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In Vitro Lethal Effect of Ellagitannins of the Fruit Rind of *Punica Granatum* and Stem and Root Alkaloids on The Miracidia and Cercariae of *Schistosoma Mansoni*

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Abstract: *Ellagitannins of fruit rind and alkaloids of the stem and root of Punica granatum (P. granatum) were investigated for their in vitro lethal effect on miracidia, and cercariae of Schistosoma mansoni (S. mansoni). All the three investigated constituents were lethal to both Schistosome larvae at concentrations ranging between 0.39-50 ppm. At 0.39 ppm of the tested active constituents, the killing time of 50% of Schistosoma larvae ranged between 30.0 to 135 min, and 43 to 380 min, for miracidia and cercariae, respectively. While stem alkaloids were generally more active against cercariae, root alkaloids were more effective against miracidia. At 3.125 ppm, the LT_{100} for cercariae and miracidia were respectively, 50 and 120 min for the stem alkaloids, and 300 and 30 min for root alkaloids. Ellagitannins were also more effective against miracidia than cercariae. At a concentration of 6.25 ppm, ellagitannins killed 50% of the cercariae and miracidia after 42 and 3 min, respectively. Ellagitannins could be prepared cheaply from pomegranate fruit rind which is produced in large amounts during the commercial production of pomegranate juice. Therefore, ellagitannins could be used as an affordable method for the control schistosomiasis.*

Keywords: *Punica granatum, ellagitannins, alkaloids, Schistosoma mansoni, miracidia, cercariae.*

I. Introduction

Human schistosomiasis or bilharziasis is an important parasitic disease.[1] It has been associated with the ailment of mankind for several thousands of years. Eggs of Schistosoma were detected in Egyptian mummies ageing thousands of years.[2]

It is estimated that 200 million people are infected with Schistosoma, and 600 million are at risk of infection.[3] Therefore, schistosomiasis is considered the second most important parasitic disease after malaria in terms of the overall morbidity and mortality.[4] Schistosoma infection is considered as a major public health problem in tropical and subtropical regions of Africa, Asia and South America since it affects young people and diminishes their productivity.[5]

There are three main species causing the disease in man; namely *S. mansoni*, *S. haematobium* and *S. japonicum* 5. In Saudi Arabia both *S. mansoni*, *S. haematobium* are endemic. According to the Ministry of Health, the prevalence of schistosomiasis in Saudi Arabia was 2.9/100,000 persons in 2004.[6] The highest prevalence was reported in Jazan, Bishah, Aseer, Al-Bahah and Taif. *S. mansoni* is more prevalent in Taif, Al-Bahah, Aseer, Bishah, Najran, Makkah Al- Mukarramah and Al-Medina[6-8], where they are presumably, transmitted by rodents, baboon monkeys and infected humans.[9, 10]

When the eggs of the Schistosoma parasite in faeces reach water, the miracidia hatch and invade suitable freshwater snails which act as intermediate hosts. In the snail, miracidia develop into sporocysts. Cercariae develop into sporocysts and emerge from the snail in water during daylight. When cercariae reach their final host, they penetrate the skin, leaving the tail behind and develops into adult worms.[3]

Various methods have been applied to control bilharziasis through, breaking the life cycle of the parasite.[3,11] In the past, the use of chemical compounds to control snails, miracidia or cercariae, was common. However, today, these chemicals are not recommended because of their adverse effects on the environment, since they are toxic to man, animals and plants.[13] Therefore, there is a continuous effort to find natural alternatives, which are usually, effective, cheaper and less hazardous to the environment.[3]

Many plants have been found to possess cercaricidal and miracicidal effects. One of the best-studied cercaricide and meracicide plants is *Phytolacca dodecandra* (Endod).[12] This plant has soapy berries and occurs

throughout Sub-Saharan Africa.[13] Endod has both cercaricide and meracicide properties which can favourably be compared with niclosamide.[14] According to a report by the World Health Organization, Endod does not have mutagenic or carcinogenic properties against a variety of plants and animals.[14] In addition, the active saponins are readily biodegradable and therefore, they are environmentally acceptable.[15]

