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RESEARCH ARTICLE

Mosquitocidal triterpenes from the stem of *Duranta repens*

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Abstract

Two triterpenes, β-amyrin and 12-oleanene 3β, 21β-diol, were isolated as a mixture from the chloroform soluble fraction of an ethanol extract of *Duranta repens* Linn (Verbenaceae) stem. The structures of the two compounds were confirmed by analysis of their IR, ^{1}H-NMR, ^{13}C-NMR and LC-MS spectral data. The mixture of β-amyrin and 12-oleanene 3β, 21β-diol (compound **1**) was highly effective against the larvae of the mosquito, *Culex quinquefasciatus* Say (Diptera: Culicidae), as a mosquitocide.

Keywords: *Mosquitocidal; β-amyrin; 12-oleanene 3β; 21β-diol; Duranta repens* Linn

Introduction

Culex quinquefasciatus Say (Diptera: Culicidae) is a potential vector of *Wuchereria bancrofti* (Filarioidae), Otto Wucherer and Joseph Bancroft, the causative agent of human lymphatic filariasis (HLF) all over the globe, including Bangladesh (Birley, 1993; Ahmed, 1994; Pailey et al., 1995). Mosquito-transmitted malaria, filariasis, yellow fever, dengue fever and Japanese B-encephalitis have great impact on public health in many countries in south and southeast Asian countries including Bangladesh (Bang, 1985; Shope, 1997). Hill (1997) estimated the number of people attacked by mosquito-borne diseases per year to be 100 million with mortality in more than a million cases. So, in order to prevent the transmission of mosquito-borne diseases, it is necessary to control mosquitoes using traditional insecticides, but in recent years it has been found that many mosquito species have already developed resistance to a number of chemical insecticides. In Bangladesh, *C. quinquefasciatus* is completely resistant to diazinon, fenitrothion, malathion, and primiphos-methyl and DDT (Georghiou & Lagunes-Tejeda, 1991; WHO, 1992). An on going search for alternative pest control as well as vector control strategies have been developed by several researchers (Pimental et al., 1992; Heckman, 1993; Coats, 1994; Mulrennan, 1995; Sugiyama et al., 1996; Peng et al., 1998).

Plant products or plant-derived compounds were reported as promising alternatives to synthetic insecticides in controlling insect pests (Rahuman et al., 2000). Vector control experts evaluated 344 plant species for their insecticidal, repellent, growth inhibiting, ovicidal activities and concluded with the suggestion that the plant products would be advantageous for field use in mosquito larvae control programs (Sukumar et al., 1991). In order to search for plant-derived compounds to be effective against mosquito larvae, a number of compounds have been reported including (5*E*)-ocimenone from *Tagetes minuta* Linn (Compositae) (Maradufu et al., 1978), rotenone from *Derris elliptica* Benth (Fabaceae) (Ameen et al., 1983), azadirachtin from *Azadirachta indica* A. Juss (Meliaceae) (Schmutterer & Ascher, 1987), capillin from *Artemisia nilagirica* Linn (Asteraceae) (Banerji et al., 1990), quassin from *Quassia amara* Linn (Simaroubaceae) (Evans & Kaleysa Raj, 1991), neolignans from *Piper decurrens* Linn (Piperaceae) (Chauret et al., 1996) and goniothalamin

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from *Bryonopsis laciniosa* Linn (Cucurbitaceae) (Kabir et al., 2003).

El-Naggar and Mosallam (1987) reported that the extracts from *Duranta repens* Linn (Verbenaceae) had antifeedant and insecticidal properties not only against the larvae of *Culex pipiens* Linn and *Spodoptera littoralis* Boised (Lepidoptera: noctuidae) but also against the adults of *Musca domestica* Linn (Muscidae) and *C. pipiens*, respectively. Castro and co-workers (1996) also reported that the fruits of *D. repens* were evaluated as antimalarials by oral and subcutaneous administration to mice infected with *Plasmodium berghei* Anka (Plasmodiidae). The chloroform extract of the fruits of the *D. repens* were reported to exhibit antifeedant activity against *Heliotis armigera* Hubner (Reoviridae), a polyphagus pest (Patil et al., 2002). The aerial part of *D. repens* showed antiviral activity against Hepatitis A virus (Lobna et al. 2007). But information on the larvicidal activity of isolated compounds or extract of *D. repens* against *C. quinquefasciatus* is not available to date which has encouraged us to perform the present study including the isolation and structure elucidation of the bioactive compounds from this plant.

