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Brine Shrimp Lethality Test of Soluble Proteis from *Biomphalaria pfeifferi* Snail as Preliminary for Vaccine Development for *Schistosoma mansoni*

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Abstract*: The study tested for in vivo Brine Shrimp Lethality Test (BSLT) of soluble protein extracts from Biomphalaria pfeifferi snail. The proteins from foot and digestive gland were processed and their concentration determined by microtitre technique. Concentrations were 1.4 mg/ml for foot and 1.4 mg/ml for digestive gland extracts. Cytotoxicity of the proteins was evaluated in terms of Lethality concentration (LC_{50}) using10µg/ml,100µg/ml and 1000µg/ml concentrations of the proteins. Ten Brine Shrimps larvae (nauplii) were placed in duplicate tubes of each concentration. After 24 hours the surviving Brine Shrimps larvae were counted and LC_{50} was determined by Finney computer program at 95% confidence interval. The results showed foot and digestive gland proteins had a LC_{50} of 71.43µg/ml and 52.18µg/ml respectively. The results imply bioactive components are present in the proteins with probable larvicidal activity. As per the results, the proteins can be used as schistosomiasis vaccine candidates.*

Key words*: Brine Shrimp Lethality Test, Schistosoma mansoni, Biomphalaria pfeifferi.*

I. Introduction

Schistosomiasisis a parasitic disease distributed in approximately 78 nations in the American, Asian, and African continents [2]. In Africa, it is prevalent in sub-tropical and tropical regions, particularly, in poor communities that have no access to adequate sanitation and safe drinking water. It is estimated that more than 90% of those that require schistosomiasis treatment are inhabitants in Africa. The transmission of the disease is multifaceted owing to several factors which include the presence of intermediate hosts (snails) bearing the *Schistosoma mansoni* (*S. mansoni*) parasite[10], inadequate health education among the populations at risk, absent or deficient household or environmental basic sanitation systems, and the presence of the definitive host of the parasite in the environment. Reference [1] shows that the World Health Organization (WHO) recommends that the control of schistosomiasis should entail basic sanitary services, health education, and chemotherapy, the disruption of the parasite's lifecycle, and the reduction or elimination in the population of the mollusc intermediate host particularly, in the endemic regions utilizing molluscicidal agents. Reference [4] shows a common bioassay technique that identifies a broader spectrum of bioactivity existent in crude extracts has been identified as the Brine Shrimp Lethality Test (BSLT). In this method, a small amount of the material is used in the test, and is cost effective. The objective of this technique is to offer a front-line screen, which can be supported by more expensive and more specific bioassays once there is isolation of the active compounds. It seems that BSLT is extrapolative of pesticidal and cytotoxicity activity. From its initiation in 1982, [5] articulates that this lethality test of in vivo nature has been efficiently used for guiding bioassay fractionation of antitumor and active cytotoxic agents for instance, the ent-kaur-16-en-19-oic acid from *Elaeoselinum foetidum*, cis-annonacin from *Annona muricata*, and trilobacin from the *Asiminatriloba* bark.

II. Control of Schistosomiasis

Reference [12] reported that of the different species of trematodes which infect humans, schistosomes have been identified as the most rampant, and the different types of schistosomiasis still pose considerable public health concerns. Even though chemotherapy is the foundation of the control of schistosomiasis presently, only two pertinent drugs (praziquantel and oxamniquine) are currently accessible. There is speculation that oxamniquine production might be halted since the demand for the drug is diminishing. If there is withdrawal of oxamniquine, the only accessible antischistosomal drug left would be praziquantel, with very serious consequences in that there is development of resistance to the drug by target parasites [11]. Even though chemotherapy decreases the transmission, it hardly, if ever, eradicates it in endemic populations. Reference [7] shows that consequently, there may be quick occurrence of re-infection to pre-treatment intensities. One way of accomplishing a long-term solution is developing a vaccine [6], of which none of the various strategies for developing one has been successful.

Reference [9] shows the biological interventions for different diseases such as schistosomiasis, have attained more approval in current times, being a substitute to the application of chemical agents, which are frequently regarded as less friendly to the environment, less inclined to result in sustainability, and more expensive.

