Molluscicidal Effects of Aqueous and Ethanolic Extracts of Lemongrass (*Cymbopogon Citratus*) Leaf against the Different Developmental Stages of *Biomphalaria Pfeifferi*

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ABSTRACT: The molluscicidal activities of aqueous and ethanolic extracts of *Cymbopogon citratus* leaf against adult, juveniles and egg-masses of *Biomphalaria pfeifferi* were investigated. Ten adults each of *Biomphalaria pfeifferi* were exposed to a serial dilution of 40, 80, 160, 240, 320, 400, and 480ppm distilled water extracts and 20, 40, 80, 160, 240, 320ppm ethanolic extracts. Twenty juveniles of uniform size each were exposed to 8, 20, 28, 40, 80, 120, and 160ppm of aqueous and ethanolic extracts. Thirty eggs each were exposed to 8, 20, 28, 40, 60, 80, and 100ppm of aqueous and ethanolic extracts. Exposure period was 24hrs for adult and juvenile stage while 48 hrs for the egg stage. The lethal concentration LC_{50} against eggs, juveniles and adults were 73.27, 64.60 and 140.74ppm respectively for *Cymbopogon citratus* aqueous extract, 42.85, 43.87 and 61.79ppm respectively for *Cymbopogon citratus* ethanolic extract. The lethal concentration LC_{90} against eggs, juveniles and adults were 182.37, 244.42 and 254.92ppm respectively for *Cymbopogon citratus* aqueous extract, 113.20, 166.31 and 159.47ppm respectively for *Cymbopogon citratus* ethanolic extract. The plant extracts caused significant mortality rates of the different stages of *B. pfeifferi* ($P<0.05$). The result obtained show that *Cymbopogon citratus* is a promising plant molluscicide candidate and deserves further studies in order to identify and characterize its molluscicidal components.
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INTRODUCTION

Schistosomiasis, a disease of various mammals, including man is caused by blood flukes of the genus *Schistosoma* (Brown, 1980). It is still a major helminth infection at the beginning of the 21st century and an important public health problem in many non-industrialized countries. It is the second major parasitic disease in the world after malaria (Carter Center, 2010). Schistosomiasis is endemic in 74 countries (WHO, 1985), affects 200 million people, 800 million being exposed to the risk of the infection (Gryseels et al., 1991; Capron et al., 2002). Twenty-two million people are infected in Nigeria including more than sixteen million children making the country one of the most endemic for this disease in the world (Carter Center, 2010).

The transmission cycle of *Schistosoma* species require specific freshwater snails as intermediate hosts (Engels et al., 2002; Zhou et al., 2008). It is generally considered that snail control is one of the most rapid and effective means of reducing transmission of schistosomiasis (Klump and Chu, 1980; McCullough et al., 1980; Fenwick et al, 1982; Manal, 2010). At present, the most reliable method of achieving drastic reductions in snail population density in short term is through the use of molluscicides (McCullough et al., 1980). Many different chemical molluscicides have been used in the past, but at present only one molluscicide, bayluscide is used (McCullough et al., 1980; Osman, 2000). However, the high cost of Bayluscide, its impact on the community and its environmental effects have stimulated interest in search for alternative molluscicides of plant origin, in the control of human schistosomiasis. The use of plant molluscicides is attractive due to the economic advantage of cultivating the plants locally instead of importing synthetic compounds (Ndamba et al., 1994). Other methods of snail control such as biological methods or drying of the canals also proved unsatisfactory. Plant molluscicides are selectively active, easily biodegradable, inexpensive and available in endemic areas (WHO, 1983).

Interest in plant molluscicides began in 1930 to develop natural substances that could be used by communities (Mozley, 1952; Kloos and McCullough, 1982). Many plants were tested as molluscicides all over the world, as indicated in a review by Kloos and McCullough (1987) who reported 571 species of plants studied, in addition to approximately 500 plants tested in China (Kuo, 1987) and 344 in Brazil (Jurberg et al., 1985).

