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Effects of *Psidium Guajava* Leaves Extract on the Viability of *Echinococcus Granulosus* Protoscolices *in Vitro* and *in Vivo*

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Abstract

Echinococcosis is a common parasitic disease in humans and ruminants; it is considered as a health and economic problem in most parts of the world. The present study aims to evaluate the *in vitro* and *in vivo* scolicidal effects of *Psidium guajava* extracts on the protoscolices from hydatid cysts of *Echinococcus granulosus*. Protoscolices were aseptically aspirated from the livers of naturally infected sheep. Various concentrations of the extract were used for 6-48hrs. Eosin staining (0.1%) was used to determine the viability of protoscolices. The findings showed that the extract at the concentrations of 100 mg/ml killed 100% protoscolices after 48 hrs. However, the concentrations of 75 and 50 mg/ml were not effective in killing the protoscolices after 48 hrs. of exposure. The results of this investigation demonstrated that *P. guajava* could be used as a natural source for the production of new scolicidal agents.

Keywords: *Echinococcus granulosus*, protoscolices, *Psidium guajava*, leaves extract.

INTRODUCTION

Hydatid is a common parasitic disease in humans and ruminants; it is a health and economic problem in most parts of the world [1]. Hydatidosis is particularly prevalent in rural areas where cattle, especially sheep and dogs, are abundant. The parasite needs to complete its life cycle to a host of sheep, goats, camels, pigs, horses, donkeys, monkeys and other ruminant animals to survive [2]. It is transmitted from carnivores to humans and herbivorous animals [3], where it develops to the larval stage of *E. granulosus*. This parasite, which belongs to the phylum Platyhelminthes of the class Cestoda, infects the small intestine of carnivores [4].

Guava (*Psidium guajava* Linn.) is a plant that belongs to the family Myrtaceae. It is originated in tropical South America and grows wild in Bangladesh, India, Thailand, Brazil, Florida, West Indies, California, and several other countries [5]. *P. guajava* is a small tree which is 10m high, with thin, smooth, patchy, peeling bark. The leaves are opposite, short-petiolate. The blade is oval with prominent pinnate veins, 5–15 cm long. The flowers are somewhat showy; the petals are whitish and up to 2 cm long; the stamens are numerous [6].

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Guava is an important plant that is used traditionally for medicinal purposes. It is used as an important food as well as a medicinal plant in tropical and subtropical countries; therefore, it has also the name of the poor man's apple. Native to tropical areas from Southern Mexico to Northern South America, guava trees have been grown by many other countries that have tropical and subtropical climates, thus allowing widespread production around the world [7]. India is the major world producer of guava [8].

The phytochemical analysis shows the presence of flavonoids, tannins, saponins, triterpenes, glycosides, sterols, alkaloids, carbohydrates, phlobatannins, terpenoids, and polyphenol in the plant extract [9].

Guava contains dietary fiber, protein, calcium, phosphorus, potassium, copper, iron, vitamin A, vitamin B1, vitamin C, vitamin B2, vitamin B3, and folic acid [6]. Guava has many benefits in our lives. Its fruits are used as food, while its wood is used in construction. Its leaves, roots, and seeds are also used in the treatment of many diseases. Its extracts showed active antibiotic effects [10, 11]. Also, these extracts were used against insects [12] and against various types of human parasites, where it showed activity against toxoplasmosis [13], *Trypanosoma brucei* [14], and malaria [15].

It was noted through studies conducted on the extracts of guava leaves that they have effects against various types of cancer, including those of colon, rectum, benign breast [16], ovary, lung, prostate, and kidney, as well as leukemia [17]. In addition, the extracts of leaves prevented tooth decay and are used as an anti-inflammatory agent against teeth gums and acne [18, 19]. Leaf extracts also showed activities against fungi [20, 21]. Guava leaves also have anti-ulcer activities by protecting the mucosa of the stomach through the action of flavonoids [22]. They also have the ability to heal wounds, inhibit diarrhea, and relieve menstrual pain, along with a broad-spectrum of other medicinal uses, including their use as anticonvulsant [19].

The importance of this study is that it shows both the *in vitro* and *in vivo* effects of the alcoholic and aqueous extracts of *Psidium guajava* leaves on the vitality of the protoscolices of *Echinococcus granulosus* of sheep origin.

MATERIALS AND METHODS

Preparation of plant extracts

Aqueous extract

The leaves of the plant under study were obtained from the campus of Dr. Rafiq Zakaria College for Women, Aurangabad, India, adapting the method followed by Mehdi *et al.* [23]. The aqueous extract of the leaves of *P. guajava* plant was prepared by mixing 40 g of leaves powder and 400ml of distilled water. The mixture was stirred by a magnetic stirrer for 24 hours. Then, the mixture was filtered through four pieces of medical gauze and centrifuged at a speed of 3000 rpm for 10 minutes. After that, the mixture was placed in Petri dishes and dried in an oven at 40 C°. Finally, the extract was scraped and collected in clean glass vials and kept in refrigerator for use [24].

