Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.7, No.18, 2017

Brine Shrimp Lethality Test and Characterization of Snail Soluble Proteins of Biomphalaria pfeifferi as a Candidate for Vaccine Development against Schistosoma Mansoni

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This work was supported by the National Commission of Science, Technology and Innovation funding; Science, Technology and Innovation (ST&I) Grant: Women Scientists/Researchers. NCST/5/003/W/2ND/CAL

Abstract
Schistosomiasis infects about two hundred million people around the world. Currently, treatment for *Schistosoma mansoni* infection is by use of Praziquantel™. Challenges associated with Praziquantel™ use include drug resistance, rapid re-infection and high cost. A longer lasting solution would be a vaccine to enhance the use of drugs. Unfortunately, no human vaccine for schistosomiasis is available. Snails that are the intermediate host of *S. mansoni* have been discovered to have common proteins with the schistosome worms. A research conducted in mouse model showed that two candidate vaccines derived from *Biomphalaria pfeifferi*, RT (soluble proteins from the rest of snail tissue) and DG (soluble proteins from the digestive gland), were protective against *S. mansoni* based on increased immune responses, worm reduction and reduced pathology. Both of them met the World Health Organization criteria of over 40% protection. It is important to ensure that substances being used as vaccines are both efficacious and non-toxic and therefore are safe. There are a number of common product safety tests and the simplest being brine shrimp lethality test. This work tested for in vivo Brine Shrimp Lethality Test (BSLT) of DG and RT. For BSLT, DG and RT were processed and their concentration determined by microtitre technique. Concentrations for both DG and RT were 1.4 mg/ml. Evaluation of the cytotoxicity of DG and RT was done in in terms of Lethality concentration (LCD_{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 LCD_{50} was determined by Finney computer program at 95% confidence interval. The LCD_{50} for DG was 3988.73 µg/ml and RT was 4158.06 µg/ml. The average percentage mortality in all the three different concentrations for the both soluble proteins was less than 50%. DG and RT had a LCD_{50} of over 1000µg/ml. The toxicity results for the two soluble proteins at the three different concentrations shows that both proteins are non-toxic and are therefore safe vaccines. After a product is found safe and efficacious, it is essential to determine its structure. This study also characterized DG and RT using Gas chromatography-mass spectrometry (GC-MS) and ultraviolet (UV) spectroscopy. Chemical identification and characterization of DG and RT using the GC-MS spectrum established the presence of different chemical compounds with varied retention times. The results from the GC-MS were confirmed by UV spectrum results which confirmed the presence of proteins.
Keywords: Schistosomiasis, Toxicity, vaccine, characterization

1 Introduction
Globally, there are over 230 million individuals affected by schistosomiasis (Vos *et al.,* 2012). Many strategies such as implementation of mass chemotherapy and other mitigating measures have been undertaken to contain the spread of schistosomiasis. Despite all these measures, the disease has continued to spread to other new regions. The most potentially effective way to control the disease would be the discovery of a vaccine, which would provide longer protection. A vaccine would aid in lowering morbidity by eliciting immune responses that would result to a decline in worm burden and female fecundity (Afzal *et al.,* 2011).

1.1 Brine Shrimp Lethality Test (BSLT)
The brine shrimp also known as *Artemia salina* Leach has been used for toxicity tests. Availability of eggs, the rapid maturation of the larvae, 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. Combined with a reference benchmark, the brine shrimp lethality test provides a

bioassay, which is reproducible, inexpensive, bench-top, simple, and more importantly, rapid (Pointier *et al.,* 2000). 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.

1.1.1 Characterization of snail soluble proteins by spectroscopy

No one technique in the present arsenal of protein structural methods is able to provide information on all aspects of protein structure. Therefore, a rational strategy is to employ a concerted approach in which the protein is examined using several structural techniques. Information obtained from different techniques can be cross-correlated to provide a more complete picture of the chemical and physical state and or bioactivity of the protein under different conditions.

