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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF EDIBLE HERBS AGAINST FISH PATHOGENIC BACTERIA

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ABSTRACT

Five selected herbs, betel vine (*Piper betle*), betel leaves (*Piper sarmentosum roxb*), turmeric (*Curcuma longa*), Indian pennywort (*Centella asiatica*) and kaffir lime (*Citrus hystrix*) were examined for their antibacterial properties against Gram positive bacteria namely, *Staphylococcus aureus, Streptococcus agalactiae* and *Streptococcus aginosus* and Gram negative bacteria namely, *Vibrio parahaemolyticus, Vibrio vulnificus, Citrobacter freundii, Escherichia coli, Edwardsiella tarda* and *Aeromonas hydrophila*. Among all herb extracts, only betel vine, turmeric and kaffir lime possessed antibacterial activities against fish pathogenic bacteria. Extracts of betel vine showed highest antibacterial activity in both form of methanol and aqueous extracts. The broadest antibacterial activity was methanol extracts which inhibited almost all bacterial species such as *V. parahaemolyticus, E. coli, E. tarda, A. hydrophila, S. aureus* and *S. aginosus* at 200 mg/ml. In determination of minimum inhibitory concentration (MIC), methanol extracts of piper betle required 1.56 mg/ml to inhibit the growth of both *V. parahaemolyticus* and *A. hydrophila*. The effect of temperature on antibacterial activity indicated the herbs tested were heat stable ranging from 37˚C to 105˚C.

INTRODUCTION

Fish reared in aquaculture facilities are exposed to numerous bacterial disease which can be treated with variety of antibiotics. Antibiotics have been widely used in aquaculture worldwide to treat fish bacterial infections such as Motile Aeromonas Septicemia (MAS), Edwardsiellosis, Vibriosis, Streptococcosis and Staphylococcosis (Frederick, 1999). Antibiotic is crucial in fish disease treatment in Malaysian aquaculture due to the fact that effective vaccines is yet to be developed. However, the extensive use of antibiotics has caused the emergence of antibiotic resistant bacteria which could be the major obstacle in successful treatment of bacterial disease in fish (Angeh, 2006). For example, *Aeromonas hydrophila* isolated from *Telapia mossambica* was reported to be resistant to streptomycin, tetracycline and erythromycin (Son *et al*., 1996). The resistance bacteria may posses R plasmids where their resistance determinants could be transferred to other bacteria (Frederick, 1999). Other than that, the spread of zoonotic disease due to bacteria should be monitored closely. The infections may occur in human through direct contact with either wild fish or fish live in captivity. For example, in 1996 and 1997 there were cases reported that new biotype of *Vibrio vulnificus* had caused 100 infections among persons during handling of the live tilapia in Israeli fish farms (Novotny *et al*., 2004). Other cases reported the infections may also occur through consumption of fish products and affected fish with *Vibrio parahaemolyticus* and *Salmonella spp* (Frederick, 1999).

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Herbs have been used in human medicine due to their antibacterial, anti-inflammatory, cytostatic, antifungal and antiviral properties centuries ago (Angeh, 2006). They contain alkaloids, phenolic compounds, diterpenoids, steroid, glycoalkaloids and other compounds that are capable to inhibit the growth of bacteria. According to Angeh (2006), Asiaticosside, extracted from *Centella asiatica* has been used traditionally in humans to treat skin diseases, leprosy, urinary and upper respiratory tract infections and malaria. Ethanolic extract of *Centella asisatica* possessed antibacterial properties against enteropathogenic bacteria which were *Aeromonas hydrophila, Salmonella typhimurium, Pseudomonas aeruginosa, Escherichia coli, Vibrio parahaemolyticus* and *Staphylococcus aureus* in human (Mamtha *et al*., 2004). Due to the emergence of resistant bacteria, the safe indigenous medicines such as herbs need to be explored as an alternative to antibiotics. Thus, the present study was carried out to screen selected herbs for their antibacterial properties and Minimum Inhibitory Concentration (MIC) value. The effect of temperature on antibacterial activities of selected herbs was also carried out.

MATERIALS AND METHODS

Preparation of crude extracts: Five selected herbs *P. betle, P. sarmentosum roxb, C. longa, C. hystrix* and *C. asiatica* were dried at 37˚C for five days, blended into powder and soaked in methanol and aqueous for 3 days. The solvents were filtered using filter paper and evaporated at 37˚C until it was completely dry. The dry crude extracts were then reconstituted

to 200 mg/ml each in their solvent respectively for antibacterial test (Daud *et al*., 2005).