Alcoholic extract

The plant leaves powder (40gm) was placed in a Soxhlet apparatus. Then, 400 ml ethyl alcohol of 70% concentration was added and the mixture was left undisturbed. The solvent was then evaporated by using a rotary evaporator [25]. The mixture was finally placed in clean Petri dishes.

The source of the hydatid cyst

The hydatid cysts were obtained from the sheep of the butchery of Al-Basateen, the city of Aden, Yemen. The cysts were then transferred to the laboratories of the Faculty of Science, University of Aden, Yemen (Figure 1).



Figure 1- Hydatid cysts in liver

Collection of protoscolices

The method of Niazi *et al.* [26] was used to obtain the protoscolices. The hydatid cyst was sterilized twice with ethyl alcohol (70%) and the cyst fluid was removed by a sterile syringe. The cyst was washed internally with pH 7.2 and the antibiotic was penicillin IU20000 and streptomycin 1 g/l. The liquid was then discarded in test tubes and centrifuged at 3000 rpm. The protoscolices were examined under a light microscope.

Evaluation of the vitality of the protoscolices

The vitality of the protoscolices were estimated by using the method of Barzinji *et al.* [27]. 20 microliters of the protoscolices were placed on a clean glass slide, to which an equal volume of the aqueous eosin stain was added and examined under the microscope. Green protoscolices were counted as vital whereas red ones were counted as dead. The vitality of the protoscolices was taken into consideration because it is an important indicator of infection severity. The percentage of live protoscolices in the sample was calculated by dividing the number of live protoscolices to the total number of calculated headings and multiplying the result by 100. The process was repeated three times and the survival rate was extracted. The percentage of vital protoscolices was calculated after each exposure period.

Laboratory animals

In this study, albino rats *Rattus norvegicus* obtained from the laboratory of the Faculty of Science, University of Aden-Yemen, were used. They were grown and reproduced in the standard conditions of the animal house, being provided with water and food *ad libitum*. The food consisted of a concentrated mash of protein and dry milk.

Implantation of protoscolices in laboratory animals

In order to determine the *in vivo* effects of the plant extract on the vitality, growth, and development of the protoscolices of sheep origin [28], rats were injected with different mixtures of protoscolices and leaf extract. The concentration and the duration of treatment were determined based on the results of the *in vitro* screening of the effects of this extract on the protoscolices. The detailed procedure applied is as follows. Protoscolices of sheep origin were treated with the alcoholic extract of *P. guajava* at 100 mg/ml for 48 hours and then injected into the peritoneum of four rats (2000 protoscolices/rat). Similarly, protoscolices were treated with the aqueous extract of *P. guajava* (100 mg/ml) for 48 hours and then injected into the peritoneum of four rats (2000 protoscolices/rat). As a control group, protoscolices not treated with *P. guajava* extracts were injected into the peritoneum of four rats (2000 protoscolices/ rat).

Anatomical examination of rats

The rats that had been injected with the protoscolices of sheep origin were treated with the extracts under study after three months. The secondary hydatid cysts were investigated in the peritoneum, liver, lungs, kidneys and other parts of the body using a magnifying lens. Pictures were taken for the rats of the two groups.

Statistical analysis

The results of the present study were analyzed by Genstat® (Version 5.2) using general treatment structure (no blocking), factorial experiment, with 3 replicates. The least significant different (LSD) test was used to test the differences between means (groups) which were considered significant at $P \leq 0.05$.

RESULTS

Effects of alcoholic and aqueous leaf extracts of *P. guajava* on the protoscolices *in vitro*.

Through the results, a clear effect of the plant extracts of *P. guajava* leaves appears in killing the protoscolices. The results of the analysis of variance shown in Table 1 reveal significant differences between the concentrations and exposure periods at the probability level $P < 0.05$. The concentration of 100 mg/ml of both aqueous and alcoholic extract showed its superiority in reducing the vitality of the protoscolices to zero at 48 hours compared with the control group, which was vital at 96.33% and 96%, respectively. The concentration of 75 mg/ml followed as the second most effective. The lowest rate of killing of protoscolices was at the concentration of 50 mg/ml at 12 hours, where the rate reached in the aqueous and alcoholic extract to 8.33% and 9.33%, respectively, compared to the control group (93.33% and 94.33%, respectively). Also, the table shows that the exposure periods have exceeded 48 hours from the other periods. It was also noticed that when the concentration and the exposure period increases, the death-rate of protoscolices increases (Figures 2, 3). The lowest rate of killing of protoscolices was at the concentration of 50 mg/ml at 6 hours, which reached 9.33% and 8.33% using alcoholic and aqueous extracts, respectively, compared to the control group.