Other examples include *Jatropha curcas* which has a lethal effect against cercariae and miracidia of both *S. mansoni* and *S. haematobium*; [16] *Tetrapleura tetraptera*, which is known as Aridan in South-West Nigeria and was used successfully to keep the transmission sites free from snails and cercariae for about 28 days; [17] *Ambrosia maritima* L. (Damsissa), an annual herbaceous plant, which is widely distributed throughout the Mediterranean region and is used on a large-scale as a safe method to control of Bilharziasis in Egypt;[18, 19] *Millettia thonningii*, was found to be lethal to molluscs, miracidia, cercariae, and adult worms of *S. mansoni*.[20-22] *P. granatum* is widely used in alternative medicine for treatment of several diseases.[23, 24] It has been demonstrated to have antibacterial, antifungal, antiprotozoal and antiviral activities.[25-27] Crude extracts of pomegranate were found to possess molluscicidal, cercaricidal and miracidal activities.[28,29] In this study, we investigated the *in* vitro efficacy of three active constituents of *P. granatum*, rind ellagitannins, stem alkaloids and root alkaloids, against cercariae and miracidia of *S. mansoni.*

II. Materials and methods

Preparation of plants for extraction

Fruit rinds, stem barks and root barks of *P. granatum* were collected from Taif, Saudi Arabia, and air dried at room temperature in the laboratory. The dried parts of the plant were ground by an electric blender.

Separation of ellagitannins

The powdered rind was percolated in water overnight in a shaking incubator. XAD-16 resin was packaged into a glass column, washed with methanol, and equilibrated with water. The aqueous extract was applied to the column. The resin was washed with water and the adsorbed ellagitannins were eluted by methanol before evaporation at 50ºC in a rotary vacuum evaporator.[30]

Separation of alkaloids:

Powdered stem and root barks were extracted with 70% ethyl alcohol. The extracts were evaporated to a thick solution using a rotary vacuum evaporator at 60^{o}C and were acidified to pH 4 before extraction with chloroform. The pH of the extracts was raised to 9 with ammonium hydroxide and extracted with several portions of chloroform. The combined chloroform extracts were evaporated under reduced pressure to obtain the crude alkaloids.[31]

Preparation of *S. mansoni* miracidia and eggs

S. mansoni eggs were separated from liver homogenates of infected mice and emulsified in 10 volumes of 10% Sodium Chloride and the sediment was washed with cold saline and stored overnight in a refrigerator. Animal handling and methodology were conducted in compliance with the Ethical Committee of Taif University. Eggs were then diluted with tap water and exposed to bright light to allow the ova to hatch and release the miracidia.[32]

Infection of snails

Biomphalaria alexandrina snails were infected individually by exposing each snail to 3-5 miracidia. The snails were maintained at 27-29 ºC for a minimum of 5 h and were then transferred to sandwich boxes containing dechlorinated tap water and kept in dark environment.[32]

Shedding of cercariae

After 3 weeks of infection, the snails were placed in a beaker containing 100 ml dechlorinated tap water and exposed to light from a 10 volt electric lamp for 4 h and the presence of cercariae inspected in water.[33]

Effect of the purified constituents on cercariae and miracidia

Tissue culture plates were used as test chambers. Twenty miracidia or cercariae were exposed to serial dilutions of the extracts. The viability of miracidia was inspected at different time intervals under a dissecting microscope.[34]

Statistical analysis

All experiments were done in triplicates and data were analysed by using SPSS 16 statistical software programme.[35]

