STUDY OF CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY FROM THE LEAVES AND TWIGS OF *Momordica charantia* L.

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TÓM TẮT

KHẢO SÁT THÀNH PHẦN HÓA HỌC VÀ HOẠT TÍNH KHÁNG KHUẨN CỦA THÂN VÀ LÁ CÂY MƯỚP ĐẮNG (*Momordica charantia* L.)

Nghiên cứu này nhằm mục đích bước đầu khảo sát hoạt tính kháng khuẩn của các cao chiết từ thân và lá cây Mướp đắng, lựa chọn cao chiết có hoạt tính tốt nhất để khảo sát thành phần hóa học nhằm tìm hiểu mối liên hệ giữa hoạt tính sinh học và cấu trúc hóa học của các hợp chất có trong cây Mướp đắng (Momordica charantia L.). Kết quả cho thấy cao chiết ethyl acetate thể hiện hiệu quả kháng khuẩn tốt theo phương pháp khuếch tán đĩa thạch trên các chủng vi khuẩn là tác nhân gây bệnh chính cho các loài thủy sản. Cao chiết ethyl acetate đều kháng lại 03 dòng vi khuẩn Aeromonas hydrophila, Aeromonas dhakensis, và Vibrio parahaemolyticus với giá trị MIC ≤ 0,3125 mg/mL. Kết quả bước đầu khảo sát thành phần hóa học cho thấy, từ cao phân đoạn ethyl acetate của thân và lá cây Mướp đắng, 03 hợp chất đã được phân lập và nhận danh là 3β,7β,25-trihydroxycucurbita-5, (23E)-dien-19-al (1), protocatechuic acid (2), và afzelin (3). Cấu trúc hóa học của các hợp chất này được xác định bằng phương pháp phổ NMR và so sánh với các tài liệu đã công bố. Đồng thời, hợp chất (1) và (3) phân lập được cũng cho hiệu quả kháng khuẩn tốt đối với khuẩn Aeromonas hydrophila. Những kết quả này cho thấy thân và lá cây Mướp đắng là một dược liệu tiềm năng chứa các hợp chất thể hiện hoạt tính kháng khuẩn tốt.

Từ khóa: *Cây Mướp đắng, kháng khuẩn, thành phần hóa học.*

1. INTRODUCTION

Momordica charantia L. (bitter melon) is a plant belonging to the Cucurbitaceae family and is widely distributed in tropical and subtropical areas around the world. This plant has been related to many therapeutic applications in folk medicine [1]. Phytochemical studies revealed the presence, in its structure, of glycosides, saponins, alkaloids, triterpenes, proteins and steroids, which are considered biologically active compounds [2, 3]. Bitter melon is a good source of phenolic compounds, including gallic acid, genistic acid, catechin, epicatechin, caffeic acid, chlorogenic acid, *p*-coumaric acid, and ferulic acid [4]. Bitter melon also has saponins, peptides, and alkaloids.

These bioactive compounds potentially impart a wide range of health benefits [5]. Bacterial resistance to antibiotics is a health problem that has spread throughout the world. Bacterial resistance against antibiotic, especially in the field of fisheries is mainly caused by the wide usage of antibiotics, allowing the bacteria to mutate to adapt against the antibiotic [6]. This problem can be handled by phytopharmaco; one of them is the bitter melon (*Momordica charantia* L.) which has antibacterial property.

Therefore, the aim of this study is to find out the antibacterial activity of the bitter melon (*Momordica charantia* L.) and investigate the chemical components of this plant.

2. EXPERIMENTAL

2.1 Chemicals and reagents

NMR spectra were recorded on a Bruker AM500 FTNMR spectrometer (Bruker, Karlsruhe, Germany) using TMS as an internal standard at Institute of Chemistry - Vietnam Academy of Science and Technology, Hanoi, Vietnam. TLC was performed on silica gel 60 F_{254} (0.063–0.200mm, Merck, Germany). The zones were detected by UV lamp at 254 or 365 nm or a solution of $FeCl_3$/EtOH or H_2SO_4/EtOH. Column chromatography was performed on silica gel (240-430 mesh, Merck, Germany), ODS (70-230 mesh, Merck, Germany).

Solvents utilized including *n*-hexane, chloroform, ethyl acetate, methanol (purity ≥ 99.0%), and ethanol 96% were purchased from Chemsol company (Vietnam).

