Molluscicidal effects of pumpkin seed extracts on *Schistosoma* vectors

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Abstract

The aim of the study was to investigate the molluscicidal effects of pumpkin seeds (*Curcurbita maxima*) on adult, juvenile *Biomphalaria*, and adult *Bulinus* snails under laboratory conditions. This study was prompted by recent reports on *Schistosoma* gaining resistance to the commonly administered drug, praziquantel. Snails were exposed to water and ethanol crude extracts for 24 hours and significant concentration-dependent mortality rates were observed. Observations of the snail mortalities continued up to 72 hours. The lethal concentration of 0.02 mg/ml killed 50% of the snails (LC50) for both the water and ethanol extracts on adult *Biomphalaria* snails. It was noted that the mortalities were not significantly dependent on the time of the snails' exposure to the extracts. There was a significant difference between the susceptibility of juvenile and adult snails to the ethanol extract ($p = 0.016$). These results suggest that pumpkin seeds have a significant molluscicidal effect on *Biomphalaria* and *Bulinus* snails. We propose that pumpkin seed extracts be considered as molluscicidal agents in a bid to control transmission of schistosomiasis.

Key words: Schistosomiasis, *Biomphalaria, Bulinus*, molluscicidal activities

Introduction

Neglected tropical diseases (NTDs) are a group of 17 major disabling conditions that are among the most common chronic infections in the world's poorest people (World Health Organisation [WHO], 2003). The NTDs afflict an estimated 1.4 billion people, whose greater population live in Africa and are among the poorest in the world, causing significant disability and impairing quality of life (Institute of Medicine, 2011). Of all NTDs, the most neglected are helminthic infections, which comprise five of the top ten NTDs in terms of Disability-Adjusted Life Years (DALYs) (Frean & Mendelson, 2013). Among these helminthic infections is schistosomiasis.

Schistosomiasis commonly known as Bilharzias is caused by a digenean trematode of the genus *Schistosoma* (Katsurada, 1904). The intermediate hosts of all digenetic trematodes are snails and schistosomes are no exemption. In Zimbabwe, the snail vectors are *Bulinus globosus* for the species *S. haematobium* and

Biomphalaria pfeifferi for *S. mansoni* (Chimbari, 2012). Despite schistosomiasis being one of the most persistent NTDs, treatment and disease control are based on the utilisation of a single drug, praziquantel (PZQ), otherwise called biltricide. Controlling or preventing morbidity in subjects using praziquantel has not been entirely successful in restricting transmission in high-risk areas as there have been recent reports of PZQ schistosomal resistance (Ismail *et al.*, 1999; Augusto *et al.,* 2017). This raises concerns about future control of the disease and demonstrates the significance of coming up with new tactics to control the disease (Wang, 2012). Optimal disease prevention can be achieved only when parasite infection or re-infection is effectually obstructed (King *et al.*, 2015). As a responsive measure, the WHO published a report of the Strategic and Technical Advisory Group for NTDs. In the light of its call to eliminate the disease by 2025, it discourses schistosomiasis management through the ecological control of the intermediate host population of Schistosoma, snails from the *Biomphalaria* and *Bulinus* genus (WHO, 2014; Augusto *et al.,* 2017).

It is, therefore, largely agreed that regulation of the snails' population is an essential part of the control of schistosomiasis (Mohamed *et al.*, 2012). Chemical, biological and physical control strategies have been used on the snails (WHO, 1967; Madsen, 1983; Fagitta & Egami, 1984). Among the chemical compounds, niclosamide is recommended by the WHO as the only chemical molluscicide to be used for snail control despite recent concerns of resistance of *Oncomelania* snails to the molluscicide (Dai *et al.*, 2014). The WHO, however, recommends further studies on plant molluscicides (Augusto *et al.*, 2017).