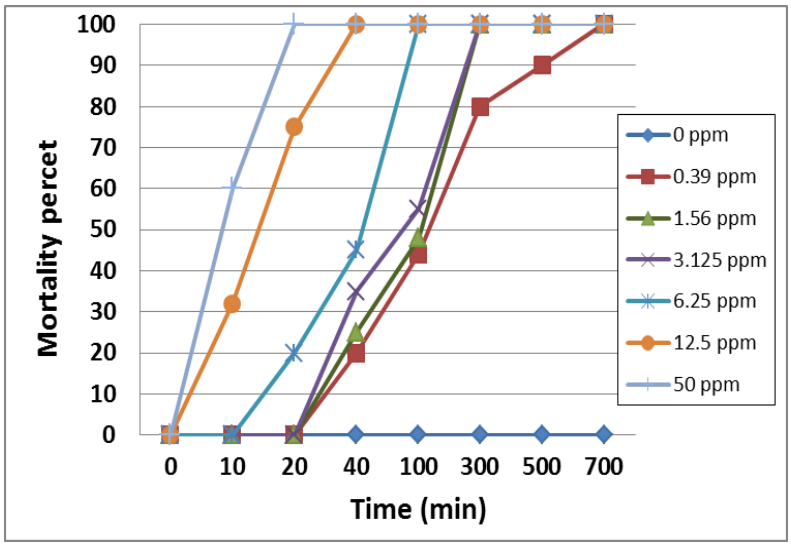


Fig. 1: The mortality rate of cercariae exposed to different concentrations (ppm) of rind ellagitannins**.**

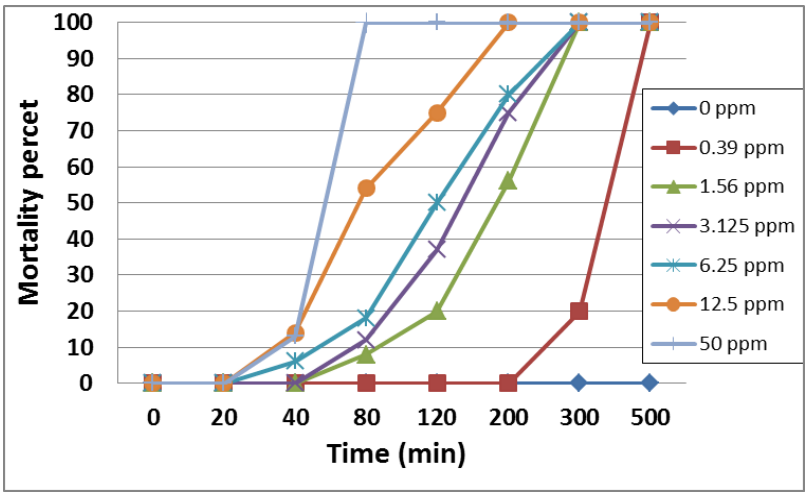


Fig. 2: The mortality rate of cercariae exposed to different concentrations (ppm) of root alkaloids

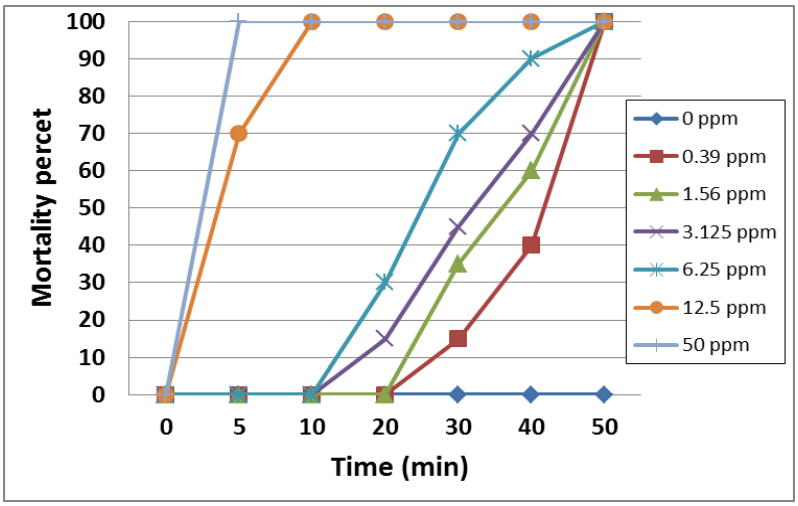


Fig. 3: The mortality rate of cercariae exposed to different concentrations (ppm) of stem alkaloids

III. Results

The rate of mortality of *S. mansoni* cercariae at different concentrations of ellagitannins and the alkaloids of the stem and root barks of *P. granatum* are shown in Figures 1-3. The mortality rate of *S. mansoni* cercariae increased by increasing the concentration of rind ellagitannins from 0.39 to 50 ppm (Fig1). At 0.39

and 50 ppm, 100% of the cercariae were killed after 700 (11.66 h) and 20 min respectively. On the other hand, 60% of the cercariae were killed after 200 min and 10 min of exposure to 0.39 and 50 ppm, respectively.
The lethal times for killing 100% of cercariae at different concentrations of root alkaloids ranged between 80 and 500 min (Figure 2). Unlike ellagitannins, which at 50 ppm killed 50% of cercariae after 8 min (Figure 1), the lethal effect of the same concentration of root alkaloids killed 50% of cercariae after 58 min.
Stem alkaloids were the most active constituents of *P. granatum* against cercariae. The time range for 100% mortality at the tested concentrations ranged between 5 to 50 min (Fig 3). At a concentration as low as 12.5 ppm, less than 10 min was required to kill 100% of cercariae.

