World Journal of Organic Chemistry, 2013, Vol. 1, No. 1, 1-5
Available online at http://pubs.sciepub.com/wjoc/1/1/1

DOI:10.12691/wjoc-1-1-1

Toxicity of Individual and Blends of Pure Phytoecdysteroids Isolated from *Vitex Schiliebenii* and *Vitex Payos* against *Anopheles Gambiae* S.S. Larvae

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Received December 31, 2012; Revised February 23, 2013; Accepted March 12, 2013

Abstract Four phytoecdysteroids: (20-hydroxyecdysone-20, 22-monacetonide (**1**), 20-hydroxyecdysone (**2**), stigmasterol (**3**), gamma-sitosterol (**4**), were investigated for toxic effect against 3rd /4th instar larvae of *Anopheles gambiae* under laboratory conditions as individual compounds and in blends. The test larvae were treated with solutions containing the phytoecdysteroids of concentrations 1, 5 and 10ppm. The blends were prepared in the ratio of 1:1. Compounds **1** and **2**, isolated from acetone extracts of *Vitex payos* caused 100% mortality at 10ppm. Compound **3** isolated from acetone leaves of *V. schiliebenii* and compound **4** isolated from acetone stem bark of *V. schilebenii* also showed potent activity against the larvae at 10ppm. At the lower concentrations, abnormal mobility and impaired development was observed. Phytoecdysteroids (20-hydroxyecdysone-20, 22-monacetonide (**1**) and 20-hydroxyecdysone (**2**) are larvicidal against *An. gambiae*. stigmasterol (**3**), gamma-sitosterol (**4**) also show potent IGR activities against *An.* gambiae. Also addition of compounds **1** and **2** to stigmasterol (**3**) and gamma-sitosterol (**4**) seperately improved the activity of the two compounds.

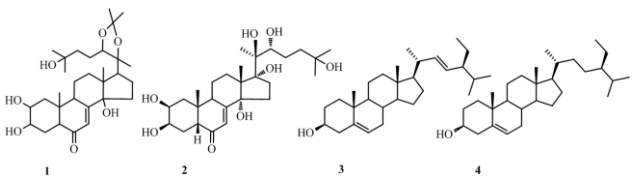
Keywords: *anopheles gambiae, phytoecdysteroids vitex schiliebenii, V. payos*

1. Introduction

Phytoecdysteroids are plant analogues of insect growth hormones whose role in insect molting is understood, but their function in plants is uncertain [1]. However, it has been suggested that these chemicals are synthesized by the plants for their defense against phytophagous insects [1]. It is therefore expected that their production will be enhanced by insect attack. In the search for bioactive secondary metabolites with potential in the control of disease vectors and/or the diseases they transmit, detrimental effects including reduced weight, molting disruption and/or mortality have been reported in insects which ingest phytoecdysteroids [2]. These structurally diverse compounds have also been reported to affect protozoa and mollusks by impairing the digestion of protein and uptake of vitamins and minerals in their gut [2,3].

One of the main benefits of phytoecdysteroids is their therapeutic effects in mammals including humans. Their claimed medical properties include adaptogenic [4], anabolic [3], hypoglycemic [5], hepatoprotective [3], immunoprotective, wound-healing and anti-tumor. Other pharmacological properties associated with phytoecdysteroids include anti-proliferative, anti-microbial and anti-oxidant activities [6]. Ecdysteroids are also considered as nutraceutical additives to food products [7].

Phytoecdysteroids are found in a wide range of species in the genus *Vitex* and may be used as taxonomic markers. An extensive literature search on the genus *Vitex* revealed the presence of ecdysteroids in a number of *Vitex* species [8] found in tropical and subtropical regions [9]. They affect a wide range of insects at low concentrations and are not harmful to human and animal cells [10]. Literature reports indicate that ingested phytoecdysteroids affect several insect species for example *Spodoptera frugiperda [11]*, *Bombyx mori* [12,13], *Lobesia botrana* [14], *Inachis io*, *Alglass urticae* [15] and *Bradysia impatiens* [16], resulting in significant growth and developmental disruption. However, some insect species such as *Heliothis virescens* [17], *H. armigera* [18], *S. litturalis* [19], *Locusta oleracea*, *Ostrinia nubilalis* [20], and *Lacanobia oleracea* [21] were reported not to be affected after phytoecdysteroid ingestion by developing a detoxifying mechanism [22].