Cymbopogon citratus was selected for this study based on previous studies, which showed that most medicinal plants have molluscicidal potency, availability of the plant locally and the plant type is

perennial rather than annual (Adewunmi and Victor, 1981; WHO 1985; Sofowora, 1993; Adetunji, 2007; El-Sherbini et al., 2009).

The aim of this study is to determine the molluscicidal potency of *Cymbopogon citratus*. There was no work found on the molluscicidal efficacy of *Cymbopogon citratus* on the developmental stages of *Biomphalaria pfeifferi*, the major intermediate host of *Schistosoma mansoni* in Nigeria.

MATERIALS AND METHODS

Field work

Snail sampling

Snails were collected from the Awba Lake, (a man-made lake) in the University of Ibadan, Ibadan, Nigeria, on the first week of November 2010. The snail collection was done early in the Morning between 8.00am-12.00 noon, using a flat dip-net scoop as described by Richie et al., (1962) and Demian et al., (1972). The collected snails were put in a sterile plastic container containing some of the lake water and sterile cotton wool. They were then taken to Parasitology Research Laboratory, Department of Zoology, University of Ibadan for identification and experimental studies.

Collection and Preparation of plant extracts

C. citratus leaf was collected from the Botanical Garden, University of Ibadan. They were taken to the laboratory in a wet sack (to avoid direct exposure to sunlight which may lead to dehydration) and rinsed to remove dust, sand and unwanted materials. Department of Botany, University of Ibadan, was consulted for identification. The leaf was dried for weeks at room temperature between 25-30^{0}C and graded into powder-like state.

Laboratory study

Maintenance of the Snails

The snail used in this study was *Biomphalaria pfeifferi*. In the laboratory, snails were identified to the species level using the snail identification key by WHO, (1971). Infected snails were identified using the shedding method described by Frandsen and Christensen (1984) briefly snail collected was placed in a beaker, half filled with dechlorinated tap water. The beakers (each with a snail) were exposed to the day light and left for one hour or more to allow cercariae to emerge. The snails that shed cercariae were gathered in one circular glass trough, half filled with dechlorinated tap water. Healthy snails were maintained in aquaria of circular glass troughs (12cm depth×30cm diameter with a capacity of about 6 litres) with stocking density of 9 snails/L of water, snails were maintained in six glass trough. Every trough (the stock aquaria) were interiorly covered with polythene bags before filling with dechlorinated tap water, a layer of clay and some gravel which has been sterilized by heating using electric cooker for at least one hour. The tap water that was used was strongly aerated for about 3 days to allow evaporation of chlorine and then, the troughs filled to two thirds.

Green *Lactuca sativa* was immersed in boiling water for about one minute and then cooled in tap water. After the removal of the mid-rib, leaves were dried and powdered. The dry powdered salad plant was used for feeding the snails three times a week. It is worth noting that the soft part of the Green leaves was the most suitable material for feeding the snail. Studies had shown that snails fed on dry ribs of the leaves cannot survive for long. The aquaria were maintained at temperature between 25-30°C. Water was changed once a week or when necessary.

Collection and Preparation of Egg-masses and Juvenile Snails

The snails were allowed to lay eggs. The polythene sheets were checked for egg-masses after 72 hours. The polythene sheet which contained egg-masses were located and isolated by cutting the plastic around each egg-mass with a scalpel (about 0.5-1.0cm from the egg-mass). Some of the egg-masses were exposed to the different extract concentrations 3-4 days after they were laid. Some egg-masses, attached to the polythene, were immersed in Petri-dishes containing clean well water to remove any debris and transferred to containers containing 200 ml of dechlorinated tap water; the dishes were covered until eggs hatched into juveniles. One week old juveniles were required for the experiment.