2.2 Sample treatment and preparation

The leaves and twigs of *Momordica charantia* L. were collected on February 2023 from Soc Trang city and authenticated by Dr. Nguyen Thi Kim Hue, a biologist at the Department of Biology, College of Natural Sciences, Can Tho University. A voucher specimen is kept under the number: MoC010323.

The sample was then washed away from muds and dust, the rotten and damaged parts were also discarded. The raw materials were left to dry in the shade at room temperature for few days and then dried in an oven at about 50°C until well-dried.

2.3 Extraction and isolation

The well-dried plant was ground into powder (3.0 Kg) which was then soaked in 96% ethanol at room temperature four times (20 L/time) and filtered. The solvent was evaporated under vacuum to give 450 g of crude extract. This extract was suspended in water followed by extraction with *n*-hexane, ethyl acetate and methanol for many times consecutively; then evaporated under vacuum conditions to give the residues of *n*-hexane (120 g), ethyl acetate (60 g), and methanol extract (90 g). The ethyl acetate fraction (60 g) was chromatographed on silica gel column using *n*-hexane, gradient of *n*-hexane/EtOAc up to 100% EtOAc and then followed by gradient of EtOAc/MeOH up to 100% MeOH to collect 08 fractions (EE1-8). Fraction EE2 was rechromatographed by column chromatography with *n*-hexane/EtOAc (9:1 to 5:5), yielding a compound that was crystallized from MeOH, to afford 50 mg of compound **1**.

Fraction EE7 was separated by a silica gel column and eluted with *n*-hexane/EtOAc (from 1:1 to 0:100, v/v) to yield 12 subfractions (EE7.1-12). Subfraction EE7.7 was further chromatographed on silica gel CC, eluted with $CHCl_3$: MeOH (from 10:1 to 5:1, v/v) to obtain three subfractions (E7.7.1-3). At last, compound **2** (10 mg) was obtained from subfraction EE7.7.2.

Similarly, rechromatography of fraction EE8 using the solvent system as stated above and fractions collected at 15 mL interval, compound **3** (35 mg) crystallized out from fraction 04 of this column.

2.4 Antibacterial activity

In the present study, the antibacterial activity of extracts and isolated compounds from *M. Charantia* was assessed using the agar well diffusion method by measuring the diameter of growth inhibition zones and minimal inhibitory concentration (MIC).

Antimicrobial activity was evaluated in triplicates, with three standard bacterial strains acquired from Research Institute for Aquaculture No. 2, Vietnam including *Aeromonas dhakensis*, *Aeromonas hydrophila* and *Vibrio parahaemolyticus* which are major pathogens for the aquaculture industry. Bacteria were grown in tryptic soy agar (TSA, Acumedia, USA), during 24 h at 37°C. From each culture, a cell suspension was prepared with an aqueous 0.85% NaCl solution and adjusted to 0.5 MacFarland turbidity. In the next step, a swab was used to inoculate bacteria on the surface of Mueller-Hinton agar (Merck, Germany) plates (95 × 15 mm). Subsequently, 50 µL of each sample (dissolved in DMSO) were deposited in 9 mm diameter holes made on the agar medium. All plates were incubated at 37°C for 24 h. After this period, those samples affording inhibition zones around the holes were considered active. Cefixim (Sigma, USA) and DMSO were employed as positive and negative controls, respectively.

To determine minimal inhibitory concentration (MIC), a broth microdilution assay was employed, using Mueller-Hinton broth (MHB, Biolife, Italy) and standard bacterial inoculums (1.5×106 CFU/mL). Two-fold serial dilutions were prepared to final concentrations ranging from 0.3 to 5.0 µg/mL for extracts and from 8 to 128 µg/mL for pure compounds. After 24 h incubation for at 37°C, the experiment was evaluated and 10 µL of sample were withdrawn from the content of each well with no visible bacterial growth and subcultured in TSA. MIC was defined as the lowest concentration of the tested substance that prevented a visible bacterial growth [7].

2.5 Statistical analysis

The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA). Results were calculated from three independent experiments and are shown as mean ± SD, n=3. Results were considered as statistically significant when p value was lower than 0.05.

3. RESULTS AND DISCUSSION

3.1. Structural elucidation

The structures of isolated compounds were characterized by NMR spectra and comparison with literature data.

