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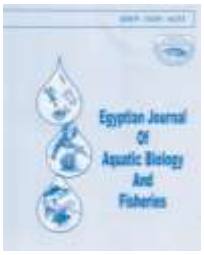

Influence of saponin fraction from *Albizia anthelmintica* on *Biomphalaria alexandrina* snail; the intermediate host of *Schistosoma mansoni* in Egypt

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ABSTRACT

Saponin fraction from *A. anthelmintica* has a promising molluscicidal effect against snails for LC_{50} of 17.6 ppm as well as a strong biocidal activity against the larval stages of *S. mansoni* (miracidia and cercariae). A high significant decrease ($p<0.001$) was recorded in infection rate and survival rate during continuous exposure of the plant; being 50±10 and 44.66±5.05 respectively compared with 81±10.1 in control groups respectively. Light microscopic investigation showed that the tested plant is responsible for the increase of snail's amoebocytes that is consider a main component of the internal defense system of the *B. alexandrina* snails. Electron microscopic examination showed remarkable apoptotic effects, nuclear chromatin condensation, vacuolated cytoplasm and deterioration in the internal organs as endoplasmic reticulum. It is concluded that exposure of *B. alexandrina* snails to saponin fraction from *A. anthelmintica* may be used as a promising molluscicidal and biocidal against the *S. mansoni* parasite.

INTRODUCTION

Schistosomiasis is one of the most important neglected tropical diseases. Despite effective chemotherapeutic treatments, this disease continues to afflict hundreds of millions of people, at least 218 million people required preventive treatment for schistosomiasis and More than 66.5 million people were reported to have been treated for schistosomiasis in 2015 (WHO, 2017). Understanding the natural intermediate snail hosts of schistosome parasites is vital to the suppression of this disease. Fresh water snails *Biomphalaria alexandrina* represents the intermediate host for *Schistosoma mansoni*. Control of *S. mansoni* by moluscicides plant origin was used for this purpose to reduce of schistosomiasis transmissioin in infected areas. Molluscicides from plant origin aregaining increased attention as they seem to be less expensive, more available, have low toxicity to non target organisms and have no side effect for other aquatic organisms and safe for all environment components (Ibrahim *et al*., 2004**).** There is a great interest in the use of molluscicides from plant origin by local community in self-supporting system of schistosomiasis control programe.

Such molluscicides are cheap, safe, available, rapidly biodegradable and probably easily applicable with simple techniques appropriate to developing countries.

Plant molluscicides have been regarded as possible alternatives to the costly and environmentally hazardous molluscicides currently available (Oliveira-Filho *et al*., 2010). More than 1000 plant species have been screened for molluscicidal activity, and some plants acts a great potential value as molluscicides, such as Cryptostegia grandiflora (Elsayed, *et al*., 2011)**,** *Yucca desmetiana* (Diab *et al*, 2012), *Nerium indicum* (Dai *et al*, 2011), *Eucalyptus globules* (Al-Sayed *et al*, 2014), *Saraca asoca* (Dos Santos *et al*, 2014), *Pueraria peduncular*is (Yang *et al*,. 2017) and *Moringa oleifera* (Ibrahim and Abdalla, 2017). A few major effective active compounds have been identified, such as saponins and alkaloids.

Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are important in human and animal nutrition. These structurally diverse compounds have been observed to kill protozoans and mollusks. The molluscicidal activity of saponins were first observed by Lemma (1965) who noticed the toxic effects of extracts of unripe berries of *Phytolacca dodecandra* on river snails in Ethiopia. Efforts were then mounted to utilize this property of saponins to control diseases such as schistosomiasis. Saponins extracted from many other sources were also seen to have similar molluscicidal properties, for example purified *Sesbania sesban* saponins at 3–25 mg/kg (Dorsaz *et al*. 1988) and purified saponin mixtures from *Maesa lanceolata* at above 5 parts per million (Sindambiwe *et al*. 1998) have been found to be active against *Biomphalaria glabrata*. The molluscicidal activity of the saponins may be due to their characteristic detergent effect on the soft body membranes of the molluscs.

The genus Albizia (Fabaceae) comprises about 150 species that are widely distributed in Africa and South America. *Albizia* species were reported as rich in phenolic compounds, saponins, and triterpenoidal saponins. Saponin glycosides with diverse pharmacological activities were isolated from different Albizia species (Zhang, *et al*, 2011, Singab *et al* 2015). Still now there are no studies on the activity of *Albizia anthelmintica* extracts against snails.