In this study, the author reports the effects of four phytoecdysteroids namely (20-hydroxyecdysone-20,22-monacetonide (**1**), 20-hydroxyecdysone (**2**), Stigmasterol (**3**), Gamma-sitosterol (**4**) on 3rd and 4th instar larvae of *An. gambiae* under laboratory conditions. The compounds were isolated from two *Vitex* species namely, *V.*

schiliebenii and *V. payos* collected from the Kenyan coastal region.

2. Materials and Methods

2.1. Plant Materials

Leaves and stem bark of *V. schiliebenii* and root bark of *V. payos* used in this study were collected from the Kenyan coastal region in 2009. The plants were authenticated at the field by a botanist from the National Museum of Kenya (NMK), who preserved a voucher specimen (GMN/22) at the museum. The materials were air-dried at room temperature under the shade for three weeks and ground into powder in an electric miller.

2.2. Extraction of Plant Materials

Each powdered material was extracted three times using acetone (5-fold volume) for 24h with occasional stirring at room temperature. The extracts were filtered and concentrated to dryness using a rotary evaporator at 40 ℃. The extraction process was repeated three times and the combined extracts were stored at 4 ℃. This procedure was repeated with methanol in the same proportions and for the same periods of time.

2.3. Isolation of Phytoecdysteroids

2.3.1. From Acetone Extract of *V. payos* Root Bark

Powdered root bark (30g) of *V. payos* was extracted using acetone and the extract (8.0g) was subjected to column chromatography on silica gel (105g) eluting with 100% dichloromethane and gradually increasing acetone to 100% then methanol to 30%. The 60% acetone eluent gave a mixture of two compounds, which were separated by repeated preparative Thin Layer Chromatography (PTLC) on silica gel (eluting with dichloromethane-acetone, 1:1) to give 20-hydroxyecdysone-20, 22-monacetonide (**1**) (16mg of a white amorphous powder which was soluble in methanol, with melting point 257-259^{o}C; lit. 256 ℃, [23]) and impure 20-hydroxyecdysone (**2**). This was further subjected to repeated PTLC (eluting with dichloromethane-acetone, 4:1) followed by sephadex LH_{20} (DCM: MeOH 1:1). Ten milligrams of need-like crystals of melting point 234-236 ℃ (lit. 230-233 ℃, [24] were obtained which were soluble in methanol.

2.3.2. Acetone Extract of *V. schiliebenii* Leaves

The acetone extract (10g) was subjected to column chromatography on silica gel (eluting with 100% dichloromethane and gradually increasing acetone to 100%). The 40% acetone eluent gave a mixture of two compounds, which were subjected to repeated column chromatography using DCM: MeOH to yield gamma sitosterol (**4**) (6mg with a melting point of 142-144 ℃) which was then re-crystallized using the same solvent into a white star shaped crystal soluble in DCM. The other compound was too little to be analyzed.

2.3.3. Acetone Extract of *V. schiliebenii* Stem Bark

Sixteen grams (16g) of the extract was subjected to column chromatography on silica gel with Dichloromethane: Acetone gradient (100:0 - 0:100). The 50% acetone eluent gave a mixture of two compounds (47mg), which were separated by repeated column chromatography followed by PTLC. The fraction yielded two compounds 20-hydroxyecdysone (**2**) (16mg) isolated as a crystalline solid and stigmasterol (**3**) (25mg of white crystals with a melting point of 165-7 ℃) lit. 163-6 ℃, [25].