3.1.1 Compound 1

Compound **1** was obtained as colorless needles, m.p. 190-192°C

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 9.90 (1H, s, H-19); 5.93 (1H, d, *J* = 5.5 Hz, H-6); 5.61 (1H, m, H-24); 5.60 (1H, m, H-23); 4.02 (1H, d, *J* = 5.0 Hz, H-7); 3.57 (1H, s, H-3); 2.59 (1H, m, H-2b); 2.20 (1H, m, H-11b); 2.18 (1H, m, H-22b); 1.97 (1H, m, H-16b); 1.96 (1H, s, H-16a); 1.75 (1H, m, H-22a); 1.72 (1H, m, H-1b); 1.69 (1H, m, H-2a); 1.61 (1H, m, H-11a); 1.57 (1H, m, H-1a); 1.54 (1H, m, H-20); 1.40 (1H, m, H-15b); 1.40 (1H, m, H-12b); 1.38 (1H, m, H-15a); 1.31 (1H, s, H-12a); 1.31 (3H, s, H-26); 1.28 (3H, s, H-27); 1.27 (3H, s, H-28); 1.10 (3H, s, H-29); 0.97 (3H, d, *J* = 5.5 Hz, H-21); 0.94 (3H, s, H-18); 0.84 (3H, s, H-30).

^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 209.7 (C-19); 147.3 (C-5); 140.9 (C-24); 125.8 (C-23); 124.0 (C-6); 77.1 (C-3); 71.2 (C-25); 66.9 (C-7); 51.3 (C-17); 51.1 (C-8); 51.1 (C-9); 50.8 (C-14); 46.6 (C-13); 42.3 (C-4); 40.3 (C-22); 37.7 (C-10); 37.6 (C-20); 35.7 (C-15); 30.1 (C-2); 30.1 (C-12); 30.0 (C-26); 29.8 (C-27); 28.5 (C-16); 27.8 (C-29); 26.0 (C-28); 23.3 (C-11); 22.2 (C-1); 19.2 (C-21); 18.8 (C-30); 15.4 (C-18).

3.1.2 Compound 2

Compound **2** was obtained as brown powder, m.p. 201-203°C

^{1}H-NMR (500 MHz, acetone-d_6), δ_H (ppm): 7.53 (1H, d, *J* = 2.0 Hz, H-2); 7.48 (1H, dd, *J* = 8.5 and 2.0 Hz, H-2′); 6.90 (1H, d, *J* = 8.5 Hz, H-5).

^{13}C-NMR (125 MHz, acetone-d_6), δ_C (ppm): 167.6 (C-7); 150.7 (C-4); 145.6 (C-3); 123.6 (C-6); 123.1 (C-1); 117.5 (C-2); 115.7 (C-5).

3.1.3 Compound 3

Compound **3** was obtained as a yellow solid, m.p. 177-179°C.

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 7.78 (2H, d, *J* = 8.5 Hz, H-2′, 6′); 6.95 (2H, d, *J* = 8.5 Hz, H-3′, 5′); 6.39 (1H, d, *J* = 2.0 Hz, H-8); 6.22 (1H, d, *J* = 2.0 Hz, H-6); 5.39 (1H, d, *J* = 1.5 Hz, H-1″); 4.24 (1H, dd, *J* = 3.5 and 1.5 Hz, H-2″); 3.73 (1H, dd, *J* = 9.0 and 3.5 Hz, H-3″); 3.34 (1H, m, H-4″); 3.34 (1H, m, H-5″); 0.94 (3H, d, *J* = 5.5 Hz, H-6″).

