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In vitro Scolicidal Effect of Ethonolic Extracts of *Juglans regia* and *Carica papaya* on Hydatid Cysts of Sheep and Goats from North Western Himalayas

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Abstract

Over a century ago, there are anecdotal stories of the use of therapeutic antihelminthic plants, such as papaya (*Carica papaya*) against cestode infections. Various studies have explored the efficacy of traditional plants in the inactivation of protoscolices and have reported the scolicidal effect of plants. Further, these plants have relatively lower side effects compared to chemo-therapeutic agents and are suggested to be used for treatment of this disease in humans as well. Thus, in the present study the in vitro scolicidal effect of *C. papaya* and *J. regia* on *Echinococcus granulosus* protoscolices was explored from sheep, goat and human cysts in Jammu region of North Western Himalayas. The ethanolic extracts of *J. regia* and *C. papaya* showed significant scolicidal activity against *E. granulosus*, under in-vitro conditions with reference to the known standard drug "Praziquantel". Against *J. regia*, highest mortality was observed at 30 mg/ml concentration at different exposure time as 10 min. (88.58%), 20 min. (91.24%), 30 min. (93.16%) and 40 min. (96.64%). Against *C. papaya*, highest mortality was observed at 30 mg/ml concentration at different exposure time as 10 min. (82.95%), 20 min. (85.83%), 30 min. (90.23%) and 40 min. (92.95%).

Keywords: *Echinococcus granulosus;* Hydatidosis; Praziquantel; Scolicidal Effect; *Juglans regia*; *Carica papaya*

Introduction

In the north-western Himalayan region of India, small ruminant rearing is the sole source of livelihood for pastoral nomadic tribes [1]. Among these, hydatidosis is considered a serious health hazard. Treatment of echinococcosis in humans involves surgery to incise cysts or liver resection and has the high risk of secondary echinococcosis infection due to spillage of protoscolices into the peritoneal cavity during the surgery. Therefore, long term chemotherapy with parasiticides is prescribed to inactivate cyst's content; however most of these parasiticides are associated with adverse side effects in the host. Other than this next generation therapeutic methods which includes destruction of germinative cell population which has the role of only known source of new cells for metacestode growth and regeneration, it would be of clear interest to develop methods to deplete them, not only as a research tool but also for the search for new therapeutic options against echinococcosis; ionising irradiation, the most usual approach in other flatworms could be used and also treatment with specific inhibitors against enzymes that are important for proliferating cells is more efficient and results in strong depletion of the germinative cells [2].

There are anecdotal stories of the use of therapeutic antihelminthic plants, such as papaya (*C. papaya*) against cestode infections [3]. Various studies have explored the efficacy of traditional plants in the inactivation of protoscoleces and have reported the scolicidal effect of plants viz., *Ajowan, Zataria multiflora*, Allium spp., Mentha spp. and *Berberis vulgaris* on *E. protoscoleces* [4]. Further, these plants have relatively lower side effects compared to chemotherapeutic agents and are suggested to be used for treatment of this disease in humans [5,6]. Thus, in the present study the in vitro scolicidal effect of *C. papaya* and *J. regia* on *E. granulosus* protoscoleces was explored.

Materials and Methods

The present study was conducted in the Division of Veterinary Public Health & Epidemiology, Faculty of Veterinary Sciences & Animal Husbandry (F.V.Sc & A.H.), Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-Jammu) and in collaboration with the Division of Veterinary Parasitology, F.V.Sc & A.H., SKUAST-Jammu. The study was designed under three major heads *viz.* studies on prevalence of cystic echinococcosis (CE), molecular characterization of cystic echinococcosis from small ruminants and to check the scolicidal effect of *C. papaya* and *J. regia* on *E. granulosus* protoscoleces.

The scolicidal effects of *C. papaya* and *J. regia* on *E. granulosus* protoscolices were evaluated as described by Gangwar, et al. [7] with slight modifications. The scolicidal effect of the plants extracts were evaluated on the basis of viable and non-viable protoscolices after treatment with the extracts at different concentration.

