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Molluscicidal Activity of Butanol Fraction of *Meryta denhamii* Flowers Against *Lymnaea natalensis* and *Biomphalaria alexandrina*

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Abstract: The molluscicidal activity of methyl extract of *Meryta denhamii* fruits and flowers against *Lymnaea natalensis* and *Biomphalaria alexandrina* snails were studied. The two extracts exhibited similar effects on *Biomphalaria snails*, LD_{50} was 85 mg/L for both flowers and fruits. While flowers extract was more active against *Lymnaea snails*; LD_{50} were 72.4 & 100 mg/L for flowers and fruits, respectively. The molluscicidal activity of flower fractions (Butanol, chloroform, petroleum ether and ethyl acetate) was screened. Butanol fraction was the most potent one against the two snail species, LD_{50} were 26.4 & 39.8 mg/L and LD_{90} were 70.8 & 79.4 mg/L for *L. natalensis* and *B. alexandrina*, respectively. While ethyl acetate fraction was inactive. Biochemical assays showed elevation in the glucose concentration and protein content in both haemolymph and soft tissues of treated snails. Although, glycogen content in soft tissues increased in treated *L. natalensis* it was decreased in treated *B. alexandrina*. Three monodesmosidic saponins were isolated from butanol fraction of *Meryta denhamii* flowers. Their structures were established by spectroscopic methods (^{1}HNMR and ^{13}NMR) as oleonolic acid-3-O -α- arabinopyranoside, oleonolic acid-3-O-β-glucopyranoside and hedragenin-3-O-α-rhamnopyranoside In conclusion,flowers butanol fraction of *M denhamii* could be successfully used as a botanical molluscicide.

Key words:*Meryta denhamii* · Flowers and fruits · *L. natalensis* · *B. alexandrina* Butanol fraction · Saponins

INTRODUCTION

Currently, there is an increased attention for the use of new molluscicides which are highly effective, rapidly biodegradable, less expensive, readily available and probably easily applicable with simple techniques than synthetic molluscicides. Therefore, plant molluscicides could be appropriate for snail control measures against fascioliosis and schistosomiosis in endemic areas [1-5]. These botanical molluscicides are of economic importance, especially in developing countries [6]. Also, there is a continuous need to search for new plant species with ideal molluscicidal properties [7, 8]. In Egypt, screening of local plants for molluscicidal activity has received increasing attention [9-14].

In order to promote energy production, gastropods categorize primarily carbohydrates, which are stored in certain tissues as glycogen and transported in the haemolymph as glucose [15]. Molluscicides greatly affect the metabolic activities of the snail intermediate hosts [9]. They act on different enzymes, chiefly those of respiration and carbohydrate metabolism [16, 17]. In addition to, they act on glucose level, lipid and protein content in snail tissues and haemolymph [5,18-20].

Oreopanax species (Araliaceae) are ornamental trees cultivated in public gardens in Egypt and was found to possess molluscicidal activity [21, 22]. Saponine with oleanane skeleton were isolated from *Oreopanax* species [23 - 25] and was proved to have molluscicidal activity [25].

As a part of investigating *Oreopanax* species, *Meryta denhamii*, family Araliaceae was selected in the present study which was designed to evaluate the molluscicidal activities of flowers and fruits of *M. denhamii* methanol extract. Preparation of fractions from flowers extract was performed and their molluscicidal activity was evaluated. Also, Biochemical parameters were estimated within snails treated with butanol fraction. In addition to isolation and identification of saponine found in butanol fraction.

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MATERIALS AND METHODS

General: Mass spectra were measured on MS Finningan Mat SSQ 7000, 70 ev. mass spectrometer. NMR spectra were run using Jeol TMS Route instrument at 300 and 90 MHz for measuring ^{1}H and ^{13}C NMR, respectively.

α-amyrin, β-sitosterol, oleanolic acid, ursolic acid, hedragenin and echinocystic acid were used as authentic reference sterols and triterpenes. Glucose, galactose, rhamnose, glucuronic acid, galacturonic acid, arabinose and xylose were used as authentic reference sugars.

Plant Material: The flowers and fruits of *M. denhamii* were collected from Faculty of Agriculture, Ein Shams University.

