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Potential Application of *Hibiscus sabdariffa* L. (Malvaceae) Aqueous Extract for Assessment of Viability of Protoscolices from Hydatid Cysts

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Abstract

Cystic echinococcosis (hydatidosis) is a serious zoonotic parasitic disease of global public health and economic importance. It is caused by infection with hydatid cysts, the larva of a tapeworm of canines, particularly dogs. The canine hosts become infected with the adult parasite when they ingest with viscera fertile hydatid cysts containing protoscolices (PSCs). Generally, the mere presence of PSCs in a hydatid cyst has been considered an indication of infectivity to the host. There is no tangible measure, however, to assess the degree of this infectivity apart from feeding experimentally to the host (dogs) a known number of PSCs and counting at necropsy the number of adult worms that develop in the intestine. This approach, however, though technically feasible, has several limitations not least among which is the potential error in finding such very small worms in the intestine and the health hazard from exposure to infective eggs released by patent worms. To avoid such risks, a number of commercial stains have been used in *in vitro* tests to assess the viability of PSCs (defined as the capacity of being alive), a property closely linked to infectivity. *Hibiscus sabdariffa* is a flowering plant widely cultivated in Sudan. Aqueous extracts from plant calyces have characteristic brilliant red coloration due to the presence of anthocyanins, an important group of water-soluble plant pigments. The present study examines the potential application of aqueous extract of plant calyces for assessment of viability of PSCs taking uptake/exclusion of plant pigment as criteria. It is proposed that the application of extract can be used effectively as an objectively quantifiable low-cost assay for assessment of viability of PSCs from hydatid cysts. Performance of extract is comparable to eosin.

Subject Areas

Parasitology, Veterinary Medicine

Keywords

Hibiscus sabdariffa, Calyces, Viability, Protoscolices, Hydatid Cysts

1. Introduction

Cystic echinococcosis (hydatidosis) is a serious zoonotic helminthic disease of global economic and public health concern. The disease results from invasion of tissues with hydatid cysts, the infective metacestode (larval) stage of the canine tapeworm *Echinococcus granulosus sensu lato* species complex. The cysts develop in a broad spectrum of domestic and wild angulates belonging to different groups, including Bovidae, Equidae and Camelidae as intermediate hosts and humans as an aberrant host [1]. The definitive canine hosts, mainly dogs, become infected with the adult tapeworm when they ingest with viscera fertile hydatid cysts containing protoscolices (PSCs). There is no tangible measure, however, to assess the degree of this infectivity apart from feeding experimentally to the definitive host (dogs), a known number of PSCs and counting at necropsy the number of adult worms that develop in the intestine. This approach, however, though technically feasible, has several limitations not least among which is the potential error in finding such very small worms, typically 2 - 7 mm in length, in the intestine and the health hazard from exposure to infective parasite eggs and environmental contamination at necropsy. To avoid such risks, a number of commercial stains such as eosin, Giemsa stain, trypan blue or methylene blue [2] [3] [4] have been used in *in vitro* tests to assess the viability of PSCs (defined as the capacity of being alive), a property closely linked to infectivity. There has been a remarkable increase of interest in natural product research over the past few decades for application of these products in various fields such as medicine, agriculture, nutraceutical or cosmetics industries. *Hibiscus sabdariffa* L. (Malvaceae) (also known as roselle) is a flowering plant widely cultivated in Sudan mainly for export of the calyx. It is locally known as "Karkadai". Aqueous extracts from calyces have characteristic brilliant red coloration due to the presence of anthocyanins [5] [6], an important group of water-soluble plant pigments commonly found in various fruits and vegetables [7]. The present study examines the potential application of aqueous extract of *H. sabdariffa* calyces for assessment of viability of PSCs from hydatid cysts taking uptake/exclusion of plant pigment as criteria.

2. Materials and Methods

2.1. Source of 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. PSCs were recovered by centrifugation of cyst fluid at 2000 rpm for 2 min at 25˚C and the supernatant was discarded.

