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Effects of triterpenes from *Ardisia* cf. *elliptica* (subgenus Tinus) and sterols from A*rdisia pyramidalis* Cav Pers on *Artemia salina* and *Danio rerio* toxicity and caudal fin regeneration

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ABSTRACT

*The dichloromethane extract of the air-dried leaves of Ardisia cf. elliptica (subgenus Tinus) afforded mixtures of α-amyrin (**1a**), β-amyrin (**1b**) and bauerenol (**1c**), while Ardisia pyramidalis leaves yielded spinasterol (**2**) and spinasteryl acetate (**3**). The mixtures of triterpenes (**1a-1c**) at ratios of 1:1:1, 2:2:1 and 2:2:3 and the sterols (**2** and **3**) were tested for their toxicity on Artemia salina and Danio rerio embryos. Compounds **1a-1c** (1:1:1) (LC_{50} = 0.60 mg/ml), **1a-1c** (2:2:1) (LC_{50} = 0.60 mg/ml), **1a-1c** (2:2:3) (LC_{50} = 0.54 mg/ml), **2** (LC_{50} = 0.50 mg/ml) and **3** (LC_{50} = 0.52 mg/ml) were found toxic to A. salina nauplii after 48h of exposure. Teratologic manisfestations such as axial deformation, pericardial edema and yolk sac edema were observed in embryos treated with **1a-1c**, **2** and **3**. The mixtures of **1a-1c** (1:1:1) and **1a-1c** (2:2:1) were further tested for D. rerio caudal fin regeneration. A shorter caudal fin regeneration length was observed in D. rerio young adults exposed to **1a-1c** (1:1:1) and **1a-1c** (2:2:1). The results of the current study present implications on the toxicity and teratologic manifestations in relation to previously observed angiosuppressive activity of **1a-1c**, **2** and **3**.*

Keywords: *Ardisia* cf. *elliptica*, *Ardisia pyramydalis*, Myrsinaceae, bauerenol, α-amyrin, β-amyrin, spinasterol, spinasteryl acetate, brine shrimp toxicity, zebra fish caudal fin regeneration

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INTRODUCTION

The genus *Ardisia* Swartz belongs to the family Myrsinaceae. *Ardisia* has 68 recorded species in the Philippines, 60 of which are endemic [1]. The genus *Ardisia* is a good source of health promoting compounds and potent phytopharmaceuticals [2]. Some of its already identified medicinal uses are anti-inflammatory [3], antioxidant [4], antimicrobial [5], antiviral [6], cytotoxic [7], and antitumor [8]. Recently, spinasterol, spinasteryl acetate, and a mixture of α-amyrin, β-amyrin and bauerenol in a 2:1:2 ratio, squalene, lutein and triglycerides were isolated from the dichloromethane extract of *A. pyramidalis* [9]. Different ratios of three triterpenes, α-amyrin, β-amyrin and bauerenol of *A.* cf. *elliptica* have been identified as the major components of this species [10]. Our recent findings demonstrate the anti-angiogenic potential of triterpenes from *A.* cf. *elliptica* [10] and sterols from *A. pyramidalis* [9]. The mixture of bauerenol, α-amyrin and β-amyrin at 3:1:2 ratio from *A. squamulosa* was found to be angio-suppressive by restricting blood vessel branch point formation, capillary sprout elongation and blood vessel density [11]. Another recent study reported that triterpenes from *Ardisia* cf. *elliptica* (§Tinus) limit vascular density and

promote von Willebrand factor expression on duck chorioallantoic membrane [12]. Despite the numerous bioactivities of this genus, there has been very limited information on the biological activities of indigenous *Ardisia* species in the Philippines. In the current study, we report the toxicity potential of two indigenous *Ardisia* species and their effects on tissue regeneration in amputate *D. rerio* caudal fin.

EXPERIMENTAL SECTION

General Experimental Procedures

The NMR spectra were recorded on a Varian VNRMS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh); TLC was performed with plastic backed plates coated with silica gel F_{254}; plates were visualized by spraying with vanillin sulfuric acid and warming.

Sample Collection

Fresh leaves of *Ardisia* cf. *elliptica* (subgenus Tinus) was collected from an ultramafic low altitude area along primary thickets (cogon grassland) at the foot of Mt. Pulido, Brgy. Pacalat, Mangatarem, Pangasinan, Philippines (15°44'16.20 N: 120°16'111.27 E). The sample genus was identified at the National Herbarium, Philippine National Museum by Domingo S. Madulid in collaboration with John Pipoly of the Urban Horticulture/Climate Change Extension, Broward County Extension, Florida. Species identification was not confirmed due to the sheer complexity of the genus *Ardisia* Swartz composing of several tribes and subgenera. For the purpose of identification and verification of future results, a morphologic description and photographic reference of *Ardisia* cf. *elliptica* was presented in our previous report on the angio-suppressive property of the triterpenes isolated from this species [10]. *Ardisia pyramidalis* leaves were collected from Hidden Valley Springs, Alaminos Laguna. The plant sample was identified by Emelina H. Mandia of the Biology Department, De La Salle University Manila and verified by Domingo S. Madulid.