Molluscicidal plant extracts may offer affordable, locally produced, biodegradable and effectual control means in the rural parts of low-income countries where schistosomiasis is prevalent (Brachenbury, 1998). Extensive investigations may help in understanding their properties and safety as molluscicides. Pumpkins are known not only for the fruit but also for many health benefits and thus have been used for a long time in traditional medicine in many countries such as Turkey and China (Young *et al.*, 2012). Pumpkin seeds have been used in different parts of the world as a traditional medicine for treatments of gastrointestinal parasites as anthelmintic, urinary dysfunctions, hyperplasia of prostate, dysuria, cardiovascular disease, enuresis and lowering blood glucose (Medjakovic *et al.,* 2016). Among the studies that have been done on pumpkin seeds, their anthelmintic potential has proved to be a success on *S. mansoni.* However, data on their molluscicidal effects on the vectors snails is scarce. A successful trial of pumpkin seeds as a molluscicide would mean a double impact on both the vectors and the cercarial stage of the *S. mansoni* parasite. The impetus of this investigation was mainly based on the high cost of synthetic molluscicides such as niclosamide in Zimbabwe, their low availability as well as the time taken by the chemical compounds to degrade in the environment. Therefore, assessing the molluscicide potential of methanol and water extracts of natural compounds on the planorbid snails from the *Biomphalaria* and *Bulinus* genus would open potential cost-effective noteworthy alternatives in the control of schistosomiasis.

Materials and Methods

Study site

The bioassays of this study were carried out in the biology laboratory and the extraction process of the seeds was done in the chemistry laboratory at Chinhoyi University of Technology, Zimbabwe.

Collection of pumpkin seeds and vector snails

Pumpkins were bought from a local supermarket in Chinhoyi. They were washed thoroughly and cut to separate the seeds from the fruit. Snails were randomly sampled in October in Murombedzi particularly

from Madzorera dam using a sweep net. They were kept in open plastic bottles and covered with moist cotton wool to keep them alive before reaching the laboratory.

Preparation of pumpkin seeds ethanolic extracts

About 685g of pumpkin seeds were sun-dried for 72 hours to a moisture content of 12.4%. Approximately 600g of the seeds were milled into a fine powder using a mortar and pestle. In order to obtain the ethanolic crude extract, the maceration technique was used. Approximately 900ml of ethanol was added to 300g of refined pumpkin seed powder and left in a dark cupboard for 7 days. At the end of this period, the mixture was filtered on 0.1mm Whatman filter paper grade using an EC vacuum pump (WP6122050) and then concentrated to dryness using Buchi rotary evaporator (R-200) at 78^{o}C in order to obtain pure crystals of the extract. The crystals obtained were weighed and a total yield of 5g was obtained. The crystals were dissolved in distilled water. The resulting solution of 100mg/ml concentration was considered as the pure extract.

Preparation of pumpkin seeds water extracts

Approximately 600ml of water was added to 300g of fine pumpkin seed powder and left in a dark cupboard for seven days. The mixture was filtered on 0.1mm Whatman filter paper grade using an EC vapour pump (WP6122050) and the filtrate was concentrated to dryness on the Buchi rotary evaporator and 8g of crystals were obtained. The crystals were dissolved in 80ml distilled water and the solution of 100mg/ml concentration was considered as the pure extract.

Snail rearing

The snails were reared under laboratory conditions in plastic aquaria of 5L holding capacity measuring 13X12cm. The aquaria were provided with fresh water, from the dams from which the snails were taken, after every two days. No mud, sand, nor any other substratum was put in the aquaria. The laboratory in which they were kept was maintained at a room temperature of 25^{o}C with natural fluctuations of +/-2^{o}C for the duration of the research. The snails were fed on oven-dried lettuce leaves *ad libitum* and kept for five days before being used to allow them to acclimatise to laboratory conditions.

Shedding of snails

Snails were shed to certify that they were not infected by cercariae, thus ensuring the use of healthy snails only (El-sherbini *et al.*, 2009). After being exposed to the dark for eight hours during the night, snails were placed in 300ml plastic bottles filled with non-chlorinated water and placed in direct sunlight for 8 hours. Thereafter, a drop of water from each of the bottles was transferred to a microscope slide and observed for the presence or absence of cercariae. A snail was considered to be immobile if it was entirely withdrawn into its shell. Snails that were unresponsive to forceful, mechanical stimulation or probing were considered dead.

Molluscicidal activity assay

During the test process, the snails were kept under normal diurnal lighting and room temperature. They were organised into two classes, established on their developmental stage and shell diameter, juveniles (below 45mm) and adults (above 45mm) (Ciomperlik *et al.*, 2013). Preliminary molluscicidal assay tests were done to determine the minimum effective concentration. A range of six concentrations were assayed - 20%; 40%; 60%; 80% and 100% of the 100mg/ml ethanol and water extract solutions. A lethal effect in a two-hour period among all the concentrations was observed and serial dilutions of the lowest concentration (20%) were used for the molluscicidal assays. A maximum of six serial dilutions of 20% of the pure water and ethanol extracts were made as *per* WHO guidelines (WHO, 1983). The final concentrations of the water and ethanol extract serial dilutions were 20mg/ml; 2mg/ml; 0.2mg/ml; 0.02mg/ml; 0.002mg/ml and 0.0002mg/ml.