2.2. Preparation of Extract

Crude, newly harvested *H. sabdariffa* sun-dried calyces of 35 g were obtained from a local Sudanese market in Khartoum. They were soaked in distilled water to give a 12.5% w/v mix. The preparation was kept in a refrigerator at 4˚C for 24 hours. By this time, water extraction is expected to be complete [8]. The preparation was subsequently strained through a fine mesh and strained fluid was centrifuged at 2000 rpm at 25˚C for 3 min to obtain a clear working solution. pH of the working solution was determined using pH meter (AD8000 Bench Meter, Adwa Instruments, Hungary).

2.3. Treatment

2.0 ml of the working solution was added to PSCs 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 at 25˚C and the supernatant was discarded leaving a trickle of fluid with the pellet. PSCs were transferred with micropipettes to glass slides, covered with cover glass slip and examined under light microscopy. To verify the criteria by which viability is assessed, control tests were performed with dead PSCs using PSCs previously exposed to hot water in a water bath at 60˚C for 5 min. Results by Moazeni and Alipour-Chaharmahali [9] indicate that exposure of PSCs to temperatures at 50˚C, 55˚C, or 60˚C for 5, 2 or 1 min, respectively, is 100% lethal to PSCs. For comparative purposes, PSCs were treated with eosin 0.1% aqueous solution, a method widely used for assessment of viability of PSCs from hydatid cysts [10] [11] [12]. According to Miman *et al.* [2], use of the stain at a concentration of 0.1% - 1% is ideal for assessment of viability of PSCs. Imaging was performed with a digital camera (OPTICAM 4083. B1) fitted to an OPTICA Srl B-193 light microscope (Ponteranica, Italy) at magnification 100×.

2.4. Statistical Analysis

Experiments were designed as an observational study with viability of PSCs being assessed in terms of uptake/exclusion of plant pigments by viable (alive) or dead (control) PSCs. Each of the experimental and control tests was replicated 5 times. Results were expressed as cumulative frequencies of nominal variables in 2 × 2 contingency tables and tested for statistical difference by the Fisher exact probability test.

3. Results

Figure 1 shows the gross morphology of normal (viable) PSCs upon recovery

from camel hydatid cysts.

Tests with *H. sabdariffa* calyx extract showed that viable (alive) PSCs did not take up plant pigment when treated with the extract (Figure 2). Control PSCs previously subjected to thermal death, however, were permeable to the extract and they acquired the pigment distinctive color (Figure 3). The outcome of treatment (uptake /exclusion of plant pigment) differed significantly (p 0.0079; two-tailed) when viable (alive) and dead (control) PSCs were exposed to extract.

Comparative tests indicated that viable PSCs exposed to eosin did not take up the stain (Figure 4). Dead PSCs, in contrast, were permeable to the stain and acquired the distinctive stain red color (Figure 5). There is simultaneous depletion of the stain from the medium associated with influx of the stain into the dead PSCs as indicated by fading of the medium color.

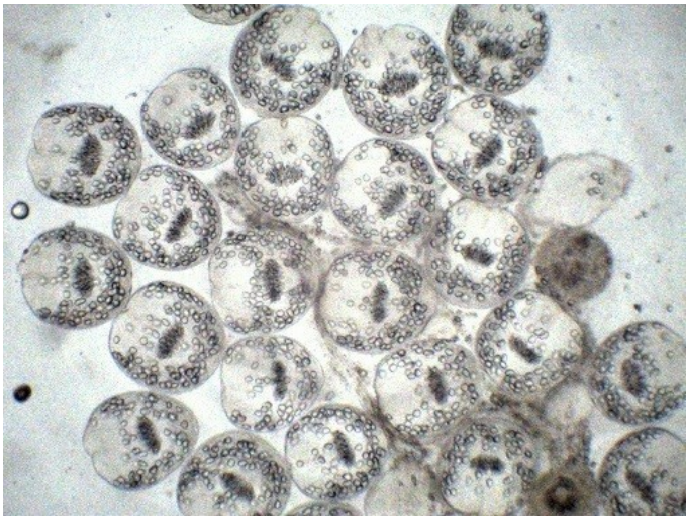

Figure 1. Normal invaginated PSCs showing the characteristic rostellar hooklets and calcareous corpuscles (100×).

