Short Communication

Methanolic Activities of Selected Weeds on Bacteria Isolated from *Macrobrachium rosenbergii* Larvae

Najiah Musa[1*] Nadirah Musa[1] Wan Nurhafizah Ibrahim[1] Mohd Zahrol Ariff Shariat[1]
Arief Izzairy Zamani[1] Muhd Ranzi Abdullah[1] Tee Lea Wee[1] Mariam Marip[2]
Laith Abdul Razak[1] Amar Sakhawi Awang Soh[1]

Abstract

The methanolic crude extracts of three weeds, Soapbush or Koster's Curse (*Clidemia hirta*), Spanish Flag or West Indian Lantana (*Lantana camara*), and Indian rhododendron (*Melastoma malabathricum*) were studied for their antibacterial properties. The crude extracts were tested against ten bacterial isolates from giant freshwater prawn larvae, *Macrobrachium rosenbergii*. *Clidemia hirta* extract showed the best MIC and MBC values against *Vibrio alginolyticus* which were 0.39 mg/ml and 6.25 mg/ml, respectively. Following that, three lowest concentration of methanolic crude extracts; 0.195 mg/ml, 0.39 mg/ml, and 0.78 mg/ml, were added to *V. alginolyticus* culture and observed for morphological changes via Scanning Electron Microscope (SEM). The cell wall of *V. alginolyticus* was disrupted at 0.195 mg/ml of methanolic crude extract. Antioxidant activities of *C.hirta* was also studied qualitatively and quantitatively and compared to the standard reference, Vitamin C. Thin Layer Chromatography (TLC) performed on the *C. hirta* extract indicated the presence of eight separation compound bands. The phytochemical of *C. hirta* extract was positive for tannin, flavonoid, saponin, terpenoid and negative for steroids compounds.

Keywords: *Clidemia hirta, Lantana camara, Melastoma malabathricum,* phytochemical analysis, SEM, *Vibrio alginolyticus*

[1]*Department of Aquaculture, Faculty of Fisheries and Aqua- Industry, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia*
[2]*Terengganu Fisheries Department, Chendering Fisheries Park, 21080 Kuala Terengganu, Terengganu*
**Correspondence author E-mail: najiah@umt.edu.my*

บทคัดย่อ

ผลของสารสกัดสมุนไพรด้วยเมธานอลต่อแบคทีเรียที่แยกจากลูกกุ้งก้ามกราม (*Macrobrachium rosenbergii*)

Najiah Musa [1] Nadirah Musa[1] Wan Nurhafizah Ibrahim[1] Mohd Zahrol Ariff Shariat[1]*
Arief Izzairy Zamani[1] Muhd Ranzi Abdullah[1] Tee Lea Wee[1] Mariam Marip[2] Laith Abdul Razak[1]
Amar Sakhawi Awang Soh[1]

การศึกษานี้มีวัตถุประสงค์ เพื่อศึกษาฤทธิ์ในการต้านแบคทีเรียของสารสกัดเมธานอลจากสมุนไพร 3 ชนิด ได้แก่ Soapbush หรือ Koster's Curse (*Clidemia hirta*), Spanish Flag หรือ West Indian Lantana (*Lantana camara*) และ Indian rhododendron (*Melastoma malabathricum*) สารสกัดที่ได้นำมาทดสอบกับแบคทีเรีย 10 ชนิดที่แยกได้จากลูกกุ้งก้ามกราม (*Macrobrachium rosenbergii*) พบว่าสารสกัดจาก *Clidemia hirta* มีค่า MIC และ MBC ต่อ *Vibrio alginolyticus* ดีที่สุด คือ 0.39 มก./มล. และ 6.25 มก./มล. ตามลำดับ จากข้อมูลดังกล่าวความเข้มข้นต่ำที่สุด 3 ค่าได้แก่ 0.195, 0.39 และ 0.78 มก./มล. จึงถูกเลือกมาใส่ในจานเลี้ยง *V. alginolyticus* และศึกษาการเปลี่ยนแปลงรูปร่างด้วยกล้องจุลทรรศ์อิเล็คตรอนแบบส่องกราด (Scanning Electron Microscope) พบว่าผนังเซลล์ของ *V. alginolyticus* ถูกทำลายเมื่อใช้สารสกัดเมธานอลเข้มข้น 0.195 มก./มล. นอกจากนั้นยังศึกษาฤทธิ์ในการต้านอนุมูลอิสระแบบคุณภาพและปริมาณของ *C. hirta* เปรียบเทียบกับสารมาตรฐาน คือ ไวตามิน ซี ผลการทดสอบสารสกัด *C. hirta* ด้วยเทคนิคโครมาโตกราฟีแบบแผ่นบาง (Thin Layer Chromatography) พบว่าประกอบด้วยแถบสารประกอบ 8 ชนิด และการทดสอบสารพฤกษเคมีของสารสกัด *C. hirta* พบประกอบด้วยแทนนิน ฟลาโวนอยด์ ซาโปนิน เทอร์ปีนอยด์ และไม่พบสารประกอบกลุ่มสเตียรอยด์