Isolation

Air-dried leaves of *A.* cf. *elliptica* (440 g) were ground in a blender, soaked in 1.8L of CH_2Cl_2 for three days and then filtered. The filtrate was concentrated *in vacuo* to afford a crude extract (8.5 g) which was chromatographed in increasing proportions of acetone in CH_2Cl_2 at 10 % increment by volume. The 30% acetone in CH_2Cl_2 fraction was rechromatographed (3×) using 5% EtOAc in petroleum ether to afford a mixture of **1a-1c** (150 mg, 2:2:1). This mixture (80 mg) was rechromatographed using 5% EtOAc in petroleum ether (2×) to afford a mixture of **1a-1c** (52 mg, 1:1:1). The 40% acetone in CH_2Cl_2 fraction was rechromatographed in 5% EtOAc in petroleum ether (6×) to afford a mixture of **1a-1c** (95 mg, 2:2:3).

The air-dried leaves of *A. pyramidalis* (400 g) were ground in a blender, soaked in 1.6L of CH_2Cl_2 for three days and then filtered. The filtrate was concentrated under vacuum to yield a crude extract (5.0 g) which was chromatographed over silica gel column gradient eluted with acetone in CH_2Cl_2 at 10 % (by volume) increment. The CH_2Cl_2 and 10% acetone in CH_2Cl_2 fractions were combined and rechromatographed over silica gel column with petroleum ether (b.pt. 35-60 ^{0}C) as eluent. The more polar fractions were rechromatographed (5×) with 2.5% (by volume) EtOAc in petroleum ether as eluent to yield **3** (6 mg). The 20%-30% acetone in CH_2Cl_2 fractions were separated over silica gel column (7×) using 5% (by volume) EtOAc in petroleum ether as eluent to yield **2** (8 mg).

Preparation of Test Substance

In the *A. salina* assay, the mixture of α-amyrin, β-amyrin and bauerenol (**1a-1c**) with ratios of 2:2:1, 2:2:3 and 1:1:1 from *A.* cf. *elliptica* was dissolved in dimethylsulfoxide (DMSO) in sterile Phosphate Buffered Saline (PBS) to obtain 0.1 mg/ml. The stock solution was then diluted with instant sea water (Mai Mix, Quezon City) (0.02g/ml) to obtain 0.01, 0.02, 0.04 and 0.08 mg/ml at a final DMSO concentration of 0.01% v/v, 0.02 %v/v, 0.04% v/v and 0.08% v/v. In the *D. rerio* embryo toxicity assay, the stock solution was reconstituted with sterile embryo media to a final concentration of 0.006 mg/ml (**1a-1c**, 1:1:1), 0.016 mg/ml (**1a-1c**, 2:2:1), 0.031 (**1a-1c**, 2:2:3), 0.1 mg/ml (**2**) 0.063 mg/ml and (**3**) at 0.01% DMSO. In the caudal fin regeneration assay, **1a-1c** (1:1:1) and **1a-1c** (2:2:1) were dissolved in DMSO as mentioned earlier. The stock solution was reconstituted in system water to a final concentration of 10µg/ml at 1% final DMSO concentration.

Artemia salina Lethality Assay

Brine shrimp (*Artemia salina*) toxicity assay [13] was performed. *A. salina* dried cysts (Sera®Artemia-Mix, Great Salt Lake, USA) were soaked in fully aerated tap water. The lethality test was performed with strong aeration under a continuous light regime. Approximately 8h after hatching, the phototrophic metanauplii (Instar II) were collected with a pipette and concentrated in a vial. Ten nauplii were transferred to each well containing various concentrations of the test samples (n=10, 3 replicates). Absolute (instant sea water) and negative controls with variable DMSO

concentrations were also assigned. Toxicity was determined after 12, 24, 36, and 48 hpt. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The number of survivors was counted and percentage of deaths was calculated as:

$$\%M = \frac{\text{average number of dead individuals}}{\text{Initial number of living individuals put in every concentration}} \times 100$$

Since the larvae did not receive food, the dead larvae in each treatment were compared to the dead larvae in the control to ensure that the mortality observed in the bioassay is due to the bioactive substance and not to starvation. The index of mortality was corrected using Schneider-Orelli's formula in the event that death in the control was detected. The corrected index of mortality (%MI) was calculated as:

$$Corrected\ \%MI = \frac{Mortality_{observed} - Mortality_{control}}{100 - Mortality_{control}} x\ 100$$

Then finally, the median lethal concentration (LC_{50}) was determined.

Embryo Toxicity Screen

Danio rerio toxicity screen was performed according to the procedure [8] modified from the earlier procedure. Viable embryos at 64-1000 cell stage [15] with intact chorion and normal appearance at age 2-3h post fertilization (hpf) were randomly selected and assigned into each well containing the test samples (n=6, 3 replicates). Ethanol (1% v/v) was used as positive control based on the published LC_{50} values of ethanol on *D. rerio* embryo (1.98% v/v) after 45h treatment [15]. Observations were made after every 24h until the end of a 120h observation period at a constant humidity (80%) and temperature (30°C). Mortality and signs of abnormal development such as number of heart beats, cephalic pigmentation, body length, yolk sac edema, pericardial edema, axial deformity and abnormal head morphology based on the descriptions of published results and atlases [14, 16-17] were noted.