Determination of Saponins by Haemolytic Index Method: Disodium hydrogen phosphate, potassium hydrogen phosphate and sodium chloride were used for preparation of isotonic buffer. The ability of the extracts to haemolyse red blood corpuscles compared with 0.05% w/w of white saponin of Merck was used to determine saponin content of both flowers and fruits [26,27].

Extraction and Isolation: About 900 g of powdered air-dried flowers of *M. denhamii*, was extracted with cold methanol till exhaustion. After stripping of the solvent under reduced pressure, the residue (68.9 g) was suspended in water and fractionated by successive extraction with suitable volumes of petroleum ether, chloroform, ethyl acetate and *n*-butanol.

The butanol fraction of the flowers (17.1 g) was fractionated on VLC column (10 × 7 cm, silica gel G 60). Gradient elution was carried out with chloroform: ethyl acetate mixtures, ethyl acetate and ethyl acetate: methanol mixtures. Fractions (200 ml each) were collected and monitored by TLC on precoated silica gel plates using chloroform: methanol [9:1 (S_a), 8:2 (S_b) and 7:3 (S_c)] as solvent systems. The chromatograms were visualized under UV light at 254 and 366 nm, before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde-sulphuric acid spray reagent [28]. Fractions (12and13) showed three major spots which gave violet to blue color with *p*-anisaldehyde-sulphuric acid spray reagent. These fractions were refractionated on silica gel using chloroform: methanol (95:5) as eluent. Subfractions (4-6) and (9-11) were finally purified on sephadex LH-20 using methanol as eluent to give two compounds (S_1 and S_2).

Subfractions (12and13) were refractionated on sephadex LH-20 using methanol as eluent, then reversed phase Rp-18 using methanol: water mixture as eluent which afforded one compound (S_3).

Compound (S_1): White amorphous powder, 20 mg, R_f = 0.72 in S_a, no fluorescence in UV, violet color with *p*-ainsaldehyde, ^{1}H and ^{13}C NMR.

Compound (S_2): White amorphous powder, 32 mg, R_f = 0.65 in S_a, no fluorescence in UV, violet color with *p*-anisaldehyde, ^{1}H and ^{13}C NMR.

Compound (S_3): White amorphous powder, 16 mg, R_f = 0.42 in S_a, no fluorescence in UV, blue color with *p*-anisaldehyde, EI/MS of its aglycone m/z(%): 149(100), 174(60.2), 57(54), 71(52.9), 167(41.6), 189(8.2), 203(2.8), 248(1.9) and 472(M^+, 0.12). ^{1}H and ^{13}C NMR spectra.

Acid Hydrolysis of Saponins: The assay was performed according to Gunzinger *et al.*[29].

Molluscicidal Activity Assay: The methanol extracts of both fruits and flowers and the fractions from flowers were tested for molluscicidal activity against two snails species as outlined by WHO [30]. Snails were immersed in glass aquaria contain dechlorinated tap water (10 snails/250 ml). Serial concentrations from methanol extracts of *Meryta denhamii* and prepared fractions (240, 120, 60, 30, 15 mg/L) were done using 5 replicates from each concentration. Untreated snails were maintained under the same experimental conditions and were used as control. LC_{50} and LC_{90} were determined after 24h exposure. The snails' haemolymph was collected at 24 and 48h post treatment [31]. Snails' tissues were homogenated [32] at 24, 48h. post treatment. Control snails haemolymph and tissues were treated in the same way.

Biochemical Assays of Some Snail Components:

- Glucose concentration was colorimetrically assayed in both tissues and haemolymph [33].
- Protein content of both haemolymph and tissues was also determined [34].
- Glycogen was estimated in snail tissues by the method of Nicholas *et al.*[35].

Statistical analysis of data was carried out according to Litchfield and Wilcoxon[36].