Materials and methods

Test insects

C. quinquefasciatus larvae were reared at 27° ± 1°C, 40–60% relative humidity and a 12:12 h light:dark photoperiod. Single egg rafts were placed in a number of 600-mL glass beakers (Duran®, Mainz, Germany) containing approximately 450 mL distilled water. The larvae were fed powdered Brewer's yeast (Red Star®, Milwaukee, Wisconsin (WI)) at 10, 20, 40 and 80 mg per beaker everyday for first, second, third and fourth instars (different stages of a insect life), respectively. Water was changed everyday to avoid scum formation, which might create toxicity.

Test plant

Stems of *D. repens* were collected in June 2003 from the adjoining areas of Rajshahi University Campus, Bangladesh. The plant was identified by A.T.M. Nadiruzzaman, Department of Botany, University of Rajshahi, Bangladesh, where a voucher specimen (No. Alam 78, collection date 19.09.1997) has been deposited.

Extraction and isolation

The sun-dried and ground plant materials (1 kg) were macerated with ethanol (5 L). The concentrated ethanol extract was fractionated with diethyl ether and chloroform. The solvents were evaporated by rotary evaporator at 40°C and under reduced pressure to obtain ethanol (90 g), diethyl ether (20.8 g) and chloroform (15.6 g) extract as semisolid mass. The chloroform soluble fraction (5 g) was subjected to a column chromatography over silica gel eluting with *n*-hexane and ethyl acetate of increasing polarity, which gave a total of 33 fractions. On the basis of TLC (Thin Layer Chromatography) profile, the fractions were combined together. Compound **1** (480 mg) was obtained as amorphous powder from the fractions 4–15 eluted with *n*-hexane-ethyl acetate (2:1) by preparative TLC (mobile phase; *n*-hexane: ethyl acetate = 5:1) as visualized as pink spot when sprayed 1% vanillin in concentrated H_2SO_4.

Bioassay

The larvicidal effect of compound **1** was determined according to the protocol described by the WHO (1975). The stock solution was prepared dissolving 10 mg of compound **1** in 1 mL of dimethylsulphoxide (DMSO). Then twenty-five laboratory reared first, second, third and fourth instars larvae were released into 100 mL glass beakers separately, containing 50 mL distilled water to which 50, 100, 200 and 400 µL stock solutions were added using capillary micro-pipettes (Wiretrol® II, Drummond Scientific Company, BROOMALL, Pennsylvania (PA)) to get the desired test concentrations (w/v), i.e. 10, 20, 40 and 80 ppm. Each concentration has three replications and three types of control were maintained: distilled water; distilled water + food medium and distilled water + solvent (DMSO), respectively. Each replication used 25 larvae. Control was raised similarly. The experiment was performed at 27° ± 1°C and 40-60% relative humidity. Brewer's yeast was supplied as a larval food during the test periods for larval feeding. The cumulative mortality data were determined according to Abbott's (1925) formula and subjected to Probit analysis (Busvine, 1971) for LC_{50} values.

Results and discussion

Compound **1** isolated from the chloroform fraction of ethanol extract of the stem of *D. repens* as a white amorphous powder, decomposed between 121°-125°C. IR spectrum showed O-H stretching band between 3445~3888 cm^{-1} and C-O- stretching vibration at 1099 cm^{-1}. The C-H and >C=C-H stretching vibrations observed between 2877~2924 cm^{-1} and >C=C< stretching showed a strong band at 1689 cm^{-1}. Although the TLC examination of compound **1** showed a single spot, but LC-MS and the NMR data (both ^1H and ^{13}C) suggested that it was not a single one. The ^1H-NMR spectrum (500 MHz, $CDCl_3$) of compound **1** showed

two triplets (J = 3.6 Hz) at δ_H 5.26 and 5.30 that suggested the presence of two oleanane-type triterpenes having double bond at C_{12}–C_{13}. Comparison of ^{1}H and ^{13}C-NMR data with the published data and from LCMS, it was confirmed that the compound **1** is a mixture of two triterpenes, β-amyrin (major) and 12-oleanene 3β, 21β-diol (minor) in the ratio of 3:1 determined from the peak intensity.