Test organisms*:* Nine fish pathogenic bacteria species were used in the study. They were *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus anginosus*, *Vibrio parahaemolyticus, Vibrio vulnificus, Citrobacter freundii, Edwardsiella tarda, Escherichia coli* and *Aeromonas hydrophila.* All bacterial cultures were obtained from Fish Disease Laboratory of Universiti Malaysia Terengganu (UMT). The bacteria were subcultured into trypticase soy broth (TSB) (Oxoid, Germany) and incubated at 37˚C for 18 h. Then, the bacterial concentration was adjusted to 1 x 10^7 cfu/ml. The optical density (OD) at 540 nm of each culture was measured with Elisa reader (Bio-Rad, Japan) (Daud *et a*l., 2005).

Table 1. Inhibition zone (mm) of selected herbs using methanol and aqueous extraction against fish pathogenic bacteria

Bacteria	Betel vine		Betel leaf		Indian pennywort		Kaffir lime		Turmeric		OTC
	Methanol	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol	Aqueous	Methanol	Aqueous	
V.parahaemolyticus	20	14.5	-	-	-	-	8	-	11	-	35
V. vulnificus	-	13	-	-	-	-	-	-	-	-	-
E. coli	17	-	-	-	-	-	-	-	-	-	18.2
E. tarda	18	-	-	-	-	-	-	-	-	-	-
S. aureus	19	-	-	-	-	-	-	-	9	-	27
S. agalactiae	-	-	-	-	-	-	-	-	-	-	-
S. anginosus	18.5	13.5	-	-	-	-	-	-	-	-	-
C. freundii	-	-	-	-	-	-	-	-	-	-	-
A. hydrophila	18	-	-	-	-	-	-	-	-	-	11.35

Table 2: Minimum inhibitory concentration of herbs extracts against fish pathogenic bacteria

Herbs extracts against bacteria	MIC value (mg/ml)
P.betle methanol against *V.parahaemolyticus*	1.56
P.betle methanol against *E.coli*	6.25
P.betle methanol against *E.tarda*	3.125
P.betle methanol against *S.aureus*	3.125
P.betle methanol against *S.arginosus*	3.125
P.betle methanol against *A.hydrophyla*	1.56
P.betle water against *V.parahaemolyticus*	12.5
P.betle water against *V.vulnificus*	12.5
P.betle water against *S.anginosus*	12.5
C.longa methanol against *V.parahaemolyticus*	6.25
C.longa methanol against *S.aureus*	6.25
C.hystrix methanol against *V.parahaemolyticus*	6.25

Antibacterial activity*:* The antibacterial activity was determined by disc diffusion method (Daud *et al*., 2005). 20 µl of each extracts was impregnated onto the sterile paper disc (6mm) and left dry for 24 h. The paper disc-containing extracts were then sterilized under ultraviolet (UV) for 20 min prior to antibacterial testing. The paper disc-containing extracts were placed onto Mueller Hinton Agar (MHA) (Oxoid, Germany) that was previously layered with bacterial suspension. The 30 µg/disc of Oxytetracycline (OTC) was used as positive control. The tests were carried out in triplicate. The plates were incubated at 37 ˚C for 24 h. The antibacterial activity is determined by measuring the diameter of inhibition zone around the paper discs (Bauer al., 1966). The extracts were sterilized for 20 min and two-fold serial dilutions were made with TSB to give concentrations ranging from 0.1 to 200 mg/ml. Then, 5µl of bacteria were added into each concentration and the OD was measured by Elisa reader. The tubes were left in laminar flow for 48 h to keep in sterile condition. The OD after 48 h was measured as no change in OD reading and less than the double reading of OD indicate the MIC value (Mutthu *et al*., 2005).

Heat stability*:* Each extract was heated in the temperature ranging from 37˚C, 47˚C, 57˚C, 67˚C, 77˚C, 87˚C, 97˚C and 105˚C for 15 min. Following that, they were reconstituted and impregnated onto the paper discs (6mm) for antibacterial test. The effects of temperature were determined by measuring the diameter of inhibition zone around the paper discs.