คำสำคัญ: *Clidemia hirta Lantana camara Melastoma malabathricum* การทดสอบสารพฤกษเคมี *Vibrio alginolyticus* กล้องจุลทรรศ์อิเล็คตรอนแบบส่องกราด

[1]*Department of Aquaculture, Faculty of Fisheries and Aqua- Industry, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia*
[2]*Terengganu Fisheries Department, Chendering Fisheries Park, 21080 Kuala Terengganu, Terengganu*
***ผู้รับผิดชอบบทความ E-mail:** najiah@umt.edu.my*

Introduction

Bacterial infection is a major cause of mortality in intensive cultures of Giant freshwater prawn, *M. rosenbergii* larvae (Al-Harbi and Uddin, 2004). *Vibrio spp*. was mostly isolated from prawn larvae during epizootics of mid–cycle larval disease (Brock, 1983). They were reported to be the causative agent of luminescent vibriosis which caused high mortalities in *M. rosenbergii* hatcheries (Jayaprakash et al., 2006). Among the *Vibrio spp*., *Vibrio alginolyticus* has been described as a principal pathogen of both penaeids and nonpenaeids (Mohney et al., 1994; Lee et al., 1996). For instance, in India antibiotic resistance in Giant freshwater prawn farming has been reported with more than 95% of bacterial isolates resistant to erythromycin and oxytetracycline (Sahul-Hameed et al, 2003). This issue has led to the use of alternative treatment from natural sources like plant extract (Alzoreky and Nakahara, 2003).

Koster's Curse (*Clidemia hirta*), Straits Rhododendron (*Melastoma malabathricum*) and Lantana Weed (*Lantana camara*) are commonly known as weeds. There were previous studies suggesting antibacterial and antioxidant properties in *M. malabathricum* (Susanti et al., 2007) and *L. camara* (Joseph et al, 2005). At the moment, *C. hirta* has not fully been explored for its compounds that are responsible for these purposes. In this study, the biological activities of *C. hirta, M. malabathricum* and *L. camara* were examined for their antibacterial properties against *Vibrio spp*. The weed with the best antibacterial properties was further examined for antioxidant properties (Mensor et al., 2001), phytochemical chemistry (Chopra et al., 2007), as well as its potential dosage for disruption of bacterial cell (Mengoni et al., 2004).

Materials and Methods

Weed materials and extraction: Fresh *C. hirta, M. malabathricum* and *L. camara* were collected in Terengganu area, Malaysia. The extraction was done according to Abdul et al. (1995).

Bacterial culture: Ten bacterial species were used, namely *Vibrio alginolyticus, Vibrio cholerae, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas putida, Aeromonas hydrophila, Morganella morganii, Acinetobacter baumannii, Acinetobacter lwoffii*, and *Stenotrophomonas maltophilia*. These bacteria were previously isolated and identified from diseased *Macrobrachium rosenbergii* larvae. They were cultured in Tryptone Soy Broth (TSB) (Oxoid, England) for 24 hours at 270C in an incubator shaker (Certomat, UK). All bacterial cultures were adjusted according to 0.5 McFarland Standard equivalent to $1.5x10^8$ CFU/ml for the next experiment.