The hydatid fluid of cysts was aseptically transferred into the 15 ml centrifugation tube and left to set for half an hour. The protoscolices were settled down at the centrifugation tube. The supernatant was then removed and the yielded protoscolices were washed three times in normal saline. The viability of protoscolices was confirmed from their motility characteristics under light microscopy. The live protoscolices were finally transferred into a dark container containing normal saline and stored at 4°C until use.

Extract Preparation

A total of 500 gram dried powder was extracted. The following method was used for preparation of methanolic extract of *C. papaya* and *J. regia*. 100g of dry powder was added to 400 ml of pure methanol and mixed gently for one hour using a magnetic stirrer. The obtained solution was left at room temperature for 24 h. The solution was stirred again, filtered and the solvent was removed by evaporation in a rotary evaporator. The remaining semisolid material was then freeze dried. The obtained residue was placed in a sterile glass container and stored in the refrigerator at 4ºC for later use. Total 6.8 gram and 10.6 gram of dried extract was obtained from 500 gram of dried powder of *C. papaya* and *J. regia*.

The scolicidal tests were carried out based on Moazen, et al. [8]. In this study, three concentrations (10, 20 and 30 mg/ml) of the *C. papaya* and *J. regia* for 10, 20, 30, and 40 min were obtained. For preparation of *C. papaya* and *J. regia* solution with 10, 20 and 30 mg/ml concentration, 0.1, 0.2 and 0.3 grams of each dried extract was dissolved in 10 mL of normal saline, respectively. The dried extract was dissolved in 1% dimethyl sulphoxide (DMSO) before use. Two milliliters of each concentration was placed in a test tube, a drop of protoscolex rich sediment was added to the tube and mixed gently. The tube was then incubated at 37°C for 10, 20, 30 and 40 min. At the end of incubation time, the upper portion of the solution was removed with a pipette avoiding settled protoscolices. Then two milliliters of 0.1% eosin stain was added to the remaining settled protoscolics and mixed gently. After 15 min, the upper portion of the solution was discarded. The remaining settled protoscolices were then smeared on a manual scaled glass slide, covered with a cover glass (24 x 50 mm) and examined microscopically for viability. The percentages of dead protoscolices were determined by counting a minimum of 350 (mostly more than 500) protoscolices. At least 1500 protoscolices with no exposure to extract was considered as control group in each experiment. The experiments were performed in triplicate.

Eosin stain with 0.1% concentration (1 gr of eosin powder in 1000 mL of distilled water) was used for the viability test of protoscolices. After 15 min of exposure, the unstained protoscolices and the stained protoscolices were accepted as potentially viable and dead, respectively.

Results

The ethanolic extracts of *J. regia* showed significant scolicidal activity against *E. granulosus*, under in-vitro conditions with reference to the known standard drug "Praziquantel" (Table 1). At 10 mg/ml concentration, the mortality rate was observed for different exposure time as 10 min. (28.57%), 20 min. (36.74%), 30 min. (47.74%) and 40 min. (56.40%). At 20 mg/ml concentration, the mortality rate was observed for different time exposures as 10 min. (66.99%), 20 min. (74.17%), 30 min. (80.81%) and 40 min. (86.60%). At 30 mg/ml concentration, the mortality rate was observed for different time exposures as 10 min. (88.58%), 20 min. (91.24%), 30 min. (93.16%) and 40 min. (96.64%). Based on our preliminary in-vitro vitality/viability assessment of protoscolices of hydatid cysts after treatment with extract of *J. regia*, indicate the significant loss in the viability, morphological tegumental alterations including disintegration of protoscolices. Positive control "Praziquantel" showed mortality rate at different exposure time as 10 min. (96.09%), 20 min. (97.02%), 30 min. (97.65%) and 40 min. (100%).