Caudal Fin Regeneration Assay

Caudal fin regeneration assay [18] was performed. Six weeks old wild type red zebrafish (*D. rerio*) were maintained according to established protocols in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines of De La Salle Health Science Institute, Dasmarinas, Cavite. The fish were acclimatized in an aerated holding tank at 28°C [19-20]. The *D. rerio* were exposed to standard lighting conditions with 14 hours light and 10 hours dark photoperiod and three times a day feeding with commercial feed rations (34% crude protein). The distal end of the caudal lepidotrichia was amputated (~0.5 cm) after anesthetizing the fish with 0.2 mg/mL Lidocaine (Astra Zeneca) in system water. The fish was immediately put in treatment solutions with 16µg/mL of either **1a-1c** (1:1:1) or **1a-1c** (2:2:1) in 1% DMSO supplemented with 5 units Penicillin and 5 µg Streptomycin per milliliter of system water for 60 minutes (n=6, 3 replicates). The fish were subsequently exposed daily to 1.056 µg/mL of test substance in 1% DMSO and system water. After 14 days, the fish were anesthetized and the degree of caudal regeneration was evaluated by measuring the caudal regenerated length and quality of the mesenchymal cells along the blastema. The regenerated caudal fin was photographed and analyzed by Image J analysis 1.40g open access software (Wayne Rasband National Institutes of Health, USA).

All animal handling procedures were in accordance with the existing policies and guidelines of the Philippine Association of Laboratory Animal Science (PALAS) for care and use of laboratory animals and with Administrative Order 40 of the Bureau of Animal Industry relative to the Rep. Act. No. 8485.

Statistical Analysis

The results were analyzed by one way analysis of variance and Probit analysis using IBM SPSSS Statistics ver. 19 for Windows. The results were considered significant at $P \leq 0.05$. Post hoc analysis was performed at 95% Tukey's test at $\alpha = 0.05$. Means are reported as Mean ± SEM.

RESULTS

Structure Identification

The dichloromethane extracts of the air-dried leaves of *A.* cf. *elliptica* afforded triterpenes **1a-1c** by silica gel chromatography. These compounds were identified by comparison of their ^{13}C NMR data with those reported in the literature for bauerenol (**1a**) [21], α-amyrin (**1b**) and β-amyrin (**1c**) [22]. The ratios of the three triterpenes were determined from the integrations of the olefinic proton resonances at δ 5.39 for **1a**, δ 5.11 for **1b** and δ 5.16 for **1c**. These triterpenes were tested for their toxicity potential on *A. salina* cysts and nauplii, as well as *D. rerio* embryos and adults.

Silica gel chromatography of the dichloromethane extract of the air-dried leaves of *A. pyramidalis* afforded spinasterol (**2**) and spinasteryl acetate (**3**). The structures of **2** and of **3** were identified by comparing their ^{13}C NMR data with those reported in the literature for spinasterol [23] and spinasteryl acetate [24], respectively.

Effects of 1a-1c, 2 and 3 on *A. salina* Nauplii Lethality

Free swimming nauplii were collected and randomly assigned into assay wells containing the test samples. Incidence of deaths in each well was recorded within a 48h observation period. Nauplii in the environmental control groups showed an incidence of 3.3% mortality at 12h progressing to 20% until the end of the 48h window. A correction factor was used to eliminate the effects of other factors contributing to the deaths observed in each treatment. Lethality observed at 0.1% v/v concentration of DMSO indicates that 6.7% died at 24hpt progressing to 50% at 48hpt. The mortality index however was negligible from 12hpt until 36hpt after the percent mortality has been normalized. A mortality index of 37.5% at 48hpt was observed. Increasing the concentration of DMSO in each treatment also increased the mortality index to 53.3% at 0.2-0.4% v/v, and 60% at 0.8% v/v at the end of 48hpt. Exposure to **1a-1c** (1:1:1) demonstrated zero lethality in the corrected mortality index until 36hpt. There was very low mortality index in groups administered with **1a-1c** (1:1:1) observed at 36hpt in all concentrations tested (4.2%, 8.3%, 4.2% and 8.3%, respectively). Similar to these results were obtained in the DMSO treatment, where a high mortality index was obtained at the end of 48hpt (50%, 37.5%, 50% and 50%, respectively) in those groups exposed to **1a-1c** (1:1:1) after 48h observation, suggesting a median lethal concentration (LC_{50}) of 0.50 mg/ml. Treatment with **1a-1c** (2:2:1) showed concentration dependent mortality from 4.2% (0.1mg/ml) progressing to 12.5% when the concentration was increased to 0.8 mg/ml at 36hpt. After 48h, a concentration dependent corrected mortality index was obtained (29.2%, 33.3%, 41.7%, and 50% respectively), suggesting an LC_{50} of 0.60 mg/ml. There was a negligible toxic effect in the first 24h of observation, but increased at 48h obtaining 25%, 33.3%, 50% and 50% at doses of 0.1, 0.2, 0.4 and 0.8 mg/ml, respectively suggesting a higher degree of toxicity with LC_{50} of 0.54 mg/ml. Compound **3** demonstrated very toxic property showing high incidence of deaths within the 12h observation period. The number of deaths progressed to 79.2%, 83.3%, 87.5% and 100% mortality index at 48h obtaining an LC_{50} of 0.52 mg/ml. Compound **2** shared similar effects with **3** demonstrating early incidence of mortality at 12h. The incidence of deaths progressed to a much higher index of mortality (79.2%, 87.5%, 87.5 and 100%) at 48h observation period suggesting an LC_{50} similar with that of **3**.

