THE CHEMICAL COMPOSITION AND IMMUNO-MODULATORY POTENTIAL ON STRIPED CATFISH (PANGASIANODON HYPOPHTHALMUS) OF EUPHORBIA HIRTA L. AND PSIDIUM GUAJAVA L.

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Le Thi Bach[1], Nguyen Trong Tuan[1], Quach Thi Hong Dung[1], Truong Huynh Kim Ngoc[1], Bui Thi Buu Hue[*1], Le Tien Dung[2], Truong Quynh Nhu[3], Nguyen Thanh Phuong[3], Nguyen Minh Duy[3]

1. Department of Chemistry, College of Natural Sciences (CNS), Can Tho University

2. Institute of Applied Materials Science, Vietnam Academy of Science and Technology

3. College of Aquaculture and Fisheries (CAF), Can Tho University

Email: ltbach@ctu.edu.vn; btbhue@ctu.edu.vn

TÓM TẮT

NGHIÊN CỨU THÀNH PHẦN HÓA HỌC VÀ HOẠT TÍNH TĂNG CƯỜNG HỆ MIỄN DỊCH CÁ TRA CỦA CÂY CỎ SỮA LÁ LỚN (*Euphorbia hirta* L.) VÀ LÁ ỔI (*Psidium guajava* L.)

Nghiên cứu nhằm phân lập các hợp chất có trong cây Cỏ sữa lá lớn (Euphorbia hirta L.) và lá ổi (Psidium guajava L.) để bổ sung thêm thành phần hóa học của hai loài cây này. Kết quả đã phân lập được các hợp chất taraxerol (1) và taraxerone (2) từ cao n-hexane của Cỏ sữa lá lớn và hợp chất avicularin (3) từ cao ethyl acetate của lá ổi (Psidium guajava L.). Cấu trúc hóa học của các hợp chất đã được xác định bằng các phương pháp phổ nghiệm: ^{1}H-NMR, ^{13}C-NMR, DEPT-NMR và so sánh với các tài liệu đã công bố. Ngoài ra, hoạt tính tăng cường hệ miễn dịch cá tra của cao tổng ethanol và 3 hợp chất phân lập được cũng đã được khảo sát. Kết quả cho thấy Cỏ sữa lá lớn (Euphorbia hirta L.) và Ổi (Psidium guajava L.) là các ứng viên tiềm năng trong việc tăng cường hệ miễn dịch cá tra (Pangasionodon hypophthalmus).

Từ khóa: *Cỏ sữa lá lớn, Ổi, khả năng tăng cường miễn dịch*

1. INTRODUCTION

The striped catfish (*Pangasionodon hypophthalmus*) is a migrant species that is extensively cultured as a key commercial fish in Vietnam. Nowadays, intensive catfish production leads to increasing pressure on aquaculture to lessen or eradicate the use of feed antibiotics, resulting in new research to figure out safe and efficient natural alternatives. Recent studies indicated that the common trend of supplementing fish with plant extracts could effectively improve aquatic immunomodulation and disease resistance.

Euphorbia hirta L., a species belonging to genus Euphorbia, Euphorbiaceae family, has been found a lot in the Mekong Delta of Vietnam. The leaves of *E. hirta* are found to contain flavonoids, polyphenols, tannins, sterols, alkaloids, glycosides and triterpenoids [1,2]. Some pharmacological investigations showed that *E. hirta* and its active components possessed a wide range of bioactivities such as anti-inflammatory, antifungal, antibacterial,

antidiarrheal, antioxidant, antiasthmatic, antitumor, antimalarial, larvicidal and diuretic [3,4].

Psidium guajava L., a species belonging to the Myrtaceae family, has been found to be rich in nutrients, including vitamins and minerals that are significant for human health. Moreover, the investigation of biological activities indicated that *P. guajava* exhibited many bioactivities such as anti-inflammatory, antifungal, antibacterial, antidiarrheal, antioxidant, antidiabetic effects [5,6].