Concentration	Exposure time (min.)	Total protoscolices (Mean± SE)	Dead protoscoloices (Mean± SE)	Mortality rate of extract (%)
Juglans regia (10 mg/ml)	10	462± 3.81	132± 2.07	28.57
	20	479± 2.56	176± 2.64	36.74
	30	530± 3.86	253± 2.82	47.74
	40	422± 1.86	238± 2.77	56.4
Juglans regia (20 mg/ml)	10	627± 2.89	420± 3.80	66.99
	20	542± 2.47	402± 2.73	74.17
	30	521± 2.01	421± 3.05	80.81
	40	612± 1.90	530± 2.39	86.6
Juglans regia (30 mg/ml)	10	662± 2.27	551± 2.68	88.58
	20	582± 3.05	531± 2.88	91.24
	30	629± 3.07	586± 3.07	93.16
	40	595± 4.00	575± 2.58	96.64
Praziquantel (1 µg/ml)	10	487± 4.19	468± 3.07	96.09
	20	638± 2.39	619± 2.77	97.02
	30	586± 2.22	581± 1.61	97.65
	40	477± 2.00	477± 2.93	100
0.9% PBS	10	549± 3.49	8± 1.39	1.46
	20	581± 3.13	6± 1.03	1.03
	30	493± 2.63	18± 2.06	3.65
	40	614± 2.96	22± 2.46	3.58

Table 1: Scolicidal effect of *Juglans regia* on protoscolices at different concentrations and exposure time.

The ethanolic extracts of *C. papaya* showed significant scolicidal activity against *E. granulosus*, under in-vitro conditions with reference to the known standard drug "Praziquantel" (Table 2). At 10 mg/ml concentration, the mortality rate was observed for different exposure time as 10 min. (26.87%), 20 min. (33.33%), 30 min. (45.03%) and 40 min. (51.59%). At 20 mg/ml concentration, the mortality rate was observed for different time exposures as 10 min. (58.78%), 20 min. (66.51%), 30 min. (75.93%) and 40 min. (77.49%). At 30 mg/ml concentration, the mortality rate was observed for different time exposures as 10 min. (82.95%), 20 min. (85.83%), 30 min. (90.23%) and 40 min. (92.95%). Based on our preliminary in-vitro vitality/viability assessment of protoscolices of hydatid cysts after treatment with extract of *C. papaya*, indicate the significant loss in the viability, morphological tegumental alterations

including disintegration of protoscolices. Positive control "Praziquantel" showed mortality rate at different exposure time as 10 min. (95.73%), 20 min. (96.28%), 30 min. (97.26%) and 40 min. (100%).

Concentration	Exposure time (min.)	Total protoscolices (Mean± SE)	Dead protoscoloices (Mean± SE)	Mortality rate of extract (%)
Carica papaya (10 mg/ml)	10	510± 2.72	137± 2.39	26.87
	20	489± 4.15	162± 2.06	33.33
	30	413± 2.94	186± 2.65	45.03
	40	663± 3.83	342± 3.48	51.59
Carica papaya (20 mg/ml)	10	541± 2.30	318± 2.58	58.78
	20	430± 3.51	286± 1.77	66.51
	30	586± 1.63	445± 2.01	75.93
	40	422± 3.03	327± 2.79	77.49
Carica papaya (30 mg/ml)	10	569± 2.06	472± 3.02	82.95
	20	487± 2.16	412± 1.77	85.83
	30	532± 2.09	480± 3.10	90.23
	40	610± 2.81	567± 2.03	92.95
Praziquantel (1 µg/ml)	10	562± 2.22	538± 2.48	95.73
	20	511± 2.82	492± 2.43	96.28
	30	474± 2.35	461± 2.29	97.26
	40	672± 1.88	672± 2.04	100
0.9% PBS	10	488± 2.39	7± 1.79	1.43
	20	652± 3.16	19± 1.94	2.91
	30	597± 3.94	16± 2.08	2.69
	40	583± 2.03	9± 1.65	1.54

Table 2: Scolicidal effect of *Carica papaya* on protoscolices at different concentrations and exposure time.