The aim of the present study was to evaluate the effect of saponin isolated from A.anthelimintica as a plant origin on susceptibility of B. alexandrina snails to infection with S. mansoni and on the count and morphology changes of hemocytes and as a possible safe method of schistosomiasis control.

MATERIALS AND METHODS

Snails

Biomphalaria alexandrina snails were collected from different water courses at Giza Governorate, Egypt, and transferred in plastic bags to the laboratory. Snails were reared in de-chlorinated water (25°C ± 1) (Liang *et al*., 1987) at Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. Healthy snails free from trematode infections were used in the experimental testes.

Plant Material

The leaves of *A. anthelmintica* BRONGN were collected in January 2013 from the Zoo, Giza, Egypt. They were kindly authenticated by Mrs. Therease Labib, the taxonomy specialist in El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (PHG-P-AA-2013) was deposited with the Herbarium of the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

Molluscicidal activity

A stock solution of 20 ppm was prepared from saponin fraction from Albizia anthelmintica extract on the basis of w/v using de-chlorinated water (pH 7.0 to 7.5). A series of concentrations was prepared on the basis of volume/volume (WHO 1965). Three replicates were used, each of ten snails (5 to 7 mm/L, for each concentration (LC_{10}, LC_{25}, LC_{50}, $LC9_0$). Exposure and recovery periods were 24 h each; at 25 ± 1°C. For each test, 3 replicates of control snails were maintained under the same experimental conditions in de-chlorinated water. The effectiveness of saponin fraction from Albizia anthelmintica has been expressed as LC_{50} and LC_{90} (Litchfield and Wilcoxon, 1949). The used concentrations were calculated through a computer program (IPM SPSS Statistics program, version 20 for Windows), employing the probit analysis (Finney, 1971).

Exposure of snails to saponin and to shistosome mansoni

Four groups of *B, alexandrina* snails (5-7 mm) were exposed for *S. mansoni* mircidia of (8 miacidia /snail), Ffirst group represent control group, other groups were exposed to 20ppm of saponin of *A. antheletica* at the same time of miracidial exposure these groups represent exposed group, three replicates (each replicate of 10 snails/L in glass container) were prepared for each group. After that, snails were transferred to clean de-chlorinated water (25 ± 1°C) and daily fed with oven dried lettuce leaves throughout the pre-patent and patent periods (Massoud *et al*., 1973). A control group of three replicates, each 10 snails/L was exposed to miracidia concurrently with the experimental snails and treated similarly till cercarial emergence. Dead snails were removed daily and surviving snails were individually examined once weekly for cercarial shedding 24 days post-miracidial exposure. The number of snails survived at the first shedding and the number of infected snails were calculated. The survival and infection rates were compared with that of control using chi -test of excel

Miracidicidal and cercaricidal activity

Ten milliliters of water containing approximately 100 freshly hatched miracidia, freshly shed cercariae was mixed with 10 mL of 40ppm extract obtain a concentration of 20 ppm (Mostafa & Gawish 2009). Approximately equal numbers of miracidia, cercariae in 20 mL of dechlorinated water were used as the control. Three replicates were used for each test. Microscopic observation of the movement and mortality of the miracidia and cercariae were performed every 15 min. The organisms were considered dead when their motion ceased completely. The dead organisms were then counted.

Hemocytes investigation:

Light microscopy:

Hemolymph samples were collected from each group of exposed snails by removing a small portion of the shell and inserting a capillary tube into the heart. The hemolymph pooled from 10 snails and collected in a 1.5 ml Eppendorf tube according to Michelson (1966) and kept in an icebox for microscopic examinations. Blood films were stained with Fleishmann, Giemsa stains and examined according to Mossalem *et al*. (2013). The number of each blood cell type was calculated, represented as a percentage per 100 of cells and photographed by Primo Star ILed, AxioCam ERcS5 digital Camera.

Electron microscopy:

The collected hemolymph were centrifuged and the sedimented cells were fixed in 4% glutaraldhyde with sodium cacodylate, Two hours later , the cells were postfixed in 2% osmium tetraoxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin according to the technique of (Grimaud *et al*.,

1980). Semi-thin and ultra- thin sections were cut with a Leika ultra microtome. Ultra- thin sections were contrasted with uranyl acetate and lead citrate stains then examined by Phillips EM 208 Electron Microscope.

Statistical analysis:

The data are presented as mean ± standard deviation). The means of the different groups were compared globally using the student's t- test (Sokal and Rohlf, 1981).