Table 1-Effect of the alcoholic and aqueous extract of *P. guajava* on the origin of sheep protoscolices *in vitro*

Extract	Concentration	Control	Time /Hour				Means
			6 hrs	12 hrs	24 hrs	48 hrs	
			M%	M%	M%	M%	
Alcoholic Extract	100mg/ml	96.00	18.00	35.33	66.00	100.00	63.07
	75mg/ml	95.33	14.33	24.00	49.00	97.67	56.07
	50mg/ml	94.33	9.33	16.00	36.67	90.33	49.33
Mean		95.22	13.89	25.11	50.56	96.00	56.15
Aqueous Extract	100mg/ml	96.33	16.33	33.33	61.00	100.00	61.40
	75mg/ml	95.33	11.67	23.67	45.33	95.00	54.20
	50mg/ml	93.33	8.33	16.00	31.67	87.33	47.33
Mean		95.00	12.11	24.33	46.00	94.11	54.31
Con*T	100mg/ml	96.17	17.17	34.33	63.50	100.00	62.23
	75mg/ml	95.33	13.00	23.83	47.17	96.33	55.13
	50mg/ml	93.83	8.83	16.00	34.17	88.83	48.33
Means of Time		95.11	13.00	24.72	48.28	95.05	
LSD 5%	EX=1.726, C=2.113, T=2.728, EX*C=2.989, EX*T=3.859, C*T=4.726, EX*C*T= 6.683.						
CV	7.4						

Least Significant Difference (LSD); EX (Extract); T (Time); C (Concentration); CV (Coefficient of Variation); M (Mortality).

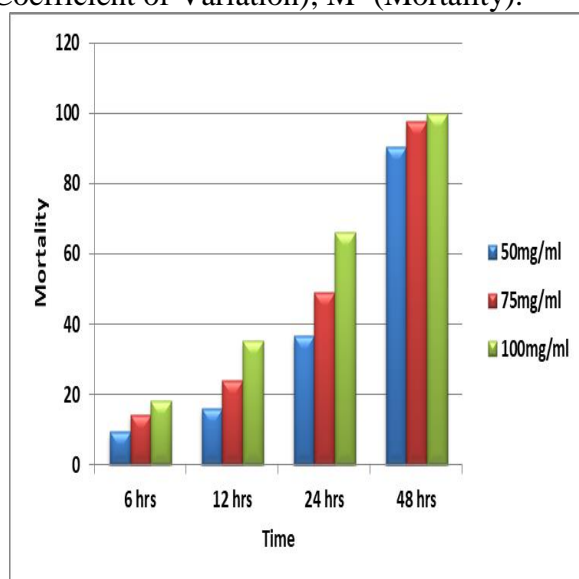


Figure 2- Effect of alcoholic extract of *P. guajava* on sheep origin protoscolices *in vitro*.

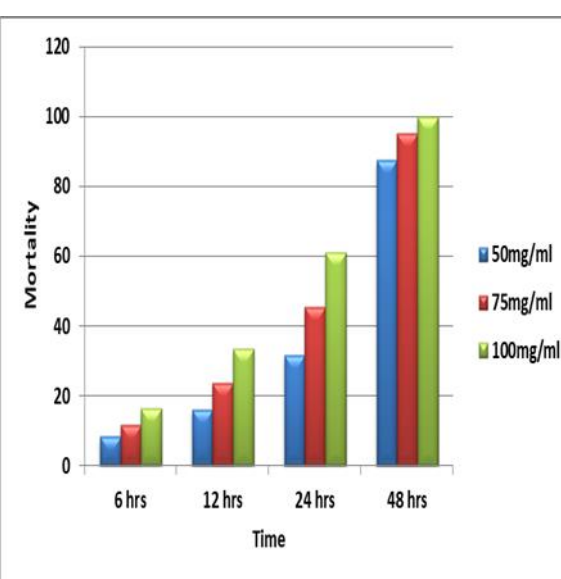


Figure 3- Effect of aqueous extract of *P. guajava* on sheep origin protoscolices *in vitro*.