A treatment consisted of three snails (three snails *per* container) of each life stage and thus fifty-three individuals of each group were used *per* trial. Each group was exposed to the test molluscicide along with three snails of each same life stage as controls. A 0.1 dilution of Thunder was used as positive control and plain dam water as a negative control. A second positive control of absolute ethanol was used to factor into

consideration the effects of residual ethanol in the ethanol extracts. The treatments used 10ml of the six dilutions of pumpkin seeds extracts in 90ml medium. The medium used was dam water from which the snails were sampled in 300ml plastic bottles. This was done in order to reduce the number of limiting factors that could affect the snails' metabolism during the trial experiment. Each treatment and the control were carried out in triplicate. The duration of exposure to the molluscicide dilutions and control was three days. After the first 24h, the number of molluscs withdrawn into their shells, immobile and unresponsive to vigorous action was recorded. In order to ensure that the snails were indeed dead, they were placed in distilled water and observed for a two-hour period. Snails were deprived of food during the molluscicidal assays.

LC 50 determination and Statistical analysis

The minimum concentration required to kill 50% of the snails (LC50) values were determined using Graph pad Prism version 7.0 software (Finney, 1971) with 95% confidence limit. Mortality percentages were expressed and plotted against the log-transformed values of the extract concentrations. The non-linear regression lines obtained from this data were used to determine the LC50 values.

One-way analysis of variance (ANOVA) and independent T-tests were used to determine the significant differences between mean mortality values using version IBM SPPS (Statistical Package of Social Sciences) software. Tests for normality were done using Kolmogorov Smirnov tests. Results with $p < 0.05$ were considered to be statistically significant.

Results

Our results show that the water and ethanol extracts of pumpkin seeds have a significant molluscicidal activity. The results further showed that there was no significant difference between the survival rates of juvenile and adult *Biomphalaria* snails exposed to water extracts ($p = 0.208$; CI = 95%). Pumpkin seeds water extract also had a molluscicidal activity against *Bulinus* snails. There was no significance in the difference of species exposed to the water extracts (i.e. *Biomphalaria* and *Bulinus*) ($p = 0{,}665$; CI = 95%. It was also observed that there was no significant dependence of mortalities on the time of the exposure of snails to the extracts (Figs 1 and 2). This observation was made for all the snail classes used in the study.

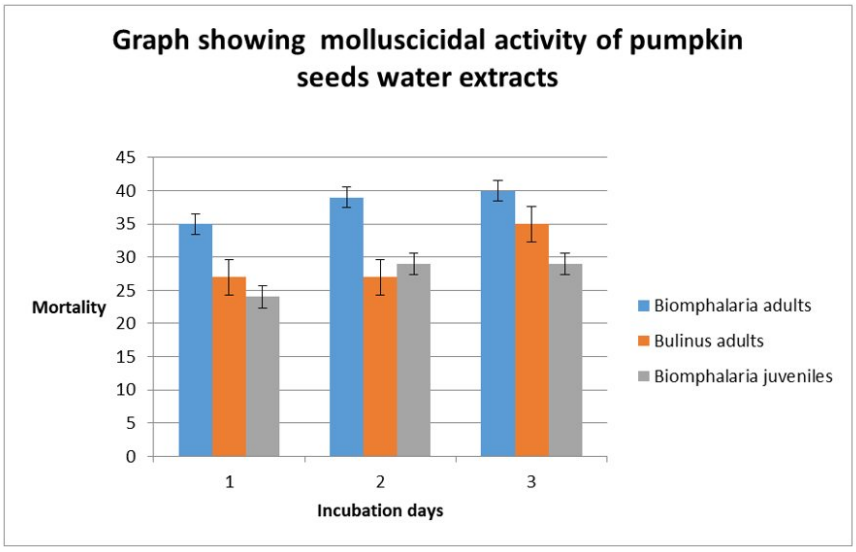

Fig 1: The molluscicidal activity of water extracts of pumpkin seeds on *Biomphalaria* and *Bulinus* snails over a three-day period.