Our previous study demonstrated that plant extract-based diets of *E. hirta* and *P. guajava* were effective to stimulate immune responses in striped catfish (*P. hypophthalmus*) [7]. Therefore, in this study, we investigated the chemical investigation as well as the immunomodulatory potential of some isolated compounds from *E. hirta* and *P. guajava*.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Solvents utilized including *n*-hexane, ethyl acetate, *n*-butanol, methanol, and ethanol 96% were purchased from Chemsol, purity ≥ 99.0%. Silicagel gel 60 (0.063–0.200mm, Merck), was used for column chromatograph. TLC F_{254} plate (Merck) was used for thin layer chromatography.

2.2. Sample treatment and preparation

The whole plant of *E. hirta* and the leaves of *P. guajava* were collected in March 2021 in Can Tho city, Vietnam. The plants were authenticated and voucher specimens were kept under normal conditions. The plants were then washed with water away from mud and dust; the rotten and damaged parts were also discarded. The plant materials were left to dry in the shade at room temperature for some days and then dried in an oven at about 60°C until well-dried.

2.3. Extraction and isolation

The well-dried plants were ground into powder which was then soaked in 96% ethanol at room temperature four times (4 × 20 L) and filtered. The filtrate were concentrated under vacuum at 50°C, to give crude ethanol extract. The residues were extracted with *n*-hexane, ethyl acetate, *n*-butanol, and methanol, successively. The extracts were concentrated to yield the corresponding *n*-hexane, ethyl acetate, *n*-butanol and methanol extracts.

The *n*-hexane extract of *E. hirta* (100g) was first subjected to flash column chromatography (CC), eluted in a gradient system consisting of *n*-hexane and EtOAc (100:1-0:100) to obtain eight fractions (HE1-8). Fraction HE3 was re-subjected on silica gel CC, eluted with *n*-hexane: EtOAc (50:1-0:100) and 14 subfractions (HE3.1-14) were collected. Subfraction HE3.3 was further chromatographed on silica gel CC, with *n*-hexane: EtOAc (50:1-0:100) to obtain four subfractions (HE3.3.1-4). Then, subfraction HE3.3.3 was further chromatographed on silica gel CC, with *n*-hexane: EtOAc (80:1-50:1) to obtain four subfractions (HE3.3.3.1-4) and at last compound **1** (120 mg) and compound **2** (140 mg) were obtained.

The ethyl acetate extract of *P. guajava* was subjected to flash column chromatography (CC) on silica gel and eluted with various proportions of *n*-hexane and ethyl acetate (100:0- 0:100) to obtain 15 fractions (EE1-15). Fraction EE5 was further separated on a silica gel column, eluted with $CHCl_3$: MeOH (50:1-1:1) to yield eight subfractions (EE5.1-8). Finally, compound **3** (50 mg) was obtained from subfraction EE5.6.

2.4. Experimental fish

Total 150 striped catfish juveniles (body weight = 50 ± 5 g) were acclimated to laboratory conditions for 15 days at 28 ± 2°C in composite tank (2000 L). Fish were fed twice (9 am and 3 pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described by Biswas et al. with slight modifications [8]. Briefly, few randomly sampled animals were examined for the presence of any abnormal lesions or parasites

on body surfaces and internal organs [9]. Further, smears head kidney from the same fish were cultured on tryptic soy agar plate (TSA, Merck) for 24–48 h at 28°C for existence of any bacterial pathogens. Any colonies presented on TSA plate were used to perform PCR for detecting 16s RNA genes of commonly bacterial pathogens (*A. hydrophyla, E. ictaluri and Flavobacterium columnare*) in striped catfish [10]. Healthy fish which did not present any pathogenic bacteria, were used for experiment.

2.5. Isolation of head kidney leukocytes (HKLs)

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-µm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH 7.4, SigmaAldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 µg mL^{-1} streptomycin +10,000 U mL^{-1} penicillin (Invitrogen).

After washing, HKLs were removed from residual erythrocytes by incubating them 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen).

Viable cells were adjusted to 5×10^6 cells mL^{-1} after enumeration using trypan blue stain and seeded in wells of a 24 or 48-well plate.