Effects of 1a-1c, 2 and 3 on *D. rerio* Embryo Hatchability

Hatching frequency was observed in embryos administered with **1a-1c**, **2** and **3**. The onset of hatching was observed within the 48h window which was comparable to the normal values for hatching period of 48-72h [14]. Embryos in the environmental control group showed a steady hatching rate beginning at 5.5% (2dph) progressing up to 94.4% at the end of the observation period (Fig. 1). Embryos administered with DMSO and ethanol had 24h delay to the onset of hatching. Hatching in the DMSO group started at 3dph with 38.9% hatchability progressing up to 83.3% at 5dph. Treatment with ethanol resulted to severe inhibition of hatching frequency. The relatively small size and polar nature of ethanol made it possible to traverse the chorion much faster than the nonpolar molecules in the other treatments. A hatching frequency of 5.5% at 3dph until 5dph was observed. The embryos in the treatments as well as in other replicates died while inside the chorion. A hatching frequency of 16.6% was observed in groups treated with **1a-1c** (1:1:1) at 2dph and hatching was completed at 3dph with a frequency of 83.3% starting from 3dph until 5dph. Similar pattern but much higher hatching frequency (33.3%) was observed in embryos administered with **1a-1c** (2:2:1) at 2dph which progressed to 88.9% starting form 3dph until 5dph. Similar pattern but different degree of hatching frequencies were also observed in embryos administered with **1a-1c** (2:2:3), **2** and **3**. A hatching frequency of 5.5% at 2dph was observed in embryos exposed to **1a-1c** (2:2:3), **2** and **3**. The frequency of hatching in **1a-1c** (2:2:3) progressed to 83.3% at 3dph until 5dph similar to **2** with 88.8% starting at 3dph until 5dph. Compound **3** on the other hand obtained the highest hatching frequency of 94.4% starting at 3dph until 5dph.

Effects of 1a-1c, 2 and 3 on Survival Rate of *D. rerio* Embryos

Survival rates of embryos exposed to each sample were observed over a 120h period (5dph). Embryos in the environmental control group (embryo media) did not have any incidence of mortality throughout the entire period, indicating that the rearing conditions were ambient for embryo survival. The DMSO control group however showed 94% survival rate at 2-4dph which further decreased to 88.9% at 5dph (Figure 2A). Since an incidence of mortality was observed in the DMSO control group, data corrections were performed. The ethanol treated group demonstrated severe effects on embryo survival similar to earlier reports [15, 17]. Survival rate started to decline beginning at 3dph (50%) which further worsened until termination of the experiment (5dph). A mortality of 47.03% was noted at 2dph progressing up to 81.33% at 5dph (Figure 2B). Early onset of lethality was noted in groups administered with all ratios of **1a-1c**. Incidence of deaths was observed at 1dph (5.6%). The survival rate of embryos administered with **1a-1c** (2:2:3) however remained stable until 4dph (94.4%) and had showed a mortality of 0.11% at the end of the treatment period. The administration of **1a-1c** (1:1:1) showed 5.93% mortality at 3dph which progressed to 11.76% (4dph) and 6.30% (5dph) towards the end of the observation period. Exposure to **1a-1c** (2:2:1) on the other

hand demonstrated 5.93% mortality at 3-4dph progressing up to 6.3% at the end of observation period. The effects on lethality were minimal to negligible within a 10% allowable mortality rate due to other existing conditions such as variable independent responses of test animals to the test samples. Treatment with **2** and **3** demonstrated 100% embryo survival at the beginning of the experiment which remained stable until 2dph. Sudden decrease in survival was noted in embryos administered with **3** with 35.28% mortality at 3dph progressing up to 62.54% until 5dph. Compound **2** on the other hand, had 17.69% mortality at 3dph progressing up to 100% mortality at the end of the observation period. The high incidence of mortality indicates severe levels of toxicity of **2** and its derivative. The 48h delay possibly indicates that **2** and **3** are still penetrating and accumulating their effects inside the chorionic environment. The time of hatching only started to happen within the 48h window keeping the embryos protected inside the chorion. The chorion might have played a significant role in the prevention of the detrimental effects of the test substances to the embryo.

