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MOLLUSCICIDAL ACTIVITY OF *AZOLLA PINNATA*

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ABSTRACT

For isolation of active components of *Azolla pinnata*, different extracts were obtained using petroleum ether, chloroform and water. The molluscicidal effect of each extract was examined separately on both healthy and infected *Biomphlaria alexandrina* snails. The different extracts exhibited significant molluscicidal activities. After twenty four hours, aqueous fraction showed activity (LC_{100}) at concentration of 500 ppm while the petroleum ether fraction demonstrated activity (LC_{100}) at 100 ppm, put the chloroformic fraction showed LC_{75} at concentration of 80 ppm after 24 hrs. Further fractionation of the active chloroformic part on silica gel column chromatography yielded an active eluate with LC_{90} at concentration of 30 ppm after 24 hr. Also, most extracts showed more killing effect on the infected snails than the noninfected ones. It could be concluded that fractionation of *A. pinnata* leads to production of more active molluscicide at lower concentrations.

INTROUCTION

At present, over 200 million patients in about 70 tropical and subtropical countries are victims of schistosomiasis, most of them are located in the third world countries[1]. In order to break the schistosomal life cycle, considerable success has been achieved by the use of chemotherapy as drugs for patients as well as intensive use of synthetic molluscicides against the snails intermediate hosts[2] However, the high cost (about $ 200 million per year in Egypt), toxicity to non target organisms and deleterious longterm effects in the environment represent a major disadvantage of those synthetic chemicals.

Therefore, search for a plant with a molluscicidial activity had received great attention from many authors in different laboratories. Marston and Hostettmann (1985)[2] mentioned that the previously reported active plants (more than 70 plants) showed several difficulties including rare distribution, minor phytochemical constituents (nanogram/kg), generally toxic and dominant growth in the non endemic area as well as occurrence of many active plants which are hard to cultivate or propagate.

Certainly, in Egypt where bilharzial infection threatening up to 25% of population with limited low national budget for health, there is a great demand for natural, vegetable, inexpensive ecologically safe molluscicide, in order to interrupt the parasite life cycle and prevent human infection.

In a previous publication by El-Sadawi et al. (1993)[3] reported on *A. pinnata* as a successful aquatic plant that can be used as an excellent natural molluscicide.

Azolla pinnata R.Br. is an aquatic plant fern with global distribution from Southeast Africa to India, China and Japan. This plant floats freely on water surface and multiplies vegetatively forming a mate within 24 hrs. Morphologically, it is formed of three-cordate leaves, with minute root like structure. It has been traditionally used for many years in several far eastern countries (Thailand, Korea, Philippine) as nitrogen fixer and as fertilizer in rice farm production [4,5]

Obviously, the current available literature indicated no records on previous work utilizing *Azolla pinnata* as a molluscicide for bilharzial snail intermediate hosts.

So, the present work aims to study the effect of several extracts of *A. pinnata* at different concentrations on the bilharzial snail vectors followed by fractionation of the active part through several chromatographic methods in order to obtain a target commercial molluscicide.

MATERIAL AND METHODS

1- Plant collection:

The whole plant *Azolla pinnata* R. Br. (Azollaceae) was collected from the local irrigation canals of Abou Kaber vicinity, 20 km North of Zagazig, Sharkiya Governorate, Egypt. Plant was identified by Dr. Ragab Abdel-Fattah, Department of Botany, Faculty of Sciences, University of Zagazig.

The plant was sundried and powdered to suitable size for extraction and bioassay purposes.

2- Preparation of *Acacia* extracts:

i- One hundred gram of powdered *Acacia pinnata* were extracted with water (2 x 200 ml) at 60°C-70°C for one hour till exhaustion[10]. The final extract was concentrated at reduced pressure (under vacuum, 45°C) then the obtained syrupy residue was subjected to drying by lyophilization (4.5 g). This residue was given the designation (A).

ii- One hundred gram of powdered *A. pinnata* was exhaustively extracted by petroleum ether at 60°C-80°C. The final extract was concentrated under reduced pressure to give 6 g residue, designated (B).

iii- One hundred gram of powdered *A. pinnata* was exhaustively extracted by chloroform in a Soxhlet extraction apparatus and the final extract was concentrated under reduced pressure to give 8 g residue, designated (C).