Extracts bioassay

A stock solution was prepared by dissolving 10 grams of dry powdered *Cymbopogon citratus* leaves distilled water. The weight dry powdered parts were soaked in 450ml (22,222ppm) of distilled water for 24 hours with occasional vigorous shaking, using magnetic stirrer for the first 6 hours. Then, the suspension was filtered using filter paper. The marc was washed with several portions of distilled water to adjust the volume of the solution; using volumetric flasks to 500ml (20,000ppm). The plant extract was used immediately after the extraction, to ensure their freshness. The same procedure was repeated with 70% ethanol, to obtain ethanolic extract. After extraction, the solvent was removed by evaporation and the volume adjusted to 500ml.

Molluscicidal Potency Test of Plant Extracts on Adult Snails

The molluscicidal potency test was carried out according to the standard method prescribed by

WHO, (1971). The different volumes of 0.0 (control), 1, 2, 4, 6, 8, 10 and 12ml from the stock solution of the aqueous extract of the plant were added to an equal volume of dechlorinated tap water of 500ml in plastic trough containers (10 cm depth×17cm diameter), to have a working solution. Then the concentration of each solution was calculated in part per million (ppm): 0.0, 40, 80, 160, 240, 320, 400, and 480ppm respectively.

For the ethanolic extract the different volumes of the stock used were 0.0 (control), 0.5, 1, 2, 4, 6, and 8ml. Each was added to an equal volume of dechlorinated tap water of 500ml in plastic trough containers (the same as in aqueous extract), to have a working solution. Then the concentration of each solution was calculated in part per million (ppm): 0.0 20, 40, 80, 160, 240, 320ppm. Ten adults of uniform size and were immersed in each trough containing the solution. In each set up, the snails were prevented from crawling out of the trough by means of a fine mesh white cloth tied to trough by rubber band. The snails were not fed during the course of the experiment, it had been observed that healthy snails live up to 5days or more without food, provided other environmental conditions are constant (Adetunji and Salawu, 2010). After 24 hours of exposure to the plant extracts, the snails were transferred to fresh dechlorinated water and maintained there for another 24 hours. Moulliscicidal test with the plant extract doses were separately repeated twice. Death of the snails was determined and confirmed by the lack of reaction to irritation of the foot with a blunt wooden probe to elicit typical withdrawal movements and absence of heartbeat observed under the microscobe, thereafter, mortality counts were recorded.

Molluscicidal Potency Tests of Plant Extracts on Juvenile Snails

The different volumes of 0.0 (control), 0.2, 0.5, 0.7, 1.0, 2.0, 3.0 and 4.0ml from the stock solution of both extracts of the plant were each added to an equal volume of dechlorinated tap water of 500ml in a plastic trough containers (10 cm depth×17cm diameter), to have a working solution. Then the concentration of each solution was calculated in part per million (ppm): 0.0, 8, 20, 28, 40, 80, 120, and 160ppm respectively. About twenty juvenile snails of uniform size (one week old) were immersed in each trough containing the solution, After 24 hours of exposure to the plant extracts, the juveniles were transferred to fresh dechlorinated water and maintained there for another 24 hours. Moulliscicidal test with this plant extracts dose were separately repeated twice and there was no feeding. Thereafter, mortality counts were recorded after careful observation under the microscope.

Molluscicidal Potency Tests of Plant Extracts on the Snail eggs

The different volumes of 0.0 (control), 0.2, 0.5, 0.7, 1.0, 1.5, 2.0 and 2.5ml from the stock solution of the both extracts of this plant were added to an equal volume of 500ml dechlorinated tap water in plastic trough containers (10 cm depth×17cm diameter), to have a working solution. Then the concentration of each solution was calculated in part per million (ppm): 0.0, 8, 20, 28, 40, 60, 80, and 100ppm respectively. Thirty snail eggs and were immersed in each trough containing the solution, After 48 hours of exposure to the plant extracts, the eggs were transferred to fresh dechlorinated water and maintained there for another 24 hours. Moulliscicidal test with this plant extracts dose were separately repeated twice. Thereafter; mortality counts were done under the microscope and recorded.