RESULTS

Antibacterial Activity and Minimum Inhibitory Concentration (MIC): The result of the antibacterial test indicated that the crude methanolic and aqueous extracts of betel vine, turmeric and kaffir lime showed different size of the inhibition zones depending on the bacterial species (Table 1). For aqueous extracts, only betel vine showed the antibacterial properties. The methanol and aqueous extracts of Indian pennywort and betel leaf were inactive against all bacterial species tested. The crude methanol extracts of betel vine showed the broadest antibacterial activity by inhibiting growth of almost all bacterial species studied. Of all extracts tested, betel vine showed the highest antibacterial activity against *Vibrio parahaemolyticus* (20 mm) and *Staphylococcus aureus* (19 mm). The inhibition zone of methanol extracts of betel vine was ranging from 18 to 20 mm which indicated the strongest antibacterial activity. However, the aqueous extract of betel vine was only capable of inhibiting *V. parahaemolyticus*, *V. vulnificus* and *S. arginosu*s with moderate activity (13 to 14.5 mm). Only the aqueous extract of betel vine showed antibacterial activity against *V. vulnificus* compared to the methanol extract of betel vine. The antibacterial activity against *V. parahaemolyticus* was the highest (20 mm). The MIC values of all extracts showed different inhibitory concentrations ranging from 1.56 to 12.5 mg/ml (Table 2). Of all, the lowest value of MIC was from methanol extract of betel vine against *A. hydrophila* and *V. parahaemolyticus* which were both at 1.56 mg/ml. The MIC of methanol extract of betel vine to inhibit *V. parahaemolyticus* was 1.56 mg/ml compared to the aqueous extract of betel vine and methanol extract of turmeric and kaffir lime which were 12.5, 6.25 and 6.25 mg/ml respectively. The methanol extract of betel vine required 3.125 mg/ml to inhibit the growth of *S. aureus* compared to the methanol extract of turmeric which was 6.25 mg/ml. Other than that, *S. anginosus* was inhibited at 3.125 mg/ml by methanol extract of betel vine rather than aqueous extract of betel vine at 12.5 mg/ml.

Heat stability study: The effect of temperature on the antibacterial activity of methanol and aqueous extracts of herbs indicated that at various temperature ranges from 37 to

105˚C the antibacterial activities remained relatively unaffected.

DISCUSSION

The present study indicated that the highest antibacterial activity was obtained from betel vine in both forms of methanol and aqueous extracts. This is perhaps due to the presence of hydroxychavicol in betel vine which is the main active compound (Nalina and Rahim, 2007). According to them, the hydroxychavicol has antibacterial activity and fatty acids which can act as anionic surfactants and possessed antibacterial potential. Betel vine also contains allylphenols which have been reported to exhibit strong antibacterial effects on obligate oral anaerobes (Ramji *et al*., 2002). Besides, the methanolic extract of betel vine showed higher than its aqueous extract. This may indicate that different solvents may have different extraction capacities and different spectrum of the phytoconstituents (Doughari *et al.,* 2007). In the present study, kaffir lime could inhibit the growth of *V. parahaemolyticus*. The inhibitory effect on bacteria might be due to the active compound which is citronellal (Dusanee *et al*., 2006). From the previous study, the active compounds of kaffir lime peels which are ß-pinene and limonene had greater antibacterial activity against some strains of *Salmonella* rather than kaffir lime leaf (Dusanee *et al*., 2006). Other than that the inhibitory of turmeric against bacterial species might be due to the various sesquiterpenes and curcuminoids which have been extracted from the turmeric leaves (Rambir *et al*., 2002). This herb also contains turmerone and curlone which have been reported containing excellent antibacterial activity against various species of *Shigella* and many Gram positive bacteria (Sanjay, 2007). Bacteria differ in their resistance to a given herb and the effect of herbs may be inhibitory or biocidal (Dusanee *et al*., 2006). The antibacterial actions of herbs are due to the impairment of a variety of enzyme systems involving in the production of energy or synthesis of structural components in microbial cells (Nanasombat and Lohasupthawee, 2005). In addition, this may be also due to cell wall injury and alteration in the cytoplasmic membrane permeability, resulting in the loss of cytosol and finally cell death (Daud *et al*., 2005). The result from temperature study may indicate that some active compounds of herbs are heat stable. The high temperature resistance may explain why the human traditional medicinal of some herbs still achieve the desired effects of cure instead of boiling practice at high temperature (Doughari *et al*., 2007). Thus, in this study, methanol extract of betel vine showed the highest and broadest antibacterial properties which could inhibit almost all bacteria tested. This information is important as herbs might serve as an alternative to antibiotics available to treat bacterial diseases in aquaculture in the future. More studies on these herbs such as screening for bioactive compounds and toxicity test are under way.

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