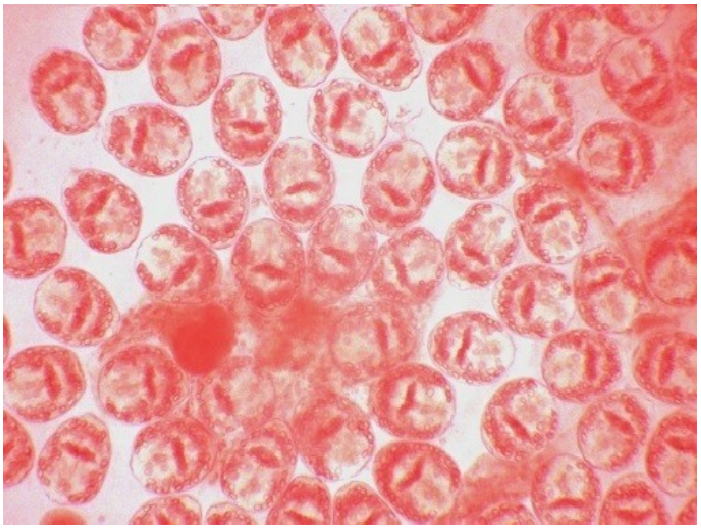

Figure 2. Viable (alive) PSCs exposed to *H. sabdariffa* calyx aqueous extract (100×).

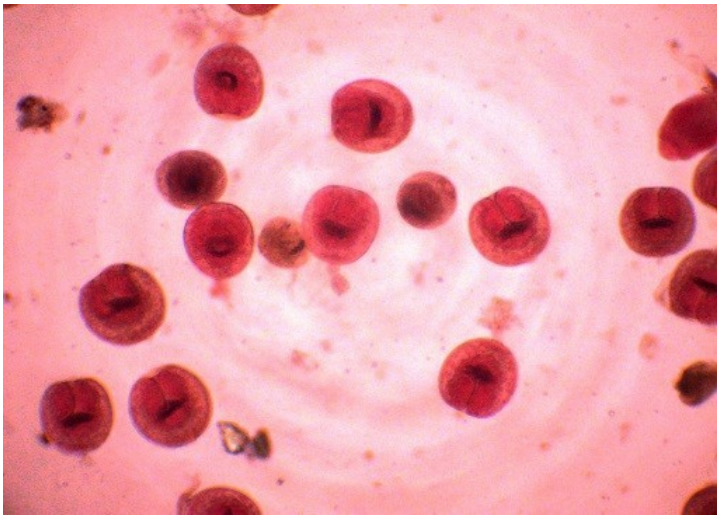

Figure 3. Dead PSCs exposed to *H. sabdariffa* calyx aqueous extract (100×).

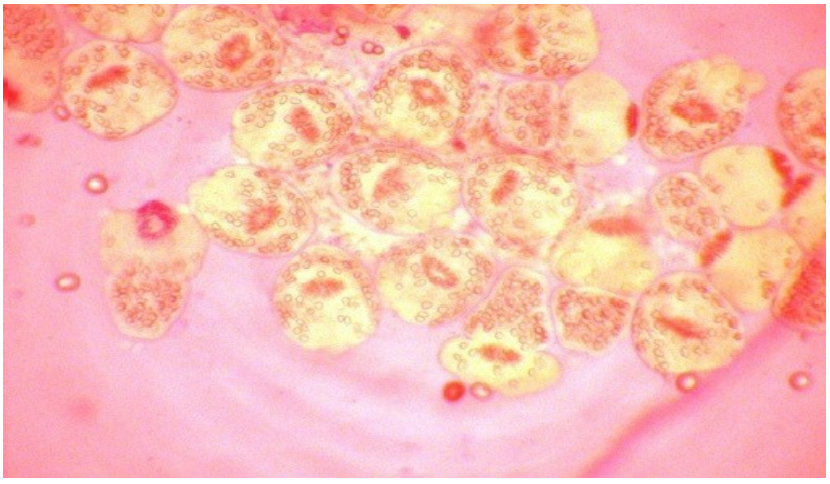

Figure 4. Viable (alive) PSCs exposed to eosin stain (100×).

