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Analgesic triterpenes from *Ardisia* cf. *Elliptica* (Subgenus: Tinus) (Myrsinaceae)

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ABSTRACT

*The dichloromethane extract of the air-dried leaves of Ardisia cf. elliptica (subgenus Tinus) afforded a mixture of bauerenol (**1a**), α-amyrin (**1b**) and β-amyrin (**1c**). Their structures were identified by NMR spectroscopy. The mixtures of triterpenes (**1a-1c**) at ratios of 2:2:1 and 1:1:1 were tested for their analgesic property using the hot plate, tail flick and acetic acid-induced writhing assays. Results from the hot plate assay indicate that 5, 10, and 50 mg kg^{-1} body weight (BW) of **1a-1c** (2:2:1) had analgesic activity comparable to the positive drug control (Diclofenac) at 75 min. Both ratios of **1a-1c** were found not effective in the tail flick assay. The mixture (2:2:1) was found most effective in the acetic acid writhing assay. Reduced analgesic activity however was observed in the other mixture (1:1:1).*

Key words: *Ardisia* cf. *elliptica* (subgenus Tinus), Myrsinaceae, bauerenol, α-amyrin, β-amyrin, analgesic

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INTRODUCTION

The genus *Ardisia* Swartz belongs to the family Myrsinaceae. *Ardisia* has 68 recorded species in the Philippines [1], 60 of which are endemic. These are primarily distributed in Mindoro, Polilio, Samar, Panay, Mindanao, Palawan, Leyte, Biliran, Nueva Ecija, Laguna, Bicol, Ilocos Sur, Ilocos Norte, Sambali, Negros Occidental, Negros Oriental, Cebu, Agusan, Pampanga, Batangas, Cagayan and some other areas in Luzon. It is commonly known as *Tagpo*. *Ardisia* leaves are eaten as vegetable, used as greens for salad, or cooked with meat or fish [2]. The flowers and fruits may be cooked and used as flavoring for fish. Young leaves are also eaten by ruminants, while the fruits are eaten by monkeys and wild pigs and birds [2]. *Ardisia pyramidalis* fruit approximate analysis [3] revealed high fiber content (37.99%), crude protein (13.50%), crude fat (0.41%) and some minerals such as Ca (0.96%), P (0.21%), K (1.90%) and N (2.16%). Spinasterol, spinasteryl acetate, 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* [4].

The genus *Ardisia* is a good source of health promoting compounds and potent phytopharmaceuticals [5] . Recent studies revealed high anti-cancer properties in a number of *Ardisia* species tested. The polyphenolic compounds and flavonoids present in *A. compressa* tea were cytotoxic on human colorectal carcinoma (HT-29 and Caco-2. LC–MS) by catalytic inhibition of DNA topoisomerase [6]. The aqueous extracts (tea) of *A. compressa* leaves was found to be cytotoxic on HepG2 cells by the inhibition of topoisonmerase II acting as catalytic inhibitors [7], *in-vitro* anti-oxidant defense against 1-nitropyrene and benomyl-induced cytotoxicity in rat hepatocytes [6, 8], and anti-carcinogenesis in the liver [8]. In addition, *A. compressa* also has hepatoprotective property [9]. *A. arboresence* was found to have antipyretic properties [10], while *A. colorata* has been found to be hepatoprotective, mucolytic and antidiarrheal [11]. A number of biological activities have been reported for *A. crenata* where it was found to induce uterine contraction [12], platelet aggregation and induce blood pressure lowering [13], cAMP inhibition [14], antiviral [15] and antithrombin activity. The methylene chloride:methanol extracts from the leaves of *A. crenata* was found to have antithrombin activity [16]. A novel compound, ardisenone, was isolated from twigs and leaves of *A. iwahigensis* [17]. Further, ardisenone was found to have significant cytotoxic activity in murine cells and later was identified anticancer as well. In addition to this, our recent findings indicate the anti-angiogenic potential of triterpenes from *A. pyramidalis* [4]. In addition, Philippine indigenous *A. squamulosa* was identified to have effects on spermatogenesis in rats [18] as well as an angiosuppressive activity of a hexane fraction from its methanolic extract on duck chorioallantoic membrane [19]. The angio-suppressive effects of a mixture of triterpenes (bauerenol, α-amyrin and β-amyrin) at different rations were recently reported [20]. Despite the numerous bioactivities of this genus, there has been very limited information on the biological potentials of indigenous *Ardisia* species as possible sources of phytopharmaceuticals. In the current study, we report the isolation and analgesic potential of mixtures of triterpenes from the dichloromethane extract of the leaves of *Ardisia* cf. *elliptica* (subgenus Tinus).