2.4. Liebermann-Burchard Test for Steroids

The isolated compounds were examined for the presence of steroids using Liebermann-Burchard test. Acetic anhydride (2ml) was added to the compounds (2mg) and the mixture was thoroughly heated and stirred for 2 minutes on a water bath and allowed to stand at room temperature. Sulphuric acid (2ml) was gently added to 1ml acetic acid layer. The blue to green color of the upper layer suggested the presence of phytosterols.

2.5. Mosquito

Larvae of *An. gambiae* Giles *s.s.* used in bioassays were obtained from a colony maintained at the International Centre of Insect Physiology and Ecology (ICIPE) Insect Mass Rearing Unit. This strain of mosquitoes originated from ICIPE's Thomas Odhiambo Campus (Mbita Point) near Lake Victoria in 2003. Larvae were allowed to emerge from eggs in plastic containers filled with distilled water and were transferred to larger pans (37 × 31 × 6) at densities of 200-300 at 2nd instar stage. They were fed on Tetramin® fish food (Terta GmbH, Germany) and the water temperature was maintained at 28±2 ℃ throughout larval development.

2.6. Larvicidal Assays

Larvicidal and insect growth regulatory (IGR) activities of the individual and blends of the four phytoecdysteroids (20-hydroxyecdysone-20, 22-monacetonide (**1**), 20-hydroxyecdysone (**2**), stigmasterol (**3**) and gamma-sitosterol (**4**) were conducted in accordance to the World Health Organization method [26]. Batches of twenty freshly moulted late 3rd and early 4th instar larvae of *An. gambiae* s.s. were transferred by means of dropper to glass beakers containing 100ml of tap water. Appropriate volume of stock solution where the pure compounds or blends were dissolved in 5% dimethylsuphoxide (DMSO) was added to 100ml water in the glass beakers to obtain 1, 5, 10ppm dose levels. The blends were mixed in the ratio of 1:1. Three replicates were set up for each concentration and two negative controls (treated with DMSO-distilled water) were set up simultaneously. Larval mortality, abnormal behavior and/or morphological deformations were recorded at 24h intervals until the death of the last larva or emergence to adult. The bioassay room was kept at a temperature of 30 ℃, an average humidity of 78 % and a photo period of 12 hours of light and 12 hours of darkness. The larvae were fed on Tetramin® fish food (Terta GmbH, Germany) at about 1mg per beaker every 24h.

2.7. Calculation of Larval Mortality

The average number of larvae or pupae collected for each replicate of each treatment and the control were

recorded after 24h. Percentage larval mortality was estimated for each treatment according to the formula:

$$\% \text{ Mortality} = \frac{Y \times 100[26]}{Z}$$

Where: Z=Initial number of larvae introduced into each test beaker and Y=Mean death defined by the difference between the mean test deaths and the mean control deaths.

3. Results

Chromatographic separation of the acetone extracts of *V. payos* root bark, *V. schiliebenii* leaves and *V. schiliebenii* stem bark yielded four known phytoecdysteroids [(20-hydroxyecdysone-20, 22-monacetonide (**1**), 20-hydroxyecdysone (**2**), stigmasterol (**3**), gamma-sitosterol and (**4**)]. The identification was done by physical, spectroscopic and chemical analysis as well as literature data comparisons [8,27,28] (Table 1). These compounds were tested for larvicidal and/or IGR activities and the results revealed that they were toxic to *An. gambiae* larvae. Compounds 20-hydroxyecdysone-20, 22-monacetonide (**1**) and 20-hydroxyecdysone (**2**) caused 100% mortality at 10ppm (Table 2). Similarly, high mortality was obtained at 1 and 5 ppm (≥ 80%) with about 15-18 % showing impaired development. Stigmasterol (**3**) and gamma-sitosterol (**4**) caused 55±2.5 and 65±2.4 % mortality respectively at 10ppm (Table 2). A blend of 20-hydroxyecdysone-20, 22- monacetonide (**1**) and 20-hydroxyecdysone (**2**) was moderately active (65±2.1%) at 1ppm but the activity was high at 5 and 10 ppm (85±2.9 and 90±2.5) respectively. These results therefore indicated a slight drop in the activity of the resulting blend. On the other hand, a combination of stigmasterol (**3**) and gamma-sitosterol (**4**) which were less active improved the activity of the individual compounds. Addition of 20-hydroxyecdysone-20, 22-monacetonide (**1**) to stigmasterol (**3**) and gamma-sitosterol (**4**) improved the activity of the two compounds to 90 and 100% at 1 and 10 ppm while individually each caused 25±2.4, 55±2.5 and 35±2.5 65±2.4% mortality, respectively. It was also interesting to note that addition of 20-hydroxyecdysone (**2**) to stigmasterol (**3**) and gamma-sitosterol (**4**) also improved their activity as shown in (Table 2).