Effect of the aqueous and alcoholic extracts of *P. guajava* on the protoscolices *in vivo*

After performing the above mentioned experiments related with the effects of alcoholic and aqueous extracts of *P. guajava* plant on the vitality of the protoscolices *in vitro* and the observation of the mortality rate of the protoscolices *in vitro*, the protoscolices treated with the extracts under this study were injected into the peritoneum of the laboratory rats to check the effect of these substances on the mortality of the protoscolices *in vivo*. Three months later, the rats were dissected to investigate the presence and growth of secondary hydatid cysts (Figures 4, 5). The hydatid cysts were clearly visible in the laboratory rats injected with the non-treated extracts using the substances used in this study, as compared to the control group (Figure 6).



Figure 4- Rat treated with alcoholic extract of *P. guajava*.

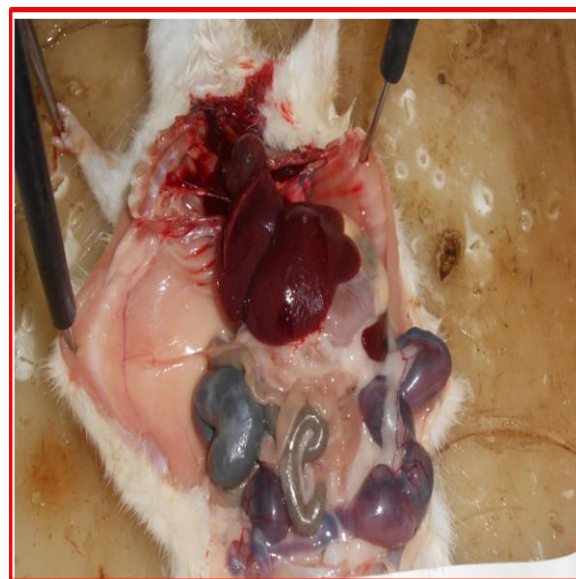


Figure 5- Rat treated with aqueous extract of *P. guajava*.



Figure 6-Control group.

DISCUSSION

The results of this study were similar to the results obtained by Mustafa in terms of time. When he used aqueous extraction of *Cyperus longous* plant at concentration of 20% after passing 48 hours, the protoscolices were killed [29].

In addition to that, these results are similar to the result obtained by Al-Hasnawi *et al.*, when they used the boiled broccoli extract at a concentration of 0.3 g/ml, as it led to the complete death of protoscolices of human origin after 168 hours of treatment [30]. The results of this study were superior to the results of the study conducted by Xing *et al.*, who used NaASO₂ at a concentration of 20 µM. They obtained a complete death rate for protoscolices after 6 days [31]. They also outperformed the results of a study conducted by Khalaf *et al.*, who used alcohol extract of *Cladophora crispola* at a concentration of 300 µg/ml that led to complete death of the protoscolices after 3 days of treatment [32].

In the previous studies, guava leaf acetone extraction showed killer activities against *Hippobosca maculate* fly adults [12]. Ethanol extraction of guava leaves inhibited the growth of *Trypanosome bruci* [15]. The leaf extract works as an anti-malaria agent due to its inhibitory activity, while guava oil works against Toxoplasmosis caused by the growth of *Toxoplasma gandhi* [13]. Guava leaf extracts were also used against trypanosomiasis and *Hymenolepis dimlnuta* in mice [33, 34].

The mortality rate of the protoscolices treated by *P. guajava* plant extracts can be attributed to its inclusion of active substances, such as alkaloids whose effect is a consequence of their reaction with the metabolic protein required for the vitality of the protoscolices. This leads to the destruction of the cell wall and its proteins till the protoscolices die [35]. The mortality rate of the protoscolices treated with aqueous and alcoholic extracts of *P. guajava* leaves can also be attributed to the tannins which may penetrate the cell membrane and block the active sites of some enzymes inside the cell, which are necessary for the growth of the parasites [36]. The death of the parasite can be due to phenol substance which has an effect on the acetyl cholinesterase enzyme that controls the flexibility and permeability of the cell membrane. Phenols make the membrane lose permeability and regulation for some substances, which results in passing of various toxic substances without regulation, leading to the parasite death [37]. The death of the parasites is attributed to flavonoids, which can reduce sugars, leading to a reduction of carbohydrate metabolism and thus a decrease in ATP [38].

CONCLUSIONS

Through the results obtained from the current study, the following can be inferred. The *Psidium guajava* extract has a clear effect in the reduction of the vitality of the protoscolices following *in vitro* treatment for a short period of time. Lack of growth of secondary hydatid cysts in the rats programmed with the protoscolices treated with the *Psidium guajava* extracts in this study suggests the ability of the plant extracts to kill the protoscolices completely.

Ethics approval

Institutional guidelines for the care and use of animals were followed. All procedures performed in the study and involved animals were in accordance with the ethical standards of the institution or practice at which the study was conducted (date 16/08/2018).

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Conflict of interest

There was no conflict of interest among the authors in presenting this article for publication.

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