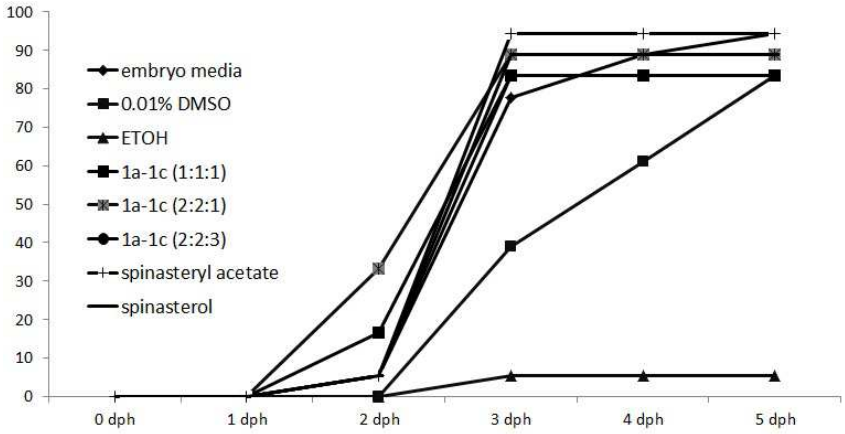

Figure 1. Effects of test samples on hatching frequency of D. rerio embryos

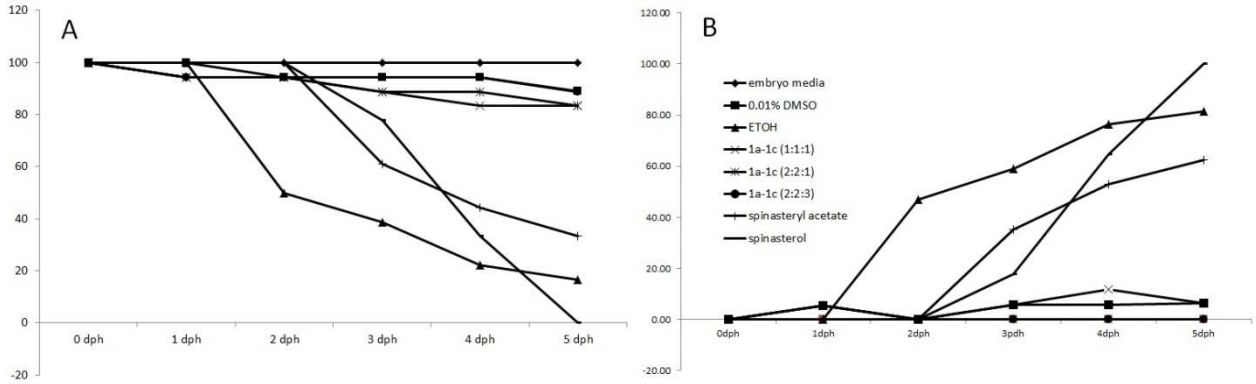

Figure 2. (A) Observed survival rate and (B) corrected mortality rate of D. rerio embryos over a 5dph (120h) observation period

Effects 1a-1c, 2 and 3 on *D. rerio* Embryo Morphology

Embryos exposed to different samples from *A. pyramidalis* and *A.* cf. *elliptica* demonstrated signs of abnormal morphology observed at end of 5dph. Three morphologic abnormalities were observed at the end of the treatment period namely: axial deformity, pericardial edema and yolk sac edema (Figure 3). DMSO treated embryo demonstrated signs of axial deformity (35.7%) and yolk sac edema (100%) similar to earlier reports [9]. Ethanol exposed embryos demonstrated 100% pericardial edema and 67% yolk sac edema concomitant to previous reports [15]. Embryos exposed to **1a-1c** (1:1:1) showed axial deformation (13.3%) and yolk sac edema (100%). Exposure to **1a-1c** (2:2:1) displayed axial deformation (26.7%) and yolk sac edema (100%). A more produced malformation was observed in those embryos exposed to **1a-1c** (2:2:3) displaying signs of axial deformation (13.3%), pericardial edema (26.7%) and yolk sac edema (100%). Treatment with **3** demonstrated axial deformity (83.3%), pericardial edema (66.7%) and yolk sac edema (100%). Compound **2** demonstrated less severe degree of axial deformity (50%) as compared to **3**, but a more pronounced pericardial edema (100%) and yolk sac edema (100%).

