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Differential responses of *Vibrio* sp. to young and mature leaves extracts of *Terminalia catappa* L.

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Abstract

Young and mature leaves of *Terminalia catappa* of alcoholic and aqueous extracts were evaluated for *in vitro* antibacterial activity against *Vibrio* sp. isolated from aquatic animals. Young leaves of *T. catappa* showed higher antibacterial activity when compared to mature leaves against *Vibrio parahemolyticus*, with methanolic and aqueous extracts exhibited the largest inhibition zones, 23 and 24 mm, respectively as determined by disc diffusion technique. Ethanolic extract of young leaves showed the lowest MIC and MBC at 3.13 mg/ml and 6.25 mg/ml, respectively. Both alcoholic and aqueous extracts of young and matures leaves exhibit variations in protein, RNA as well as pyrine and pyrimidines leakage of *Vibrio* sp. Cell membrane disruption is proposed as the mechanism of action of *T. catappa* leaves extract against *Vibrio* sp.

Introduction

Tropical almond or scientifically known as *Terminalia catappa* of combretum family is broadly distributed in tropical and subtropical region in Asia and South America (Hutchinson and Dalziel, 1972). Many studies have reported the pharmacological effects of *Terminalia* sp. (Shridhar, 2007; Anwarul Hassan *et al*., 2008; Gurvinder *et al*., 2008). In aquaculture, *Terminalia catappa* or locally known as ketapang has showed some potentials as an antibacterial agent (Nair and Chanda, 2008; Shinde *et al*., 2009). For example, ethanol extract of *T. catappa* showed antibacterial activity against nine bacterial strains with minimum inhibitory concentrations (MIC) ranging from 0.25 to 16 mg/mL (Kloucek *et al*., 2005). However, to the best of our knowledge, less information is available on the comparison of various extracts (alcoholic and aqueous) of this plant as well as their mechanism of action onto bacterial cell.

Vibriosis is one of the most prevalent bacterial fish diseases worldwide, characterized by hemorrhagic septicemia and ulcerative external lesions. This disease has been reported in a wide range of marine and estuarine fish species (Liao *et al*., 1996) as well as shellfish and crustacean (Lagana *et al*., 2011) causing mortality and high economic loss (Goarant *et al*., 1999). In Malaysia, *Vibrio* sp. has been detected from cultivated freshwater fish (Noorlis *et al*., 2011). The causative agents of vibriosis include *Vibrio harveyi*, *V. parahaemolyticus*, *V. cholerae* and others. In aquaculture, antibiotics have been used for prophylaxis purpose (Rodgers and Furones, 2009). However, the indiscriminate use of antibiotics in aquaculture has led to the arising cases of antibiotic resistant incidences. For instance, in Malaysia, the occurrence of antimicrobial resistance incidence of *Vibrio* sp. was first reported by Son *et al*. (1998) from cockles, *Anadara granosa* which has further intensifying the search for alternative antibiotics particularly of natural remedies (Kolkovski and Kolkovski, 2011).

Therefore, our objectives were to evaluate the antibacterial activity of *T. catappa* leaves of methanol, ethanol and aqueous extracts on *Vibrio* sp. *in vitro* as well as their mechanism of action onto membrane of *Vibro* sp. by measuring cell protein and nucleic acid leakage. In addition, antibacterial activities of mature and young leaves of *T. catappa* were also compared. As such, the information obtained regarding the effect of various extracts of *T. catappa* leaves on antibacterial activities as well as their mode of action will help to develop it as an alternative biodegradable and safe antimicrobial agent for aquaculture use in the future.

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Materials and Method

Plant materials and extraction

Fresh leaves of *Terminalia catappa* L. was collected from beach area of Pantai Teluk Ketapang (N 05° 22.629'; E 103° 07.146'), Terengganu. Leaves of different age were sampled from the top (3rd, young leaves, and 9th, mature leaves) and were collected following Unyayar and Cekic (2005). The leaves were washed under running tap water and dried at 50°C in ventilated oven before ground to fine powder. A total of 100g dry leaves powder was soaked in either 500 ml of ethanol or methanol overnight while aqueous extraction was kept for 16 h at room temperature (Sharma *et al*., 2009) and stored in airtight containers in the dark till use (Tetyana *et al*., 2002).

