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Characterization of Immunogenic Soluble Crude Proteins from Biomphalaria Pfeifferi Against Schistosoma mansoni

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Abstract
Schistosomiasis is the second most prevalent tropical disease in the world after malaria. Chemotherapy and molluscicides which are the main methods of control do not give lasting protection as the disease continues to spread to other new regions. Previous studies have demonstrated the immunogenic properties of the digestive gland (DG), foot parts (FT) and the rest of body tissue (RT) soluble protein of *Biomphalaria pfeifferi* against *Schistosoma* parasite and therefore possible candidates for vaccine development against the parasite. However, information about the chemical composition of the soluble proteins is scanty. The objective of this study was to characterize and determine chemical composition of the DG and FT soluble proteins from *Biomphalaria pfeifferi.* A total of twelve compounds were identified using GC-MS. *N-tert*-butyl methylamine and penicillamine were present in both DG and FT soluble proteins. Butylamine S, valine, amino heptanoic acid, 1,1-dimethylamino-1 butane and valienamine were present in the DG soluble protein but were missing in the FT extracts. Tert-butylamine, heptylamine, cycloheptane methylamine, erythro-*O*-methylthreonine and leucine were present in the FT soluble protein but missing in the DG extracts. FTIR analysis showed N-H stretch at 3100 cm^{-1}, C=O stretch at 1700cm^{-1}, N-H bending at 1600cm^{-1} and O-H peak at 3500 cm^{-1} while UV absorption occurred at 240-300 nm thus confirmed the presence of amino acids in the soluble protein extracts. Results from this study justifies medicinal activity of *Biomphalaria pfeifferi* soluble crude protein extracts. Further studies involving isolation of individual constituents in the crude soluble proteins and subjecting them to bioassay is highly recommended.
Keywords: Schistosomiasis; *Biomphalaria pfeifferi;* Protein extracts; Chemical compounds
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1. Introduction

Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes (trematode worms) of the genus *Schistosoma* (Jauréguiberry *et al.* 2010). It is the second most prevalent tropical disease in the world after malaria and World Health Organization estimated the annual death rate at 200,000 globally (WHO 2016). The disease is found mainly in developing countries in Africa, Asia, South America, the Middle East, and the Caribbean. More than 207 million people, 85% of whom live in Africa, are infected with schistosomiasis, and an estimated 700 million people are at risk of infection in 76 countries where the disease is considered endemic (WHO 2016). In Kenya, prevalence of the disease ranges from 5% to over 65% in different communities and contributes to significant morbidity. It is also estimated that 16 million people in 56 out of 158 districts are at risk of the disease and over 9.1 million are infected in the country (Odhiambo *et al.* 2014). Schistosomiasis mostly affects poor and rural communities, particularly agricultural and fishing populations. Women doing domestic chores in infested water, such as washing clothes, are also at risk and can develop female genital schistosomiasis. Inadequate hygiene and contact with infected water make children especially vulnerable to infection (WHO 2016). Currently, chemotherapy and molluscicides are used to control schistosomiasis, however, they do not give lasting protection as the disease continues to spread to other new regions and risk of re-infection. Vaccine would be cost-effective alternative way for the control of schistosomiasis.

Nature has provided a large source of medicinal agents and traditional medicine systems continues to play an essential role in healthcare, with about 80% of the world's population still relying on traditional medicines for primary healthcare (Owolabi *et al.* 2007; Opiyo 2009, 2019, Ndirangu *et al.* 2020a, 2020b). The search for anti-infective agents from natural origin has led to the discovery of novel drug candidates used against diverse diseases (Manguro *et al.* 2009, 2010a, 2010b; Opiyo 2011a, 2011b, 2015, 2017; Jeruto *et al.* 2017; Ochung *et al.* 2018; Makenzi *et al.* 2019a; 2019b). Over 50% of all modern clinical drugs are of natural product origin (Newman *et al.* 2003; Ochung *et al.* 2015; Ochieng *et al.* 2013, 2017; Njoroge & Opiyo 2019a, 2019b). Continued research aimed at determination of chemical composition of natural products is important for the discovery new sources of bioactive principle for drugs and vaccine development.