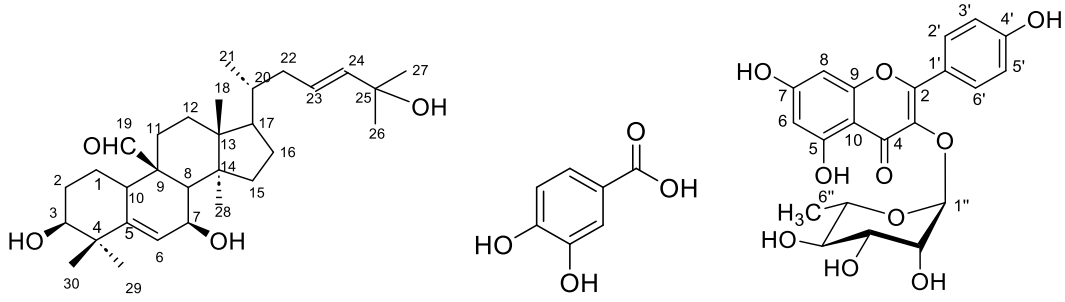
^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 179.6 (C-4); 166.0 (C-7); 163.2 (C-5); 161.6 (C-4′); 159.3 (C-9); 158.6 (C-2); 136.2 (C-3); 131.9 (C2′, 6′); 122.7 (C-1′); 116.5 (C3′, 5′); 105.9 (C-10); 103.5 (C-1″); 99.9 (C-6); 94.8 (C-8); 73.2 (C-4″); 72.2 (C-2″); 72.0 (C-3″); 71.9 (C-5″); 17.7 (C-6″).

Compound (**1)** was obtained as colorless needles. The typical ^{13}C-NMR signals of CHO group (δ_C 209.7) and two isolated C=C bonds (δ_C 124.0 (d) and 147.3 (*d*); δ_C 125.8 (m) and 140.9 (m), in addition to the resonances of seven Me groups (δ_C 15.4, 18.8, 19.2, 26.0, 27.8, 29.8, and 30.0), seven CH_2 groups (δ_C 22.2, 23.3, 28.5, 30.1, 30.1, 35.7, 40.3), six CH groups (δ_C 37.6, 37.7, 51.1, 51.3, 66.9, and 77.1), and five quaternary C-atoms (δ_C 42.3, 46.6, 50.8, 51.1, and 71.2) revealed that (**1)** is a cucurbita-5,23-dienetype triterpene. Moreover, a downfield shift of two Me groups at δ_H 1.28 (3H, s, H-27), and δ_H 1.31 (3H, s, H-26), indicating that C-25 position [δ_C 71.2] was the site of hydroxyl group. From these evidences and by comparison with literature data [8], the structure of compound **(1)** was identified as 3*β*,7*β*,25-trihydroxycucurbita-5, (23E)-dien-19-al.

The ^{1}H-NMR spectrum of compound (**2)** demonstrated two protons doublets at δ 6.90 (1H, *J* = 8.5 Hz) and δ 7.53 (1H, *J* = 2.0 Hz) and one double doublet proton signal at δ 7.48 (1H, *J* = 8.5/2.0 Hz). The appearance of two doublets and their coupling constant values are further in agreement with the presence of a trisubstituted aromatic nucleus in 1, 3, 4-positions. The ^{13}C-NMR exhibited 7 carbon signals. The DEPT- 135 spectrum showed that it possessed 4 quaternary carbons. The ^{13}C-NMR spectrum showed a downfield signal at δ 167.6 clearly assigned to carbonyl carbon. Therefore, the structure of compound (**2**) was established to be 3,4-dihydroxybenzoic acid or protocatechuic acid [9].

The ^{1}H-NMR spectrum of compound (**3)** displayed that protons in B ring gave a doublet signal (2H, d, *J* = 8.5 Hz) at δ 7.78 and 6.95, suggested the presence *p*-disubstituted benzene ring. Protons in ring A were observed at δ 6.39 (1H, d, *J* = 2.0 Hz) and 6.22 (1H, d, *J* = 2.0 Hz), respectively, suggested the presence of *meta*-aromatic protons at H-8 and H-6. In addition, there is the presence of a secondary methyl group with a doublet signal at δ 0.94 (3H, d, *J* = 5.5 Hz) and the signals of oxymethine groups at δ_H 3.34-4.24. The ^{13}C-NMR spectrum showed 21 carbon signals, which were classified by their chemical shifts and the DEPT spectra as one oxygenated sp^3 methylene, four oxygenated sp^3 methines, one anomeric carbon, six sp^2 methines, eight sp^2 quaternary carbons and one carbonyl group. Based on these observations, it can conclude that compound **3** is consist a flavonoid skeleton of kaempferol as an aglycone moiety and rhamnoside as a sugar unit. The anomeric proton had a coupling constant of 1.5 Hz, conforming the *α*-orientation of L-rhamnosyl moiety. The position of *α*-L-rhamnosyl moiety in **3** was identified at C-3 on the basis of HMBC correlations. Based on the above spectral data, structure **3** was identified as kaempferol-3-*O*-*α*-L-rhamnoside consistent with previous report [10], which was reported for the first time from *M. charantia*.