For isolation of active components of *A. pinnata*, about 5 kg of *A. pinnata* were successively extracted by cold percolation with petroleum ether (30 L) at 60°C-80°C, chloroform (20 L), methanol (15 L) and finally with water (15 L) one week each. Each extract was concentrated under reduced pressure at 45-55°C to give total residues of 56 g, 50 g, 17 g and 160 g of petroleum ether, chloroform, methanol and water respectively.

Snail Bioassay:

This bioassay including laboratory breeding of *Bulinusdrinus* snails was conducted according to WHO reports (WHO, 1965)[9]. For obtaining the infected *Bulinusdrinus* snails, the liver of mice infected with *S.mansoni* cercariae obtained from hatching eggs, (After 8-12 weeks of infection), was cut into pieces and placed in 200 ml Eberbach container and placed in a blender connected to autotransformer. Tissues were homogenized for 5-10 sec. at low speed.

The suspension was poured into a tiered column of sieves. The eggs were collected from the bottom sieve, washed and transformed into Petri dish containing distilled water and put under direct illumination at 25°C to allow eggs hatching.

The snails were put in Petri dish containing miracidiae which were picked up by Pasteur pipette under dissecting microscope with a dose of 8-10 miracidiae for each snail. The dishes were maintained at 25- 27°C for 3-5 hrs. to ensure maximum penetration by miracidiae before being transferred to plastic trays.

From each powdered extract (A, B and C) 20, 30, 100, 200, 300, 400 and 500 mg were dissolved in one litre of dechlorinated water by the aid of ultrasonic bath at room temperature for 15 min. to obtain the desired concentrations expressed as parts per million (ppm). The same concentration for powdered extract (B and C) were also dissolved in dechlorinated water using 1.5% (v/v) Tween-80 as emulsifier. The solutions were placed in specimen jars containing 20 healthy snails two weeks age. In other jars, each containing 20 infected snails two weeks age were exposed to the same solutions.

Two control jars (without plant extracts) were prepared, one containing snails in dechlorinated water and the other containing snails in dechlorinated water with Tween-80. The test jars as well as the control jars were kept at 24°C for 24, 48, 72 and 96 hrs. After that, the snails were examined to separate the dead ones. The signs of snail death were determined according to WHO rules (1965)[9].

The lethal effect of the extracts at the different concentrations was listed in tables (1-5). Each concentration was tested twice. The most active extract with highest lethal effect on the snails was chloroformic extract which then submitted to column chromatography (Merck, 70-230 mesh, 200 g silica-gel, 25 g. extract, 5 x 75 cm) then eluted with petroleum ether (60°-80°C). The polarity was gradually increased with chloroform and then methanol. Five hundred ml of each fraction were collected then screened by thin layer chromatography and concentrated under vacuum to yield four major pooled fractions designated as 1,2,3 and 4. The lethal effect of each fraction was observed on snail groups (20 snails each) for a period of 24 hr at concentrations of 80 and 30 ppm. as in tables (4, 5).

RESULTS

Lethal effect of A. pinnata extracts at different concentrations*:

At a concentration of 500 ppm of all extracts, all tested snails (100%) died within 24 hours.

On the other hand, all tested snails died within 24 hrs. at 400 ppm of both petroleum ether and chloroformic extracts while aqueous extract showed 70% mortality , as shown in table (1).

Chloroformic extract was the only one (table 1) that at 20 ppm still having lethal effect (60%) after 72 hrs. This effect was higher than that of other extracts which showed lethal effects of 40% and 25% for both petroleum ether and aqueous extracts respectively at the same concentration within the same period. On the other hand, table (1) showed that the highest lethal effect of aqueous and petroleum ether extracts after 96 hours were 50% at 200 ppm and 70% at 80 ppm. respectively.

* N.B. Snails were observed to escape up towards the jar walls till death.

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Table (1): Lethal effect of different extracts of *A. pinnata* at different concentrations on healthy *B. alexandrina* (20 snails each) for different periods.