After isolation of striped catfish HKLs, five hundred µL of cell suspension (5×10^6 cells mL^{-1}) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A were added to each well of 48-well plate. Afterward, leukocytes stimulation was carried out with samples to reach final concentrations at 10 and 100 µg mL^{-1} for extracts; 10 and 50 µg mL^{-1} for pure compounds. Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was realized in triplicates. The humoral immune response was assessed for 24 hrs at 28°C in a humidified atmosphere of 5% CO_2. Collected leukocyte membranes were disrupted by 50 µL lysis buffer (50 mM tris HCl, 150 mM NaCl, 0.1% Triton X 100, PMSF 0.1 µg mL^{-1}). Samples were centrifuged at 2000 g for 10 min to remove debris. Supernatants were collected for immune assays.

2.6. Immune parameters

2.6.1. Lysozyme assay

The lysozyme assay protocols were adapted from Ellis and Milla et al. [11,12]. Briefly, 0.3 µg mL^{-1} *Micrococcus lysodeikticus* (Sigma) was suspended in phosphate buffer, pH 6.2. In 96 wells microplates, the lysozyme activity was measured after mixing 30 µl of cell culture supernatant with 120 µL of *M. lysodeikticus* suspension. The difference in absorbance at 450 nm was monitored in every 30 s, between 0 and 30 min. The absorbance measurements were used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.6.2. Complement assay

The alternative complement pathway (ACH50) was assayed using rabbit red blood cells (RRBC, Biomerieux, Craponne, France). The assay protocols were adapted from Sunyer and Tort [13] and Milla et al. [12]. Briefly, 3 µL of RRBC suspension (3%) diluted in veronal buffer (Biomerieux) were mixed with serial dilutions of cell culture supernatant (60 mL of total volume). After incubation for 120 min at 28°C, the samples were centrifuged at 2000 g for 10 min at room temperature. The spontaneous hemolysis was obtained by adding 60 mL of veronal buffer to 10 mL of RRBC. The total lysis was obtained by adding 60 mL of distilled water to RRBC. The absorbance of these samples was then measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.6.3. Total immunoglobulin assay

The total immunoglobulin (Ig) concentrations of samples were measured by the method of Siwicki and Anderson [14], modified by Milla et al. [12]. Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma). Serums were mixed with 12% PEG solution for 2 h at room temperature under constant shaking. After centrifugation at 1000 g for 10 min, the supernatant was collected and assayed for its protein concentration. The total immunoglobulin concentration was calculated by subtracting this value from the total protein concentration in the plasma before precipitation with PEG.

2.7. Statistical analysis

All data were expressed as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Tukey's test was run to find out any difference in immune parameters. Results were considered as statistical significant when p value was < 0.05.

3. RESULTS and DISCUSSION

3.1. Structural elucidation

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

3.1.1. Compound 1

Compound **1** was characterized as white powder, m.p. 282-284°C; $[\alpha]_D^{30}$ = + 9.2 (c 0.1, $CHCl_3$)

^{1}H-NMR (500 MHz, $CDCl_3$), δ_H (ppm): 5.53 (1H, *dd*, 8.5 and 3.5 Hz, H-15); 3.19 (1H, *dd*, 10.5 and 3.5 Hz, H-3); 1.09 (3H, *s*, H-27); 0.97 (3H, *s*, H-23); 0.95 (3H, *s*, H-29); 0.92 (3H, *s*, H-25); 0.91 (3H, *s*, H-28); 0.91 (3H, *s*, H-30); 0.82 (3H, *s*, H-26); 0.80 (3H, *s*, H-24).

^{13}C-NMR (125 MHz, $CDCl_3$), δ_C (ppm): 158.1 (C-14); 116.9 (C-15); 79.1 (C-3); 55.5 (C-5); 49.3 (C-9); 48.7 (C-18); 41.3 (C-7); 39.0 (C-8); 38.7 (C-4); 38.0 (C-17); 37.7 (C-1); 37.7 (C-12); 37.5 (C-10); 36.7 (C-16); 35.8 (C-13); 35.1 (C-19); 33.7 (C-21); 33.3 (C-29); 33.1 (C-22); 29.9 (C-28); 29.8 (C-26); 28.8 (C-20); 28.0 (C-23); 27.1 (C-2); 25.9 (C-27); 21.3 (C-30); 18.8 (C-6); 17.5 (C-11); 15.4 (C-25); 15.4 (C-24).