MATERIALS AND METHODS

General Experimental Procedures

^{13}C NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 150 MHz. 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.

Plant Material

Fresh leaves of *Ardisia* cf. *elliptica* (subgenus Tinus) were collected from a chromafic low altitude area along primary thickets (cogon grassland) at the foot of Mt. Pulido, Barangay Pacalat, Mangatarem, Pangasinan, Luzon Island, Philippines (15°44'16.20 N: 120°16'111.27 E). The genus of the plant sample was identified at the National Museum, Manila by Dr. Domingo S. Madulid in collaboration with Dr. John Pipoly of the Urban Horticulture/Climate Change Extension, Broward County Extension, Florida. Species identification was not done yet due to the sheer complexity of the genus *Ardisia* composing of several tribes and subgenera. For the purpose of identification and verification of future results, a morphologic description and photographic reference of *Ardisia* sp. was presented [20].

Extraction and Isolation

Air-dried leaves of *Ardisia* cf. *elliptica* (subgenus Tinus) (1 kg) were ground in an osterizer and then soaked in dichloromethane (DCM) for 3 d and filtered. The filtrate was concentrated *in vacuo* to afford a crude extract (21 g) which was chromatographed in increasing proportions of acetone in DCM at 10 % increment. The 30% acetone in DCM fraction was rechromatographed (3x) using 5% ethyl acetate in petroleum ether to afford a mixture of **1a-1c** (295 mg, 2:2:1). This mixture (185 mg) was rechromatographed using 5% ethyl acetate in petroleum ether (2x) to afford a mixture of **1a-1c** (114 mg, 1:1:1). The procedure was repeated several times to get enough samples for the bioassay. The ratios of the three triterpenes were determined from the integrations of the olefinic proton resonances at δ 5.39 for bauerenol, δ 5.11 for α-amyrin and δ 5.16 for β-amyrin.

Preparation of test substance

The mixture of bauerenol, α-amyrin and β-amyrin (**1a-1c**) with a ratio of 2:2:1 and another mixture of **1a-1c** (1:1:1) from *Ardisia* cf. *elliptica* (subgenus Tinus) was dissolved in dimethylsulfoxide (DMSO) and diluted by sterile

phosphate buffered saline (PBS) to obtain a final DMSO concentration of 0.1%. The volume of the suspension was computed per gram body weight (BW) of the individual animal following three dosages (5, 10, and 50 mg kg^{-1} BW) of the mixtures.

Experimental animals

A total of 169 ICR mice (6-8 wk old *Mus musculus* L.) with an average weight of 29.22 ± 4.50 g were obtained from the Experimental Animal House of the Bureau of Food and Drugs, Muntinlupa City were used. The experimental animals were acclimatized for 1 wk followed by a 2 h post acclimatization period. The animals were randomized in 30.48 x 20.32 x 12.7 cm plastic cages fitted with a wire mesh cover at 5 mice per cage. The animals were fed twice a day at morning and afternoon feeding with sterilized beef meal dog chow (Beef Pro Puppy, Northwest Pet Products Inc.) with 32% crude protein and 20% crude fat content. Sterile water was made available to the mice *ad libitum*. Body weights and behavioral indicators of toxicity such as jumping, grooming, curiosity and overall activity were also noted during the acclimatization period. The test animals were subjected to a 13-h fasting period prior to the bioassay.

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.