Bacteriological analysis

Samples of various tissues from diseased mudcrab, oyster, giant freshwater prawn and grouper were aseptically removed and were blended separately for homogeneity by the use of mortar and pestle. One g sample was weighed and added to 10 mL of distilled water aseptically. Ten-fold serial dilutions of the homogenates; 0.1 mL of 10^{-2}, 10^{-3} and 10^{-4} dilutions was made and Pour Plate Method (PPM) was used for microbial enumeration. Each dilution was plated by pipetting 1 mL of dilution into sterile Petri dishes of previously prepared blood agar, cytophyga agar, GSP, XLD and McConkey agar (Difco, USA) and incubated for 24 h at room temperature. Colonies were selected at random and subcultured to obtain pure isolates on fresh plates containing nutrient agar (NA) and then incubated at 37°C for 24 h. The stock cultures obtained were labelled carefully and was used for bacterial isolation and identification.

Isolation and identification of Vibrio spp.

Phenotypic characteristics, Gram staining and oxidase production were determined for isolates according to APHA (1970), Holt *et al*. (1994), Whitman (2004) and Najiah *et al*. (2008). Further identification was carried out using a commercial Identification System kit (BBL) following manufacturers instruction.

Antimicrobial tests

Antimicrobial tests were performed using disc diffusion assays (Bauer *et al*., 1966). Bacterial cultures in tryptic soy broth (TSB) incubated for 18 h at 30°C were centrifuged, washed twice with phosphate buffer saline (PBS) and resuspended in 0.85% sterile physiological saline. Bacteria concentration was adjusted to 0.5 McFarland Standard with biophotometer at 600 nm (OD600) using physiological saline, swabbed on the surface of Mueller-Hinton agar (Oxoid, UK) using sterile cotton swab and the 6 mm sterile disc of extracts (2mg/disc) were placed on it. Plates were incubated at 3^{0o}C for 24 h. Antimicrobial activity were detected by measuring the inhibition zone around the discs (in mm). Erythromycin (E 15 µg/disc), chloramphenicol (C 30 µg/disc), and tetracycline (TE 30 µg/disc) (Oxoid, UK) were used as positive controls while free solvents were used as negative controls.

Determination of MIC and MBC

Minimum inhibition concentrations (MICs) were determined using broth dilution methods. Approximately, 100 µl of sterile TSB were added to all wells in 96 microtitre plates. Leaves extracts ranged from 100 mg/ml to 0.19 mg/ml were prepared by using two-fold dilution, with the addition of 10 µl of bacterial culture (1 x 10^8 CFU/ml) and incubated for 24 h at 30°C. The MIC was defined as the lowest concentration that inhibit bacterial growth. It was then read at 540 nm by using microplate reader based on two-fold increment of the optical density. An aliquot of bacterial cultures (100 µl) were also spread plate on the tryptic soy agar, TSA to determine the minimum bactericidal concentrations, MBCs (i.e. no growth of bacteria detected).

Bacterial protein leakage and nucleic acids measurements

The extract concentration equal to the highest MIC of 50 mg/ml was selected in the study of bacteria cell membrane disruption. Bacteria cells in 1 ml trypticase soy broth of 24 h incubation were harvested in sterile, centrifuged (12,000 x g, 4°C 10 min), washed twice with PBS and adjusted to 0.5 Mcfarland Standard in steriled physiological saline. A mixture of 90 µl of extracts was then added to 10 µl of bacterial suspensions. The suspensions were filtered through 0.2 µm Minisart® syringe filter (Sartorius Stedim, Germany) and measured for nucleic acids at 0, 10, 20, 30, 40, 50 and 60 min at the wavelength of A_{260} nm and A_{280} nm as described by Henie *et al*. (2009). Leaves extracts without bacterial suspensions served as controls. The release of protein concentrations was determined using Bradford assay kit following Bradford (1976) at 595 nm with Elisa Reader.