Spectroscopy involves measurement of the contact between the molecules of a substance with electromagnetic emission. Light in the near-ultraviolet (UV) and observable limit of the electromagnetic spectrum has energy of around 150– 400 kJ mol. Electrons are elevated from the ground position to an aroused position by light energy. When the absorption of light is measured as a function of its frequency or wavelength, a spectrum is attained. Molecules having electrons in delocalized aromatic arrangements frequently absorb light in the near-UV (150–400 nm) or the visible (400–800 nm) areas (Parvez & Severcan, 1999). Electromagnetic radiations are formed by the alternation of electric charge and magnetic field existing in on the atom. Different kinds of electromagnetic radiation exist; for example light (visible), ultraviolet, infrared, X-rays, radio waves, cosmic rays and microwaves.

1.1.2 Mass spectroscopy (MS)

Determination of the structure of primary and high order proteins is commonly being done by mass spectrometry. The test is based on the capability of mass analysis techniques that detects changes in protein conformation under differing conditions. The advantage of mass spectroscopy is that carrying out of the experiments can be done on proteins alone without the modifying substance, chemical modification or combing the sample with proteolytic digestion (Richard, 2004).

Determination of protein structure using mass spectroscopy started around 1960. The technique is based on one basic principle that determination of the mass of an ion in vapour form is a possibility. The sample is ionized into a beam of ions to a vapour form and then the ions are separated on the basis of their mass to charge ratios *(m/e* or *m/z* values). The mass spectrum is recorded as a plot of *mle* of ions versus their relative abundances. The importance of this method in characterization of organic compounds is that it gives exact molecular masses, exact molecular formulae and can also demonstrate particular structural units and their attachment points within a molecule. This clearly provides a full picture of the molecule structure (Yadav, 2005).

1.1.3 Ultraviolet spectroscopy

This technique involves taking records of the absorption of radiations of the ultraviolet and visible regions of the electromagnetic spectrum. The ultraviolet area ranges from 10 to 400 nm. The region is subdivided into 3 main regions; the near ultraviolet (200-400 nm) the far or vacuum ultraviolet (10-200 nm) and the visible (400 to 800 nm). The absorbed electromagnetic radiations within these regions evoke elevation of an electron from a lower to higher electronic energy level (Yadav, 2005). Ultraviolet spectroscopy is used in analytical chemistry for the quantitative determination of different analytes, such as highly conjugated organic compounds and biological macromolecules.

2 Materials and Methods

2.1 Snail soluble proteins

Snail soluble proteins were extracted from the digestive gland (DG) and the rest of the snail body tissues (RT) of *Biomphalaria pfeifferi* snails collected from Mwea town in Kirinyaga County, Kenya. *Biomphalaria pfeifferi* snails were preferred in this study as they are the intermediate host for *Schistosoma mansoni*. The snails were transferred to the malacology laboratory at the Institute of Primate Research (IPR), Nairobi where they were maintained under suitable conditions.

2.1.1 Preparation of snail soluble proteins

Preparation of snail soluble proteins was done using *Biomphalaria pfeifferi* F1 snails. Digestive gland (DG) and the rest of the snail body tissues (RT) were obtained from the snail under microscopy. Each snail was placed on a petri dish and a pair of forceps was used to separate it from its shell by crushing. The digestive gland (DG) and the rest of the body tissue(RT) were teased out using a scalpel and placed in individually labelled Eppendorf tubes containing phosphate buffered saline (PBS x1). The extracted samples were then ground using a mortar and pestle. A fine homogenate of the 2 samples was obtained after sonication and thereafter centrifugation for 1hr at 14,000g at 40°C was conducted. Protein concentration was assayed using Bradford method (1976). This method utilized bovine serum albumin, BSA, (Biorad Co.) as a standard protein. BSA and the soluble proteins were serially diluted. The dye was added and incubated. The samples were read using Enzyme Linked Immunosorbent Assay (ELISA) reader at 630nm wavelength. A standard curve for BSA was drawn, various

concentration of the soluble proteins were read off the curve and average concentration obtained. Protein concentration was adjusted to 1mg/ml. The soluble protein was aliquoted and sterilized by exposure of UV light (10 minutes, 5cm from a 30 watt ultra violet OSRAM bulb). The aliquots were stored at -20°C