RESULTS DISCUSSION

The Molluscicidal activity of saponin fraction from *A. anthelmintica* against *B. alexandrina* snails is more affected for snails with lethal concentration LC_{50} = 17.6 ppm Table (1).

Table 1: Molluscicidal activity of saponin fraction from *A. anthelmintica* on *B. alexandrina* snail

Concentration	LC_{10}(ppm)	LC_{25}(ppm)	LC_{50}(ppm)	LC_{90}(ppm)
Saponin of A.anthelmintica	9.9	13.6	17.6	25.3

A high significant ($p<0.001$) decrease in infection rate and survival rate during continuous exposure of plant for one month showed (50%) than control (81%) for infection rate and 44.66% for survival rate when compared with control 96% Table (2).

Table 2: Effect of a saponin fraction from *A. anthelmintica* continuous exposed for one month on *B. alexandrina* snail's infection rate and survival rate.

Sample type	Infection rate	Survival rate
	M±SD	M±SD
Control	81±10.1	96 ±1.0
Infected	50±10***	44.66±5.05***

Saponin fraction from *A. anthelmintica* had strong miracidicidal activity (100% inhibition) through 1/2h exposure and highest cercaricidal activity 70 & 100% after ½ h minutes & 1h exposure, respectivelyTable(3).

Table 3: Effect of a saponin fraction from *A. anthelmintica* on *S. mansoni* miracidia and cercariae.

Plant	Miracidicidal activity %		Cercaricidal activity%	
time	1/2h	1h	1/2h	1h
Control	0	0	0	0
A. anthelmintica	100	0	70	100

Examination of *B. alexandrina* hemolymph by light and electron microscopy revealed three types of different cells classified according to their shape and granular contents. These cells are: - Granulocytes, Amoebocytes and Hyalinocyte as shown in Fig (1). Morphological alter of hemocytes category Amoebocytes, Granulocytes and Hyalinocytes which are key immune elements in *B. alexandrina* snails. Volume size, shape and their number showed very high significant rework as decreased at two types as follows 23.3%,6.6% Granulocytes, Hyalinocytes in compared with control 50.3%, 23% plus high increased in Amoebocytes 70% in link to control 25% table(4). Histological transform as remarkable apoptotic changes represented by nuclear chromatin condensation, eccentric nucleus and fragmentation, vacuolated cytoplasm, deterioration in the internal organs as endoplasmic reticulum as shown in Fig (1 & 2)

Table 4: Percentage of hemocytes in the hemolymph of *B. alexandrina* snails exposed to saponin fraction from *A. anthelmintica* for one-month infection with *S. mansoni*.

Hemocyte type / Sample type	**Amoebocyte (%)**	**Granulocyte (%)**	**Hyalinocyte(%)**
Control	25	50	23
Exposed	70***	23***	6.6***

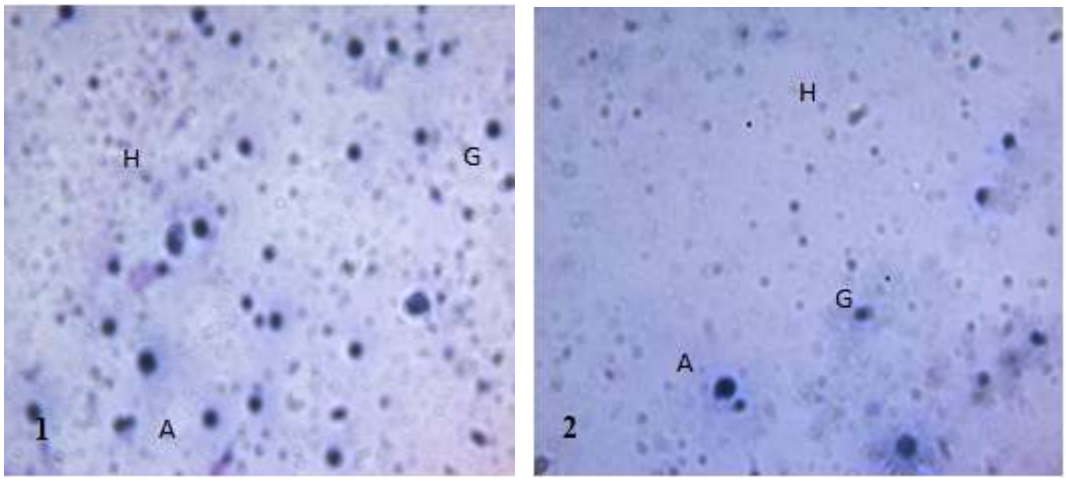

Fig. 1: light microscope digital cameara showing Three types of hemocytes of Biomphalaria alexandrina hemolymph A Amoebocyte, H Hyalinocyte, G Granulocyte 1: Control wih large number of haemocytes 2: after treated with saponin fraction from *A. anthelmintica* for one month infection with *S. mansoni* with decrease in hemocyte number .