Azolla extract \ Concentration ppm		400		300		200		100		80		20	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Aqueous	24 hrs	14	70	8	40	8	40	8	40	6	30	5	25
	48 hrs	15	75	10	50	9	45	8	40	6	30	5	25
	72 hrs	17	85	11	55	9	45	9	45	7	35	5	25
	96 hrs	17	85	12	60	10	50	9	45	7	35	6	30
Petroleum ether	24 hrs	20	100	16	80	14	70	12	60	10	50	6	30
	48 hrs	--	--	16	80	14	70	12	60	12	60	8	40
	72 hrs	--	--	16	80	14	70	12	60	13	65	8	40
	96 hrs	--	--	16	80	14	70	12	60	14	70	9	45
Chloroformic	24 hrs	20	100	18	90	15	75	13	65	12	60	11	55
	48 hrs	--	--	18	90	16	80	13	65	12	60	11	55
	72 hrs	--	--	18	90	16	80	15	75	13	65	12	60
	96 hrs	--	--	18	90	16	80	15	75	15	75	12	60

No. = number of dead snails

Table (2): Lethal effect of *A. pinnata* extracts emulsified in Tween-80* at different concentrations on healthy *B. alexandrina* (20 snails each) for different periods.

Type of extract \ Concentration ppm		200		100		80		20	
		No.	%	No.	%	No.	%	No.	%
Petroleum ether	24 hrs	20	100	20	100	14	70	11	55
	48 hrs					16	80	11	55
	72 hrs					17	85	12	60
	96 hrs					17	85	12	60
Chloroform	24 hrs	15	75	14	70	15	75	15	75
	48 hrs	16	80	16	80	15	75	15	75
	72 hrs	17	85	16	80	17	85	16	80
	96 hrs	17	85	16	80	17	85	16	80

* **N.B.** Tween-80 alone showed percentage death (30%) of snails.

Table (3): Lethal effect of *A. pinnata* extracts at different concentrations on infected *B. alexandrina* (20 snails each) for different period.

Type of extract \ Concentration ppm		400		300		200		100		80	
		No.	%	No.	%	No.	%	No.	%	No.	%
Aqueous	24 hrs	18	90	13	65	12	60	10	50	8	40
	48 hrs	18	90	13	70	12	60	10	50	8	40
	72 hrs	19	95	14	70	13	65	10	50	9	45
	96 hrs	19	95	14	70	13	65	11	55	10	50
Chloroformic	24 hrs	20	100	20	100	16	80	14	70	13	65
	48 hrs	--	--	--	--	16	80	15	75	13	65
	72 hrs	--	--	--	--	16	80	15	75	14	70
	96 hrs	--	--	--	--	17	85	15	75	14	70

No. = number of dead snails

Table (4): Lethal effect of chloroformic column fractions on healthy *B. alexandrina* (20 snails each) for 24 hrs.

Fractions \ Concentration ppm solvent		80		30	
		No	%	No	%
1	PE-CF 1:0	10	50	6	30
2	2 : 1	20	100	18	90
3	1 : 1	8	40	6	30
4	1 : 2	0	0	0	0

No. = number of dead snails PE = Petroleum ether CF = Chloroform

Table (5): Lethal effect of chloroform column fraction No. 2 at concentration of 30 ppm on healthy and infected *B. alexandrina* (20 snails each) for different period.

Exposure period (hours) \ Type of snail	Healthy		Infected	
	No.	%	No.	%
24	18	90	19	95
48	19	95	20	100
72	19	95	--	--
96	19	95	--	--

No. = number of dead snails

Using Tween 80 as emulsifier for *A. pinnata* extracts, petroleum ether extract was highly effective (LC_{100}) at higher concentrations (200 ppm and 100 ppm) than chloroformic extract while the reverse was observed at lower concentrations (20 ppm) as shown in table (2).

Studying the lethal effect of *A. pinnata* extracts on infected snails showed that the highest lethal effect of aqueous extract (95%) was obtained at 400 ppm after 72 hrs. while death of all snails (100%) was observed at 300 ppm of chloroformic extract within the first 24 hrs. (table 3).

From the previously obtained data, chloroformic extract was found to be the most active one with significant lethal effect. Therefore, it was submitted to further fractionation on silica gel column chromatography. As a result, 4 major fractions (1,2,3 and 4) were obtained and subsequently tested for their lethal effect.

Table (4) showed that chloroformic column fraction No. 2 eluted by PE:CF (2:1) demonstrated highest lethal effect as 100% and 90% at 80 ppm and 30 ppm, respectively.