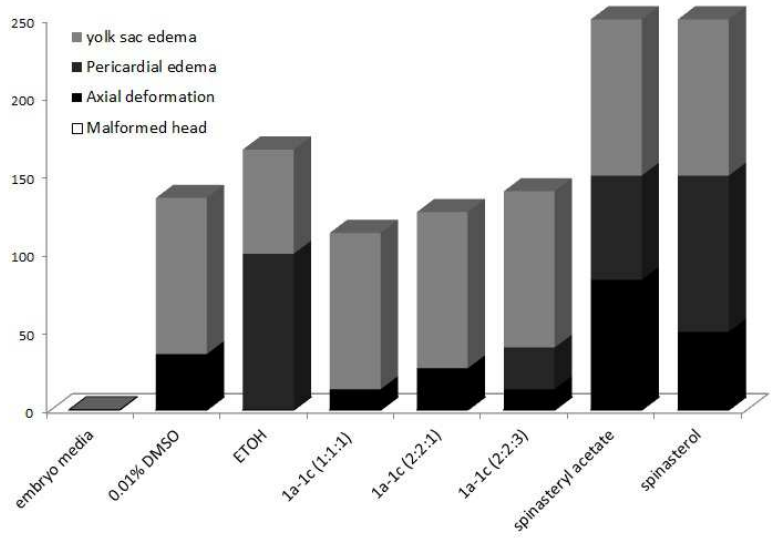

Figure 3. Effects of 1a-1c, 2 and 3 on morphology of D. rerio embryo at 5dph

There was no incidence of malformed head or any signs of abnormalities in the cephalic region. Heart rate was significantly (P=0.0001) affected by the application of ethanol, **1a-1c** (2:2:3) and **2**. Treatment with ethanol significantly (P=0.0001) decreased the heart rate (123.33±12.67 bpm) of *D. rerio* embryo compared to the heart rate of the control (171.06±2.88 bpm) similar to the published values [17] (Table 1). Compared to the other ratios, the effects of **1a-1c** (2:2:3) are significantly (P=0.002) lower than the effects of **1a-1c** (2:2:1) (171.5±4.70 bpm). The effects on heart rate of **1a-1c** (2:2:3) however was not significantly (P=0.11) different with that of **1a-1c** (1:1:1) (164.5±3.90). The ratios of baurenol in **1a-1c** (2:2:3) and **1a-1c** (1:1:1) are higher as compared to **1a-1c** (2:2:1) indicating that the difference in bauerenol concentration in each ratio has an effect on the heart rate of the embryos. The effects of **3** on heart rate were found similar (P=0.157) with those of the effects of embryo media (167.67±2.70). Compound **2** significantly (P=0.0001) decreased (133.33±4.43 bpm) the heart rate of *D. rerio* embryos, obtaining the lowest heart rate among all treatments.

Table 1. Effects of 1a-1c, 2 and 3 on heart rate, pigmentation, and body length of *D. rerio* embryos

Treatment	Heart rate *(bpm)*	Cephalic Pigmentation *(mpdcr)*	Body Length *(mm)*
Embryo media	167.67±2.70	14±0.66	3.16±0.05
DMSO	171.06±2.88	13.43±0.54	2.99±0.10
Ethanol	123.33±12.67**	---	---
1a-1c (1:1:1)	164.5±3.90	13.0±0.61	3.09±0.08
1a-1c (2:2:1)	171.5±4.70	13.67±0.82	3.08±0.08
1a-1c (2:2:3)	148.71±3.20*	18.07±0.52**	3.56±0.09*
2	133.33±4.43**	---	---
3	151.27±5.70	14.50±2.23	2.97±0.22

**means are significantly different the control at P≤0.05; **means are significantly different to the control at P≤0.001; beats per minute (bpm); melanocyte per dorsal cephalic region (mpdcr) embryos died before 48h (---)*

The degree of cephalic pigmentation in *D. rerio* embryos in groups administered with embryo media was 14±0.66 melanocytes in the dorsal cephalic region. Similar effects were found in those groups administered with DMSO (13.43±0.54), **1a-1c** (1:1:1) (13.0±0.61), **1a-1c** (2:2:1) (13.67±0.82), and **3** (14.50±2.23). On the other hand, the administration of **1a-1c** (2:2:3) has significantly (P=0.0001) increased (18.07±0.52) the melanocyte frequency on the dorsal cephalic regions of the embryo.

The average rostrocaudal length of embryos in the embryo media group at 48hpf was 3.16±0.05 mm. The rostrocaudal length of embryos in those groups administered with DMSO, **1a-1c** (1:1:1), **1a-1c** (2:2:1) and **3** were not significantly (P>0.05) different with the length obtained in the control, indicating that treatments of the above mentioned substances did not affect the body length of embryos. Exposure to **1a-1c** (2:2:3) on the other hand significantly (P=0.12) increased the rostrocaudal length of embryos (3.56±0.09mm).

