Potential application of Hibiscus sabdariffa L. (Malvaceae) and Lawsonia inermis L. (Lythraceae) aqueous extracts for assessment of viability of protoscolices from hydatid cysts

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Abstract

Background

A number of commercial stains have been used to assess the viability of protoscolices (defined as the capacity of being alive) that develop in hydatid cysts, the infective stage of the tapeworm Echinococcus granulosus (s.l.). There has been a remarkable increase of interest in natural product research over the past few decades for application of these products in a diversity of fields. Hibiscus sabdariffa L. (Malvaceae) is a flowering plant widely cultivated in the Sudan. Aqueous extracts from plant calyces have characteristic brilliant red colouration due to the presence of anthocyanins, an important group of water-soluble plant pigments. Lawsonia inermis L. (Lythraceae), commonly called "Henna" or "Mehndi ", is famous for having dyeing properties due to the presence of lawsone, a red-orange pigment extracted from plant leaves. Objective was to assess the viability of protoscolices of camel origin following exposure to aqueous extracts of H. sabdariffa calyces or L. inermis leaves taking advantage of the kinetically distinct molecular transfer systems of the Echinococcus protoscolex for uptake of materials across the tegument (external body covering).

Results

Viable protoscolices are capable of exclusion of plant pigment obtained from either H. sabdariffa or L. inermis whereas dead protoscolices uptake the pigment in both occasions. Performance is comparable to eosin.

Conclusions

It is suggested that extracts of both plants can be used effectively as a cheap and a readily available natural material for assessment of viability of protoscolices. Regarding the remarkable diversity of functions of the tapeworm tegument, it is proposed that the Echinococcus protoscolex can be employed as an experimental model in studies concerned with tapeworm natural protective responses in hostile environments, for instance upon exposure to antiparasitic drugs or host's body defences.

Background

Cystic echinococcosis (hydatidosis) is a serious helminthic zoonotic disease of economic and public health concern resulting from tissue invasion with hydatid cysts the infective metacestode (larval) stage of the tapeworm Echinococcus granulosus sensu lato (s.l.). The cysts develop in a broad
spectrum of domestic and wild angulates belonging to different groups, including Bovidae, Cervidae, Equidae and Camelidae as intermediate hosts and humans as an aberrant host [1]. The definitive hosts, mainly dogs, become infected with the adult tapeworm when they ingest with viscera fertile hydatid cysts containing protoscolices. Various commercial stains such as eosin, Giemsa stain, trypan blue and other stains [2,3] have been used in *in vitro* tests to assess the viability of protoscolices (*defined as the capacity of being alive*) in order to avoid various problems [4] associated with animal experimentation involving the canine (dog) definitive host. There has been a remarkable increase of interest in natural product research over the past few decades for application of these products in a diversity of fields such as medicine, agriculture, nutraceutical or cosmetics industries. *Hibiscus sabdariffa* L. (Malvaceae) (also known as roselle) is a flowering plant widely cultivated in the Sudan mainly for export of the calyx. It is locally known as “Karkadai”. Aqueous extracts from calyces have characteristic brilliant red colouration due to the presence of anthocyanins [5, 6], an important group of water-soluble plant pigments commonly found in various fruits and vegetables [7]. This attractive natural colouration found wide application in the food industry as alternative to synthetic dyes. *Lawsonia inermis* L. (Lythraceae), commonly called “Henna” or “Mehndi “, is a widespread plant universally famous for having dyeing properties [8]. It is commonly used for personal adornment especially body art and in industry. The plant has also been described to have medicinal benefits [9, 10]. Henna’s characteristic staining properties stem from the compound 2-hydroxy-1, 4-naphthoquinone, known as lawsone; a red-orange pigment extracted from plant leaves [11]. The present study examines the potential application of aqueous extracts of *H. sabdariffa* calyces and *L. inermis* leaves for assessment of viability of protoscolices from hydatid cysts taking uptake/exclusion of plant pigment as criteria.