Characterization of β-amyrin

The LC-APCI-MS showed a broad peak which gave two molecular ion $[M+H]^+$ at *m/z* 427.3 and at *m/z* 443.1, corresponding to the molecular formula $C_{30}H_{50}O$ and $C_{30}H_{50}O_2$ respectively. The ^{1}H-NMR spectrum showed the presence of an olefinic proton (H-12) at δ_H 5.30 and eight methyl group protons between 0.79-0.94 and a multiplet at δ_H 3.23 for an oxygenated proton (H-3). The H-12 (δ_H 5.30) coupled with the protons at δ_H 1.89 in the COSY experiment and was assigned as H-11 protons. In the HSQC experiment, H-12 showed direct correlation with a carbon at δ_C 124.2 (C-12). The ^{13}C-NMR data of an authentic β-amyrin (Ndom et al., 2001) were compared to those of this part of compound **1** and found almost identical. Thus this part of compound **1** was identified as a major compound, β-amyrin, a common triterpene of plant origin. Though the values of H-12 and C-12 were slightly higher than those of authentic sample, which might be due to its existence as a component in a mixture (Table 1 and Figure 1).

Characterization of 12-oleanene 3β, 21 β-diol

The NMR spectrum of the minor compound showed many similarities to those of 12-oleanene- 3β, 22 β-diol (Rahman, 2002). The H-12 of the diol appeared as a triplet due to the coupling with H_2-11 at δ_H 5.26 (J = 3.6 Hz) and H-3 at δ_H 3.27 (*m*). The proton at δ_H 0.74 (H-5)

Table 1. ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) data of compound **1**.

	Proton value $^\delta$H		Carbon value $^\delta$C		Long range coupling			
					1a		**1b**	
H/C	**1a**	**1b**	**1a**	**1b**	^2J	^3J	^2J	^3J
1			38.2	38.5				
2			28.8	28.8				
3	3.23 (*m*)	3.27 (*m*)	80.6	80.5		23,24		23,24
4			40.3	40.2				
5	2.21 (1H,*brd*,*J*= 10.6Hz)	0.74 (1H,*br d*, *J*=11.0)	56.8	54.2	4	25		25
6			19.9	19.2				
7			33.9	33.3				
8			40.3	40.4				
9	1.50~1.60 (*m*)	1.55 (1H, *m*)	49.1	49.2	11,8	26	11	26
10			38.5	35.4				
11	1.89 (*m*)	1.92 (1H, *m*)	24.8	24.6	12		12	13
12	5.30 (1H,*t*, *J*= 3.6Hz)	5.26 (1H, *t*, *J*=3.6Hz)	124.2	127.4		14	13	14
13			145.1	145.1				
14			40.2	39.3				
15			25.7	25.0				
16			29.6	29.2				
17			34.5	38.6				
18	2.01 (1H,*dd*, *J*=5.0 & 15.0 Hz)	2.19 (1H,*bd*,*J*= 10.6Hz)	49.5	42.7	13	12	13	12,20
19			47.4	47.4				
20			32.1	32.2				
21		3.27* (*m*)	35.5	80.2			20	
22		1.62 (2H,*bd*,*J*=10.0 Hz)	35.4	44.2				18
23	0.79, s, 3H	0.80, s, 3H	29.7	18.5		3		3
24	0.77, s, 3H	1.01, s, 3H	17.0	29.6		3,5		3,5
25	0.91, s, 3H	0.96, s, 3H	17.1	17.1		5		5
26	0.92, s, 3H	0.99, s, 3H	18.5	16.9		9,14		9,14
27	0.86, s, 3H	1.15, s, 3H	25.1	25.2	14	15		13
28	0.88, s, 3H	0.89, s, 3H	29.7	22.7		16		22
29	0.79, s, 3H	1.09, s, 3H	33.7	27.4		21		19
30	0.94, s, 3H	0.92, s, 3H	22.7	34.6		19,21		21

*overlap; **1a**, β-Amyrin; **1b**, 12-Oleanene 3, 21-diol.

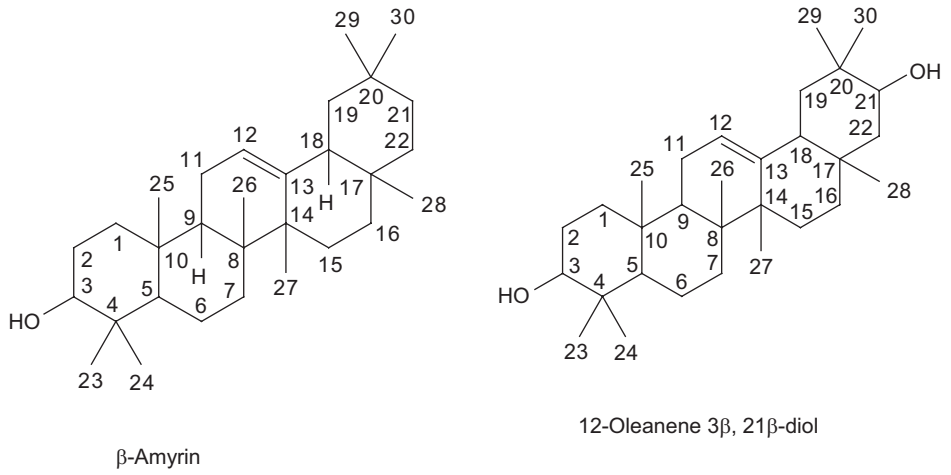

Figure 1. Structure of compound **1**.