RESULTS

Molluscicidal activity of alcoholic extract of flowers as well as fruits was assessed against *L. natalensis* and *B. alexandrina* (Table 1). The two extracts exhibited similar effects on *Biomphalaria snails*, LD_{50} was 85 mg/L for both flowers and fruits, whilst flowers extract was more active against *Lymnaea snails*, LD_{50} was 72.5 and 100 mg/L for flowers and fruits, respectively. Based on calculated LD_{50}, flowers extract was selected for phytochemical studies. Different fractions of flowers extract (Butanol, chloroform, petroleum ether and ethyl acetate) were screened for molluscicidal activity (Table 2). Butanol fraction was the most active one, LD_{50} were 26.4 & 39.8 mg/L and LD_{90} were 70.8 & 79.4 mg/L for *L. natalensis* and *B. alexandrina*, respectively, Due to the promising effect of butanol fraction, it was chosen for carrying out the biochemical studies and data are shown in tables (3and4). Butanol fraction caused increase in the tissues glycogen, concentration of glucose and protein content in both haemolymph and snails' soft tissues of *L. natalensis* (Table 3). In *B. alexandrina*, all previous components were increased except glycogen content in soft tissues was decreased and the reduction percentages were 29.6 and 50 after 24h and 48h of treatment, respectively (Table 4).

Percentages of saponins present in flowers and fruits of *M., denhamii* was found to be 1.87 and 1.12%, respectively. Three triterpenoidal saponins were isolated from the butanol fraction, their ^{1}H and ^{13}C NMR spectral data are compiled in tables 5 and 6.

Table 1: Molluscicidal activity of *M. denhamii* flowers and fruits methanol extract

	L. natalensis		*B-alexandrina*	
Extracts	LC_{50} mg/L	LC_{90} mg/L	LC_{50} mg/L	LC_{90} mg/L
Methanol extract of flowers.	72.4	125	85	155
Methanol extract of fruits	100	165	85	138

Table 2: Molluscicidal activity of fractions prepared from flowers of *M. denhamii* methanol extract

	L. natalensis		*B-alexandrina*	
Fractions	LC_{50} mg/L	LC_{90} mg/L	LC_{50} mg/L	LC_{90} mg/L
Butanol	26.4	70.8	39.8	79.4
Chloroform	306	1258	-	-
Petroleum ether	236.7	931.8	-	-
Ethyl acetate	-	-	-	-

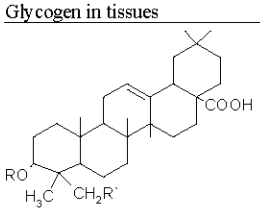
Table 3: Effect of butanol fraction (26.4 mg/L) on glucose level, protein and glycogen content on *L. natalensis* at 24h and 48h post treatment

	Non treated snails Mean ± SD		Treated snails Mean ± SD			
Biochemical Components	24h.	48h.	24h.	Change %	48h.	Change %
Protein in haem	139.5±1.8	121.6±3.5	161.14±1.2	15.5	143.5±1.1	18
Protein in tissues	73±0.68	82.0±0.5	112±2.36	53.42	109.5±1.4	33.46
Glucose in haem	34.8±1.39	31.75±0.94	37.5±3	7.76	34.16±2.2	7.5
Glucose in tissues	19.7±0.36	28.5±0.25	32.6±0.5	65.48	38±1.9	32.99
Glycogen in tissues	12.8±0.22	7.2±0.35	22.4±3.1	75	11.84±3.5	64.4

Haem.=haemolymph.

Table 4: Effect of butanol fraction (39.8 mg/L) on glucose level, protein and glycogen content on *B. alexandrina* at 24h and 48h post treatment

	Non treated snails Mean ± SD		Treated snails Mean ± SD			
Biochemical components	24h.	48h.	24h.	Change %	48h.	Change %
Protein in haem	114.4±4.9	105±5.2	191±5.7	66.9	178.3±1.76	69.8
Protein in tissues	162.3±1.7	146±2.9	170±3.1	4.7	161±5.5	10.27
Glucose in haem	37.6±1.5	38.4±1.5	38.6±3	2.6	45.3±5.1	17.96
Glucose in tissues	51.9±0.4	69.7±2.4	56.3±0.36	8.47	74.1±1.2	6.3
Glycogen in tissues	10.8±0.5	8±0.37	7.6±0.32	29.6	4±0.39	50



R R` S_1 Arabinose H S_2 Glucose H S_3 Rhamnose OH

Table 5: ^{1}H NMR spectral data of compounds S_1, S_2 and S_3 in DMSO (δ ppm)