Compound **1** was obtained from *n*-hexane extract as white powder, m.p. 282-284°C (MeOH). IR spectrum (KBr, ν_{max}, cm^{-1}): 3435 (-OH), 2941, 2869, 1642, 1457, 1381, 1030. The ^{13}C-NMR and DEPT spectra of **1** displayed 30 carbon signals, including 1 carbinol carbon >CH-OH at δ_C 79.1 (C-3), one tertiary olefinic carbon =CH- [δ_C 116.9 (C-15)], one quaternary olefinic carbon =C< [δ_C 158.1 (C-14)], 8 methyl carbons -CH_3, 10 methylene carbons, 3 methine carbons >CH- [δ_C 55.5 (C-5); 49.3 (C-9) and 48.7 (C-18)], and 6 quaternary carbons. The ^{1}H-NMR signals suggested the presence of triterpene skeleton – taraxerane, consisting of one hydroxyl group and one double bond. From HMBC, 2 methyl groups at C-29 and C-30 correlated with each other and also correlated with C-20, C-19 and C-21. Both methyl groups H_3-26 and H_3-27 correlated with the olefinic quaternary carbon at C-14. In addition, protons H_3-26 also correlated with C-8, C-9 and C-7. Protons H_3-27 also correlated with carbons at C-13, C-18 and C-12. Besides, olefinic proton at δ_H 5.53 corresponed with three quaternary carbons at C-8, C-13 and C-17; so the double bond must be at C-14 and C-15. Proton H_3-28 corresponded with four carbons at C-17, C-18, C-16 and C-22. Methyl proton H_3-25 correlated with three carbons at C-9, C-5 and C-1. Two methyl groups C-23 and C-24 correlated with each other and also correlated with carbon at C-4, C-5 and C-3. The double of doublet signal (J = 8.5 and 3.5 Hz) appeared at δ_H 5.53 is assigned to the olefinic proton H-15. The broad doublet signal (J = 10.5 and 3.5 Hz) was found at δ_H 3.19 could be assigned to proton H-3. This allow to identify compound **1** as *β*-taraxerol through the comparison of physical and spectral data with the published data [15].

3.1.2. Compound 2

Compound **2** was characterized as a white solid, m.p. 238-240ºC; $[\alpha]_D^{30}$ = + 12.3 (c 0.1, $CHCl_3$)

^{1}H-NMR (500 MHz, $CDCl_3$**)**, δ_H (ppm): 5.56 (1H, *dd*, 8.5 and 3.5 Hz, H-15); 1.14 (3H, *s*, H-27); 1.09 (3H, *s*, H-23); 1.08 (3H, *s*, H-25); 1.07 (3H, *s*, H-24); 0.96 (3H, *s*, H-29); 0.92 (3H, *s*, H-28); 0.91 (3H, *s*, H-30); 0.83 (3H, *s*, H-26).

^{13}C-NMR (125 MHz, $CDCl_3$), δ_C (ppm): 217.5 (C-3); 157.6 (C-14); 117.2 (C-15); 55.8 (C-5); 48.8 (C-9); 48.7 (C-18); 47.6 (C-4); 40.7 (C-19); 38.9 (C-8); 38.4 (C-1); 37.8 (C-17); 37.7 (C-10); 37.6 (C-13); 36.7 (C-16); 35.8 (C-12); 35.1 (C-7); 34.1 (C-2); 33.6 (C-21); 33.4 (C-29); 33.1 (C-22); 29.9 (C-28); 29.7 (C-26); 28.8 (C-20); 26.1 (C-23); 25.6 (C-27); 21.5 (C-24); 21.4 (C-30); 20.0 (C-6); 17.5 (C-11).