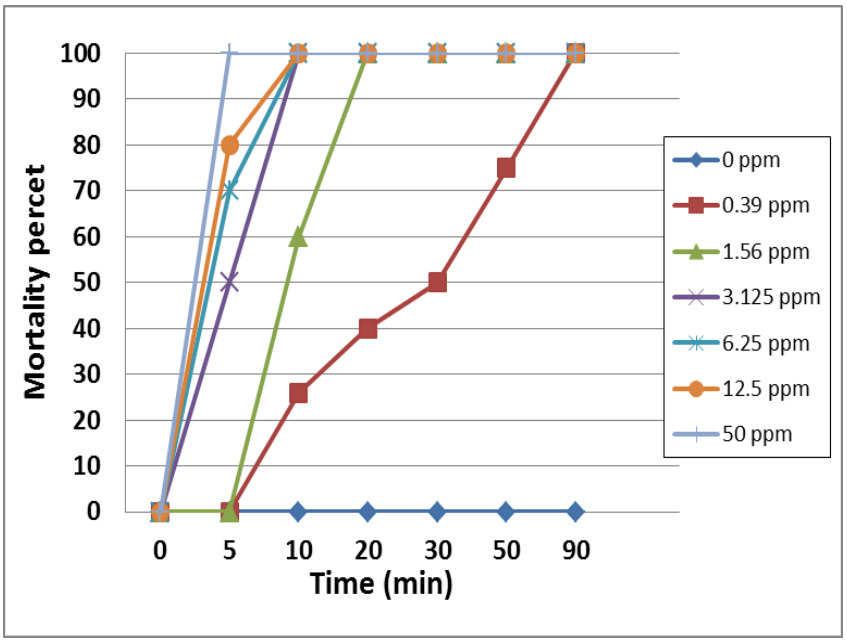


Fig. 4: The mortality rate of miracidia exposed to different concentrations (ppm) of rind ellagitannins

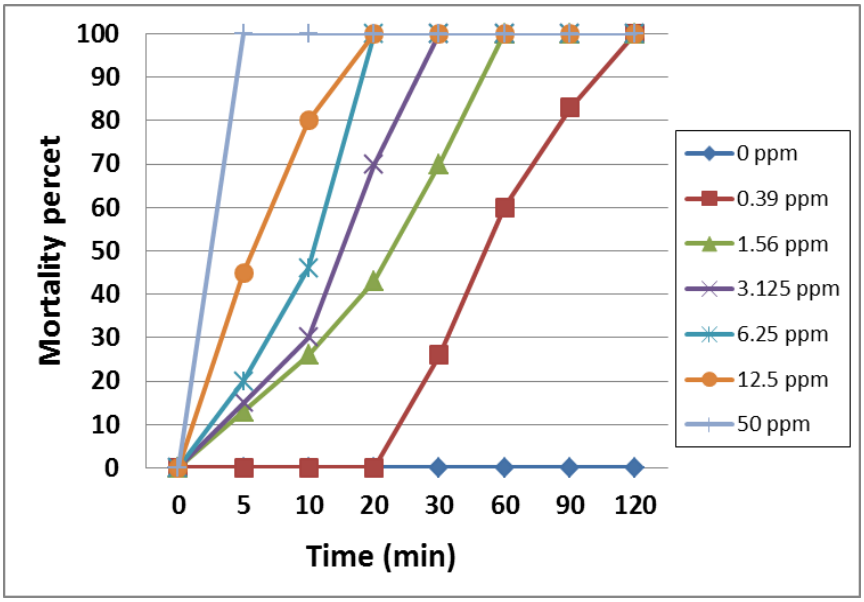


Fig. 5: The mortality rate of miracidia exposed to different concentrations (ppm) of root alkaloids