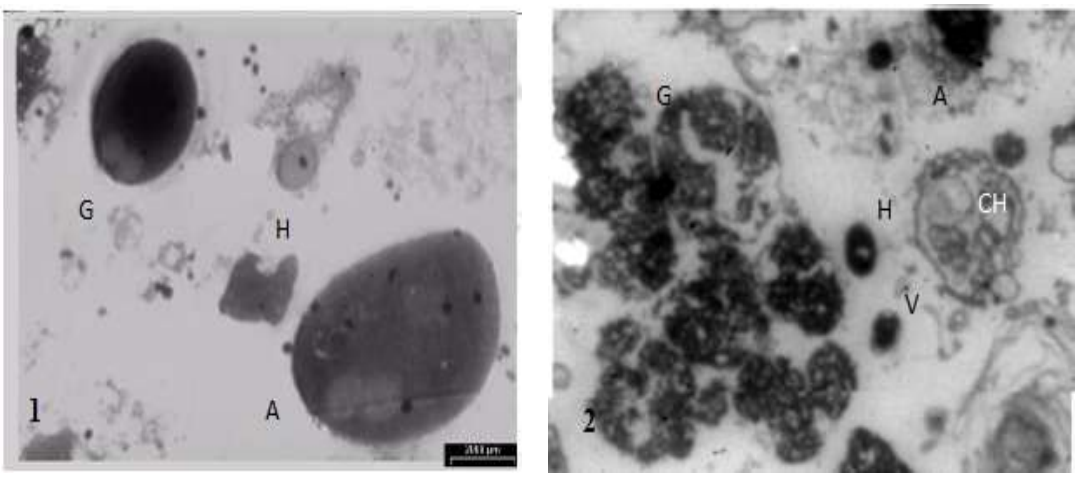

Fig. 2: Ultrastructure of hemocytes 1- Normal hemocytes of *B. alexandrina* illustrate A amoebocyte, G granulocyte and H hyalinocyte 2 – hemocytes of *B. alexandrina* after exposed to saponin fraction from A. anthelmintica for one month infection with S. mansoni for one month point up CH nuclear chromatin condensation, eccentric nucleus and fragmentation, VC vacuolated cytoplasm, deterioration in the internal organs as endoplasmic reticulum.

DISCUSSION

The *Albizia* members in Africa are used in folk medicine for the treatment of rheumatism, cough, diarrhea and injuries (Watt, and Breyer 1962). Phytochemical studies carried out on *Albizia* species led to the isolation of several triterpene glycosides, flavonoids, alkaloids and miscellaneous compounds. *Albizia anthe-lmintica* is cultivated widely in Africa and Asia. East Africans widely use *A.*

anthelmintica to control helminth parasites in human and animal medicine in Sudan (koko, 2000) and Ethiopia (Desta, 1995). In the present study, plant was tested for molluscicidal effect, the extract of plant found to be more toxic to schistosomiasis vector snail *B. alexandrina*. The killing effect of the plant due to the presence of the active component saponins. (Tadros *et al*., 2008) found that the steroidal saponin-containing fraction from methanolic extract of the plant *Dracaena fragrans* had a considerable molluscicidal activity against *B. alexandrina* and *Bulinus. truncatus* snails.

The results of the present study indicate that maintenance of snails in LC_{50} concentration of *A. anthelmintica* for one month results a significant reduction of the infection rate and survival rate for treated snails versus control ones. This may be explained by the deterioration of physiological parameters of snails making them unsuitable for the parasite development. These results agree with other authors **(**Al Shakaway *et al*, 1996; Gawish and El Bardicy, 2004**,** Gawish, 2008**)** who reported the toxic molluscicidal effectiveness of various plants against schistomiasis vector snails and their susceptibility to infection with schistosome miracidia**.** (El- Ansary *et al*., 2000) **r**eported that *Ambrosia maritima* caused a remarkable decrease in cercarial shedding from *Biomphalaria* treated with the plant powder. Moreover (Massoud *et al*., 2004**)** found that no *S.mansoni* cercariae were produced from *B.alexandrina* snails treated with LC_{20} of oleo resin from the plant *Commiphora molmol* (Myrrh), whereas, those exposed to LC_{10} showed a considerable reduction in the infection rate and cercarial production.