However, there was a significant difference between the mortality responses of juvenile and adult *Biomphalaria* to ethanol extracts (p=0,016; CI 95%) with adults showing greater mortality rates than juveniles as shown in Fig 2.

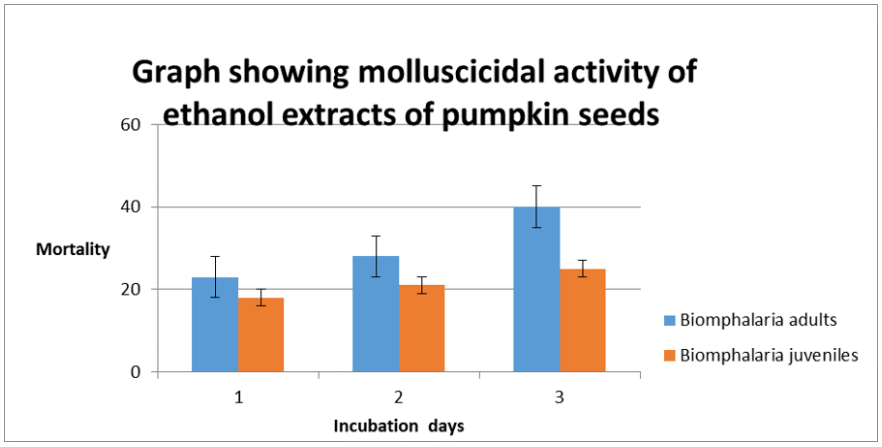

Fig 2: The molluscicidal activity of ethanol extracts of pumpkin seeds on *Biomphalaria* and *Bulinus* snails over a three-day period.

The results also showed that there was no significant difference between the effects of the water and ethanol extracts on adult *Biomphalaria* (p = 0.875; CI=95%) as is also shown by the mortalities of the snails in the graph given below (Fig 3).

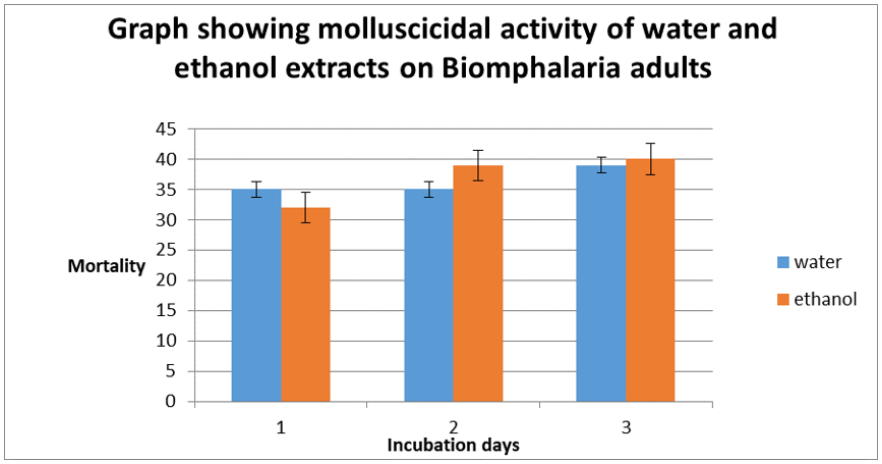

Fig 3: The molluscicidal activity of water and ethanol extracts on *Biomphalaria* adults.

The difference in the molluscicidal activity of water and ethanolic extracts on *Biomphalaria* juveniles was not significantly (p=0,231 C1=95%) although ethanol extracts seemed to have had greater effect than water extracts as shown in Fig 4.