2.1.2 Toxicity Test

Toxicity test for the two snail soluble proteins DG and RT was done using brine shrimp (*Artemia salina*). Brine shrimp eggs (JBL Novo Temia, Germany) were hatched in a shallow rectangular dish filled with commercial sea salt (Sera premium Brine-Sea Salt, company). A plastic divider with several 2 mm holes was clumped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was dark while the smaller one was illuminated. A Lamp was positioned above the uncovered side to attract hatched shrimps. After 48 h the eggs hatched and matured as nauplii. Three different concentrations (10 µg/ml, 100 µg/ml and 1000 µg/ml) of the two test samples, DG and RT, were prepared and dispensed into vials. Ten brine shrimp larvae (nauplii) were collected by pipette from the light side and added into each vial and incubated at room temperature for 24 h. The numbers of surviving nauplii in each vial were counted under a stereoscopic microscope after 24 h. The experiments were conducted in five replicates for each concentration.

2.1.3 Analysis of DG and RT snail soluble protein using the gas chromatography-mass spectroscopy

Gas chromatography-mass spectrometry (GC-MS) analysis of soluble proteins isolated from *B. pfeifferi* was done using a gas chromatography mass spectrometry (GC-MS) machine (GCMS-QP2010SE) joined to gas chromatography (GC-2010 plus) both manufactured by Shimadzu Corporation (Kyoto, Japan). The machine had two components; the chromatography component and the mass spectrometer component, equipped with MS fused silica capillary column (30m ×0.25mm internal diameter and film thickness of 0.25µm). In GC-MS spectroscopic detection, ionization energy of 70 eV was used in the electron ionization energy. Pure helium gas (99.9%) was used as carrier gas and was set to between 5-9 bars and the chromatography vacuum for mass transfer line and interface temperature oven programmed to heat at 40°C, 60°C, 100°C, 130°C and 170°C with 1 min interval. Diluted samples (1/100 v/v in methanol and water of 1 µl were loaded in cuvettes which were inserted in an auto injector (A0C-201) also manufactured by Shimadzu Corporation (Kyoto, Japan) in the spit and the separation performed in the gas chromatography. Analysis was then done using the mass spectrometer. Relative percentage of the chemical constituents in the soluble proteins were captured and resolved using the GC-MS real time analyser software (GC-MS Shimadzu Lab Solution) during the process and later using GC post-run analyser with a built in library to compare the molecular masses to known compounds.

2.1.4 Analysis of DG and RT snail soluble protein using ultraviolet (UV) spectroscopy

UV spectroscopy was performed using standard process. Using soluble protein extracts, a concentration of 1.4 mg/ml of ddH_2o was prepared and each transferred into a clean cuvette (precision cells Inc., NY, USA). This was placed in a UV spectrophotometer (UV-1800, Shimadzu corporation, Kyoto, Japan) and scanned between wavelength ranges of 200nm and 700nm. The data sets obtained were used to plot the chromatogram using Origin software.

2.1.5 Statistical analysis

Lethality assays were evaluated by Finney computer statistical program to determine the LC50 values and 95% confidence intervals. Significance level/probability level used in calculation was $p<0.05$.

3 RESULTS

Table 1 shows analysis of lethal concentration 50 (LD_{50}) of DG and RT against *Artemia salina* nauplii larvae.A summary of average mortality and percentage average mortality for both DG and RT that was recorded for nauplii that were incubated for 24 h in the concentration of 10 µg/ml, 100 µg/ml and 1000 µg/ml are shown. In DG, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%, in the concentration of 100 µg/ml was 10% while it was 26% in the concentration of 1000 µg/ml. In RT, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%; in the concentration of100 µg/ml was 16% while it was 32% in the concentration of 1000 µg/ml. The average percentage mortality in all the three different concentrations for the both soluble proteins was less than 50%.The LCD50 for DG was 4158.08 µg/ml while for RT was 3988.74 µg/ml.