Most of the plants screened against schistosomiasis cercariae and miracidia were generally effective at levels less than that of their molluscicidal ones. Regarding the miracidicidal and cercaricidal activities, the present study showed that 100% inhibition for miracidia and cercarial after 1/2 hour and one-hour exposure to the concentration of LC_{50} of *A. anthelmintica* respectively. Methanol extracts of plants *Callistemon citrinus*, *Punica granatum* and *Pumpkin* killed 100 % of the treated *S. mansoni* miracidia and cercariae after 30 minutes of exposure to LC_{90} (Ammar *et al*, 2016). However dry powder of *Calendula micrantha* 100 ppm were killed miracidia and cercariae within 2 and 24 h of exposure, respectively (El-Emam, 1986).

The *Biomphalaria* immune response is mounted both by cellular effectors (i.e. via the hemocytes (Nacif-Pimenta *et al*, 2012), and humoral factors (Mitta *et al*, 2012), acting independently or together to fight invading microbes or parasites (Coustau *et al*, 2015) Many snail humoral factors have been carefully characterized in the *Biomphalaria* genus to identify the resistance mechanisms to schistosome infection. The first line of defense is mediated by circulating phagocytic cells known as hemocytes (also known as amoebocytes) found in the hemolymph of the snail. These cells have an important role in phagocytosis and encapsulation reactions. The use of molluscicides in the control of fresh water snails is now approaching a highly developed state and plants having molluscicidal properties were found to suppress the total number of snail's hemocytes. In the present study three main types of hemocytes were detected in the hemolymph of untreated *B. alexandrina* by light & electron microscopy. The main cell was granulocytes (50%) followed by amoebocytes (25 %) and hyalinocytes (23 %). Granulocytes and hyalinocytes of snails treated with the tested plant decreased to 23% and 6.6% respectively. This reduction may be due to the fact that snails become unhealthy and change in physiological parameter as the result of continuous exposure to the tested plant. The reduction in hemocytes after treatment with plant could also be a result from a direct action of the plant on these cells. The increase in the number of amoebocytes 70% is of specific importance to

snails since it represents the first line of defense. The immune response of these blood cells is mediated by infiltration around the parasite, encapsulation, and phagocytosis. This result agrees with the findings of (Martin *et al*, 2006, Souza & Andrade**,** 2006 and Mossalem *et al*. 2014**),** On the other hand, the number of circulating hemocytes was highly variable in *Biomphalaria* and mainly dependent on parameters such as the method and site of hemolymph extraction besides the physiological conditions of the snail (Sminia, 1981).

CONCLUSION

Exposed of tested snails to LC_{50} of saponin fraction from of *A. anthelmintica* showed strong activity against intermediate host snail and larval stages of *S. mansoni*. It is evident thatsaponin fraction from of *A. anthelmintica*, May be used for snail control which is one of the efficient methods to control this parasite. and evaluated in the field to determine its potential use as natural products.

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ARABIC SUMMARY

تأثير مستخلص الصابونين من نبات اللبخ *Albizia anthelmintica* على قواقع *Biomphalaria alexandrina* العائل الوسيط لطفيل *Schistosoma mansoni* فى مصر

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تتم دراسة تأثير مستخلص الصابونين من نبات اللبخ ***Albizia anthelmintica*** على اللأطوار الحرة لطفيل ***Schistosoma mansoni*** ، نسبة العدوى، معدل البقاء على قيد الحياة للقواقع وعلى شكل وعدد خلايا الهيموسيت بواسطة الميكروسكوب الضوئى والالكترونى.

وأثبتت الدراسة انخفاض كبير فى معدل الاصابة بطفيل ***Schistosoma mansoni*** وكان الانخفاض ذات قيمة معنوية ($P\leq0.001$) وكذلك انخفاض فى معدل البقاء خلال التعرض المستمر لمستخلص النبات.

اوضحت الدراسة بواسطة كلا من الميكروسكوب الضوئى والالكترونى ان تعريض القواقع الى المستخلص ادى الى تدمير المصنع المسؤول عن زيادة الخلايا الأميبية الحلزونية التي تعتبر عنصرا أساسيا في نظام الدفاع الداخلي للقواقع بالاضافة الى تغييرات فى الانسجة متمثلا فى تكثف الكروماتين النووي ، السيتوبلازم المفرغ والتدهور في الأعضاء الداخلية. في الختام يمكن استخدام جزء سابونين من نبات اللبخ كمبيد حيوى ضد العائل الوسيط للبلهارسيا المعوية ويرقات الطفيل.