**Methods**

**Protoscolices**

Hydatid cysts were obtained from the lungs of naturally infected camels slaughtered in a local market in Gezeira State, Central Sudan. Cysts were transported intact to the parasitology laboratory, Faculty of Veterinary Medicine, University of Khartoum. They were grossly examined for any evidence of
pathological changes such as caseation or calcification. Those with a tender texture, apparently containing fluid, were washed thoroughly from debris, dissected free from host adventitia and slit open to recover cystic fluid. Protoscolices were recovered by centrifugation of cyst fluid at 2,000 rpm for 2 min. The supernatant was discarded.

Preparation of Extracts

Crude, newly harvested *H. sabdariffa* sun-dried calyces of 35 g were obtained from a local Sudanese market in Khartoum known for selling *H. sabdariffa* (locally known as Karkadai) and other indigenous plants for domestic use. They were soaked in distilled water to give a 12.5 % w/v mix. The preparation was kept in a refrigerator for 24 hours. By this time, water extraction is expected to be complete [12]. The preparation was subsequently strained through a fine mesh and strained fluid was centrifuged at 2,000 rpm for 3 minutes to obtain a clear working solution. *L. inermis* aqueous extract was prepared in a similar manner using a 10 % w/v crude leaves mix. In both occasions, pH of the working solution was determined using pH meter (AD8000 Bench Meter, Adwa Instruments, Hungary).

Treatment

2.0 ml of either *H. sabdariffa* or *L. inermis* working solution was added to protoscolex sediments in test tubes. The contents were agitated with slight movements and incubated for 5 min at room temperature. Tubes were centrifuged at 1500 rpm for 2 min and the supernatant was discarded leaving few drops with the pellet. Protoscolices were transferred with micropipettes to glass slides, covered with cover glass slip and examined under light microscopy. For comparative purposes, protoscolices were treated with eosin 0.1% aqueous solution, a method widely used for assessment of viability of protoscolices from hydatid cysts [13, 14, 15]. According to Miman et al. [2], use of the stain at a concentration of 0.1–1% is ideal for assessment of viability of protoscolices. To verify the criteria by which viability is assessed, control tests were performed with dead protoscolices using protoscolices previously exposed to hot water in water bath at 60 °C for 5 min. Results by Moazeni and Alipour-Chaharmahali[16] indicate that exposure of protoscolices to temperatures at 50, 55, or 60 °C for 5, 2 or 1 min, respectively, is 100% lethal to protoscolices.

Results
The Figure 1 shows the gross morphology of normal (alive) *Echinococcus* protoscolices upon recovery from camel hydatid cysts.

Control tests indicated that normal protoscolices exposed to eosin did not take up the stain (Fig. 2) and they maintained an unchanged color. Protoscolices subjected to thermal death following exposure to hot water, however, were permeable to the stain and appeared red in color (Fig. 3). Tests with *H. sabdariffa* calyx extract showed that normal protoscolices did not take up plant pigment (Fig. 4) whereas dead protoscolices acquired the pigment distinctive color (Fig. 5).

A similar result was obtained when *L. inermis* leaf extract was applied to normal or dead protoscolices (Figs. 6 and 7, respectively).

**Discussion**

Among parasitic helminths, tapeworms have an exceptionally highly dynamic and a metabolically active body cover (tegument) capable of performing digestive, absorptive and protective functions [16,17,18] providing a surface for interaction with the environment in which the worms reside.

According to Pappas [19], four distinct mechanisms, viz pinocytosis, diffusion, active transport and facilitated diffusion, are implicated in nutrient uptake by tapeworms. The *Echinococcus* protoscolex, in particular, has been shown to have kinetically distinct molecular transfer systems for uptake of materials across the tegument [20]. Such properties enable the viability of protoscolices to be assessed taking uptake/exclusion of exogenous material as criteria. Results show that viable protoscolices are capable of exclusion of plant pigment obtained from either *H. sabdariffa* calyces or *L. inermis* leaves whereas dead protoscolices uptake the pigment in both occasions. The actual mechanism, by which either function is performed at the tegument cell level, however, remains to be defined.