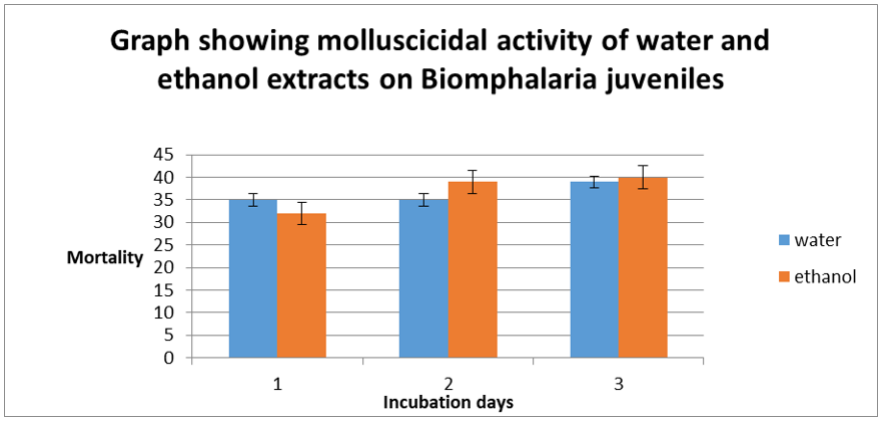

Fig 4: The molluscicidal activity of water and ethanol extracts on *Biomphalaria* juveniles

The mortalities of *Biomphalaria* adult snails were concentration- dependant and the molluscicidal activity of water extract decreased with concentration with 0.0002mg/ml showing no activity at all as indicated in Fig 5.

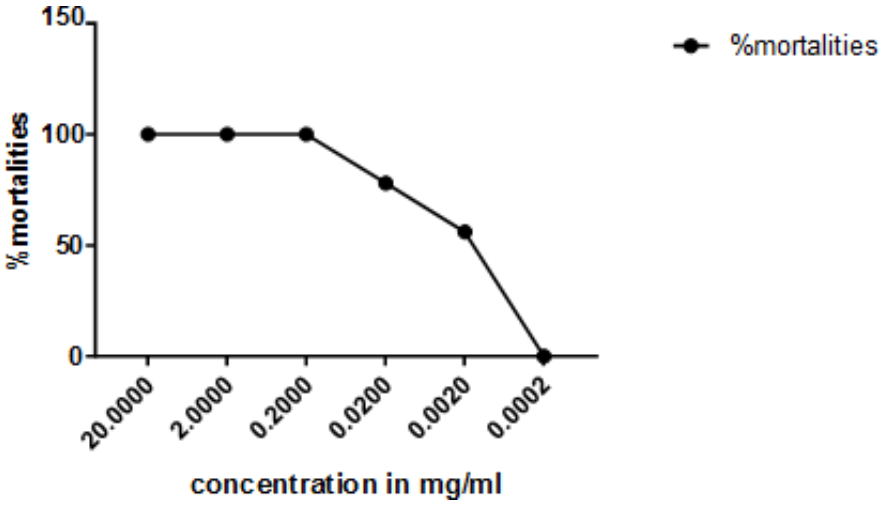

Fig 5: The concentration- dependent mortalities of *Biomphalaria* adults exposed to water extracts of pumpkin seeds.

The mortalities of *Biomphalaria* adult snails exposed to ethanol extracts were concentration-dependent and the molluscicidal activity decreased with dilution concentration as shown by 0.0002mg/ml showing no activity as indicated by the curve in Fig 6.

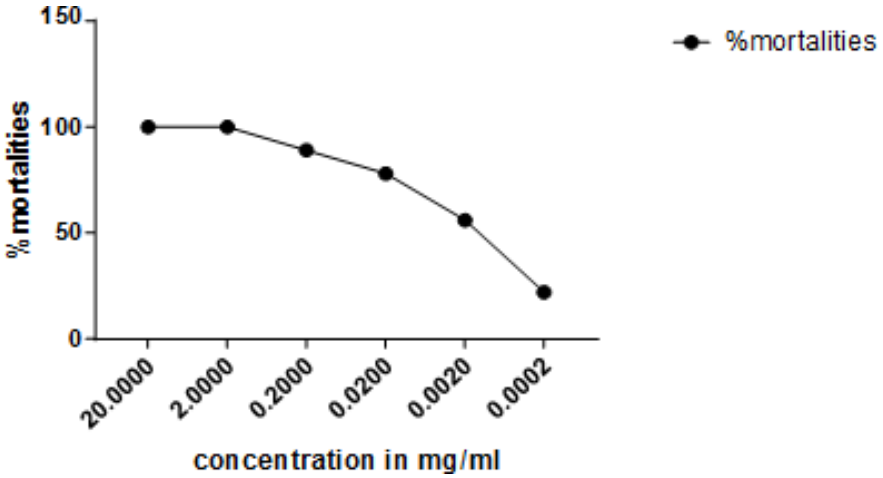

Fig 6*:* The concentration- dependent mortalities of *Biomphalaria* adults exposed to ethanol extracts of pumpkin seeds.