Snails have been used both as a food and as a treatment for a variety of medicinal conditions (Ulagesan & Kim 2018). Snail meat has medicinal value and is used to treat ailments such as whooping cough, anaemia, asthma and high blood pressure (Ebenebe 2000). Extracts from fresh water and land snail species namely *Achatina fulica*,

Cryptozona *bistrialis*, *Pila globosa*, *Pila virens*, *Bellamya dissimilis*, *Bithynia (Digoniostoma) pulchella* and *Melanoides tuberculate* showed antibacterial and antifungal activities (Ulagesan & Kim 2018). Extracts from African giant snail (*Archachatina marginata*) caused agglutination of certain pathogenic bacteria and exhibited anti-tumor properties (Lawal *et al.* 2015). Haemolymph of *A. marginata* showed hepatoprotective effect and produce a dose dependent effect on haematological and biochemical parameters when administered to albino rats (Lawal *et al.* 2015). Haemolymph from two species of giant African land snails (*Archachatina marginata* and *Achatina achatina* showed shaemagglutination potential (Abiona *et al.* 2014). Soluble proteins from *Biomphalaria pfeifferi* showed larvicidal activity against *Artemia salina* Leach (Kobia 2017).

Common antigens between different species of *Schistosoma* and their intermediate host have been reported (Khattab *et al.* 2010) providing a reason why the parasite is able to develop in this host. Soluble proteins from digestive gland (DG), foot parts (FT) and the rest of *Biomphalaria pfeifferi* body tissue (RT) showed immunogenic properties against the *Schistosoma* parasite (Kobia 2017; Kuria *et al.* 2019). Immunization of BALB/c mice with the soluble proteins from *Biomphalaria pfeifferi* caused worm reduction when the mice were challenged with the parasite (Kuria *et al.* 2012; Kobia 2017; Kuria *et al.* 2019). The soluble proteins also stimulated the production of effective cytokines, immunoglobulins and reduced hepatic pathology (Kuria *et al.* 2019). Despite the fact that the soluble proteins from *Biomphalaria pfeifferi* exhibit immunogenic effect and hence possible candidates for vaccine development to be used to control schistosomiasis, information about the chemical composition of the proteins is scanty. The objective of this study was to determine the chemical composition of the DG and FT soluble proteins from *Biomphalaria pfeifferi.*

2. Materials and Methods

2.1 Collection and maintenance of snails

Biomphalaria pfeifferi snails were collected from irrigation canals in Mwea in Kirinyaga sub-county in Kenya which is located between latitudes 0°36'0"S and 0°44'0'S' and longitudes 37°16' 0"E and 37°28' 0"E. The snails were carried in plastic containers lined with damp cotton wool and transported to the Malacology Laboratory at the Institute of Primate Research (IPR) in Kenya. The snails were screened for schistosomes by exposing them to light (100 watts bulb shielded with glass to safeguard the snails from the heat) for two hours to check if any was shedding cercariae at interval of 30 minutes. Screening was carried out for five consecutive weeks and snails that were negative were housed in a temperature-controlled snail room at 26-28°C, with 12 hours of light and 12 hours of darkness. Sand and gravel from the area where the snails were collected was sterilized by heating at 150°C for 12 hours then cooled and layered in plastic tanks. Snail water (chlorine free water from IPR well) was placed in the tanks three quarter full. Daphnia (tiny and semitransparent freshwater crustacean) was added for aeration and the screened cercariae negative snails were transferred into the tanks. The water was changed twice a week. Dried soft lettuces (dipped in hot water and dried in the oven) were placed into the snail tanks for the snails to feed on as described by Yole *et al.* (1996).

2.2 Preparation of snail soluble crude proteins

Foot and digestive gland were obtained from *B. pfeifferi* snails under a dissecting microscope. The snails were placed on a petri dish and exposed from its shell by crushing the shell using strong forceps. Using a scarpel the foot and the digestive glands were incised and placed in labeled eppendorf tubes containing phosphate buffered saline (PBS x1) as described in our laboratory (Kuria *et al.* 2012). The samples were homogenized using a glass motor and pestle. The homogenate was centrifuged for one hour at 14,000 g at 4°C and resulting supernatant assayed for protein concentration. Protein assay estimation was done in serial doubling dilution in microtitre plate where the BIO-RAD dye was added to the aliquots, then incubated for 1 hour. The protein concentrations were determined using ELISA reader at wavelength of 595nm and was found to be 1.44 mg/ml for both foot and digestive gland soluble proteins. The protein extracts were stored at -20°C until required.