Results and Discussion

Seven and 4 strains of *Vibrio alginolyticus* and *V. parahaemolyticus*, respectively, were identified following phenotypical tests, gram staining and oxidase production from tissue samples of diseased aquatic animals namely giant fresh water prawn, mud

Table 1. Characteristics of *Vibrio* sp. isolates

Isolate	Organism	Origin	Morphology	Oxidase Test	Indole Test	Blood Hemolysis	ONPG	Vibriostat 0/129 (10µg)	Vibriostat 0/129 (150 µg)
Va_1	*V. alginolyticus*	Giant fresh water prawn	1-2mm, yellow, mucoid	-	+	α	-	-	-
Va_2	*V. alginolyticus*	Giant fresh water prawn	0.5-1mm, white yellow, cream	-	-	γ	-	+	+
Va_3	*V. alginolyticus*	Giant fresh water prawn	1.5-3mm, yellow, mucoid	-	+	α	-	+	-
Va_4	*V. alginolyticus*	Giant fresh water prawn	1-3mm, yellow, mucoid	-	-	α	-	+	-
Va_5	*V. alginolyticus*	Mud crab (gut) MHK Akua, Pagar Besi	0.5-2mm, yellow, mucoid	-	+	γ	-	-	-
Va_6	*V. alginolyticus*	Giant fresh water prawn	1-4mm, yellow, mucoid	-	+	α	-	-	-
Va_7	*V. alginolyticus*	Giant fresh water prawn	1.5-4mm, yellow, mucoid	-	-	α	-	+	-
Vp_1	*V. parahemolyticus*	Mud crab	1-2mm, green, mucoid	-	+	α	-	-	-
Vp_2	*V. parahemolyticus*	Oyster	1-3mm, green, mucoid	+	+	β	-	+	+
Vp_3	*V. parahemolyticus*	Mud crab (gut) MHK Akua, Pagar Besi	1-2mm, green, mucoid	-	+	α	-	-	-
Vp_4	*V. parahemolyticus*	Grouper (liver) Tanjung Demong N 5°24.606', E 103°05.502'	0.5-2mm, green, mucoid	-	-	β	-	-	-

Table 2. Antibacterial activities of *Terminalia catappa* young and mature leaves extracts against *Vibrio* sp by disc diffusion technique (mm)

	Inhibition zone (mm)[a]									
	Ethanol		Methanol		Aqueous					
Isolate	Young	Mature	Young	Mature	Young	Mature	Erytromycin	Chloramphenicol	Tetracycline	Control
Va_1	13.67±1.15	19.33±0.58	13.33±3.21	17.33±0.58	15.33±3.06	17.33±0.58	16.67±0.58	37.0±2.65	34.0±1.0	-
Va_2	14.0±3.61	14.0±4.0	18.33±0.58	12.33±2.08	15.0±1.0	12.33±2.08	19.0±1.73	29.67±0.58	29.67±1.53	-
Va_3	17.67±4.51	16.33±1.53	18.67±2.31	14.67±4.16	16.33±3.06	12.67±4.16	17.67±0.58	36.67±2.89	28.67±1.15	-
Va_4	15.33±0.58	17.33±2.08	17.33±1.53	17.33±2.08	14.0±1.73	17.33±2.08	18.0±1.73	35.33±2.31	29.0±1.73	-
Va_5	18.0±1.73	13.67±2.52	16.0±3.61	11.33±0.58	15.67±2.89	11.33±0.58	16.33±2.31	32.33±1.53	29.33±1.53	-
Va_6	19.33±0.58	14.67±3.21	17.0±3.61	13.0±1.0	19.33±6.43	13.0±1.0	17.0±1.73	32.33±3.21	28.0±1.73	-
Va_7	19.0±3.0	10.67±0.58	15.33±1.15	9.67±0.58	16.33±0.58	9.67±0.58	16.33±2.31	31.0±7.81	26.0±5.20	-
Vp_1	14.67±3.21	16.0±1.73	18.33±3.78	13.33±2.89	17.0±3.61	11.33±2.89	17.0±1.73	33.67±3.79	27.67±5.51	-
Vp_2	16.0±4.0	16.33±4.04	19.0±3.0	14.33±3.79	22.33±5.51	11.33±3.79	19.67±0.58	35.33±1.53	30.33±1.53	-
Vp_3	17.67±4.04	14.67±0.58	23±1.73	12.67±3.06	24.0±1.73	12.0±3.06	16.33±1.53	35.67±1.53	26.67±2.89	-
Vp_4	12.33±2.08	12.33±2.08	12±1.73	12.0±1.73	15.0±0.0	12.0±1.73	16.0±1.73	33.33±2.31	27.0±2.65	-

[a] Values are mean ± standard deviation in triplicates

crab, oyster and grouper (Table 1).