Effects 1a-1c on *D. rerio* Caudal Fin Regeneration

Caudal regeneration along the dorsal lepidotrichia of *D. rerio* administered with **1a-1c** was significantly (P=0.009) reduced. Caudal fins of fish exposed to **1a-1c** (1:1:1) and **1a-1c** (2:2:1) obtained average regenerated lengths of 0.72±0.13mm and 0.87±0.03 mm which were found significantly different (P=0.007 and P=0.091, respectively) from that of the negative control (1.26±0.13 mm) (Figure 4). The degree of regeneration observed in those fish exposed to **1a-1c** (1:1:1) was found similar (P=0.671) with that of the **1a-1c** (2:2:1) group (Table 2). A highly significant (P=0.0001) inhibition of caudal fin regeneration was observed along the medial lepidotrichi where fish exposed to **1a-1c** (1:1:1) were found to have the shortest regenerated caudal length (0.47±0.09mm) which was highly significantly different (P=0.0001) with that of the control (0.86±0.04mm). The effect of **1a-1c** (1:1:1) however was found similar (P=0.264) with **1a-1c** (2:2:1) (0.63±0.03mm). Along the ventral lepidotrichia, the caudal regeneration length of fish exposed to each ratio of **1a-1c** was found significantly (P=0.0001) shorter than that of the control (1.17±0.07 mm). Further, the regeneration length of **1a-1c** (1:1:1) (0.47±0.09mm) was shorter (P=0.003) than those fish exposed to **1a-1c** (2:2:1) (0.91±0.02mm), indicating that the highest activity of **1a-1c** on inhibiting caudal regeneration is at the ventral lepidotrichia.

Table 2. Effects of mixtures of 1a-1c at 1:1:1 and 2:2:1 ratio on caudal fin regeneration of adult *D. rerio*

Treatment	Total Regeneration Length (mm)		
	Dorsal	Medial	Ventral
DMSO (5%)	1.26±0.13	0.86±0.03	1.17±0.13
1a-1c (1:1:1)	0.72±0.38**	0.47±0.03**	0.47±0.03**
1a-1c (2:2:1)	0.87±0.9**	0.63±0.67**	0.91±0.07*

**means are significantly different the control at P≤0.05; **means are significantly different to the control at P≤0.001*

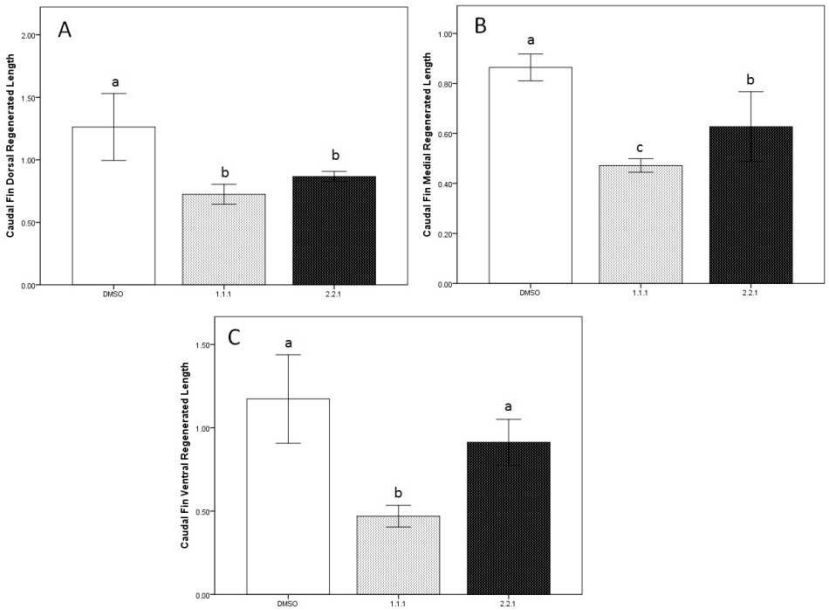

Figure 4. Caudal fin regenerated length along the (A) dorsal lepidotrichia, (B) medial lepidotrichia and (C) ventral lepidotrichia
Means followed by the same letter are not significantly different from each letter at 5% Tukey's test

Caudal circulation extended only up to the differentiated area extending until the border of the mesenchymal blastema. There were no observable differences in the blood flow of those *D. rerio* treated with **1a-1c** (1:1:1) and **1a-1c** (2:2:1) as compared to those treated with 1% DMSO. Arterial loops were noted rebounding along the blastema border. Such structure is necessary to ensure continuous supply of circulatory elements and nutrition to the regenerating tissues. Hyper pigmentation was seen more dominant in the dorsal and ventral region as compared to the medial segment. There was however lesser degree of hyperpigmentation in either ratios of **1a-1c** compared to the control group which is a consequence to less pronounced blastema proliferation.

DISCUSSION

The bioactivities of sterols and triterpenes were tested on *A. salina* and *D. rerio* toxicity as well as caudal fin regeneration. Embryos exposed to **1a-1c** demonstrated low levels of toxicity in the first 24h of observation. Increase in mortality was noted at 36h until the end of 48h. There was however a more pronounced incidence of mortality in those groups exposed to **2** and **3**. Similar results were obtained in the *D. rerio* embryo toxicity assay. Compound **2**