Carbon no.	1	2	3
Aglycone			
3	3.41(dd, J=6.9, 0.9Hz)	Overlapped by peak of solvent	3.60 (d, J=6.6 Hz)
12	5.15 (s)	5.14 (t)	5.15 (s)
23	1.23 (s)	1.23 (s)	3.45 (d,J=11.4Hz)
24	1.09(s)	1.09(s)	0.86(s)
25	0.75(s)	0.76(s)	0.86(s)
26	0.96(s)	0.95(s)	1.09(s)
27	1.23(s)	1.23(s)	1.18(s)
29	0.87(s)	0.80(s)	0.58(s)
30	0.87(s)	0.82(s)	0.71(s)
Sugar			
1	4.12(d,J=5.4Hz)	4.21(d, J=7.8Hz)	4.19(s)
2	3.24-3.40	3.04-3.66	3.07-3.67
3	3.24-3.40	3.04-3.66	3.07-3.67
4	3.24-3.40	3.04-3.66	3.07-3.67
5	3.24-3.40	3.04-3.66	3.07-3.67
6	-	3.04-3.66	1.88(d, J=4.8Hz)

Table 6: ^{13}C NMR spectral data of compounds S_1, S_2 and S_3 in DMSO (δ ppm)

Carbon no.	S_1	S_2	S_3
Aglycone			
3	87.5	81.2	--
12	--	121.1	123.9
23	28.8	28.4	--
24	--	18.4	12.9
25	--	12.9	13.4
26	--	18.8	13.8
27	25.3	24.8	28.9
28	--	177.9	171.8
29	32.2	31.4	33.2
30	--	23.8	23.2
Sugar			
1	105.5	100.7	--
2	70.9	73.4	--
3	--	76.9	--
4	67.4	70.1	--
5	64.7	76.7	71.0
6	--	61.1	18.4

DISCUSSION

The rising costs and high toxicity of synthetic molluscicides have led to an increasing interest in plants and plant-derived compounds which are lethal to the intermediate host of fascioliosis and schistosomiosis. In the present study, *Meryta denhamii* fruit and flower extracts exhibited molluscicidal activity against *L. natalensis* and *B. alexandrina*. Flower extract was the most active against *L. natalensis*, this may be due to its higher saponine content which was previously isolated from *Oreopanax* sp and exhibited moluscicidl activity [23-25].

Different fractions were prepared from flowers of *M.denhamii* and their molluscicidal activity was evaluated (Butanol, chloroform, ethyl acetate and petroleum ether). Butanol fraction was the most potent with the lowest LD_{50} and LD_{90} against two snails sp., while ethyl acetate was inactive. On the contrary, Abdel-Rahman.and Hassan [4] proved that butanol fraction of *Hedera canariensis* which belong to the same family (Araliaceae) was inactive and ethyl acetate was the most potent against *L. natalensis* and *B. alexandrina*. Although both plants belong to the same family, their effects on snails were different. This is probably attributed to different active principles of each plant.

Biochemical parameters are sensitive index to monitore changes due to xenobiotics and can constitute important diagnostic tool in toxicological studies [37]. In the present study, total protein content in both haemolymph and soft tissues of *L. natalensis* and *B. alexandrina* snails increased in response to treatment with butanol fraction. The present data are concided with Mello-Silva *et al.*[3] who recorded increase in protein content of *Biomphalaria glabrata* treated with sub-lethal contration of *Euphorbia splendens* latex which could be related to the acceleration in the gluconeogensis process. Also, Bode [38] observed an increase in protein content in *B. glabrata* treated with extract of *Tetrapleura tetraptera*. There was an increase in the number of secretory cells and reduction in the number of digestive cells in the digestive gland and also an intense autolysis of membranous structures, such as those of Golgi complex, mitochondria and endoplasmic reticulum. These alterations could lead to cell lysis, resulting in the release of a large quantity of proteins. Meanwhile, Alcanfor [39] recorded reduction in the level of protein content of *B. glabrata* treated with molluscicidal plant extract. This author also observed histological alterations in treated snails with degeneration of the digestive gland, but without elevation in the total protein level. Moreover, reduction in the total protein content was observed in *L. natalensis* treated with *Calendula micrantha* [18] as well as in *Eobania vermiculata* snails treated with carbamate pesticides [37]. They attributed this reduction to the consumption of some protein content in energy production in snails to face stress factors and this not coincided with the present data. The present results also showed an elevation in glucose level in both haemolymph and soft tissues of *B. alexandrina* and *L. natalensis*, while glycogen content in *B. alexandrina* soft tissues decreased. Glycogen is the primary source for serum glucose, known to be the most important anaerobic