Table 1 Lethal Concentration 50 of DG and RT against *Artemia Salina* nauplii

Protein	Concentration (µg/ml)	Total No. of Brine Shrimps	Mortality in 5 Replicates					Average Mortality	% Average Mortality	LC_{50} (µg/ml)
DG	10	10	0	0	1	0	1	0.4	4%	**3988.74**
DG	100	10	2	1	0	2	0	1	10%	
DG	1000	10	5	3	1	2	2	2.6	26%	
RT	10	10	1	0	1	0	O	0.4	4%	**4158.08**
RT	100	10	3	2	2	1	0	1.6	16%	
RT	1000	10	3	5	3	2	3	3.2	32%	

Key: DG- Snail soluble proteins from the digestive gland; **RT-** Snail soluble proteins from the rest of the body tissues

3.1 Establishment of chemical constituents of DG and RT snail soluble protein using GC- MS

The chemical compounds of the different soluble protein isolated from *B. pfeifferi* were identified based on GC retention time on the chromatogram column, and computer matching of mass spectra with the library standards in computer.

3.1.1 Chemical composition and characterization of the snail soluble proteins

The identification and characterization of chemical compounds of DG and RT snail soluble proteins are shown on table 1.2 and 1.3 respectively according to their elution on the capillary column. The main chemical compounds in DG were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l- Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. The main chemical compounds in RT were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentanamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

Table 1.2 Chemical Composition of Snail Digestive Gland (DG) Soluble Protein Extracts

Extracts	Peak	Retention time	Similarities (%)	Name of the compound	Molecular formula
DG extract in Methanol	1	1.625	97	Butylamine S	$C_4H_{11}N$
	2	2.195	92	N-tert- Butyl methylamine	$C_5H_{11}NO_2$
	5	3.255	83	Valine	$C_4H_{11}NO_2$
	6	4.120	74	Amino heptanoic acid	$C_7H_{15}NO_2$
	7	4.345	84	L-Valine	$C_5H_{11}NO_2$
DG extract in water	1	1.625	88	1,1-Dimethylamino-1 butane	$C_6H_{13}N$
	3	2.20	82	N-tert-Butyl methylamine	$C_5H_{13}N$
	4	2.265	73	Valienamine	$C_7H_{13}NO_4$
	7	3.26	69	Penicillamine	$C_5H_{11}NO_2S$
	8	4.345	82	Penicillamine	$C_5H_{11}NO_2S$

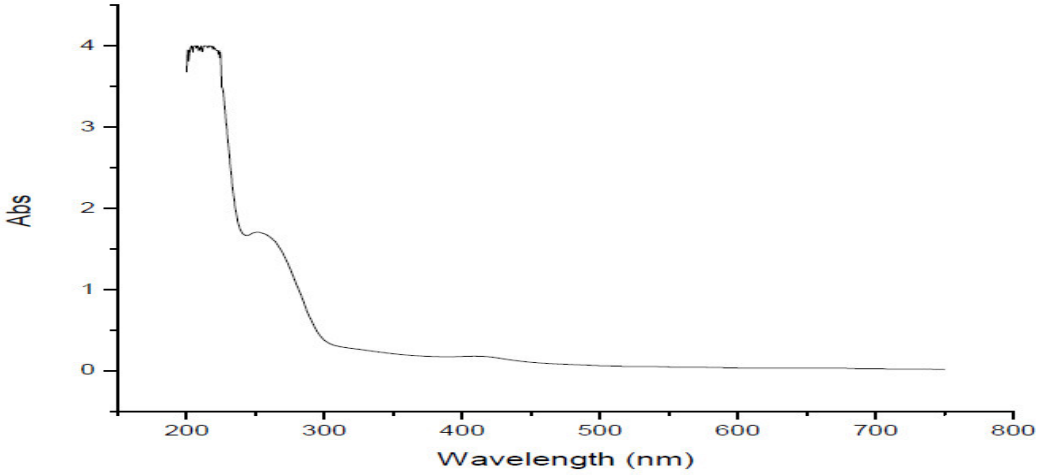
Table 1.3 Chemical Composition of the Rest of the snail Body Tissues (RT) Soluble Protein Extracts