Hole-plate Diffusion Method (Screening for Antibacterial Properties): Hole-plate diffusion method was used (Bennet et al., 1966). The best weed crude extracts which possess the best antibacterial properties was selected for further tests.

Minimum Inhibitory Concentration (MIC) Assay: MIC is considered as the lowest concentration of the sample that prevents visible bacterial growth. MIC of the weeds extract against selected bacteria was determined according to the methods of Cristina et al. (2004).

Minimum Bactericidal Concentration (MBC) Assay: MBC values were indicated by the absence of bacterial growth on the medium. The suspension from MIC test was streaked onto Mueller-Hinton Agar medium (Merck, Germany) and incubated for 24 hours at 37^0C.

Antioxidant Activity Assay: The method of antioxidant was based on Farhana et al. (2009). Antioxidant activities were determined based on their scavenging potential of the stable DPPH free radical in both qualitative (Sadhu et al., 2003) and quantitative assays. The radical scavenging activity was expressed as the inhibition percentage and monitored as per the equation following Mensor et al. (2001).

Phytochemical Analysis - Thin Layer Chromatography: TLC analysis was done according to Nalina and Rahim (2007). TLC was used to analyze the methanolic crude extract of *C. hirta* (10 mg) on silica Gel G plate (20x10 cm) (Macherey - Nagel) using a solvent ratio system.

Identification of Bioactive Compounds: Chemical tests for detection of tannins, saponin, flavonoid, terpenoid and steroid were carried out on the methanolic extract of weed via standard procedures to identify the constituents as described by Edeoga et al. (2005).

Bacterial Cell Disruption: The extracts were made in triplicate at different concentrations of 0.195 mg/ml, 0.39 mg/ml and 0.78 mg/ml, respectively. Bacterial membrane disruption was determined by the method of Suhaila et al. (2009). Cellular leakages of bacterial disruption were observed by using method of Anam et al. (2010). Then it underwent a standard process for scanning electron microscopy observation (Kelly, 1973).

Results and Discussion

Antibacterial Properties of selected weeds: The strong antibacterial properties were displayed by *C. hirta*'s leaf which was sensitive against 10 bacterial isolates (Table 1). The MIC value was found at 0.39 mg/ml (Table 2). The result for MBC indicates that there was no bacterial growth at 6.25 mg/ml for *Vibrio alginolyticus* and *Stenotrophomonas maltophila*. In this study, the crude extract of *C. hirta* showed strong antibacterial activity compared to the other weeds. *Lantana camara* was found to be inactive for its antibacterial screening properties. The result was different from Badakhshan et al. (2009) who stated that *L. camara* extracts have the best action against *Salmonella typhi* and *Bacillus cereus*. This could be due to different contents of bioactive compounds in the weed like flavonoids and tannins as well as different concentrations and methods used in each study (Kumar et al., 1997). Antibacterial result for *C. hirta* and *M. malabathricum* were alike as both originated from the same family, Melastomaceae. In general, Vibrios are very sensitive to acids and its sensitivity is varied from species to species (Koga et al., 1994). This might be the reason why it possesses the lowest value of MBC and MIC compared to the other bacterial species.

Table 1 Antibacterial properties of the methanolic crude extract of the weeds

Bacterial	Extracts							
	Clidemia hirta		*Melastoma malabathricum*			*Lantana camara*	**MeoH**[a]	**TE 10** [b]
	Leaf	Stem	Leaf	Stem	Flower	Leaf		
Proteus vulgaris	**+++**	+++	++	++	+++	+	++	+++
Pseudomonas aeruginosa	**+++**	+++	++	+++	++	+	++	+
Pseudomonas putida	**+++**	++	++	++	+	+	++	++
Vibrio alginolyticus	**+++**	+++	++	++	+++	+	++	+++
Vibrio cholerae	**+++**	++	+	++	++	+	++	+++
Acinobacter baumanii	**+++**	+++	++	++	+++	+	++	+++
Acinobacter lwoffi	**+++**	+++	++	+++	+++	+	++	+++
Aeromonas hydrophila	**+++**	+++	+	++	++	+	++	+
Stenotrophomonas maltophila	**+++**	+++	++	+	+++	+	++	+++
Morganella morganii	**+++**	+++	+	+++	+++	+	++	++