Studying the lethal effect of chloroformic column fraction No. 2 on both infected and healthy snails, a concentration of 30 ppm was prominent. The infected snails were found to be affected more than the healthy ones as shown in table (5). The lethal effect was directly related to duration of exposure.

DISCUSSION

For the last two decades, many plants have been screened for their molluscicidal activity[2]. Although several highly toxic compounds to snails have been isolated and their chemical structures were fully elucidated, yet non of them had been introduced for commercial control of schistosomal snails[8]. This is because of the rare geographical distribution of the active plants, low yield of the toxic compounds and its high cost for use.

Here, comes the great value of *Azolla pinnata*, our plant of study as it is an abundant aquatic easily propagative plant with unlimited huge source for vegetable molluscicide which is extremely safe to other aquatic and / or environmental organisms.

In this connection, peters and Meeks (1989)[9] mentioned that *A. pinnata* had low toxicity to non-target organisms, whatever the concentrations used as it acts as a nitrogen fixer.

Thus, in the present study the lethal effect of different extracts of *A. pinnata* on *B. alexandrina* snails was tested. The aqueous extract as well as other extracts had exhibited 100% molluscicidal activity at a concentration of 500 ppm within the first 24 hours.

After 24 hrs. the highest molluscicidal activity (LC_{100}) was obtained by petroleum ether and chloroformic extracts at a concentration of 400 ppm while at the same concentration the least effect (70%) was produced by the aqueous extract.

On the other hand, in comparison with other plants reported to having molluscicidal activity, Selim et al. (1987)[10] reported that *Ambrosia maritima* (Damsissa) caused death of 67.5% Lymnaea snails at concentration of 2000 ppm for 24 hrs. In addition, Abou Basha et al. (1994)[11] reported that dry powdered Damsissa was lethal (LC_{100}) for Lymnaea snails at concentration of 3000 ppm after 24 hrs. and at concentration of 100 ppm after 14 days.

In the present study, petroleum ether extract of *A. pinnata* emulsified in Tween-80 had LC_{100} of 100 ppm within 24 hrs on *B. alexandrina* snails. The same lethal effect at the same concentration within the same period was obtained by crude methanolic extract of *Dysoxylum lenticellare* leaves on *B. glabrata* by Alodesanmi et al. (1988)[12]. However, the most toxic fractions of chloroformic extract of *A. pinnata* (LC_{90}) was at concentration of 30 ppm within 24 hrs. against *B.alexandrina* snails.

In conclustion, fractionation of *A. pinnata* would lead to production of more active molluscicide at lower concentrations. Therefore, further investigations are needed for separation of active components which might have an ideal, safe molluscicidal activity with high lethal effect.

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التأثيـــر القاتل لنبات الأزولا على قواقع البلهارسيـــا

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سبق أن أثبت الباحثون التأثير القاتل لنبات الأزولا على قواقع البلهارسيا. وفى هذا البحث تم استخلاص النبات بثلاثة مذيبات هى الأثير البترولى والكلوروفورم ثم بالماء وتم اختبار التأثير القاتل على القواقع لكل من هذه المستخلصات وذلك على القواقع الصحيحة والمصابة وقد أظهرت كل هذه المستخلصات تأثيرا قاتلا على القواقع ذا دلالة إحصائية.

وقد أظهر المستخلص المائى تأثيرا قاتلا بتركيز ٥٠٠ جزء فى المليون بنسبة ١٠٠٪ ما أظهر مستخلص الأثير البترولى تأثيرا بتركيز ١٠٠ جزء فى المليون بنسبة ١٠٠٪ بينما أظهر مستخلص الكلوروفورم تأثيرا قاتلا على نفس القواقع بنسبة ٧٥٪ عند تركيز ٨٠ جزء فى المليون بعد ٢٤ ساعة من بداية التجربة.

وبتجزئة خلاصة الكلوروفورم على عمود السيلكاجيل بمذيبات متدرجة القوة أعطى جزءا أكثر فاعلية حيث أنه بتركيز ٣٠ جزء فى المليون كان التأثير القاتل بنسبة ٩٠٪ بعد ٢٤ ساعة. وقد لوحظ أن تأثير الخلاصات المختلفة كان أكثر فاعلية على القواقع المصابة منها على القواقع السليمة. ومن هنا نستطيع القول بأن استمرار تجزئة الخلاصة قد يعطى تأثيرا قاتلا على القواقع وبتركيز أقل.