2.3 Gas chromatograph - mass spectrometer (GC-MS) analysis

GC-MS analysis of DG and FT soluble crude protein extracts from *B. pfeifferi* was performed using GCMS-QP2010SE gas chromatograph mass spectrometer machine coupled to gas chromatograph (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 X 0.25 mm internal diameter and film thickness of 0.25 µm). In GC-MS spectroscopic detection, ionization energy of 70 eV was used. Helium gas (99.99%) was used as carrier gas and was set between 5-9 bar and the chromatograph 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 (Shimadzu Corporation Kyoto, Japan) in the spit and the separation performed in the gas chromatograph followed by analysis using the mass spectrometer. The relative percentage of the chemical constituents in the soluble protein extracts were captured expressed and resolved using

the GC-MS real time analyser software (GCMS Shimadzu Lab Solution) during the process and later using GC postrun analyser with an in-built library to compare the molecular masses to known compounds (NIST 2017).

2.4 Fourier Transform Infrared (FTIR) Spectrometry Analysis

The FT and DG soluble crude protein extracts were lyophilized using a lyophilizing machine. Solid potassium bromide (KBr) was dried in an oven at more than 100ºC to evaporate any water molecules in the salt. The samples of soluble protein extracts (FT and DG) were mixed with the KBr (IR grade) in the ratio of 1:100 and ground to fine powder using glass mortar and pestle. The mixture was then used to prepare thin pellets by compressing at 20 MPa using a hydraulic press machine (Biotech engineering management company limited, UK). The pellets were transferred to a pellet holder. The machine was run first without the sample to collect the background data. The pellets were then analysed using a 600 FTIR spectrometer (Biotech engineering management company limited, UK).

2.5 Ultraviolet Spectroscopy

UV spectroscopy was performed using the standard process. Briefly, using soluble protein extracts, a concentration of 1.4 mg/ml in double distilled water 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 200 nm and 700 nm.

3. Results and Discussion

The chemical components of the DG and FT soluble crude proteins from *B. pfeifferi* (Tables 1 and 2) were determined using GC-MS based on their retention time and computer matching of the mass spectra with the library standard (NIST 2017). The chemical compounds identified from the soluble crude proteins consisted mainly on amines and amino acids. The DG extracts consisted of butylamine S (97%), *N-tert*-butylmethylamine (92%), valine (83%), aminoheptanoic acid (74%), L-valine (84%) in absolute methanol, and 1,1-dimethylamino-1-butane (88%), *N-tert*-butylmethylamine (82%), valienamine (73%), penicillamine (69%) and penicillamine (82%) in water. In FT ectract, the major chemical components were found to be *tert*-butylamine (96%), *N-tert*-butylmethylamine (87%), heptylamine (91%), penicillamine (81%), penicillamine (84%) in absolute methanol and cycloheptanemethylamine (83%), *N-tert*-butylmethylamine (81%), dl-erythro-*O*-methylthreonine (73%) and leucine (70%) in water.

N-tert-butyl methylamine and penicillamine were the only compounds that were present in both DG and FT soluble crude proteins. Butylamine S, valine, amino heptanoic acid, 1,1-dimethylamino-1 butane and valienamine were present in the DG soluble protein extract but were missing in the FT extracts. Tert-butylamine, heptylamine, cycloheptane methylamine, erythro-*O*-methylthreonine and leucine were present in the FT soluble protein but missing in the DG extracts. Penicillamine is used to treat a wide range of medical conditions including copper-associated hepatitis, lead poisoning, Wilson disease and rheumatoid arthritis (Fieten *et al.* 2013). Penicillamine derivative S-nitro-N-acetyl-D, L-penicillamine has been implicated in antiparasitic action against the *in vitro* growth of *Plasmodium falciparum, P. chabaudi and P. berghei.* (Balmer *et al.* 2000) while its derivative penicillin is used as an antimicrobial agent (Ashraf *et al.* 2015). The results on chemical composition of DG of *B. pfeifferi* soluble protein are in agreement with previously reported data (Kobia 2017). Literature regarding the identified chemical composition of FT of the snail protein extracts are unavailable. This is the first report on the chemical composition of FT soluble protein of *Biomphalaria pfeifferi.*

FTIR spectra of DG and FT soluble crude protein (Figures 1 and 2) showed N-H stretch at 3100 cm^{-1}, C=O (Amide I) at 1700cm^{-1}, N-H bending (Amide II) at 1600cm^{-1} and O-H peak at 3500 cm^{-1}. FT-IR spectroscopy has been widely used to study the structure of proteins and the results in this study concurred with literature report (Singh 1999). From the UV experiment (Figure 3) the soluble protein absorbed between wavelengths of 240 to 300 nm which was in agreement with previous reports (Fohely & Suardi 2018). The FTIR and UV data confirmed the presence of amino acids in the soluble protein extracts.