Bioassays

Analgesic

The investigation of the analgesic potential of **1a-1c** with a ratio of 2:2:1 from *Ardisia* cf. *elliptica* (subgenus Tinus) was performed followed by the investigation of the same mixture but with a ratio of 1:1:1. The analgesic potential was performed using three standard pharmacologic assays namely hot plate, tail flick, and acetic acid writhing assay. The mixtures (5, 10, and 50 mg kg^{-1} BW), Diclofenac (7.14 mg kg^{-1} BW, GX International, Philippines) and PBS as the positive and negative controls respectively were orally administered to the test animals.

Hot plate assay

Hot plate assay was performed modified from the reported procedure [21,22]. The assay was performed 30 min after oral administration of the mixture. Latency reaction was observed and immediately recorded upon exposure to the heat stimulus (50°C). The response was graded and recorded as reaction time (sec) as soon as the mice began to exhibit behavioral attempts to remove its feet from the hot surface characterized by jumping or licking of the paws. The frequencies were compared to the negative control and presented as percent inhibition.

Tail flick assay

Tail flick assay was performed according to the reported procedure [23]. Tail flick assay was done on mice (n=7) by immersing one third of the mice's tail in a 50°C hot water bath 45 min after oral administration of the treatments. The time it took the mice to react by attempting to remove its tail from the thermal stimulus was recorded and presented as per cent inhibition.

Acetic acid-induced writhing assay

Acetic acid-induced writhing assay was performed according to the reported procedures [24, 25]. The test animals (n=14) were injected intraperitoneally with 1% glacial acetic acid (Ajax, FineChem, Australia) 1h after oral administration of the treatments. Abdominal stretches of the mice were counted within 10 min upon injection of the chemical stimulus and presented as percent inhibition.

Statistical Analysis

Results were analyzed using SPSS version 13 for Windows. One Way Analysis of Variance (ANOVA) was performed to determine the significant analgesic effects of the test compounds. Post hoc analysis using Tukey's Test was used (α=0.05) to determine significant differences between group variables. The results obtained were considered significant at $P < 0.05$. Results are presented as Mean±SEM of the percent inhibition.

RESULTS

The dichloromethane extracts of the air-dried leaves of *Ardisia* cf. *elliptica* (subgenus Tinus) afforded the 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**) [26], α-amyrin (**1b**) [27] and β-amyrin (**1c**) [27]. The ratios of the three triterpenes were determined from the integrations of the olefinic proton resonances at δ 5.39 for bauerenol, δ 5.11 for α-amyrin and δ 5.16 for β-amyrin.

Bauerenol (**1a**): colorless solid. ^{13}C NMR: 36.9 (C-1), 27.7 (C-2), 79.0 (C-3), 38.9 (C-4), 50.4 (C-5), 24.1 (C-6), 116.4 (C-7), 145.2 (C-8), 48.2 (C-9), 35.3 (C-10), 16.9 (C-11), 32.4 (C-12), 37.7 (C-13), 41.5 (C-14), 28.9 (C-15), 37.7 (C-16), 32.0 (C-17), 54.9 (C-18), 35.3 (C-19), 32.0 (C-20), 29.7 (C-21), 31.5 (C-22), 27.5 (C-23), 14.7 (C-24), 13.0 (C-25), 23.7 (C-26), 22.7 (C-27), 40.0 (C-28), 25.6 (C-29), 22.5 (C-30).