Extracts	Peak	Retention time	Similarities (%)	Name of the compound	Molecular formula
RT extract in Methanol	1	1.625	97	Tert Butylamine	$C_4H_{11}N$
	2	2.195	90	N-tert-Butyl methylamine	$C_5H_{13}N$
	3	2.20	91	Heptylamine	$C_7H_{17}N$
	6	4.120	76	Pentanamine	$C_5H_{13}N$
	7	4.345	84	Valine	$C_5H_{11}NO_2$
RT extract in water	1	1.625	83	Cycloheptanemethylamine	$C_8H_{17}N$
	3	2.20	83	Pentanamine	$C_8H_{19}N$
	4	2.265	82	Hexadecylamine	$C_{16}H_{35}N$
	7	3.26	82	Buten 1 amine	$C_7H_{15}N$
	8	4.345	81	Dimopheptanol-S Dimethyl amine	$C_{21}H_{29}NO$

Ultraviolet (UV) Spectrometry

Figure 1(A) and (B) shows the UV spectrum of DG and RT soluble protein extracts. The peak for DG ranged between wavelengths 210- 230nm while for the RT ranged between wavelength 250-280 nm.

A.)



B.)

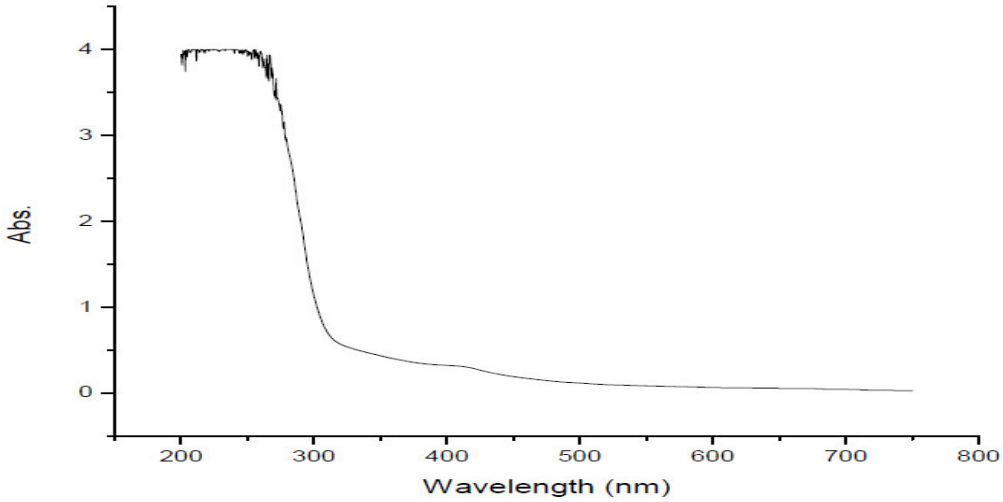


Fig 1 (A) UV spectrum of DG and (B) UV spectrum of RT soluble protein extra 7.5

4 Discussion

DG was a snail soluble protein from the digestive gland while RT was a snail soluble protein from the rest of the body tissues. In DG, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10µg/ml was 4%; in the concentration of 100µg/ml was 10% while it was 26% in the concentration of 1000 µg/ml. In RT, the percentage average mortality for the nauplii incubated for 24 h in the concentration of 10 µg/ml was 4%; in the concentration of100 µg/ml was 16% while it was 32% in the concentration of 1000 µg/ml. The average percentage mortality in all the three different concentrations for the both soluble proteins was less than 50%.

(Nguta *et al.*, 2011) gave a criteria for lethal concentration dose; LC50< 100 = Strongly/highly toxic, LC50> 100 < 500 = moderately toxic, LC50> 500 < 1000 = weakly toxic andLC_{50} > 1000 = Non- toxic. RT had LC_{50} of 4158.06 µg/ml and DG LC_{50} of 3988.73 µg/ml. DG and RT had a LC_{50} of over 1000µg/ml. The toxicity results for the two soluble proteins at the three different concentrations shows that both proteins are non-toxic and are therefore safe vaccines.

Most of the toxicity using brine shrimps has been done in plant studies. One of the studies by (Musila *et al*, 2013) on in vivo antimalarial activity, toxicity and phytochemical screening of selected antimalarial plants ;

aqueous extract of the stem bark of *Adansonia digitata* showed highest chemo-suppression of parasitaemia, ≥60% in a mouse model of *Plasmodium berghei* infected mice. Aqueous and organic extracts of *Launaea cornuta* and *Zanthoxylum chalybeum* were lethal to the brine shrimp (LD50≤1000 mg/ ml) while aqueous and organic extracts of *Adansonia digitata* and aqueous extracts of *Canthium glaucum* were non-lethal to brine shrimp (LD50≥41000 mg/ml.