Screening assay of the ethanolic, methanolic and aqueous extracts of *T. catappa* leaves showed promising antibacterial activities against all *Vibrio* strains tested. Overall, the antibacterial activity of young *T. catappa* leaves extracts were higher than mature leaves extracts against the bacteria, with the largest inhibition zone of young leaves aqueous extracts against *V. parahaemolyticus* strain, Vp3 (24.00±1.73 mm) in disc diffusion assay (Table 2). On the other hand, ethanolic extracts of mature T. catappa leaves exhibit higher antibacterial activity for V. alginolyticus, Va1 (19.33±0.58mm) as determined by disk diffusion method (Table 2) when compared to methanolic and aqueous extracts, thus suggesting the use of ethanol as preferred solvent for *T. catappa* mature leaves extraction. The antimicrobial activity of *T. catappa* leaves from methanol, ethanol and aqueous solvent suggest their ability to extract the polyphenolic compounds such as simple phenols, anthocyanins, phenylpropanoids and flavonols (Shahina *et al*., 2007).

The antimicrobial assay showed *T. catappa* leaves extracts exhibit antibacterial activity against *V. parahemolyticus* and *V. alginolyticus* strains. *Vibrio parahaemolyticus* and *V. alginolyticus* were known as an infectious agent in aquatic animals (Thakur *et al*., 2002). The MIC analysis of *T. catappa* extracts against *Vibrio* strains ranged from 3.13 to 50 mg/ml. The ethanolic of young leaves extracts present the highest antibacterial activity particularly against *V. parahaemolyticus* strain, Vp4 with MIC and MBC value of 3.13 and 6.25 mg/ml, respectively (Table 3). Our results are also in accordance with the report that *T. catappa* leaves extracts were able to inhibit 70% gram positive bacteria and 63% gram negative

Table 3. MICs and MBCs of T. catappa leaves extracts against *V. parahemolyticus* and *V. alginolyticus* (mg/ml)

Isolates	Young leaves extracts						Mature leaves extracts					
	Methanol		Ethanol		Aqueous		Methanol		Ethanol		Aqueous	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Va 1	12.5	25	12.5	50	3.13	50	12.5	50	12.5	50	50	100
Va 2	50	>100	25	>100	12.5	>100	6.25	>100	3.13	>100	6.25	>100
Va 3	12.5	25	12.5	25	3.13	25	25	50	12.5	25	25	100
Va 4	12.5	25	12.5	25	50	100	25	50	12.5	25	12.5	100
Va 5	12.5	12.5	12.5	25	25	25	25	50	25	25	25	100
Va 6	6.25	12.5	12.5	25	25	25	25	25	12.5	100	25	100
Va 7	12.5	12.5	12.5	25	25	25	25	25	12.5	50	6.25	100
Vp 1	6.25	12.5	6.25	12.5	12.5	12.5	25	25	12.5	25	25	50
Vp 2	6.25	6.25	12.5	12.5	25	25	12.5	50	12.5	50	25	100
Vp 3	12.5	12.5	12.5	12.5	6.25	25	25	25	12.5	25	25	100
Vp 4	6.25	6.25	3.13	6.25	12.5	12.5	12.5	25	6.25	6.25	12.5	100