Table 1. Chemical composition of snail digestive gland (DG) soluble proteins extracts

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

Table 2. Chemical composition of snail foot gland (FT) soluble proteins extracts

Extracts	Peak	Retention time	Similarities (%)	Name of compound	Molecular formula	Nature of compound
FT extract in Methanol	1	1.63	96	Tert- Butylamine	$C_4H_{11}N$	Amine
	2	2.165	87	N-tert-Butylmethylamine	$C_5H_{13}N$	Amine
	3	2.195	91	Heptylamine	$C_7H_{17}N$	Amine
	6	3.255	81	Penicillamine	$C_5H_{11}NO_2S$	Amine
	7	4.345	84	Penicillamine	$C_5H_{11}NO_2S$	Amine
FT extract in Water	1	1.625	83	Cycloheptanemethylamine	$C_8H_{17}N$	Amine
	3	2.200	81	N-tert-Butylmethylamine	$C_5H_{13}N$	Amine
	4	2.27	73	dl-Erythro-O-methylthreonine	$C_5H_{11}NO_3$	Amine
	7	3.265	70	Leucine	$C_6H_{13}NO_2$	Amino acid

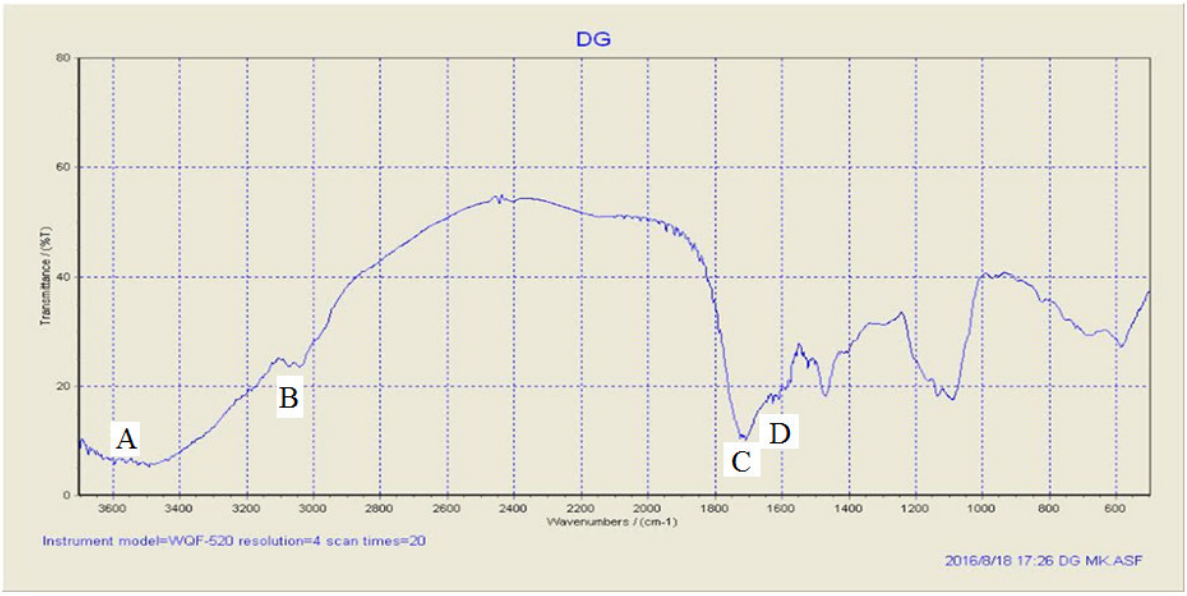

Figure 1. FTIR spectrum of DG soluble protein extract. (A) O-H stretch at 3500 cm^{-1};(B) N-H stretch at 3100 cm^{-1}; (C) C=O stretch at 1700 cm^{-1}; (D) N-H bending at 1600 cm^{-1}.