Dosage-mortality curves are the subject matter of an entire field of biometric analysis, and bioassay is one of the techniques used in this field. The obtained data were subjected to probit analysis software, BioStat 2007 Professional version 3.2, to get LC_{50}(mg/l) and LC_{90}(mg/l), probit regression graph, and chi-square. While regression equations and R square were obtained from the same software using regression analysis.

RESULTS

Table 1 shows the LC_{50} and LC_{90} values of *Cymbopogon citratus* aqueous extract on the three developmental stages of *B. pfeifferi*. The lethal concentration that killed 50% of egg, juvenile and adult stage of the snail was 73.27, 64.60 and 140.74ppm respectively, while the lethal concentrations that killed 90% of eggs, juveniles and adults stage of the snail were 182.37, 244.42 and 254.92ppm respectively. *Cymbopogon citratus* leaves aqueous extract was very potent against all stages of *B. pfeifferi* {eggs at 48 hrs (x^2=3.29, *df*=6;p<0.05)}, juveniles at 24 hrs (x^2=0.63, *df*=5;p<0.05) and adults at 24 hrs (x^2=1.38, *df*=5;p<0.05)}. The aqueous extract concentrations of *Cymbopogon citratus* leaves showed that R^2 = 0.9373, 0.8343 and 0.8771 on the eggs, juveniles and adults of *B. pfeifferi* respectively (Figure 1-3). There were strong positive correlations between mortalities observed in all stages of *B. pfiefferi* and the aqueous extract concentrations of *Cymbopogon citratus* leaves.

Table 2 presented the LC_{50} and LC_{90} values of *Cymbopogon citratus* ethanolic extract on the three stages of *B. pfeifferi*. It was noted that this ethanolic extract was more potent compared to aqueous extract (Figure 4). The lethal concentrations that killed 50% (LC_{50}) of egg, juvenile and adult stage of the snail

were 42.85, 43.87 and 61.79ppm respectively. While the lethal concentrations that killed 90% (LC_{90}) of egg, juvenile and adult stages of the snail was 113.20, 166.31 and 159.47ppm respectively. *Cymbopogon citratus* leaves ethanolic extract was very potent against all stages of *B. pfeifferi* {eggs at 48 hrs (x^2=2.39, *df*=6; p<0.05), juveniles at 24 hrs (x^2=0.63, *df*=5;p<0.05) and adults at 24 hrs (x^2=0.52, *df*=5;p<0.05)}. The ethanolic extract concentrations of *Cymbopogon citratus* leaves had R^2 = 0.9195, 0.8403 and 0.9778 on eggs, juveniles and adults of *B. pfeifferi* respectively (Figure 1-3). There were strong positive correlations between mortalities observed in all stages of *B. pfiefferi* and the ethanolic extract concentrations of *Cymbopogon citratus* leaves.

Table 1: Toxicity of the aqueous extract of *Cymbopogon citratus* on the different developmental stages of *B. pfeifferi* snails

Snail Stages	**Regression equation**	**Chi Square (p < 0.05)**	**LC_{50}(ppm)×**	**LC_{90}(ppm)×**
Eggs (72-96hrs old)	y =2.5494 + 0.0317X	3.29	73.27	182.37
Juveniles (a week old)	y =3.4811 + 0.0172X	0.63	64.60	244.42
Adults (6.0-8.0)	y =0.8998X -1.4332	1.38	140.74	254.92

× Mean lethal concentration in ppm

Table 2: Toxicity of the ethanolic extract of *Cymbopogon citratus* on the different developmental stages of *B. pfeifferi* snails

Snail Stages	**Regression equation**	**Chi Square (p < 0.05)**	**LC_{50}(ppm)×**	**LC_{90}(ppm)×**
Eggs (72-96hrs old)	y =3.0965 + 0.0386X	2.39	42.85	113.20
Juveniles (a week old)	y =3.9212 + 0.0172X	1.81	43.87	166.31
Adults (6.0-8.0)	y =2.883X - 0.1522	0.52	61.79	159.47