J. regia and *C. papaya* extracts, provoked significant loss of motility and turgidity of cysts, evagination of the protoscolices were observed after treatment, however all protoscolices were stained in 0.1% eosin test. Very similar types of morphological changes were also observed with Praziquantel, a known anti-cestodal drug. It was observed that eosin dye expressed as somewhat pink appearance, once retained by the non-viable protoscolices. The tegumental vacuolization associated with the loss of turgidity of the protoscolices were the most diagnostic feature that appeared after extract treatment. Other morphological changes observed were loss of motility, loss of hooks (free hooks can be seen in the preservation medium), and damaged germinal layers. Additionally the degenerative effect was also observed as loosening of the microtriches and hooks at the scolex region that causes the significant loss in the potential of protoscolices to attach with host tissues, leading to decrease the infection vigor of the cysts. It was also observed that the arranged scolex region of the cysts completely disintegrated. The control live protoscolices have unique diagnostic features like highly turgid soma and scolex regions, circularly arranged microtriches and hooks, uniform tegumental layer etc. But after treatment with a control drug Praziquantel there were significant changes observed such as loss of turgidity especially with the soma region, and the formation of blebs caused tegumental damages. Even detrimental changes observed when the protoscolices were treated with 10 mg/ml of extract, the contraction of soma region, formation of blebs on the tegument and the disorganization of the rosteller region, moreover with a high dose treatment 30 mg/ml caused tegumental vacuolization leading to the disruption of the tegumental layer and complete collapse of the protoscolices.

Discussion

Various synthetic anthelmintics are used to prevent the reoccurrence in surgical cases in addition to benzimidazole carbamates as viable options of inoperable symptomatic cysts. However there are serious health risks in human being

and are evident with many synthetic drugs like albendazole, nitazoxanide, flubendazole etc. Besides chemicals other alternative scolicidal agents like silver nitrate, hypertonic saline etc. have been reported significant activity but can't be considered as an ideal scolicidal agent since they have too many adverse effects [9,10]. Although most of the synthetic drugs have 100% effectiveness but also serious side effects associated with them like Cetrimide (0.5%-1.0%) causes methemoglobinemia, peritonitis, convulsions and sometimes coma [11], albendazole sulphoxide (100 mg/ml) elevate liver enzyme functions and increases solubility [12], nitazoxanide (10 mg/ml) causes cellular autolysis [13], flubendazole (10 mg/ml) causes dyspepsia and sleepiness [14], hypertonic Saline (20%) causes hypernatremia, intracraniel bleeding and myelinolysis [15], silver nitrate (20%) is toxic on absorption through cysts wall, pleura and peritoneal membrane [16]. Natural scolicidal agents will be an alternative that can be safe with no adverse associated effects. With this hypothesis a large number of medicinal plants having their ethanobotanical history as anthelmintics are used to screen for their scolicidal potential especially against *E. granulosus*, for example *Zataria multiflora* [17], *Satureza khuzistanica* [17], *Salvia officinalis* and *Thymus vulgaris* [18], *Alium sativum* (Sadjjadi *et al*. 2008), *Dendrosicyos socotrana* and *Jatropha unicostata* [19]. Additionally it is important to evaluate the dose dependent activity and the sustainability of the effect in light of this fact that many species are becoming resistant. For the first time we have reported the extract of *J. regia* and *C. papaya* for its scolicidal activity against hydatid cysts *E. granulosus*. Although this plant has been known for its medicinal properties, but most popular activity of this plant is the anti-parasitic specially anti-helminthic reported in the traditional system of medicine. In our investigations we have established the scolicidal potential of the *J. regia and C. papaya* extract with reference to the standard drug Praziquantel. Although a high dose of extract (30 mg/ml) shows significant scolicidal effect with reference to Praziquantel, but it need to remember that being an natural alternative, these are very safe for its application as scolicidal agent [20,21].

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