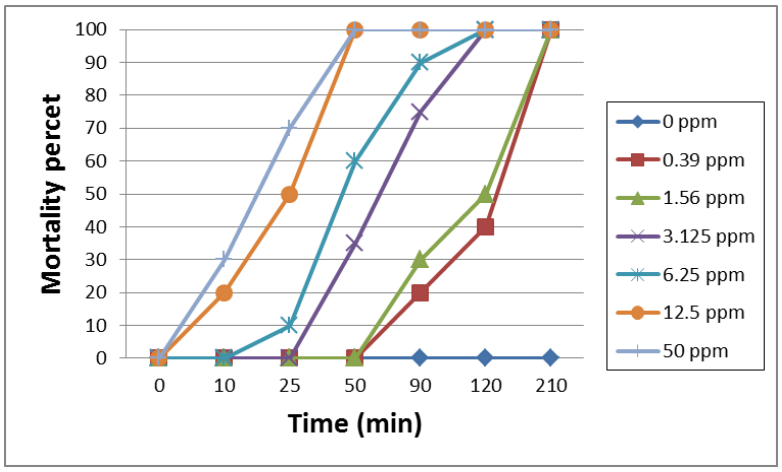


Fig. 6: The mortality rate of miracidia exposed to different concentrations (ppm) of stem alkaloids

Rind ellagitannins at concentrations ranging between 0.39 to 50 ppm, killed miracidia within 5 to 90 min (Fig.4). The lethal time for 100% of miracidia at 1.56 ppm of ellagitannins was 20 min. However, on dilution of ellagitannins to 0.39, the killing time for miracidia was prolonged to 90 min (Fig 4).

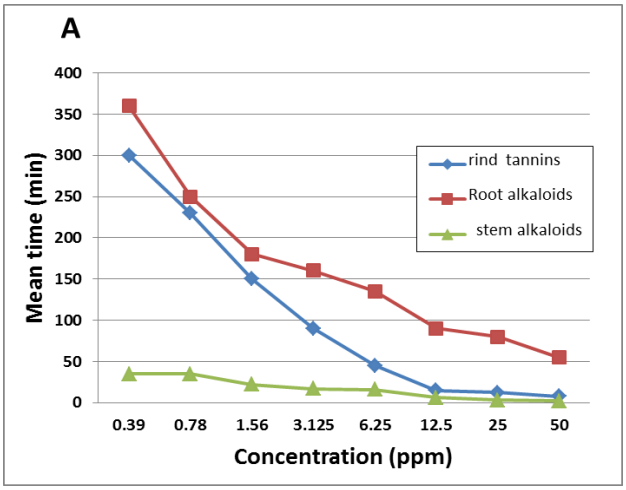
Root alkaloids were also highly lethal to miracidia, though they were relatively less active than rind ellagitannins (Fig 5). The tested concentrations of root alkaloids killed 100% miracidia in a time range between 5 and 120 min (Fig 5).

As shown in Figure 6 stem alkaloids were the least active constituent of *P. granatum* against miracidia compared to rind ellagitannins and root alkaloids. Time needed for 100% mortality of the tested miracidia, at 50 ppm and 0.39 ppm, were 50 and 210 min respectively and for 70% mortality of miracidia, at the same concentrations, were 25 and 165 min, respectively (Fig 6).

Figure 7 compares between the LT_{50} (A) and LT_{100} (B) of cercariae resulting from exposure to different concentrations of the tested active constituents. Cercariae were more sensitive to stem alkaloids as their LT_{50} ranged between 2 to 35 min and their LT_{100} ranged between 5 to 50 min. On the other hand, rind ellagitannins were intermediate in their LT_{50}, compared to the tested alkaloids. However, the activity of ellagitannins was dramatically affected by dilution especially in the case of LT_{100}, where, at concentrations of 0.39 and 0.78 ppm, tannins became markedly less active than the root alkaloids. The respective LT_{100} of ellagitannins at these concentrations were 600 and 700 min compared to 500 and 300 min in the case of root alkaloids (Fig 7).

Figures 8, compares the LT_{50} and LT_{100} of the investigated active constituents of *P. granatum* on miracidia. The most active constituent of *P.* granatum on miracidia was the rind ellagitannins and the least active ingredient was the stem alkaloids. Ellagitannins were markedly more active than both stem and root alkaloids especially at low concentrations ranging between 0.39 and 6.25 ppm.