× Mean lethal concentration in ppm

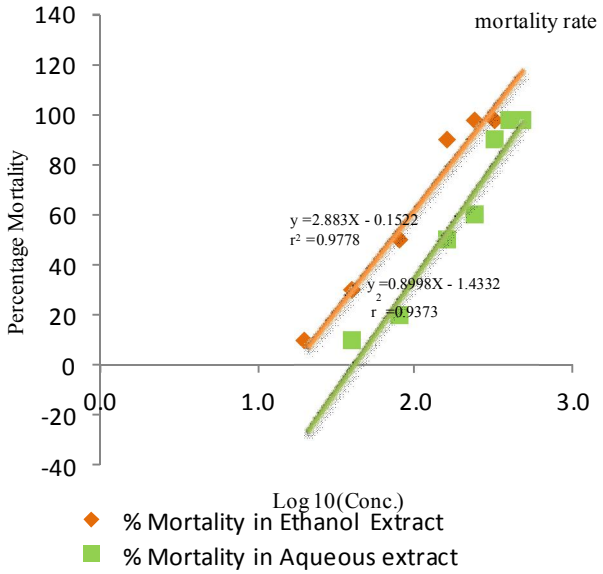

Figure 1: *C citratus* extract against adult stage of *B. pfeifferi*

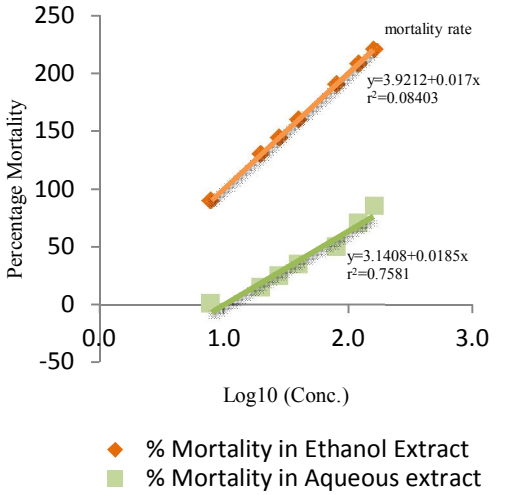

Figure 2:*C. citratus* extract against juvenile stage of *B. pfeifferi*

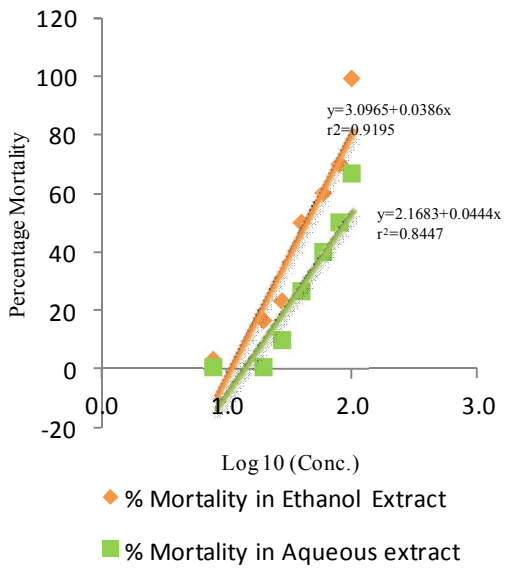

Figure 3: *C. citratus* extract against egg stage of *B. pfeifferi*

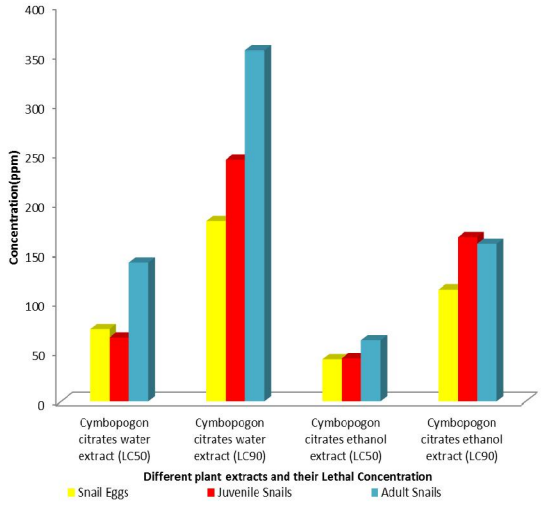

Figure 4: *C. citratus* aqueous extracts on different stages of *B. pfiefferi*

Bench side observations

On hatching (between 6-8 days), the juveniles resemble the adult in some ways; however, the shell is softer than that of the adult and it is a very small, squat, transparent shell rather than elongated spire. The juveniles' movement is very difficult to notice. The embryos of the dead eggs remained motionless in their cells, when observed under the microscope and showed no signs of development contrary to observations recorded in the control.

Snails (juveniles and adults) in the untreated troughs and control withdrew into their shell but became active after about 30-45 mins, moving around the container with their foot extended. When extracts were applied, the snails immediately or after some seconds, depending on the extracts` concentrations, withdrew into their shell. After 24hrs, the toxic effects of the active plant extract became evident in the test snails. There was either a partial retraction (withdrawal response) in the partially dead snail or no retraction in the dead snails.

There was a visible swelling of the cephalopodal mass. Development of hemorrhagic "blisters" over the ventral surface of the foot-sole was also noted. High doses of the active plant extracts caused the cephalopodal mass of each snail to become severely swollen and turgid and mucous secretion was observed over most of the foot.