Compound **2** was obtained as colourless needles with m.p. 238-240°C. The **^{1}H-NMR** spectrum showed eight singlet signals representing eight methyl groups, all of which were singlets at δ_H 0.83, 0.91, 0.92, 0.96, 1.07, 1.08, 1.09 and 1.14 for H-26, H-30, H-28, H-29, H-24, H-25, H-23 and H-27 respectively and one olefinic proton at δ_H 5.56 (1H, *dd, J* = 8.5 and 3.5 Hz). The ^{13}C-NMR of the compound **2** showed 30 carbon signals including eight methyls, ten methylenes, four methynes and eight quaternary carbons. A carbonyl was observed at δ_C 217.5. The double bond was represented by two singlets at δ_C 157.6 and 117.2 for carbons C-14 and C-15, respectively. The signal for C-14 was shifted to the lower field because it is a quaternary carbon at the ring junction of rings C and D. By comparing with literature information, compound **2** was determined as taraxerone [16].

3.1.3. Compound 3

Compound **3** was obtained as yellow powder, m.p. 216-218ºC

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 7.54 (1H, *s*, H-2′); 7.51 (1H, *d*, 8.5 Hz, H-6′); 6.92 (1H, *d*, 8.5 Hz, H-5′); 6.41 (1H, *s*, H-8); 6.23 (1H, *d*, 8.5 Hz, H-6); 5.49 (1H, *s*, H-1′′); 4.35 (1H, *d*, 2.5 Hz H-2′′); 3.92 (1H, *m*, H-4′′); 3.89 (1H, *t*, 4.5 Hz, H-3′′); 3.52 (2H, *m*, H-5′′).

^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 180.0 (C-4); 166.1 (C-7); 163.1 (C-5); 159.4 (C-2); 158.6 (C-9); 149.9 (C-4′); 146.4 (C-3′); 134.9 (C-3); 123.1 (C-6′); 123.0 (C-1′); 116.9 (C-2′); 116.5 (C-5′); 109.6 (C-1′′); 105.6 (C-10); 99.9 (C-6); 94.8 (C-8); 88.1 (C-4′′); 83.3 (C-2′′); 78.7 (C-3′′); 62.6 (C-5′′).

The ^{1}H-NMR data of compound **3** indicated that ring A is 5,7-disubstituted, as shown by two *meta*-located protons at δ_H [6.41 (1H, *s*, H-8) and 6.23 (1H, *s*, H-6)]. The observation of ABX system at δ_H [7.54 (1H, *s*, H-2′), 7.51 (1H, *d*, 8.5 Hz, H-6′) and 6.92 (1H, *d*, 8.5 Hz, H-5′)] has suggested a 3',4' -disubstituted ring B. A 3-*O*-substituted quercetin structures were indicated for compounds **3** due to the corresponding anomeric protons at δ_H 5.49 (1H, *s*) characteristic for arabinofuranosyl moiety. The structure of compound **1** were characterized as quercetin 3-*O*-α-L-arabinofuranoside (avicularin) by comparison with literature data [17].

Three compounds **1-3** were isolated and identified from the whole plant of *E. hirta* and the leaves of *P. guajava*, including taraxerol (**1**), taraxerone (**2**) and avicularin (**3**) by analysis of their NMR spectra and comparison with literature data. (Fig. **1**).

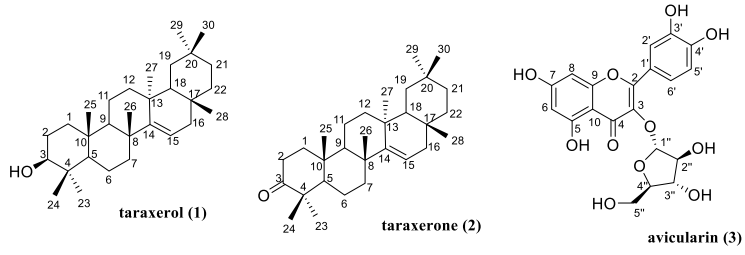

Fig 1. Chemical structures of compounds 1–3

3.2. Effects of crude extracts and isolated compounds on immune response of HKLs

3.2.1. Lysozyme activity

Crude ethanol extract of *E. hirta* L. and *P. guajava* and compounds (**1**), (**2**), (**3**) have selected to investigate on increase immune responses in a dose dependent manner in striped catfish HKLs after 24 hrs, at 10 and 100 µg/mL for extracts; at 10 and 50 µg/mL for pure compounds.

As shown in **Fig. 2**, the lysozyme levels in HKLs treated with *E. hirta* and *P. guajava* extracts after 24 hrs were increased compared to those of the control (both 10 and 100 µg/mL).