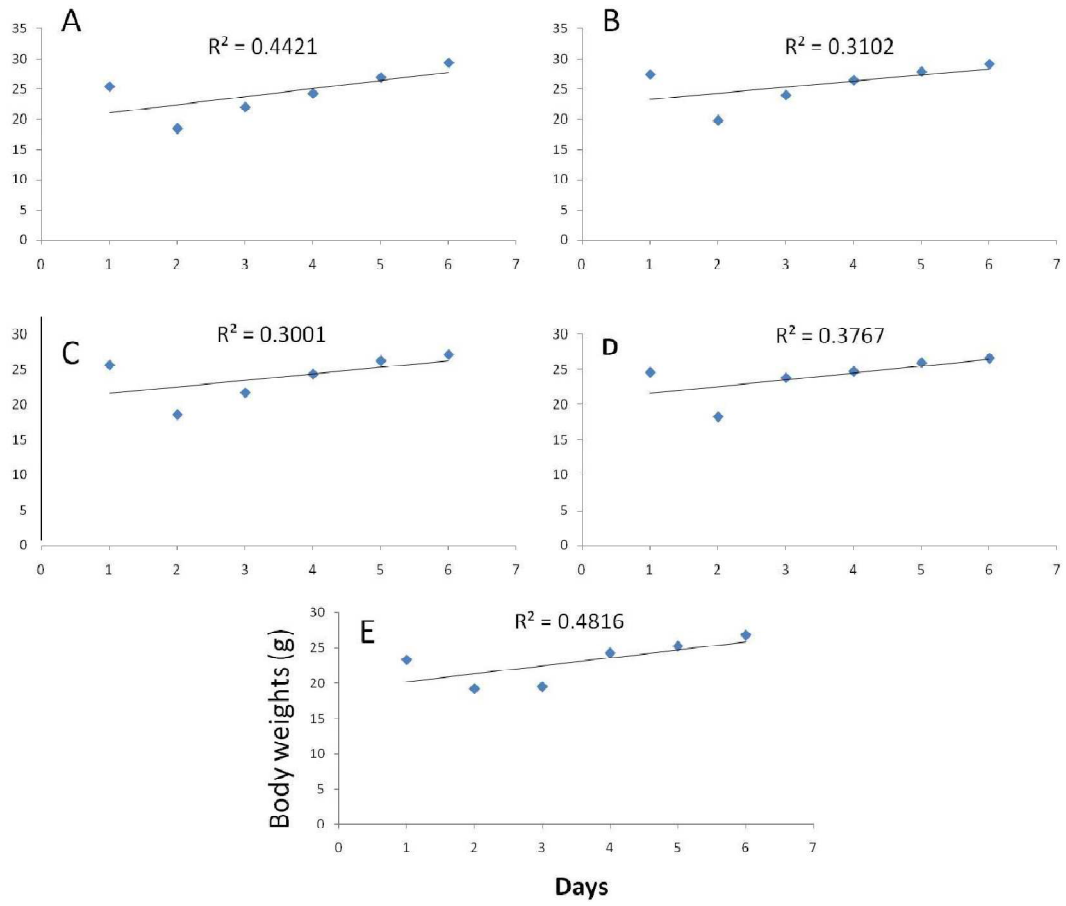

Figure 1. Body weight increase of mice during acclimatization period. Mice treated A) phosphate buffered saline (PBS), B) Diclofenac, C) 5 mg kg^{-1} body weight (BW) 1a-1c (2:2:1, D) 10 mg kg^{-1} BW 1a-1c (2:2:1) and E) 50 mg kg^{-1} BW 1a-1c (2:2:1) revealed normal increase in body over a 7-day acclimatization period

α-Amyrin (**1b**): colorless solid. ^{13}C NMR: 38.8 (C-1), 27.2 (C-2), 79.3 (C-3), 38.8 (C-4), 55.2 (C-5), 18.3 (C-6), 32.9 (C-7), 40.0 (C-8), 47.7 (C-9), 36.9 (C-10), 23.3 (C-11), 124.4 (C-12), 139.6 (C-13), 42.1 (C-14), 28.7 (C-15), 26.6 (C-16), 33.7 (C-17), 59.1 (C-18), 39.6 (C-19), 39.7 (C-20), 31.2 (C-21), 41.5 (C-22), 28.1 (C-23), 15.7 (C-24), 15.6 (C-25), 16.8 (C-26), 23.3 (C-27), 28.1 (C-28), 17.5 (C-29), 21.4 (C-30).

β-Amyrin (**1c**): colorless solid. ^{13}C NMR: 38.6 (C-1), 27.3 (C-2), 79.0 (C-3), 38.8 (C-4), 54.9 (C-5), 18.4 (C-6), 32.6 (C-7), 38.8 (C-8), 47.7 (C-9), 37.7 (C-10), 23.5 (C-11), 121.7 (C-12), 145.2 (C-13), 41.7 (C-14), 26.1 (C-15), 27.2 (C-16), 32.5 (C-17), 47.6 (C-18), 46.8 (C-19), 31.2 (C-20), 34.7 (C-21), 37.1 (C-22), 28.1 (C-23), 15.6 (C-24), 15.7 (C-25), 16.9 (C-26), 26.1 (C-27), 28.4 (C-28), 33.3 (C-29), 23.7 (C-30).