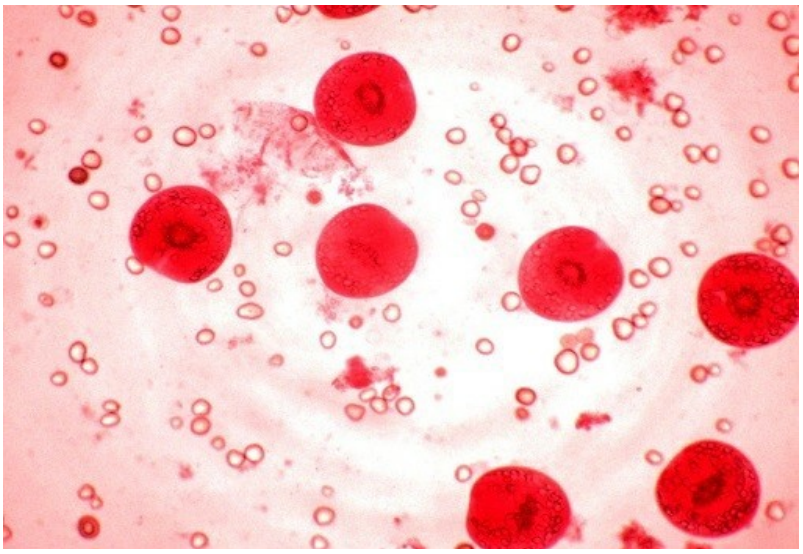

Figure 5. Dead PSCs exposed to eosin stain (100×).

4. 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 [13] [14] [15]. The *Echinococcus* protoscolex, in particular, has been shown to have kinetically distinct molecular transfer systems for uptake of materials across the tegument [16]. Such properties enable the viability of PSCs to be assessed taking uptake/exclusion of exogenous material as criteria. The results show that viable (alive) PSCs are capable of exclusion of plant pigment obtained from *H. sabdariffa* calyces. Dead PSCs, in contrast, are permeable to extract when exposed to extract over a similar period. It is proposed, therefore, that viability of PSCs can be assessed in terms of uptake/exclusion of plant pigment and in a manner comparable to that when eosin is used.

Infection with hydatid cysts has been reported in various species of animals in Sudan with different prevalence rates in camels, cattle, sheep and goats [17] [18] [19] [20]. The cysts recovered from these animals also showed different levels for fertility, cystic developmental stages and organ disposition [21] [22]. Molecular and epidemiological studies indicate that the camel strain, *Echinococcus canadensis* genotype 6, is the predominant genotype infecting both animals and humans in Sudan [19] [22]. These findings suggest that the camel is the principal intermediate host responsible for parasite transmission in this country. Assessment of viability of PSCs may, therefore, be adopted as an approach to determine the relative importance of the different species of animals in the epidemiology of the disease in this country. We propose from the present results that the application of *H. sabdariffa* calyces' aqueous extract can be used effectively as an objectively quantifiable low-cost assay for assessment of viability of PSCs to serve this purpose.

Generally, anthocyanin pigments, including *Hibiscus* anthocyanins [23], are potentially unstable; the pH being a major factor that influences pigment color variations and stability [6] [24]. The present study indicates that an *H. sabdariffa* calyx aqueous extract at pH 1.64 provides verifiable results. Further tests may be necessary to standardize a technique for protoscolex viability assay by studying as example the effect of the time of exposure of PSCs to extract and the influence of the other constituents of extract on the performance of the anthocyanin's component.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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