Bulinus adult snails were susceptible to water extracts of pumpkin seeds and their mortality was concentration-dependent although 66% mortality was observed in 0.002mg/ml which was higher than the 55% mortality in the 0.02mg/ml dilution (Fig 7).

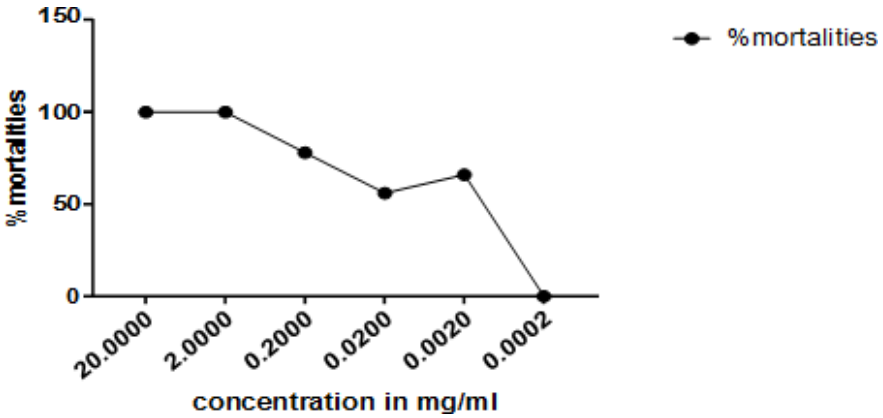

Fig 7: The concentration-dependent mortalities of *Bulinus* adults exposed to water extracts of pumpkin seeds.

Biomphalaria juvenile snails that were exposed to water extracts of pumpkin seeds did not show uniform concentration-dependent mortalities with 0.2mg/ml and 0.002mg/ml dilutions causing abnormally higher mortalities than the subsequent stronger more concentrated dilutions (Fig 8).

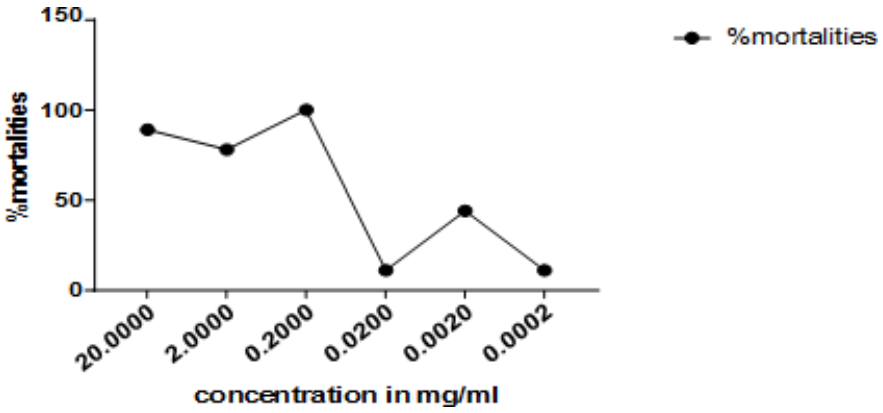

Fig 8: The concentration-dependent mortalities of *Biomphalaria* juveniles exposed to water extracts of pumpkin seeds.

Ethanol extracts of pumpkin seeds induced concentration-dependant mortalities on the *Biomphalaria* juvenile snails with the lowest concentration of 0.0002mg/ml causing 11% mortality (Fig 9).

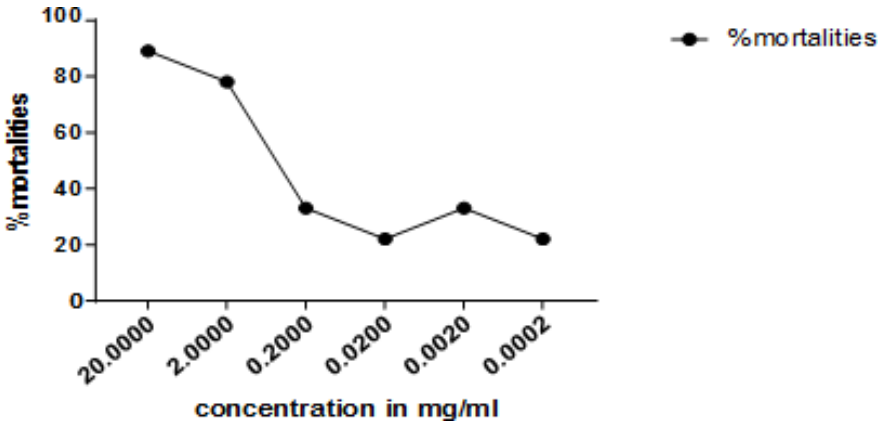

Fig 9: The concentration- dependent mortalities of *Biomphalaria* juveniles exposed to ethanol extracts of pumpkin seeds.