The analgesic property of the mixture, α amyrin, β amyrin and bauerenol, from *Ardisia* cf. *elliptica* (subgenus Tinus) was evaluated using thermal assays namely the hot plate and tail flick assays and a chemical test, the acetic acid writhing assay on ICR mice.

General Observations

There were no behavioral indicators of toxicity observed in the test animals prior to the duration of the study. This is characterized by no apparent weight loss, normal grooming, curiosity and constant movement such as climbing, digging into the beddings and playing, a fast reflex response and other behavioral manifestations [28]. This is further supported by the constant increase in bodyweights which indicates normal growth and development of mice during the acclimatization period (Figure 1).

*Analgesic Property of **1a-1c** (2:2:1)*

The data obtained from the hot plate assay reveals that treatment with **1a-1c** (2:2:1) has significantly (P = 0.039) affected the pain sensitivity of mice at 75 min post treatment (Table 1). Although statistical differences (P=0.0001) between treatments were obtained during 30 min post treatment, **1a-1c** (2:2:1) did not have sufficient potency to inhibit sensitivity to the pain stimulus. Mice administered with the positive control (Diclofenac) had delayed perception of pain obtaining the highest % maximum analgesic effect among other treatments. Mice administered with Diclofenac after 75 min post treatment showed continued inhibition of pain sensitivity. The three doses of **1a-1c** (2:2:1) on the other hand revealed no significant difference with that of the positive control.

Table 1. Analgesic property of a mixture of bauerenol, α-amyrin, and β-amyrin (1a-1c, 2:2:1) on a thermally induced stimulus (hot plate assay)

Treatment	30 min		75 min	
	Time (*sec*)	% inhibition (P = 0.0001)	Time (*sec*)	% inhibition (P = 0.039)
0.1% DMSO in PBS	2.26 ± 0.83	-11.22 ± 34.34^{ab}	1.02 ± 0.19	-3.62 ± 21.78
Diclofenac	2.64 ± 0.91	23.78 ± 18.33^{a}	1.45 ± 0.45	16.98 ± 32.88
5 mg kg^{-1} BW (2:2:1)	1.38 ± 0.14	-64.85 ± 16.13^{c}	1.40 ± 0.15	26.56 ± 7.17
10 mg kg^{-1} BW (2:2:1)	1.90 ± 0.48	-27.51 ± 36.27^{b}	1.46 ± 0.54	21.28 ± 29.63
50 mg kg^{-1} BW (2:2:1)	1.42 ± 0.20	-61.51 ± 20.85^{c}	1.33 ± 0.24	21.25 ± 13.63

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight; Means followed by the same letter superscripts are not significantly different 95% Tukey's test (α=0.05)*

Analysis on the effects of **1a-1c** (2:2:1) in the tail flick assay indicates that 5 and 10 mg kg^{-1} BW **1a-1c** (2:2:1) had no significant (P = 0.128) difference with the effects obtained in the positive control at 30 min post treatment. Table 2 presents that %maximal analgesic effect was elicited in mice administered with 10 mg kg^{-1} BW. At 75 min post treatment, mice administered with Diclofenac and 5 mg kg^{-1} BW **1a-1c** (2:2:1) showed similar analgesic effect.