Using the yeast *Saccharomyces cerevisiae* as model organism, Kwolek-Mirek and Zadrag-Teczain [21] introduced a classification of methods for assessment of viability of cells. These include stain-based methods whose mechanisms of action depend on the properties of the cell membrane.

Methylene blue, a synthetic basic dye and a redox indicator (*also called an oxidation-reduction indicator*), for instance, has been classified as one of the dyes that traverse cell membrane into both living and dead cells. Living cells are able to reduce the dye and remain colorless whereas dead cells
are unable to do so and are therefore stained blue. Similarly, a dye like phloxine B also penetrates into both living and dead yeast cells [22]. In this case, metabolically active cells pump out the dye and remain colorless, whereas dead cells passively incorporate the dye and are stained red. Although it is difficult to make comparisons between different organisms, tests with methylene blue [4] showed that viable and dead *Echinococcus* protoscolices uptake the dye as do living and dead yeast cells. Viable protoscolices are capable of reducing the oxidized dye and instantly lose the dye blue color whereas dead protoscolices fail to do so and they retain the color. According to Lumsden [23], tapeworms, with no trace of a mouth or digestive canal, with neither a respiratory nor a blood-vascular system [24], utilize the tegument alone for chemical interchange with the host; the tegument glycocalyx layer serving as a binding surface for inorganic ions and higher molecular weight organic compounds. Such properties of the tegument are translated into a remarkable diversity of functions including contact-digestion at the surface of the worm [23] and protection of the parasite against injurious elements such as host’s digestive enzymes, bile acids or components of the host’s immune reaction [17]. The present study indicates that viable *Echinococcus* protoscolices are potentially capable of exclusion of extraneous, possibly harmful material. They may, therefore, be employed as experimental model in studies concerned with tapeworm natural protective responses in hostile environments, for instance upon exposure to antiparasitic drugs or immunologically-mediated host’s body defences.

Generally anthocyanin pigments, including *Hibiscus* anthocyanins [25], are potentially unstable; the pH being a major factor that influences pigment color variations and stability [6, 26]. The present study indicates that an *H. sabdariffa* calyx aqueous extract at pH 1.64 and a *L. inermis* leaf extract at pH 3.13 provide verifiable results. Further tests may be necessary to standardize a technique for protoscolex viability assay using the extract material.

**Conclusion**

It is concluded that aqueous extracts of either *H. sabdariffa* calyces or *L. inermis* leaves can be used effectively as a cheap and a readily available natural material for assessment of viability of protoscolices from hydatid cysts. Performance of the two plant extract materials is comparable to
List Of Abbreviations

*Echinococcus granulosus* (s.l): *Echinococcus granulosus* sensu lato; *H. sabdariffa*: *Hibiscus sabdariffa*; *L. inermis*: *Lawsonia inermis*; w/v: weight/volume.

Declarations

*Ethics approval and consent to participate*: Not applicable

*Consent for publication*: Not applicable

*Availability of data and materials*: Not applicable

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Figures

Figure 1

Fig. 1 Normal invaginated Echinococcus protoscolices showing the characteristic rostellar hooklets and calcareous corpuscles (100x)
Fig. 2 Normal Echinococcus protoscolices exposed to eosin stain (Magnification 100x)
Fig. 3 Dead Echinococcus protoscolices exposed to eosin stain (100x)
Figure 4

Fig. 4 Normal Echinococcus protoscolices exposed to H. sabdariffa calyx aqueous extract (100x)
Figure 5

Fig. 5 Dead Echinococcus protoscolices exposed to H. sabdariffa calyx aqueous extract (100x)
Figure 6

Fig. 6 Normal Echinococcus protoscolices exposed to L. inermis leaf aqueous extract (100x)
Figure 7

Fig. 7 Dead Echinococcus protoscolices exposed to L. inermis leaf aqueous extract (100x)