Table 1. ^{13}CNMR spectral data for compounds 1, 2, 3 and 4 [8,27,28]

Compounds	1		2		3		4	
Position	δ (obs.)	δ (lit.)	δ (obs.)	δ (lit.)	δ (obs.)	δ (lit)	δ (obs.)	δ (lit.)
1	36.0	38.0	36.0	37.3	37.3	37.2	37.3	37.0
2	67.3	68.1	67.3	68.6	31.9	31.8	29.4	29.5
3	67.1	68.0	67.1	68.5	71.8	71.5	71.8	71.8
4	31.5	31.7	31.5	32.7	42.3	42.2	42.3	42.3
5	50.4	51.3	50.4	51.7	140.8	140.7	140.8	140.8
6	205.1	203.5	205.1	206.6	121.7	121.6	121.7	121.7
7	120.7	121.8	120.7	122.1	33.9	33.6	31.7	31.9
8	165.3	165.4	166.5	168.1	29.7	29.6	29.2	29.2
9	33.7	34.4	33.7	35.0	50.1	50.1	50.2	50.2
10	37.8	38.6	37.9	39.3	36.5	36.4	36.5	36.5
11	21.0	21.0	20.1	21.5	21.1	21.1	21.1	21.1
12	30.9	32.4	30.4	32.4	39.7	39.7	26.1	26.1
13	48.6	47.8	47.4	48.6	42.2	42.2	45.9	45.9
14	84.5	84.1	83.4	84.2	56.8	56.1	56.8	56.7
15	30.2	31.6	31.1	31.7	24.3	24.1	24.3	24.1
16	20.1	22.1	20.1	21.5	28.4	28.3	39.8	39.8
17	49.1	50.0	49.1	50.5	56.7	56.0	56.1	56.1
18	16.3	17.2	16.7	18.1	12.0	12.1	11.9	12.2
19	23.1	24.4	23.0	24.4	19.4	19.4	18.8	18.8
20	83.9	82.1	76.5	78.0	40.5	40.3	34.0	34.0
21	21.2	22.4	19.7	21.1	19.8	20.5	19.1	19.1
22	81.9	85.5	77.0	78.4	138.3	138.5	37.3	37.3
23	23.3	24.3	25.9	27.3	129.3	129.4	26.2	26.6
24	40.8	42.1	41.0	42.3	51.2	51.2	51.6	50.1
25	69.7	69.2	69.9	71.4	31.7	31.9	28.2	28.3
26	28.1*	30.0	28.3	29.1	21.1	21.2	19.4	19.4
27	27.9 *	29.8	27.6	29.7	19.0	19.8	19.8	19.8
28					26.1	25.4	23.3	23.3
29					11.9	11.9	11.8	12.0
O-C-O	106.6	106.9						
Me	27.6	27.1						
Me	29.8	29.4						

*- signals may be interchanged

Compounds 1 and 2: (500 MHz, MeOD); Compound 3: (300 MHz, $CDCl_3$); Compound 4: (400 MHz, $CDCl_3$)