Table 2. Analgesic property of a mixture of bauerenol, α-amyrin and β-amyrin (1a-1c, 2:2:1) on a thermally induced stimulus (tail flick assay)

Treatment	30 min		75 min	
	Time (*sec*)	% inhibition (P = 0.128)	Time (*sec*)	% inhibition (P = 0.506)
0.1% DMSO in PBS	2.26 ± 0.98	-22.01 ± 73.41	2.68 ± 0.80	-5.90 ± 27.13
Diclofenac	3.13 ± 0.87	20.61 ± 30.56	3.10 ± 0.82	13.42 ± 33.56
5 mg kg^{-1} BW (2:2:1)	3.35 ± 1.25	22.65 ± 33.10	2.70 ± 0.95	0.04 ± 34.29
10 mg kg^{-1} BW (2:2:1)	6.02 ± 7.85	23.69 ± 15.53	6.58 ± 9.80	10.66 ± 22.23
50 mg kg^{-1} BW (2:2:1)	2.17 ± 0.75	-16.37 ± 47.16	2.40 ± 0.56	-6.49 ± 25.01

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight*

Since it has been pointed out that the perception of pain is affected by sex differences, male and female mice were tested separately in chemical pain model. The overall sensitivity to a chemical pain stimulus in females was significantly (P=0.011) affected by the administration of **1a-1c** (2:2:1) (Table 3). Female mice administered with **1a-1c** (2:2:1) have demonstrated analgesic effects similar to that of Diclofenac. The analgesic response in those mice administered with the positive control was concomitant with those of the reported values [23-25]. A slightly higher % inhibition of pain was observed in male mice compared to the average responses seen in the female subjects that underwent the same assay. The analgesic effects observed in male mice compared to the female

however had similar analgesic response patterns as a reaction to the chemical stimulus at 75 min post treatment of the mixture.

Table 3. Analgesic property of a mixture of bauerenol, α-amyrin, and β-amyrin (1a-1c, 2:2:1) on a chemically induced stimulus (acetic acid writhing assay)

Treatment	Male		Female	
	Time (*sec*)	% inhibition (P = 0.397)	Time (*sec*)	% inhibition (P = 0.011)
0.1% DMSO in PBS	35.40 ± 42.31	0.00 ± 119.53	6.23 ± 2.45	-0.01 ± 39.40[b]
Diclofenac	13.60 ± 2.44	61.58 ± 20.43	2.50 ± 2.44	59.87 ± 39.19[ab]
5 mg kg^{-1} BW	9.80 ± 6.26	72.32 ± 17.69	1.86 ± 1.86	70.19 ± 29.93[a]
10 mg kg^{-1} BW	17.00 ± 6.36	51.98 ± 17.98	3.14 ± 4.34	49.55 ± 69.61[ab]
50 mg kg^{-1} BW	19.60 ± 18.35	44.63 ± 51.84	4.00 ± 3.87	35.79 ± 62.17[ab]

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight; Means followed by the same letter superscripts are not significantly different 95% Tukey's test (α=0.05)*

Analgesic property of **1a-1c** (1:1:1)

Further investigation was conducted on the analgesic effects of α-amyrin, β-amyrin and bauerenol mixture with 1:1:1 ratio. Table 4 presents the %analgesic effects of **1a-1c** (1:1:1) in the hot plate assay. Similar analgesic response was obtained in those mice administered with the mixture of **1a-1c** (1:1:1) compared to those administered with **1a-1c** (2:2:1). There was no positive analgesic effect observed in the animals administered with **1a-1c** (1:1:1) at 30 min post treatment of the hotplate assay. Inhibition of pain however was observed in those mice administered with **1a-1c** (1:1:1) at 75 min post treatment.

Table 4. Analgesic property of a mixture of bauerenol, *α-amyrin,* and β-*amyrin* (1a-1c, 1:1:1) on a thermally induced stimulus (hotplate assay)

Treatment	30 min		75 min	
	Time (*sec*)	%inhibition (P=0.0001)	Time (*sec*)	%inhibition (P=0.186)
0.1% DMSO	2.26 ± 0.83	-11.22 ± 34.34[ab]	1.02 ± 0.19	-3.62 ± 21.78
Diclofenac	2.64 ± 0.91	23.78 ± 18.33[a]	1.45 ± 0.45	16.98 ± 32.88
5 mg kg^{-1} BW (1:1:1)	1.59 ± 0.22	-44.03 ± 20.18[c]	1.52 ± 0.65	22.61 ± 29.59
10 mg kg^{-1} BW (1:1:1)	1.23 ± 0.27	-89.96 ± 36.93[d]	1.21 ± 0.33	10.40 ± 23.08
50 mg kg^{-1} BW (1:1:1)	1.57 ± 0.39	-50.80 ± 32.69[c]	1.10 ± 0.12	6.90 ± 91.75