After exposure to the plant extracts examined in the present study, the snails showed several behavioural responses, including the "distress syndrome" and swelling of the tissues that was not restricted to the tentacles, but involved the whole cephalopodal mass.

DISCUSSION

In the present study, *C. citratus* was screened for its molluscicidal activity. Aqueous and ethanolic extracts of this plant leaves were evaluated for molluscicidal activity against different developmental stages of *Biomphalaria pfeifferi* snails.

This study revealed that *B. Pfeiffer'*s different developmental stages (egg, juvenile and adult) were susceptible to *C. citratus* extracts` at different concentrations. *C. citratus* ethanolic extract that killed 50% (LC_{50}) and 90% (LC_{90}) of eggs were 42.85ppm and 113.20ppm respectively. The potency of *C. citratus* ethanolic extract on the eggs in the present study was much higher when compared to other plant extracts used in other works. Adenusi and Odaibo, (2009), reported that the ethanolic extract of *Dalbergia sissoo* leaves on *B. pfeifferi* egg masses**,** had LC_{50} and LC_{90} as 236.12ppm and 604.91ppm respectively. The *Cymbopogon citratus* ethanolic extract that killed 50% (LC_{50}) and 90% (LC_{90}) of juveniles were 43.87ppm and 166.31ppm respectively. And for the adults the lethal concentrations that killed 50% (LC_{50}) and 90% (LC_{90}) were 61.79ppm and 159.47ppm respectively. The potency of *C. citratus* ethanolic extract on the adults in the present study was much higher when compared to other plant extracts used in other works. Fayez, (2009) reported that the cold water extract of *Guayacum officinalis* and *Calotropis procera* on adult stage of *Biomphalaria alexandrina* had LC_{50} as 120 and 243ppm respectively.

However, *C. citratus* aqueous extract had less molluscicidal effects on the eggs (when compared to the juvenile and adult stages) of *B. pfeifferi* with (LC_{50}) and (LC_{90}): 73.29ppm and 182.37ppm

