg/mL)	0	Betel	n.i	n.i	n.i	n.i
		Turmeric	n.i	n.i	n.i	n.i
		Tuhau	n.i	n.i	n.i	n.i
	20	Betel	25.33 ± 0.47^{b}	18.00 ± 2.83^{b}	22.00 ± 1.41^{b}	23.33 ± 0.47^{b}

		Turmeric	0.00 ± 0.00[a]	9.33 ± 0.47[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
		Tuhau	9.67 ± 0.47[a]	0.00 ± 0.00[a]	9.67 ± 0.47[a]	0.00 ± 0.00[a]
	50	Betel	27.33 ± 0.94[b]	22.00 ± 1.41[b]	24.00 ± 0.82[b]	22.33 ± 0.47[b]
		Turmeric	0.00 ± 0.00[a]	8.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
		Tuhau	10.67 ± 0.47[a]	0.00 ± 0.00[a]	10.67 ± 0.47[a]	7.50 ± 0.50[a]
	100	Betel	31.00 ± 2.94[b]	22.00 ± 0.82[b]	27.33 ± 1.25[b]	24.67 ± 0.47[b]
		Turmeric	0.00 ± 0.00[a]	8.33 ± 0.47[a]	8.00 ± 0.00[a]	0.00 ± 0.00[a]
		Tuhau	13.33 ± 3.40[a]	8.33 ± 0.47[a]	13.00 ± 1.41[a]	8.00 ± 0.00[a]

*Values are expressed as Mean ± S.D

*Superscripts a and b denote significant differences among the treatments ($P < 0.05$, Duncan test)

*n.i: No inhibition observed

Antifungal activity

In this assay, three concentration levels of herbs were tested at 20, 50 and 100mg/mL at three different dipping time, which was 1 hour, 2 hours and 24 hours of the fungal agar blocks in the extract. The results showed that all the herb extracts showed 100% inhibition of the hyphae growth of *F. moniliforme* (Table 4).

Table 4: Percentage inhibition of the tested herbs against *Fusarium moniliforme*

				Extract		
				Betel	Turmeric	Tuhau
				Percentage of Inhibition (%)		
Extract Concentration (mg/mL)	Dipping Time (h)	1	20	100	100	100
			50	100	100	100
			100	100	100	100
		2	20	100	100	100
			50	100	100	100
			100	100	100	100
		24	20	100	100	100
			50	100	100	100
			100	100	100	100

Discussion

In present study, betel extract proved to have great potential in aquaculture as it gave the best results for the antioxidant, antibacterial and antifungal assay. Many previous studies have reported similar positive results from betel extract whereby for antioxidant activity, betel extracts did show high range of 20% - 90% and the activity increases with extract concentration [32, 33]. Despite the amount of extracted tested, it showed promising inhibition zones for antibacterial activity. Some researchers have reported that the reason behind the strong antibacterial exhibition of betel extract is because of its various chemical components and fatty acids [23]. For the antifungal assay, a 100% inhibition against *Fusarium moniliforme* was obtained. Ethanol crude extracts and acetone crude extracts of betel gave similar results to the present results where the extracts were tested against *Fusarium* sp. which causes wilt disease in tomato [34]. In another study on the antifungal activity, ethanol crude extract of betel exhibited a 100% inhibition against all the tested plant pathogenic fungi and they reported that hydroxychavicol in betel is the component responsible for antifungal activity [35].

Crude methanolic extract of turmeric showed promising results in present study as it has antioxidant, antibacterial and antifungal activity. For its antioxidant activity, it also shows an increasing pattern as the concentration of the extract increases however its activity is considered moderate. In previous studies, the methanolic and ethanolic extract were tested for the antioxidant activity and it resulted in the ethanolic extract having higher antioxidant activity [36]. It is also reported that ethanol is much more preferred solvent compared to methanol for the extraction of antioxidant compounds because it has lower toxicity [37]. The antioxidant activity shown by turmeric extract in present study is less than 50% which differs from other previous studies whereby most of them obtained above 70%. This is because the turmeric used in present study is in a form of dry spice. According to a previous study, dry spice turmeric has lower antioxidant properties because of the loss of its properties during the preparation process [38]. For the antibacterial activity of turmeric extract in present study, it only exhibited its properties on two bacterial strains; *V. anguillarum* and *V. harveyi*. In a previous study, turmeric leaf methanolic extract did inhibit the growth of *V. parahaemolyticus* but at a higher concentration tested, 200mg/mL [39]. Turmeric mainly contains tumerone and curlone and they have been reported to exhibit excellent antibacterial activity against various species of *Shigella* and many Gram-positive bacteria [40]. Meanwhile, the antifungal assay of turmeric extract in present study showed a 100% inhibition towards *F. moniliforme*. Previous studies have tried testing turmeric extract using various solvents however the antifungal properties are very low to zero [41].

Activities and properties of tuhau are less studied and has only a few reports. Antioxidant activity in present study showed an increasing pattern however it is the lowest compared to betel, turmeric and BHT. Results from present study is comparable to a previous study where the antioxidant activity of tuhau stems is 32.09% when 500µg/mL of extract was used [42]. In present study, tuhau showed good results on antibacterial and antifungal assay. In both assays, the extracts managed to inhibit the growth of *Vibrio* species and wider inhibition zones can be seen when higher concentrations are used and it exhibited strong antifungal properties when tested against *F. moniliforme*. The most abundant volatile component found in tuhau is borneol (29.6%) and when its essential oil was tested on four food pathogenic bacteria, its activity is comparable to present study [12]. A study on *E. elatior* reported that oxygenated monoterpenes are dominant

in the rhizomes and stem oils, which is actually also dominant in tuhau and it is associated closely with antibacterial properties [43-46, 12] However, the antimicrobial activity contradicts to another study where no inhibition zones were exhibited when they tested tuhau extracts on both Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*S. aureus*, *Bacillus subtilis* and *Bacillus spizinium*) and also fungi (*A. niger*, *T. rubrum*, *Candida albicans* and *S. cerevisiae*) [42]. In another previous study, leaf extracts of other *Etlingera* species (*E. elatior*, *E. rubrostriata*, *E. fulgens*, *E. maingayi* and *E. littoralis*) did inhibit the growth of Gram-negative bacteria but weakly inhibited Gram-positive bacteria [47]. Present results are the first to be reported about tuhau extracts having antifungal properties.

The varying results obtained from this study on antioxidant, antibacterial and antifungal compared to other studies is probably due to its extraction methods used and may also be due to the different geographical area where the plants are grown. Soil composition can influence the amount of phytoconstituents in the plants and the solvent that was used may not be suitable to extract all the oils and components from the plants well thus limiting their abilities to exhibit the properties well enough [23]. Also, the parts of the herbs used can affect results of the assay. Such as for tuhau, its leaves may contain more phenolic compounds more than its stems and rhizomes as the leaves are situated higher and are able to absorb more ultraviolet wavelengths therefore more secondary metabolites are accumulated in the vacuoles of epidermal cells compared to stems and rhizomes [48].

Conclusion

In conclusion, the *in-vitro* screening of these three-different species of local herbs has antioxidant, antibacterial and antifungal activities. Further studies on the methods of extraction of the oil from the plants to maximize the potential of the plant needs to be further elucidated.

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