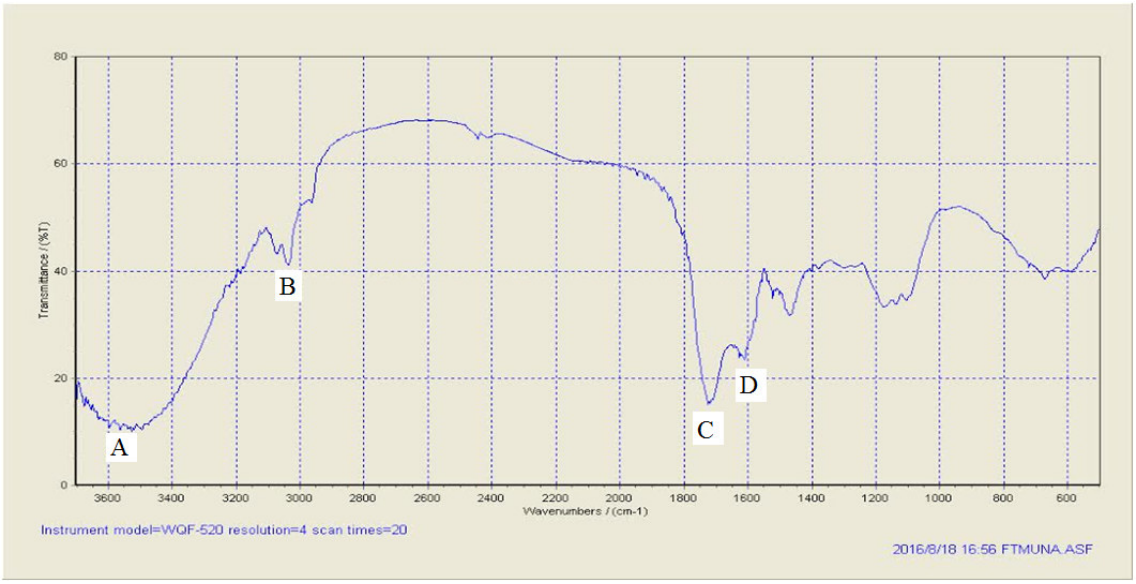

Figure 2. FTIR spectrum of FT soluble protein extract. (A) O-H stretch at 3500 cm^{-1}; (B) N-H stretch at 3100 cm^{-1}; (C) C=O stretch at 1700 cm-1; (D) N-H bending at 1600 cm^{-1}.

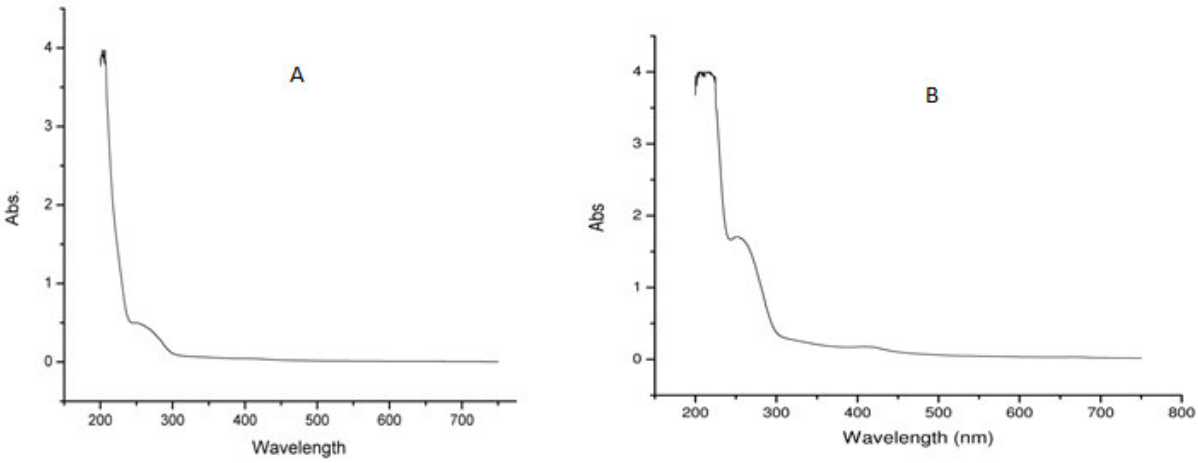

Figure 3. UV spectra of FT (A) and DG (B) soluble proteins extract of *Biomphalaria pfeifferi*

4. Conclusion

In the present study twelve chemical constituents have been identified from *Biomphalaria pfeifferi* soluble crude protein extracts by Gas Chromatogram-Mass spectrometry (GC-MS) analysis. The presence of various bioactive compounds justifies their medicinal activities. Further studies involving isolation of individual constituents in the soluble proteins and subjecting them to bioassay is highly recommended.

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Disclosure

The authors are the principal investigators in this study. They take primary responsibility for the paper, as they were in charge of the main laboratory works. The authors report no conflicts of interest in this work.

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