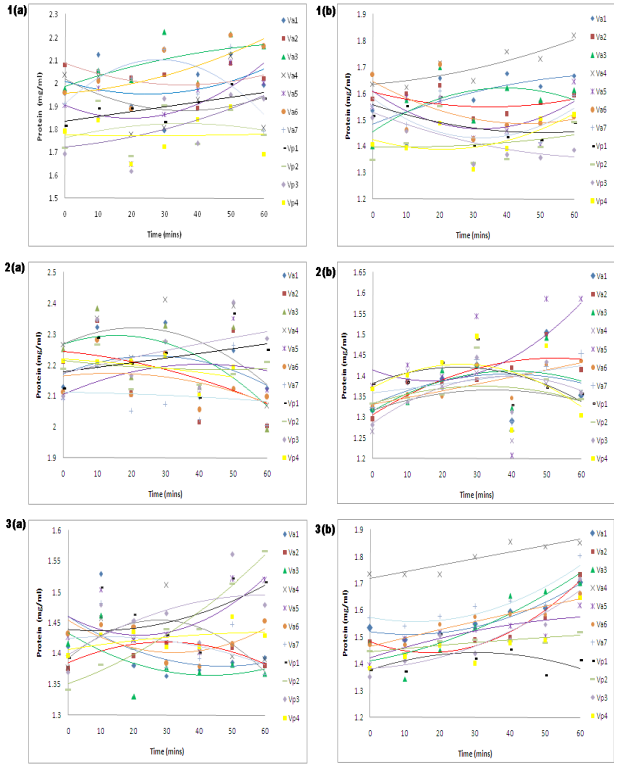

Figure 1. Protein leakage of *Vibrio* strains after treatment with *T. catappa* leaves extracts. 1:Ethanolic extracts, 2: Methanolic extracts, 3: Aqueous extracts; (a): young leaves extracts, (b): mature leaves extracts

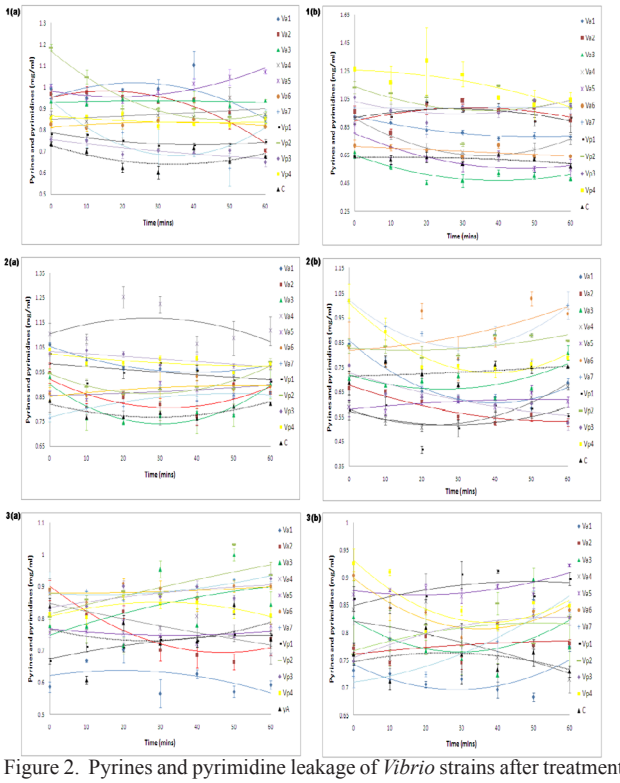

Figure 2. Pyrines and pyrimidine leakage of *Vibrio* strains after treatment with *T. catappa* leaves extracts [A_{260}nm]. 1:Ethanolic extracts, 2: Methanolic extracts, 3: Aqueous extracts; (a): young leaves extracts, (b): mature leaves extracts.

bacteria as reported by Manzur *et al*. (2011). In addition, ethanolic leaves extracts were found to inhibit *Escherichia coli* and *Salmonella typhi* at 500 µg/disc in (Muhamad and Mudi, 2011). However, Chansue and Assawawongkasem (2008) reported the MIC value of 1.5 mg/ml and 2.0 mg/ml against *V. alginolyticus* and *V. parahaemolyticus*. Study by Manzur *et al*. (2011) showed that *T. catappa* leaves extract exhibit antibacterial activity at concentration of 250 µg/disc. These reported differences may be attributed to the different extraction method and bacterial strains which yield varying amounts of bioactive compounds (Malu *et al*., 2009). In addition, different extraction methods also affect the antioxidant activity (Liu *et al*., 2008).