showed direct correlation to a methine carbon (C-5) at δ_C 54.2 in the heteronuclear single quantum coherence (HSQC) experiment and ^3J correlation to methyl carbon at δ_C 17.1. The latter methyl carbon was also correlated to H-3 by ^3J. The eight-methyl group signals were appeared between δ_H 0.80-1.15. The carbon chemical shifts of C-3 and C-12 were found to be 80.5 and 127.4, respectively, as evident from the HSQC experiment. The C-12 value of this minor component was slightly higher which was assumed to be due to its existence in a mixture.

The *J* modulated ^{13}C-NMR data were very close to those of 12-oleanene-3β, 22β-diol (Rahman, 2002) except C-21 and C-22. While C-22 was oxygenated methine (δ_C 76.9) in case of 12-oleanene-3β, 22β-diol, one methine carbon was appeared at δ_C 80.2 in compound **1**. As this chemical shift was very close to C-3 (δ_C 80.5) and two methyls existed at C-4, it was assumed that the carbon chemical shift at δ_C 80.2 must be C-21 while two methyls existed at C-20 instead of C-4. On this basis, this minor component was identified as 12-oleanene-3β, 21β-diol and the ^{1}H-NMR and ^{13}C-NMR data are presented in Table 1 and Figure 1. Mokboul et al. (1981) reported α-amyrin was isolated from *D. repens*. But both the compound β-amyrin and 12-oleanene 3β, 21β-diol are isolated for the first time from this plant and the minor one, 12-oleanene 3β, 21β-diol appeared to be a new compound.

Statistical data obtained from toxicity bioassays are presented in Table 2, which clearly showed that compound **1** (mixture of β-amyrin and 12-oleanene 3β, 21β-diol) was a potent larvicide against *Culex quinquefqsciatus*. With the increase of exposure time, the LC_{50} values of compound **1** decreased in all the instars tested. The increase in mortality with the increase of exposure period could be due to several factors, which may be acting separately or jointly. For example, the uptake of the active moiety of the compound could be time dependent, leading to a progressive increase in the titre of the plant-derived compounds tested and its effect on the larval body, the active moiety of the compound could get converted into more toxic metabolites in the larval integument and alimentary canal, resulting in time-dependent effects.

Table 2. Larvicidal action of compound **1** from *D. repens* on *C. quinquefasciatus* at different instars.

Larval instar	Exposure period (h)	LC_{50} values (ppm)	95% fiducial limits (lower-upper)	χ^2 value (d.f.)
First	3	32.58	14.35–73.96	6.4
	6	12.32	5.06–29.98	5.37
	12	7.75	2.75–21.84	0.13
Second	12	49.29	17.86–136.01	2.83
	24	16.11	8.33–31.13	0.3
Third	12	65.82	19.96–217	6.01
	24	28.63	13.07–62.7	0.19
Fourth	12	82.96	14.17–485.64	0.23
	24	26.53	16.22–43.41	0.47

Values were based on four concentrations and three replications with 25 insects each.
d.f = degree of freedom.

Antibacterial, antifungal, MIC, brine shrimp lethality, acute toxicity test on rats and insecticidal activity on *Tribolium Castaneum* (Herbst) was performed using compound **1** and showed promising antibacterial, antifungal, toxic and insecticidal activities such as MIC 32 μg/ml against *Klebsiella* sp.; 13 μg/disc zone of inhibition against *Aspergillus flavus*; LC_{50} 1.21 ppm against brine shrimp larvae and LC_{50} 90.6 μg/cm^2 against 1st instars larvae of *T. castaneum* (Herbst) (Nikkon et al., 2008). The present results also seem to be very effective against *C. quinquefasciatus*. However, more comprehensive experiments are solicited in the future and if it is possible to produce their active analogues synthetically, which might prove to be more effective and more economical.

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Declaration of interest

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