The results showed that lysozyme levels in HKLs were regulated by the plant extracts of *E. hirta* and *P. guajava* at 100 µg/mL significantly. The level of lysozyme in HKLs ranged between 1.8 and 2.5 (at 100 µg/mL of *E. hirta* and *P. guajava* extracts, respectively) times than those of the control.

At the low dose of 100 µg/mL of taraxerol and taraxerone significantly enhanced the lysozyme levels compared with control ($p < 0.05$) in HKLs, while avicularin showed no statistical influence on lysozyme activity.

3.2.2. Complement activity

The complement levels increased in cells treated with crude extract as well as some isolated compouns compared with control treatment at 24 hrs ($p<0.01$) **(Fig. 2)**. In particular, the treatment containing taraxerol 100 µg/mL was observed to possess the highest level compared to other treatments throughout the sampling time points.

3.2.3. Total immunoglobulin (Ig)

Total Ig activity was noticed to be statistically higher in crude extract treated groups at 24 hrs compared with control group ($p<0.01$). The level of total Ig was found to be the highest in the crude extract of *E. hirta* (at 100 µg/mL), increased about three times than those of the control. However, no significant changes were observed in HKLs stimulated with taraxerol and taraxerone (**Fig. 2).**

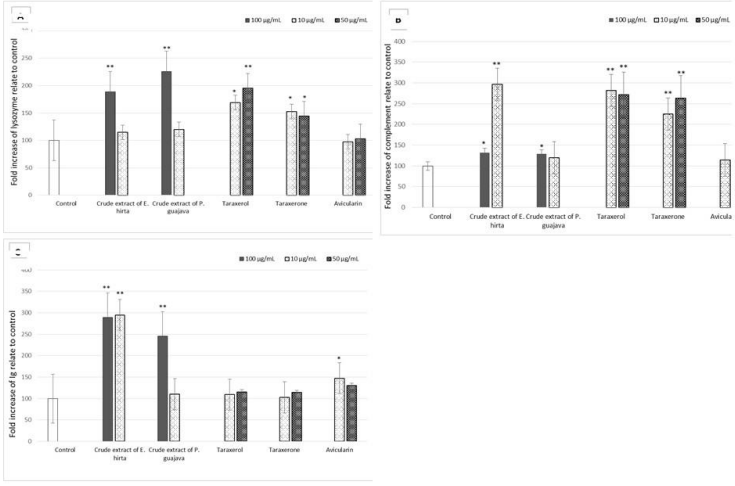

*Fig. 2. Effects of extracts and pure compounds on immune parameters of striped catfish HKLs (where: (A) The effects on lysozyme levels; (B) The effects on complement activities; (C): The effects on total Ig. Asterisk indicates significant differences between stimulated and non-stimulated cells at 24 h (*p < 0.05; **p < 0.01). Values are mean ± S.D. (n = 3)).*

Manipulaton of heath status using plant extracts has been developed as an ecological practice for sustainable aquaculture [18]. Among the plant derived products, *E. hirta* and *P. guajava* displayed immunomodulatory properties due to their presence of various biological compounds [19, 20]. In the present study, the immunomodulatory effects of *E. hirta* and *P. guajava* extracts as well as isolated compounds were assessed by evaluating their capacities to improve immune parameters. The current study revealed that the increase of lysozyme levels, complement activities, as well as total Ig was concentration-dependent. In agreement with our results, several studies also indicated that *E. hirta* and

P. guajava extracts positively enhanced lysozyme levels, complement activities and total Ig in rohu, tilapia, and common carp [21-23].

4. CONCLUSION

Phytochemical analysis of different fractions of the plant *E. hirta* and *P. guajava* led to the isolation of three compounds including taraxerol (**1**), taraxerone (**2**), and avicularin (**3**). The structures of the natural compounds were determined from the NMR spectroscopic evidences.

Both crude ethanol extracts and compounds (**1**), (**2**) and (**3**) enhanced immune responses in striped catfish (*Pangasianodon hypophthalmus*).

Our results suggest a positive contribution of *E. hirta* and *P. guajava* to increase immune responses in a dose dependent manner in striped catfish HKLs.

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