In our study, it was found that antimicrobial activity of leaves extracts was comparable to the standard antibiotic, erythromycin. However, chloramphenicol and tetracycline were more efficient as antibacterial agents when compared to erythromycin due to their higher dosage. Our results are similar to Lagana *et al*. (2011) where the *Vibrios* isolated from shrimp, mussel, oyster and seabass were 100% susceptible to chloramphenicol and 88% were susceptible to tetracycline. The majority of *Vibrio* strains isolated from moribund shrimps were also found to be sensitive against erythromycin and chloramphenicol, as reported by Thakur *et al*. (2002).

All extracts showed variations in bacterial protein leakage (Figure 1) within 1 h period. Similar trend was observed for RNA (Figure 2) as well as pyrines and pyrimidine leakage (Figure 3). Therefore, *T.*

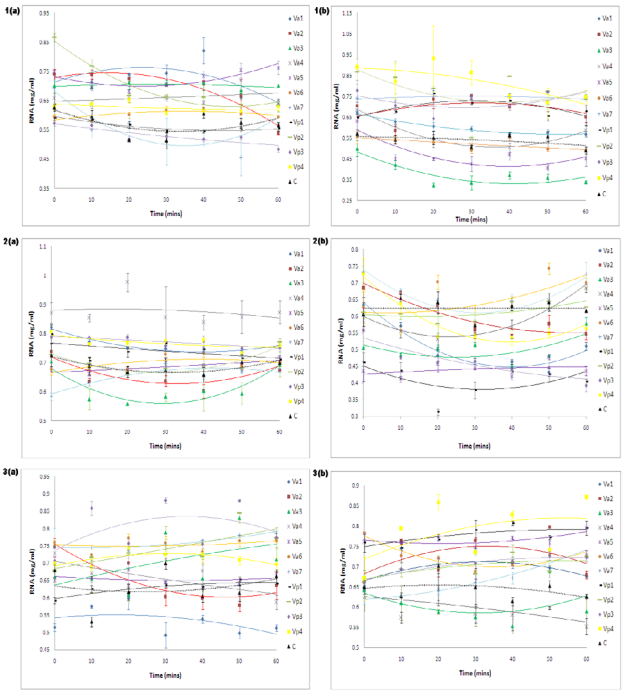

Figure 3. RNA leakage of *Vibrio* strains after treatment with *T. catappa* leaves extracts [A_{280}nm]. 1:Ethanolic extracts, 2: Methanolic extracts, 3: Aqueous extracts; (a): young leaves extracts, (b): mature leaves extracts.

catappa leaves extracts were found to enhance the bacterial protein and nucleic acid leakage within 1 h in the present study. Our finding revealed that all extracts caused increase cellular leakage of protein and nucleic acid. This could be due to the disruption of cell membrane when the bacterial were treated with *T. catappa* leaves extracts. Similar trends were also observed with pyrine and pyrimidines at 1 h after treatments. Study by Henie *et al*. (2009) demonstrated higher RNA, pyrines and pyrimidines reading of bacteria- treated with *Psidium guajava* extracts. Our results are also in accordance with Cox *et al*. (2000) where essential oil extracted from *Malaleuca alternifolia* disrupts the permeability barrier of cell membrane structures and thus induced the leakage of intracellular metabolites (Nedorostova *et al*., 2009). In addition, it has been suggested that the bacterial response to the extracts by modifying their membrane cells, exert their physiological changes after the disruption of their liphophilic membrane constituents, which further alter the bacterial function and eventually lead to cell death (Henie *et al*., 2009).

Conclusion

In conclusion, extracts of *T. catappa* leaves has potential to be used as an easily accessible source of antimicrobial agent for vibriosis in aquaculture. Age of leaves (either young or matured) and extraction agents used greatly influence the antibacterial property of *T. catappa* leaves. However, our results do not reveal the chemical compound that is responsible for aforementioned activity. Thus, further studies will be undertaken to determine the types of bioactive compounds which can be obtained from this plant as well as their effect on intracellular pH, membrane potential and ATP synthesis.

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