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight; Means followed by the same letter superscripts are not significantly different 95% Tukey's test (α=0.05)*

In the tail flick assay, administration of **1a-1c** (1:1:1) in mice did not elicit significant analgesic potency at 30 min (P=0.420) and 75 min (P=0.133) post treatment. Although Diclofenac obtained the longest time of reaction, no significant differences were noted among all treatments (Table 5). The minimal potency observed in the test animals administered with the crude sample was not evident anymore when the same mixture with a ratio of 1:1:1 was administered in mice which could probably indicate the antagonistic interaction of the three compounds at equal ratio.

Table 5. Analgesic property of a mixture of bauerenol, α-amyrin, and β-amyrin (1a-1c, 1:1:1) on a thermally induced stimulus (tail flick assay)

Treatment	30 min post treatment		75 min post treatment	
	Time (*sec*)	%inhibition (P=0.420)	Time (*sec*)	%inhibition (P=0.133)
0.1% DMSO	2.60 ± 0.98	-22.01 ± 73.41	2.68 ± 0.80	-5.90 ± 27.13
Diclofenac	3.13 ± 0.87	20.61 ± 30.56	3.10 ± 0.82	13.42 ± 33.56
5 mg kg^{-1} BW (1:1:1)	2.46 ± 0.94	-13.29 ± 70.88	2.18 ± 0.85	-24.55 ± 41.69
10 mg kg^{-1} BW (1:1:1)	2.57 ± 0.73	5.87 ± 27.93	2.11 ± 0.86	-31.71 ± 49.77
50 mg kg^{-1} BW (1:1:1)	2.48 ± 0.69	3.32 ± 24.63	2.79 ± 1.25	-0.84 ± 39.85

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight*

Acetic acid writhing assay of **1a-1c** (1:1:1)

Analysis of the analgesic property of the **1a-1c** (1:1:1) using the acetic acid writhing assay showed relatively high potential of the mixture towards inhibiting the reception of the pain stimulus in female mice (Table 6). Loss of analgesic potential was observed from female mice administered with the same sample which could probably

indicate a possible antagonistic activity of the three compounds contrary to what was observed in the effects of the crude sample.

Table 6. Analgesic property a mixture of bauerenol, *α*-amyrin, and β-amyrin (1a-1c, 1:1:1) on a chemically induced stimulus (acetic acid writhing assay)

Treatment	Male		Female	
	Time (*sec*)	%inhibition (P=0.351)	Time (*sec*)	% inhibition (P=0.001)
0.1% DMSO	35.4 ± 42.31	0.00 ± 119.53	6.23 ± 2.45	39.40 ± 10.93[b]
Diclofenac	13.62 ± 2.44	61.58 ± 20.43	2.5 ± 2.44	59.87±39.19[b]
5 mg kg^{-1} BW (1:1:1)	23.8 ± 9.93	32.77 ± 28.06	0.29 ± 0.49	7.83 ± 2.96[a]
10 mg kg^{-1} BW (1:1:1)	23.8 ± 6.76	32.77 ± 19.10	3.29 ± 1.70	27.365 ± 10.34[ab]
50 mg kg^{-1} BW (1:1:1)	10.8 ± 5.97	69.49 ± 16.88	7.0 ± 7.23	-12.36 ± 116.12[b]

**DMSO-dimethylsulfoxide; PBS-phosphate buffered saline; BW-body weight; Means followed by the same letter superscripts are not significantly different 95% Tukey's test (α=0.05)*