and its derivative **3** demonstrated their toxic effects in the embryo with a 48h delay which possibly indicate that **2** and **3** are still penetrating and accumulating their effects inside the chorionic environment. These results were concomitant with earlier findings on the toxicity of **2** and **3** from *A. pyramidalis* on duck embryonic systems [9]. The time of hatching only started to happen within the 48h window keeping the embryos, during that time, well protected inside the chorion. The chorion might have played a significant role in the prevention of the detrimental effects of the test substances to the embryo. Abnormal morphology in embryos was more severe in those groups exposed to **2** and **3**. Axial deformation, pericardial edema and yolk sac edema were primarily noted with extreme effects which indicate possible disruption of normal somitomeric development, normal bone morphogenesis, and high fluid perfusion. The incidence of pericardial edema is supported by the low heart rate observed in ethanol, **1a-1c** (2:2:3), and **2** treated embryos. Compound **3** also obtained low heart rate next to **1a-1c** (2:2:3), but the effects were not enough to attain statistical significance. The impaired circulatory function as seen in the lower heart rate of these embryos is a consequence to the hyperperfusion of fluids from blood vessels to its adjacent tissues [25]. The hyperperfusion is a consequence to high vascular pressure which might be due to a possible obstruction of blood vessels or an impaired function of the heart. These results were related to the earlier findings on the effects of **1a-1c** (1:1:1), **1a-1c** (2:2:1), **1a-1c** (2:2:3) from *A.* cf. *elliptica* [10] and **2** and **3** from *A. pyramidalis* [9] which caused impairment of vascular activity in duck chorioallantoic membrane causing ghost vessels and hyperemia. The 2:2:3 ratio of α-amyrin, β-amyrin and bauerenol found in **1a-1c** has the highest bauerenol concentration among all three ratios (1:1:1, 2:2:1, and 2:2:3) used in these assays. This implies the role of bauerenol in the observed toxicity and manifestation of abnormal morphology. Similar findings were reported on the efficacy of the three ratios of **1a-1c** from *A.* cf. *elliptica* on the inhibition of blood vessel sprouting and branch point formation resulting to lower vascular density in duck chorioallantoic membrane [10]. Bauerenol from the dichloromethane/methanol extract from the roots of *Anthocleista schweinfurthuii* was reported to have α-glucosidase inhibitory activity [26]. These inhibitors prevent the absorption of carbohydrates by competitive inhibition with α-glucosidase [27]. The action of baurenol might have prevented the metabolism of carbohydrates in the embryonic system, thereby simulating a condition similar to glycogen storage disease which involves the abnormal deposition of harmful chemicals such as glycogen in the heart, muscle, liver, kidney, spinal cord and brain later on causing pericardial edema as one of its symptoms [28]. Amputation of messenchymal layer of cells in the *D. rerio* regenerating caudal fin include fibroblasts, blood vessels, endothelial cells, axons, glial cells, osteoblasts, osteoclasts, and pigment cells [18, 29-30]. The formation of regeneration blastema starts at 36h and then undergoes active cell proliferation and differentiation during regenerative growth to replace the lost tissue [31]. Treatment with **1a-1c** (1:1:1) and **1a-1c** (2:2:1) inhibited caudal fin regeneration in young adult *D. rerio*. Between the two ratios, **1a-1c** (1:1:1) obtained a shorter regenerated length in all three regions of the caudal lepidotrichia. The inhibition of caudal fin regeneration in those groups administered with **1a-1c** implies the impairment of related factors such as the rate of cell proliferation and differentiation, development and morphogenesis of osteoblasts, osteoclasts, pigments cells, fibroblasts and blood vessels. The impairment of cell proliferation and differentiation are supported by the findings in the *A. salina* toxicity assay which shows a certain degree of toxicity at 36-48h observation period. The results in the *D. rerio* embryo toxicity assay also support the findings demonstrating characteristic toxicity at 3-5dph. Lesser degree of pigmentation was observed along the differentiation area proximal to the blastema as compared to the degree of hyperpigmentation in the DMSO treated group. Such observation is supported by the low melanocyte density in the dorsal cephalic region of *D. rerio* embryos. Melanocytes exhibit almost unlimited self-renewal capacity during regenerative processes in *D. rerio* caudal fin regeneration [31]. Low pigmentation is one of the indicators of teratologic manifestations in *D. rerio* embryos [17]. The inhibition of caudal fin regeneration in **1a-1c** (1:1:1) exposed *D. rerio* can also be associated with the effects of bauerenol. The **1a-1c** (1:1:1) ratio contains more bauerenol as compared to 2:2:1, hence the amount of bauerenol in the mixture is a limiting factor that might cause deleterious effects to developing embryonic systems.

CONCLUSION

The results of the current study present the relative toxicity of substances isolated from the dichloromethane extract of *A. pyramidalis* and *A.* cf. *elliptica* with specific reference to mortality, teratological manifestations and inhibition of regeneration. The mixture of **1a-1c** at concentration ratios of 1:1:1, 2:2:1 and 2:2:3 from *A.* cf. *elliptica* indicate the varying efficacy and synergism of these three triterpenes. The higher concentration of bauerenol in the mixture, the greater is the toxicity, teratologic manifestations (axial deformation and yolk sac edema), and inhibition of blastema cell proliferation and differentiation. Compounds **2** and **3** from *A. pyramidalis* were found toxic with primary manifestations of axial deformation, pericardial edema and yolk sac edema in *D. rerio* embryos. Proteotoxicity should be examined to determine the specific response of the organism in relation to developmental genes that might have been affected during the toxic assault.

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