Three compounds **1-3** were isolated and identified from the leaves and twigs of *Momordica charantia* L., including 3*β*,7*β*,25-trihydroxycucurbita-5, (23*E*)-dien-19-al (**1**), protocatechuic acid (**2**), and afzelin (**3**) by analysis of their NMR spectra and comparison with literature data (Figure **1**).



3*β*,7*β*,25-trihydroxycucurbita-5,(23E)-dien-19-al (1) **Protocatechuic acid (2)** **Afzelin (3)**

*Figure 1. Chemical structures of compounds **1–3**.*

3.2 *In vitro* antibacterial activity results

The bacterial infections are considered as the major cause of mortality in aquaculture. Due to the growing bacterial resistance against commercial standard and reserve antibiotics, the search for new active substances with antibacterial activity against pathogenic bacteria is of increasing importance [11]. The medicinal plants may be used as potential and promising drugs against fish pathogens in the aquaculture [12]. In this study, *M. charantia* extracts and isolated compounds were screened for antibacterial activity against three bacterial strains including *A. hydrophila*, *A. dhakensis*, and *V. parahaemolyticus* which commonly occur in aquaculture sector [13]. The extracts of *M. charantia* exhibited broad spectrum activity against the pathogenic bacteria in the range of 15.13 to 28.61 mm (Table 1). The results obtained from the disc diffusion method indicated that the ethyl acetate extract of *M. charantia* exhibited a stronger antibacterial activity against the tested fish pathogenic bacteria in comparison with the other extracts. The best antibacterial activity was determined against *A. hydrophila* (28.61 mm) among the tested pathogenic bacteria. In addition, both compound 3*β*,7*β*,25-trihydroxycucurbita-5, (23*E*)-dien-19-al and afzelin inhibited the growth of *A. hydrophila* with the value of MIC ≤ 8 μg/mL. Türker *et al.* reported that *A. hydrophila*, a Gram negative fish pathogenic bacterium, appeared to be more susceptible to the plant extracts used in their experiments [14]. Direkbusarakom *et al.* also reported that among 16 Thai traditional herbs, ethanol extracts of *M. charantia* and *P. gaujawa* displayed the highest activity against *Vibrio harveyi* and *V. parahaemolyticus*, and MIC value of *M. charantia* was found to be 1.25 mg/mL [15].

Table 1. Zone of inhibition and MIC of extracts and isolated compounds

Extracts and isolated compounds		**Diameter of growth inhibition zones at different concentrations**					**MIC (mg/mL)**
Extracts (mg/mL)		**0.3125**	**0.625**	**1.250**	**2.500**	**5.000**	
Crude extract	*A. hydrophila*	18.63^{e}±0.31	19.77^{c}±0.42	20.43^{c}±0.60	20.63^{b}±0.14	22.49^{a}±0.64	MIC≤0.3125
	A. dhakensis	19.87^{e}±0.17	20.83^{d}±0.26	21.48^{c}±0.51	21.71^{b}±0.18	24.16^{a}±0.22	MIC≤0.3125
	V. parahaemolyticus	18.34^{e}±0.16	19.46^{d}±0.24	20.49^{c}±0.32	22.71^{a}±0.09	21.90^{b}±0.40	MIC≤0.3125
***n*-Hexane extract**	*A. hydrophila*	15.13^{d}±0.22	15.29^{d}±0.10	16.43^{c}±0.25	17.66^{b}±0.29	19.58^{a}±0.24	MIC≤0.3125
	A. dhakensis	18.23^{c}±0.26	15.84^{d}±0.18	21.22^{b}±0.27	22.83^{a} ±0.34	15.69^{d}±0.25	MIC≤0.3125
	V. parahaemolyticus	17.85^{d}±0.32	18.20^{c}±0.20	19.75^{b}±0.15	19.93^{b}±0.34	20.23^{a}±0.27	MIC≤0.3125
Ethyl acetate extract	***A. hydrophila***	**21.73^{d}±0.23**	**24.37^{c}±0.32**	**26.80^{b}±0.23**	**28.33^{a}±0.34**	**28.61^{a}±0.17**	**MIC≤0.3125**
	A. dhakensis	**20.27^{e}±0.29**	**21.67^{d}±0.35**	**23.33^{c}±0.18**	**27.13^{a}±0.31**	**26.78^{b}±0.14**	**MIC≤0.3125**
	V. parahaemolyticus	**22.03^{c}±0.13**	**22.53^{c}±0.23**	**24.83^{b}±0.25**	**24.30^{b}±0.20**	**25.23^{a}±0.40**	**MIC≤0.3125**