DISCUSSION

α-Amyrin and β-amyrin are pentacyclic triterpenes which are known for their broad biological uses, some of which include antioxidant, anti-allergic, anti-inflammatory, anticarcinogenic, antiviral, antibacterial, analgesic, gastroprotective, hepatoprotective, cytotoxic, and cardioprotective properties [29]. Oral administration of a mixture of α-amyrin and β-amyrin was proven positive for analgesic activity in a chemically induced pain model but not on a thermal pain model [30]. Mice injected with α-amyrin and β-amyrin had significant reduction of orofacial pain induced by formalin or capsaicin [31] and suppressed scratching behavior induced by dextran T40 [32]. Studies indicate significantly suppressed nociceptive behaviors of mice exclusively administered with α amyrin, β amyrin [33, 34]. Bauerenol from *A. eliptica* on the other hand was reported to have antibiotic properties [5]. and bauerenol in combination with α-amyrin and β-amyrin was found to have 51% analgesic effect [35]. In the current study, the mixture of bauerenol, α amyrin, and β amyrin isolated from *Ardisia* cf. *elliptica* (subgenus Tinus) was tested for its analgesic activity using the tail flick, hot plate and acetic acid writhing assays. A minimal analgesic effect was observed in mice administered with the positive control and a very minimal to no analgesic effect was observed in mice administered with both mixtures of **1a-1c** in the hotplate and tail flick assays. The minimal potency could be attributed to three main factors such as the kind of target receptors specific for the compounds, the relative solubility of the mixture and a possible antagonistic interaction between the components of the mixture. Since the hotplate assay evaluates supraspinally mediated nociception [36] the action of **1a-1c** and Diclofenac might not be specific to elicit potent action to inhibit stimulus reception or propagation of signals along the primary areas concerning supraspinal transmission of nociceptive related impulses. The tail flick assay specifically measures centrally mediated perception of pain transduced by opioid receptors [37]. The very minimal potency of both mixtures of **1a-1c** demonstrates that the possible action of the mixtures does not concern the block of opiod receptors as seen in the tail flick assay. Factors of solubility might probably be due to certain impurities such as fatty acids in the mixture thereby contributing to a limited analgesic action of the substance rendering it incapable of eliciting proper analgesic response compared to the reported activities of the compounds. A possible antagonistic interaction may also exist along with some impurities in the mixture which may be responsible the loss of potency observed in mice.

The acetic acid writhing assay demonstrates the peripheral inhibition of pain triggered by localized anti-inflammatory response mediated by peritoneal mast cells, acid sensing ion channels and prostaglandin pathways which are activated when acetic acid is introduced into the system [37]. Although no dose relationship was observed in the experimental animal's response in the acetic acid writhing assay 5 mg kg-1 BW **1a-1c** showed comparable analgesic effect with Diclofenac, but the observed analgesic activity was lost in the **1a-1c** (1:1:1) treatment. Similar patterns were observed in both male and female treatments. The %analgesic effect obtained in for Diclofenac is also concomitant with that of the results in our previous study [23-25]. The acetic acid writhing test is sensitive in examining peripheral acting analgesics [38]. The reduced writhing frequency observed in each mouse indicates that there was desensitization of pain in the body by preventing the release and conversion of free arachidonic acid from phospholipid tissues into prostaglandins [39, 40]. via the COX-II inhibition pathway. Thus, a reduced writhing frequency indicates the inhibition of the synthesis of prostaglandin [37]. Similar responses were observed with **1a-1c** and Diclofenac which is classified as a non-steroidal anti-inflammatory drug generally inhibiting the action of cyclooxygenase as synthesis of prostaglandin precursor [41]. The mixture of **1a-1c** (2:2:1) was found to be more efficacious at 5 mg/ Kg BW potent at 1h post treatment compared to **1a-1c** (1:1:1).

CONCLUSION

Ardisia cf. *elliptica* (subgenus Tinus) share similarities in their chemical composition with previously reported indigenous species where bauerenol, α-amyrin and β-amyrin are found present in the dichloromethane extract of leaves. The analgesic property was determined to be most potent in inhibiting the reception of a chemically induced stimulus observed in the acetic acid writhing assay. A mixture of **1a-1c** with a ratio of 1:1:1 had reduced analgesic activity in mice. A receptor assay could be performed as well to determine the specific receptors involved in the inhibition of the chemical stimulus.

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