Table 1 summarises the LT_{50s} for the tested active constituents for the three investigated active ingredients on cercariae and miracidia. Generally speaking, miracidia were more sensitive to ellagitannins and root alkaloids compared to stem alkaloids. On the contrary, the latter was more active against cercariae. For instance at a concentration of 0.39 ppm stem alkaloids killed 50% of the miracidia and cercariae after 135 and 43 min respectively. On the



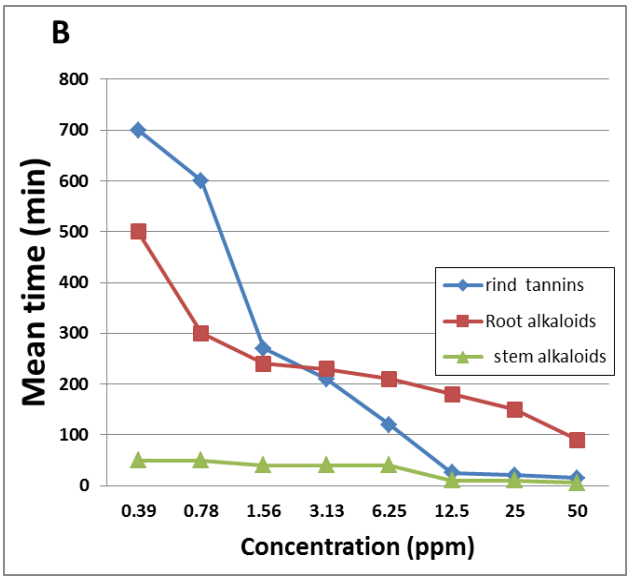


Fig. 7: Comparison between the LT_{50s} (A) and LT_{100s} (B) of cercariae of *Schistosoma mansoni* exposed to ellagitannins, root alkaloids and stem alkaloids.

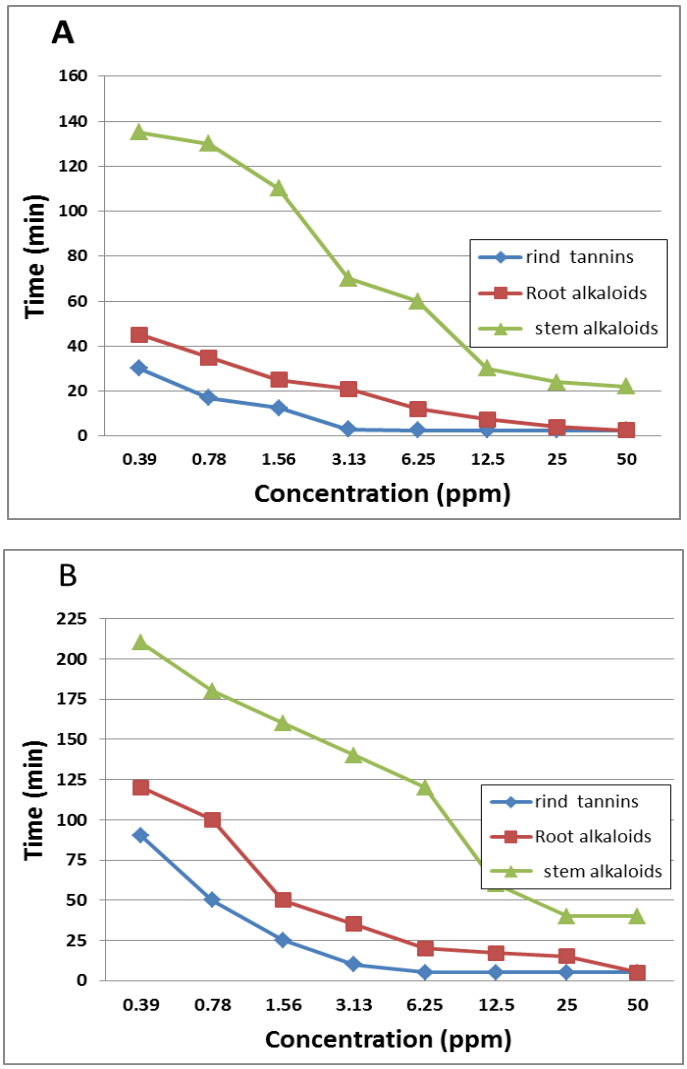


Fig. 8: Comparison between the LT_{50s} (A) and LT_{100s} (B) of miracidia of *Schistosoma mansoni* exposed to ellagitannins, root alkaloids and stem alkaloids.