respectively (Figure 1). The same extract had stronger efficacy on the juvenile stage with LC_{50} and LC_{90} at 64.60ppm and 244.42ppm respectively. This may be due to the protective covering of capsular jelly like material that covers and protects the eggs from the external environment. A similar observation was also reported for nicotinanilide by Parashar et al., (1995) against *L. auricularia* (LC90 value against 0 to 1 day and 4 to 5 days old eggs was 1.03 mg/l and 0.57 mg/l respectively) and the eggs of *Indoplanorbis exustus* (the LC90 value as 0.087 mg/l), and for the adults the lethal concentration that killed 50% (LC_{50}) and 90% (LC_{90}) of juveniles were 140.74ppm and 354.92ppm respectively. Hatil et al., (2010) had similar results when they used aqueous extract of *Cymbopogon nervatus* Leaves on *B.pfeifferi,* they had LC_{50} as 213.099ppm and LC_{95} as 506.69ppm. They also used the same aqueous extract of *Cymbopogon nervatus* leaves on *Bulinus truncatus* and had LC_{50} and LC_{95} as 237.33ppm and 331.05ppm. Fayez, (2009) also reported similar observation with cold water extract of *Guaiacum officinalis* on *Biomphalaria alexandrina,* he had LC_{50} as 110ppm, LC_{90} as 230ppm and slope as 1.65. However the present results is different from the one observed by Schall et al., (1998) where results of their study on the effect of natural latex of *E. splendens* on *B.pfeifferi*, demonstrated an effective lethal dose of 4.0ppm on adult stage of *B.pfeifferi.* This observation, where the efficacy of an extract is more on juvenile, adult and lesser on egg stages *B.pfeifferi,* may be due to the poor solvent nature of water on *C. citratus* plant or protective jelly-like nature of the egg mass of this freshwater snail.

Difference in the slope functions of the extracts was noted in the analysed mortality data and it indicates the extent to which increase in concentration of the extracts should be made, to secure an increase in mortality of the different developmental stages of *B.pfeifferi*. Chi Square (x^2) analysis shows that moluscicidal potency of both plant extracts showed significant mortality rates of the three stages of *B. pfeifferi* ($P<0.05$) at different concentrations for 24hrs exposure while 48hrs for eggs. The correlation coefficient in all cases showed that there were strong positive correlations between mortalities observed in *B. pfeifferi* and extract concentrations of this plant.

The withdrawal of each snail in the untreated water in the troughs into their shell and their subsequent activity after a few minutes was also reported in Egypt by El-Sherbini et al., (2009), the same source stated that the snail resumed normal activity after about 45 minutes. This observation may be due to slight changes in the physiochemical conditions of the water in the troughs. At the introduction of the extracts the snails withdraw into their shell again, however they start crawling out of the water, most staying at the water-air interface with their shell partially immersed the water. Similar observation was made by Adetunji and Salawu (2010), Evans et al*.,* (1986) and Brackenbury, (1999) in the pulmonate snail, *Bulinus*. This observation where the snail shells were partially immersed in the water and their soft tissue above the water-air interface was well observed by Adenusi and Odaibo (2008). The mechanism of the *B. pfiefferi* partially leaving the treated water has been found to increase the survival of *Biomphalaria straminea* exposed to sub-lethal doses of niclosamide (Sarquis et al., 1997) and *B. glabrata* exposed to *Phytolacca dodecandra* (Jurberg et al., 1988). The seemingly quick action of the ethanolic extract of *C. citratus* on *B. pfeifferi*, may be probably due to their acute toxic effects at this concentration. This is desirable, as it reduces the possibility of the escaping behavior (Sarquis et al., 1997). Similarly, immobility and lack of tactile response in snails exposed to copper sulphate and *T. tetraptera* have been reported (VanAardt and Coertze, 1981; Bode et al., 1996).

CONCLUSION

The results obtained shows that *C. citratus* on could be considered a promising molluscicide, in the control of schistosomiasis. These results need to be followed up by further investigations for comprehensive evaluation of the possible use of *C. citratus* on *B. pfeifferi*.

The screening of plants for molluscicidal activity started over 60 years ago and is continuing. The necessary research in the field of vegetable molluscicides should be encouraged. Therefore, the use of plant molluscicides might be one of the best means for the control of schistosomiasis and trematode infections in third world endemic countries.

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