energy source in anoxic tolerant mollusks [40]. The decrease in tissues glycogen may be due to increase in the rate of glycogen break down (glycogenolysis) [41], leading to increased glucose level in snails haemolymph and consequently increase its level in snails soft tissues. The present data are supported by other studies that confirm the efficiency of molluscicidal plant or plant derived compounds in the reduction of glycogen content and increase in glucose haemolymph [5,16,17,42]. In the present study, *L. natalensis* exhibited different response to butanol fraction, where there was increase in haemolymph and tissues glucose and increase in tissues glycogen content.Comparable results were observed by Radwan *et al.*[37] who recorded marked increase in glycogen content in soft tissues of *Eobania vermiculata* snails after 5 days following treatment with carbamat pesticides.

The present isolated saponins were identified as oleanolic acid 3-0-α-arabinopyranose, oleanolic acid 3-0-β-glucopyranose and hedragenin-3-0-α-rhamnopyranose based on the spectral data and published data.

Compound S_1: Upon acid hydrolysis and comparison with authentic samples, it showed an aglycon corresponding to oleanolic acid and a sugar corresponding to arabinose. ^{1}H NMR showed 7 signals assignable to 7 CH_3 groups at δ 1.23 (s, 6H, CH_3-23& 27), 1.09 (s, 3H, CH_3 -24), 0.75 (s, 3H, CH_3-25), 0.96 (s, 3H, CH_3-26) and 0.87 (s, 6H, CH_3-29& 30), signal at δ 3.41 (dd, J = 6.9, 0.9 Hz)ascribable to H-3. The olefenic proton (H-12) is represented by a triplet at δ 5.15. These signals were in agreement with oleanolic acid. Doublet at δ 4.12 (J = 5.4 Hz) was ascribed to the anomeric sugar proton. ^{13}C NMR showed the anomeric carbon at δ 105.5 and by comparing the signals of the sugar molecule with the published data [23,24,43-45], the sugar moiety was identified as 3-0-α-arabinopyranose.

Compound S_2: ^{1}H and ^{13}C NMR showed signals of the aglycone part resembling those of S_1. The signal of the anomeric proton at δ4.21 (J=7.8 Hz) indicated β-configuration [46, 47]. ^{13}C NMR showed signals at δ 100.7, 76.9, 76.7, 73.4, 70.1 and 61.1; indicating 3-0-β-glucopyranose [44, 48]. Thus, compound S_2 was identified as oleanolic acid 3-0-β-glucopyranose.

Compound S_3: ^{1}H NMR showed signals of aglycon part differing from those of S_1 and S_2. It showed signals at δ 3.45 (d, J=11.4 Hz, 3H, CH_3-23), 0.86 (s, 6H, CH_3-24& 25), 1.09 (s, 3H, CH_3-26), 1.18 (s, 3H, CH_3-27), 0.58 (s, 3H, CH_3-29), 0.71 (s, 3H, CH_3-30), 3.60 (d, J=6.6 Hz, 1H, H-3) and δ 5.15 (s, 1H, H-12 olefenic proton). These signals indicated hedragenin as aglycone which was confirmed by acid hydrolysis and EI/MS analysis [49]. The sugar moiety was identified as rhamnose by acid hydrolysis and comparison with authentic sugars.

^{1}H NMR showed the anomeric proton as singlet at δ 4.19 and the CH_3 of rhamnose as a doublet at δ 1.88 (J= 4.8 Hz, 3H) which was confirmed by the signal at δ 18.4 in ^{13}C NMR spectrum corresponding to CH_3 of rhamnose [23, 24]. Thus, Compound S_3 was identified as hedragenin-3-0- α-rhamnopyranose.

In conclusion, it is evident that the application of butanol fraction from *M. denhamii* flowers could be helpful in snails' control. Moreover, the obtained results can motivate further investigations regarding biochemical stress response in snails which is especially useful as biomarker for lethal effect of butanol fraction.

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