Table 1: Comparison between times required for killing 50% of cercariae and miracidia by rind ellagitannins, root alkaloids and stem alkaloids.

Conc. (ppm)	Rind ellagitannins		Root alkaloids		Stem alkaloids	
	cercariae	miracidia	cercariae	miracidia	cercariae	miracidia
	Time (min)					
0 .39	125	30	380	50	43	135
0.78	115	17	250	35	39	130
1.56	100	8.0	180	23	36	120
3.125	95	5.0	150	15	32	70
6.25	45	4.0	120	12	25	40
12.5	14	3.0	75	7.0	4.0	25
25.0	10	2.8	68	4.0	3.0	20
50.0	8.0	2.5	58	2.5.0	2.5	17.5

It should be mentioned that even though miracidia were relatively less susceptible to stem alkaloids and cercariae were relatively less susceptible to both ellagitannins and root alkaloids, still even at low concentrations of 0.39 ppm, 50% of both larvae were killed within 380 min (6 h) (Table 1).
Table 1 also demonstrates that rind ellagitannins and root alkaloids were markedly more active against miracidia than cercariae, at lower concentrations.

IV. Discussion

Crude extracts of different parts of the *P. granatum* including leaves, stem bark, root bark and fruit rind were previously proven to be lethal to cercariae of *S. mansoni.*[11] In this study, we investigated the lethal effect of three active constituents, namely rind ellagitannins, root and stem alkaloids of *P. granatum* against both miracidia and cercariae.

Amongst the three tested ingredients of *P. granatum* stem alkaloids were the most potent cercaricide, rind ellagitannins were the most active miracicide.

While miracidia were, generally speaking, more sensitive to ellagitannins and root alkaloids than cercariae, they were less susceptible to stem alkaloids. On the other hand, cercariae were more sensitive to stem alkaloids and ellagitannins and less sensitive to root alkaloids.

However, in spite of the relative lower activity of stem alkaloids against miracidia and root alkaloid against cercariae, they were still lethal at a low concentration of 0.39 ppm to 50% of miracidia and cercariae after 135 and 380 min respectively.

The lethal effect of the tested active constituents of *P.* granatum against miracidia is of paramount importance because each miracidium invading a snail of the genus *Biomphalaria* is transformed into a sporocyst which produces thousands of cercariae. Consequently, the killing of a miracidium aborts the production of thousands of cercariae.[5] Therefore, the data of this study suggest that the three investigated active constituents could possibly be used to control miracidia.

The extracts of plants like *Origanum compactum,*[34] *Lagenaria breviflora,*[36] *Iris pseudacorus,*[37] and *Iris germanica,*[38] were categorised as potent cercaricides and miracicides because their extracts were lethal to the larvae at concentrations below 1 ppm. Comparatively, the three investigated active ingredients of *P. granatum,* may also be categorised as potent miracicide and cercaricide.

A previous study suggested that pomegranate rind extracts are cercaricide.[11] Because, the rind lacks alkaloids,[23] Data obtained in this study suggest that rind cercaricide activity could be attributed mainly to ellagitannins

During the commercial production of pomegranate juice, large amounts of the fruit rind are produced as a by-product. Ellagitannins are abundant in fruit rind and could be cheaply purified in a rapid large scale process.[30] Because, ellagitannins are highly lethal to miracidia and cercariae, they could be used as affordable and effective components in an integrated approach to control schistosomiasis. However, ellagitannins should not be used at concentrations below 1.56 ppm because their lethal effect on both miracidia and cercariae was very much reduced.

V. Conclusion

Ellagitannins of the fruit rind of *P. granatum* and stem and root alkaloids exhibited potent lethal effects on both miracidia and cercariae of *S. mansoni* at concentrations below concentrations. Ellagitannins could be produced cheaply from the fruit rind, which is a by-product of pomegranate juice production. Therefore, ellagitannins could presumably be considered as a cheap candidate for control schistosomiasis.

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