[a] Methanol solvent used as a negative control , [b]Tetracycline antibiotic disc used as positive control
+++ : Sensitive, ++ : Intermediate, +: Resistant

Table 2 MIC and MBC of *C. hirta* against ten bacterial species

Bacteria	MIC[a] (mg/ml)	MBC[b] (mg/ml)
Proteus vulgaris	1.56	50.00
Pseudomonas aeruginosa	6.25	12.50
Pseudomonas putida	3.12	25.00
Vibrio alginolyticus	0.39	6.25
Vibrio cholerae	0.78	50.00
Acinobacter baumanii	3.12	50.00
Acinobacter lwoffi	3.12	12.5
Aeromonas hydrophila	6.25	50.00
Stenotrophomonas maltophila	1.56	6.25
Morganella morganii	6.25	25.00

[a]Minimum Inhibition Concentration,
[b]Minimum Bactericidal Concentration

Antioxidant Activities of C. hirta: TLC plate showed yellowish colouration on purple background of the TLC plate after spraying with 2, 2-diphenyl-1-picrylhydrazyl (DPPH). This shows the occurrence of chemical compounds with bleaching activity. Further quantitative analysis indicated that methanolic extract of *C. hirta* showed strongest activity with 50.2% compared to vitamin C, 20.0% at 0.4 mg/ml. Scavenging activities *C. hirta* indicate the extract is a potent scavenger of radical-related damage. Antibacterial activities are often linked to the secondary metabolites such as flavanoid compound which is one of the responsible compounds contributing to the highest antioxidant effect in some plants (Lamb and Cushine, 2005). In this study, both flavanoids and tannins were present in *C. hirta.*

Phytochemical Analysis via TLC: Eight separation bands of *C. hirta* compounds were observed using three solvents mixture, Ethyl Acetate: Methanol: Water with ratio of 7: 2: 1. Phytochemical analysis was positive for phenolic compounds such as tannins, flavanoids, saponins and terpenoids, except steroids (Table 3). The presence of flavanoids and tannins in *C. hirta* is likely to be responsible for the free radical scavenging effect observed in the extracts. Flavanoids and tannins are both phenolic compounds which act as a primary antioxidants or free radical scavengers (Ayoola et al., 2008). The presence of tannins in *C. hirta* might also be responsible for the antibacterial activity (Al-Genaidy, 1993; Chopra et al., 2007). The absence of steroids in *C. hirta* could be due to insufficient quantities of steroids in the crude extracts (Taylor et al., 2001).

SEM of C. hirta methanolic crude extract against V. alginolyticus: In SEM, at 0.195 mg/ml methanolic crude extract of *C. hirta*, the bacterial surface started to disrupt by showing a deep roughening of cell surface, the collapse of cell structure, leaving transparent look and flat, resembling of 'Ghost Cells'. SEM analysis showed that *C. hirta* was capable of inhibiting the growth of *V. alginolyticus* at 0.195 mg/ml by disrupting the cell surface. The cell was fully disrupted at the concentration of 0.78 mg/ml. No study has been carried out on the effect of these weeds against *V. alginolyticus* as observed by SEM, therefore no comparison could be made. Mangoni et al. (2004) observed that by increasing the concentration of microbial peptide, most *E. coli* cells were transparent, empty and flat, with cell debris arising from them.

Table 3 Qualitative phytochemical screening of *C. hirta* methanolic crude extracts.

Phytochemical Test	Result
Tannins	Present
Flavonoids	Present
Saponins	Present
Terpenoids	Present
Steroids	Absent

C. hirta could serve as an alternative to antibiotics especially in *M. rosenbergii* farming which is highly exposed to bacterial diseases. The present study shows that this weed possesses potential properties of antibacterial and antioxidant activities with several bioactive compounds. Further studies are needed to isolate, identify, characterize, and elucidate the structure of bioactive compounds of *C. hirta.*

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