This work demonstrates that both DG (LCD 50=3988.74) and RT (LCD 50=4158.08) are not toxic and are therefore safe. This is important since DG was found to be efficacious and it can go into vaccine development. Detailed literature review on the digestive gland soluble proteins and the rest of the snail tissues soluble proteins chemical composition investigation has shown that there is yet no publication available.

The GC-MS spectrum established the presence of different components with varied retention times. The mass spectrometer evaluates compounds eluted at various times to identify the nature and chemical structure of the compounds. These mass spectra are fingerprint of that compound which can be identified from the data library (Kanthal *et al*., 2014). Findings from this work help to predict the molecular formula and structure of the chemical compounds in the two soluble proteins. The main chemical compounds in DG were Butylamine S (97%), N-tert-Butylmethylamine (92 %), Valine (83 %), Aminoheptanoic acid (74%), L-Valine (84 %) in absolute methanol, and l, l- Dimethylamine-1-butane (88%), N-tert-Butylmethylamine (82), Valienamine (73%), Penicillamine (69%) and penicillamine (82%) in water. The main chemical compounds in RT were Tert Butylamine (97%), Tert Butylmethylanine (90%), Heptylamine (91%), Ethyledoxyl Pentylamine S (76%) and Valine (84%) in absolute methanol, and Bonzodrex Cycloheptanemethylamine (83%), Pentanamine (83%), Buten 1 amine (81%) and Dimopheptanol S Dimethylamine (81%) in water.

Among the compounds identified, penicillamine has been implicated in antiparasitic action against the in-vitro growth of *Plasmodium falciparum, Plasmodium chabaudi and Plasmodium berghei.* The compound as S-nitroso-acetyl-penicillamine, on high concentrations was cytotoxic to *Plasmodium falciparum*. At low concentrations, it had cytostatic effect and certain parasites restarted growth and division (Balmer *et al.*, 2000). The chemical compounds identified in the DG and RT snail soluble proteins by GC-MS might have some immunological significance as DG and RT caused profound immunological responses. This is a crucial step in vaccine development.

The UV spectrum of DG and RT soluble protein extracts peaks ranged between wavelengths 210--280 nm. The ultraviolent light absorbance maximum of proteins in solution is at 280 and 200nm. The principal cause for the absorbance peak at 280 nm is the amino acids with aromatic rings. The Peptide bonds on the other hand are main cause for the peak at 200 nm.

Secondary, tertiary, and quaternary structures influence absorbance. Other aspects such as pH, ionic power have the ability of also changing the absorbance spectrum (Stoscheck, 1990). The common way to measure the optical absorption of proteins is at 280 nm. The absorption of proteins at this wavelength is primarily as a result of the amino acids cysteine, tryptophan and tyrosine with their molar absorption coefficients decreasing in that order (Layne, 1957). Peaks observed for the both proteins are a further confirmation of presence of amino acids in the two soluble proteins.

5 Conclusions

This work demonstrates that both DG (LCD50= 3988.74) and RT (LCD50= 4158.08) are not toxic and are therefore safe. This is important since DG was found to be efficacious and it can go into vaccine development.The UV spectrum of DG and RT soluble protein extracts peaks ranged between wavelengths 210-280 nm. The ultraviolent light absorbance maximum of proteins in solution is at 280 and 200nm. The primary reason for the absorbance peak at 280 nm is the amino acids with aromatic rings. This confirms the identity of the molecules to be proteins as they have characteristic absorbance at these wavelengths.

Acknowledgements

We would like to thank The Institute of Primate Research, Kenya and University of Nairobi, Kabete Campus for allowing us to conduct the research in their facilities, National Council for Science Technology and Innovation for financial support and the technical support from particularly Mr. Joseph Nderitu, Mr. Sammy Kisara and Mr. Collins Otengo.

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