III. Brine Shrimp Lethality Test

The investigation for bioactive compounds that are derived from plant extracts in the chemical laboratory is frequently hindered by the lack of a rapid, simple, and suitable screening method. Reference [8] elucidated that several bioassay procedures are applied using biochemical systems, isolated tissues and whole animals. The bioassays can be quite expensive and complicated. A practical process for common toxicity screening is hence vital as a preliminary phase in the investigation of bioactive plants. As such, the brine shrimp also known as *Artemia salina* Leach has been applied for this purpose. Availability of eggs, the rapid maturation of the *nauplii*, the simplicity of hatching the eggs into larvae and the moderate easiness of sustaining a population under laboratory conditions have rendered the Brine Shrimps effective and simple animal experiment in toxicology and biological sciences. Combined with a reference benchmark, the BSLT provides a bioassay, which is reproducible, inexpensive, bench-top, simple, and more importantly, rapid.

Reference [8] adds that the biological or physiological impact to be examined in the screening process is vital. One of the easiest biological reactions is to evaluate the lethality because there is a single criterion; either alive or dead. In that situation, the statistical evaluation is moderately easy. The lethal concentration resulting in 50% mortality following 24 hour exposure is considered as the evaluation of the toxicity of the compound or extract. The selection of time, based on the solubility of the substance or extract is one of convenience because the test should be kept simple yet rapid. The application of the BSLT as an instrument of evaluating the widespread bioactivity in extracts of plants was initiated in 1982 then subsequently adopted in 1991 as a low cost, bench-top, in-house, rapid, and simple pre-screen for pesticidal and cytotoxicity activities [3]. The BSLT has been established as a method for the past 20 years hence resulting in the detection of the cytotoxic impacts of a broad variety of bioactive compounds and plants so varied in their chemical systems. This technique is currently applied globally with great reports of its success.

As a way of finding a more effective method to combat *S. mansoni*, this study aimed at employing the Brine Shrimp lethality activity of soluble protein from *Biomphalaria pfeifferi* snail to determine the toxicity which would be used in an attempt to develop a vaccine candidate against *S. mansoni* parasite.

IV. Materials and methods

A. Soluble protein extracts

Soluble proteins were extracted from foot and digestive glands of *Biomphalaria pfeifferi* snails collected from Mwea in Kirinyaga County, Kenya. This species of snails was selected because it is a known intermediate host for *Schistosoma mansoni*, the parasite that causes intestinal schistosomiasis. The snails were maintained under appropriate conditions in the malacology laboratory at the Institute of Primate Research (IPR), Nairobi.

B. Preparation of soluble protein extracts

Offsprings of *B. pfeifferi* snails maintained at IPR were used for preparation of soluble proteins. Foot and digestive glands were obtained from the snail under a dissecting microscope. Each snail was placed on a petri dish and separated from its shell by crushing the shell using a pair of forceps. Using a scalpel, the foot and the digestive glands were teased out and placed in labeled separate Eppendorf tubes containing phosphate buffered saline (PBS X1)[11]. The samples were then ground using a glass mortar and pestle. The ground samples were then sonicated to obtain fine homogenate. The homogenate was centrifuged for 1hour at 14,000g at 4^0C. The supernatant was decanted, assayed for protein concentration using a microtitre plate technique and aliquoted. The protein concentrations were read using the enzyme - linked immunosorbent assay (ELISA) reader at wavelength of 595nm [11]. The foot and digestive gland concentrations were 1.44 mg/ml respectively. Preparations of the different dilutions of the protein extracts for BSLA were each dissolved in 1ml of solvent. The final concentrations were 1000µg/ml, 100µg/ml and 10µg/ml. There were two (2) replicates in each concentration.

C. Brine shrimp hatching

Brine shrimps eggs (JBL Novo Temia, Germany) were hatched in a shallow rectangular container filled with artificial seawater prepared from commercial sea salt (Sera premium Brine-Sea Salt, company). A plastic divider with several 2mm holes was clamped in the container to make two unequal compartments. The eggs were sprinkled into the larger compartment, which was then sealed with opaque material, while the smaller compartment was subjected to light. After 48 hours incubation at room temperature (about 25^0C), the larvae (*nauplii*) hatched and swam to the lit side where they were collected using a pipette.