Table 2. Percentage mortality of *An. gambiae* larvae exposed to pure compounds isolated from *V. payos* and *V. schiliebenii* individually and in blends at 1, 5, and 10ppm

S/No.	Compound(s)	Mean % mortality/Concentration (ppm)		
		1ppm	5ppm	10ppm
1	20-hydroxyecdysone-20,22-monacetonide (1)	90±2.1	95±2.2	100±0.0
2	20-hydroxyecdysone (2)	80±2.4	90±2.0	100±0.0
3	Stigmasterol (3)	25±2.4	40±2.1	55±2.5
4	Gamma-sitosterol (4)	35±2.5	45±1.5	65±2.4
5	1 + 2	65±2.1	85±2.9	90±2.5
6	1 + 3	90±3.3	90±2.1	100±0.0
7	1 + 4	90±2.0	90±2.2	100±0.0
8	2 + 3	80±2.1	85±2.0	100±0.0
9	2 + 4	65±2.1	80±2.9	90±2.5
10	3 + 4	50±2.4	70±2.0	85±2.4
11	Control	0±0.0	0±0.0	0±0.0

4. Discussion

In the present study, 20-hydroxyecdsone (**2**) exhibited larvicidal activity against *An. gambiae* larvae. This observation compares with previous studies where 20-hydroxyecdysteroid (**2**) was used as the sole or major component in bioassays. Results indicated a range of detrimental effects on development and survival of several insect species including *Bombyx mori* [11], *Pectinophora gossypiella* [11,29], *Spodoptera frugiperda* [30], *Acrolepiopsis assectella* [29,30] and *Agrius convolvulus* [31]. The blend effect observed in the current study indicated that the activity of stigmasterol (**3**) and gamma-sitosterol (**4**) can be improved by preparing a formulation of the two compounds containing either 20-hydroxyecdysone-20, 22-monacetonide (**1**), or 20-hydroxyecdysone (**2**). In addition, the synergism observed in the blend of compounds **3** and **4** also indicated that a better formulation can be prepared by using the two compounds as a blend instead of using them as seperate compounds. Reported experiments [32,33,34,35,36] working on some plant extracts having potential larvicides also note that botanical blends provide better effect in reducing vector population. This is attributed to the adaptive value of phytochemical diversity in ecological interactions among plants and their associated herbivores and pathogens.

The purpose of this study was to elucidate the role and relative importance of steroids in *Vitex* species in controlling *An. gambiae* larvae and to use this information in guiding effective development of formulations to be used in integrated pest management programmes. Three variants of blend effects were noted from these results. First, production of a less active blend from active constituents; secondly, enhancement of the activity of a moderately active compound by an active constituent and; thirdly, synergism between moderately active compounds to give a blend that was more active than the individual constituents. The first variant was illustrated by the high lethal activity of compounds **1** and **2** while the second was illustrated by the enhancement of the activity of compounds **3** and **4** in blends with **1** and **2**. The third variant was illustrated by the combination of compounds **3** and **4**.

5. Conclusion

It can be concluded that phytoecdysteroids (20-hydroxyecdysone-20, 22-monacetonide (**1**) and 20-hydroxyecdysone (**2**) possess high larvicidal activity against *An. gambiae.* Stigmasterol (**3**) and gamma-sitosterol (**4**) also show potent insect growth regulatory (IGR) activities against *An. gambiae*. Consequently, *V. payos* and *V. schiliebeinii* have important practical implications in the search for and use of plants and their phytochemicals for mosquito larvae control.

Acknowledgements

The author is grateful to the International Centre of Insect Physiology and Ecology (ICIPE-Kenya) for providing the necessary bioassay requirements. Muhimbili University of Health and Allied Sciences is also acknowledged for providing the necessary chromatographic equipment for fractionation and purification of the crude plant materials. The author also acknowledges DAAD-NAPRECA for funding the work reported in this paper.

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