The LC50 values extrapolated from the non-linear regression line from Graph pad Prism were way below the maximum LC50 value of 40mg/L set by the WHO. The LC50 value of water and ethanol extracts of pumpkin seeds on adult *Biomphalaria* had the lowest LC values of 0.002mg/ml. The water extracts LC values on juvenile *Biomphalaria* snails and adult *Bulinus* snails were both 0.004mg/ml. The LC50 for ethanol extracts on juvenile *Biomphalaria* snails was 0.19mg/ml. These results are presented n Table 1.

Table 1 showing LC50 values of pumpkin seed extract on the five snail classes.

MOLLUSCIDE EXTRACT AND SNAIL CLASS EXPOSED	LC50 VALUES
Water extract on *Biomphalaria* adults	0.002mg/ml
Water extract on *Biomphalaria* juveniles	0.004mg/ml
Water extract on *Bulinus* adults	0.004mg/ml
Ethanol extract on *Biomphalaria* adults	0.002mg/ml
Ethanol extract on *Biomphalaria* juveniles	0.19mg/ml

Thunder control	0.000049mg/ml

Discussion

The majority of *Biomphalaria* and *Bulinus* snails, withdrawn into their shell through the initial 24 hours, were found to have died at the end of the experiment. None of the snails survived 20mg/ml extract concentration except for *Biomphalaria* juveniles. The significant difference between susceptibility of juveniles and adults to the ethanol extracts agrees with the results from previous studies by Anto *et al.* (2005); Omobhudhe (2017) and Ping *et al.* (2017). Throughout the study, high concentrations of both ethanolic and water extracts of pumpkin seeds showed substantial molluscicidal activities with LC50 values that were clear-cut below the upper threshold of 40 mg/L set for a potential molluscicide by the WHO (WHO, 1993). 100% mortalities were observed in adult *Biomphalaria* and *Bulinus* exposed to the highest concentration of both water and ethanol extracts. The susceptibility of *Biomphalaria* snails to the extracts could be attributed to the fact that they have no operculum; thus, their cephalopodia were continuously in contact with the molluscicide during the assays (Ping *et al.*, 2017).

The present outcomes might have been due to metabolic disorders, loss of muscle coordination which prompts snail's death (Labe *et al.*, 2012). As is valid for the mechanism of action of many insecticides, the activity of a molluscicide may possibly be a multi-part process, influencing more than one of the snails' internal systems. Various such reactions that provide evidence for this have been recorded in the literature, for example; decrease in heart rate, swelling of tissues and change in the water balance (McCullough *et al.*, 1980; WHO 2017).

The tested plant is generally liable for the defect of the internal defence system (Augusto *et al.*, 2017) hence the compatibility of the surviving snails to Schistosoma infection could be reduced. This reduction may be because the physiology of the snails would be altered due to constant exposure to the pumpkin seed extract (WHO, 2017). There is, however, no current motivation to trust that a common mechanism of action is responsible for these reactions (Labe *et al.,* 2012). This then offers the proposition that there could be a range of activities and the mechanism of action of molluscicides might take time to be understood. Nonetheless, further research is necessary in order to discover the constituents responsible for the molluscicidal activity of pumpkin seeds so as to produce greater quantities for inclusive laboratory and semi-field bioassays.

Our results show that the water and ethanol extracts of pumpkin seeds induced a significant molluscicidal activity. The results showed that there was no significant difference between the survival rates of juvenile and adult *Biomphalaria* snails exposed to water extracts (p = 0,208; CI = 95%). Pumpkin seeds water extract also had a molluscicidal activity against *Bulinus* snails. There was no significance in the difference of species exposed to the water extracts (i.e. *Biomphalaria* and *Bulinus*) (p = 0,665; CI = 95%. It was also observed that there was no significant dependence of mortalities on the time of the exposure of snails to the extracts. This observation was made for all the snail classes used in the study.

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