D. Bioassay

Ten 48 hour old Brine Shrimps were introduced into each of the six tubes per protein extract. Thus, there were a total of twenty (20) shrimps per dilution. Different volumes of the respective crude protein extracts (foot and digestive gland) 6.9µl, 69µl and 690µl were added on the tubes and topped up to 1ml making a concentration of 10µg/ml, 100µg/ml and 1000µg/ml respectively. The tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 hours. Analysis of the data was performed by probit

analysis method described by Finney (Finney computer program) to determine the lethal concentration to half of the test organisms (LC_{50}) at 95% confidence interval. *P* values < 0.05 were considered significant. The percentage mortality (%M) was also calculated using the formula;

% Mortality = No. of dead *nauplii* x 100

Total No. of *nauplii* in the concentration

The lethality results of the extracts were classified according to [2] where levels of toxicity can either be free of toxicity (1000 µg/ml), low toxicity (500 < LC50 < 1000 µg/ml), moderate toxicity (250 < LC50 < 499 µg/ml), and high toxicity (LC50 < 249 µg/ml).

V. Results and discussion

Table 1: The number of Brine Shrimps that survived after subjecting them to the two protein extracts and the percentage mortality.

T = Trial

Soluble Protein Extract	Concentration (µg/ml)	No. of Surviving Shrimps after 24 hours		Total No. of Survivors	% Mortality	LC_{50}
		T1	T2			
Foot	10	7	9	16	20	71.43 µg/ml
	100	8	7	15	25	
	1000	6	6	12	40	
Digestive Gland	10	9	8	17	15	52.1808 µg/ml
	100	8	6	14	30	
	1000	7	5	12	40	

The results of the study are summarised in Table 1. Based on the delineation of [2], the results obtained from this study demonstrated that 12 shrimps that were exposed to 1000 µg/ml of the protein extract were free of toxicity. On the other hand, 12 shrimps survived after 24 hours of being subjected to 1000 µg/ml concentration of the foot and digestive gland extracts. With reference to high toxicity, shrimps that were exposed to 10 - 100µg/ml concentration of the protein extracts (both foot and digestive parts) were highly toxic ($P<0.05$). During the evaluation of the larvicidal activity, both the 10µg/ml and 100µg/ml of the foot and digestive gland concentrations of the protein extracts showed some toxicity towards the *nauplii*. A closer look at the results shows that the digestive gland protein extracts result in the most cases of death of the *nauplii*, even though it is not significant ($P>0.05$)(*Table 1*). However, compared to the foot protein extracts, the digestive gland protein extracts report the best results, with most mortalities occurring in the second trial ($P<0.05$)(*Table 1*). Higher larvicidal toxicity of the digestive gland extracts confirms their higher level of toxicity, which corroborates the previous findings that high toxic concentrations are inclined to result in greater mortality rates [2].

From the results, the study indicates that the soluble crude protein extracts from the digestive glands and to some extent the foot extracts with concentrations of between 10µg/ml and 100µg/ml can be regarded as good candidates of larvicidal agents in the ongoing efforts towards development of candidate vaccines for *S. mansoni*. Although the tested fractions do not bear extreme levels of toxicity, they demonstrate apparent toxicity towards *nauplii*, which implies the existence of bioactive elements with probable larvicidal activity (*Table 1*). Nevertheless, a literature search to compare the results obtained by other scholars who might have done similar work did not yield any results. As such, while the BSLT method is not new, the element of acquiring soluble protein extracts from alternative sources other than plants is relatively new in schistosomiasis research Nevertheless, the current studies confirm that while the *B. pfeifferi* solube protein extracts show larvicidal activity towards *nauplii*, more research is required in order to identify different other compounds that may be useful in study of the larvicidal activity. This study is the first of its kind to use snail protein extracts and demonstrate their potential toxicity, which as per the results could be used as schistosomiasis vaccine candidates.

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