Extracts and isolated compounds		Diameter of growth inhibition zones at different concentrations					MIC (mg/mL)
Isolated compounds (µg/mL)		8	16	32	64	128	MIC (µg/mL)
3*β*,7*β*,25-trihydroxy cucurbita-5, (23*E*)-dien-19-al	***A. hydrophila***	**16.55^{d}±0.41**	**17.45^{c}±0.14**	**19.34^{b}±0.22**	**20.03^{a}±0.27**	**19.71^{b}±0.26**	**MIC≤8**
	A. dhakensis	14.82^{d}±0.45	17.26^{c}±0.29	19.48^{b}±0.35	19.90^{b}±0.37	21.57^{a}±0.26	MIC≤32
	V. parahaemolyticus	14.40^{c}±0.40	17.35^{b}±0.25	20.71^{a}±0.27	20.45^{a}±0.22	20.47^{a}±0.33	MIC≤8
Afzelin	***A. hydrophila***	**14.65^{e}±0.18**	**15.23^{d}±0.23**	**17.68^{c}±0.32**	**24.06^{b}±0.25**	**25.13^{a}±0.31**	**MIC≤8**
	A. dhakensis	12.11^{e}±0,12	14.17^{d}±0,96	15.13^{c}±0,14	16.43^{b}±0,16	17.31^{a}±0,18	MIC≤8
	V. parahaemolyticus	11.42^{d}±0,17	11.72^{d}±0,08	12.44^{c}±0,27	14.85^{b}±0,30	15.86^{a}±0,21	MIC≤8
Cefixim	*A. hydrophila*	22.33^{c}±0.58	23.00^{c}±1.00	26.67^{b}±2.31	30.67^{a}±1.53	31.67^{a}±0.58	MIC≤8
	A. dhakensis	-	-	-	9.67^{b}±0.58	14.33^{a}±0.58	64
	V. parahaemolyticus	-	-	-	10.00^{b}±0.00	12.67^{a}±0.58	64

Note: Values are presented as means ± SE (n=3). Different letters (a, b, c, d, e) in the same row show sinificant difference at the level of 0.05. The results were statistically analyzed using Minitab 16 software (ANOVA-Turkey's).

Similarly, Muniruzzaman and Chowdhury indicated that MIC values of *M. charantia* leaves ethanol extracts were 1.2 mg/mL against *A. hydrophila* and *Pseudomonas flourescens*, and 2.5 mg/mL against *Edwardsiella tarda*, which were fish pathogenic bacteria [16]. Previous studies have also demonstrated that *M. charantia* is very rich in triterpenes, proteins, and steroids [17]. It is speculated that the antimicrobial activities of triterpenes depend on interactions between their lipid components and the net surface charge of microbial membranes. Furthermore, the drugs might cross the cell membranes, penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity [18]. *M. charantia* is frequently used in folk medicine in Viet nam. To the best of our knowledge, we firstly report the antimicrobial activity of *M. charantia* against three pathogenic bacteria: *A. dhakensis*, *A. hydrophila* and *V. parahaemolyticus*. The results showed that all the extracts exhibited varying degrees of antimicrobial activity on the microorganisms tested. The ethyl acetate extract of *M. charantia* may be used as an alternative to antibiotics in aquaculture sector.

4. CONCLUSION

Based on the results of the present study, it can be concluded that different fractional extracts of *M. charantia* have variable antibacterial activity. The best antibacterial activity was determined against *A. hydrophila* is ethyl acetate extract. This extract of *M. charantia* showed the presence of 3*β*,7*β*,25-trihydroxycucurbita-5, (23*E*)-dien-19-al (**1**), protocatechuic acid (**2**), and afzelin (**3**). In addition, compound (**1**) and (**3**) isolated from *M. charantia* was characterized and evaluated for their antibacterial activities against *A. hydrophila* with good results. This plant may be a potential source of a natural preservative in pharmaceutical industry.

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