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Article

Study of Hydatid Cyst Fertility: Effectiveness of Pineapple (Bromelain) and Garlic Extracts in Hydatid Cycst From Kirkuk Slaughterhouse

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Abstract: Cystic echinococcosis, due to the larval stage of Echinococcus granulosus, remains a major parasitic zoonosis worldwide and continues to impose significant public health and economic burdens in endemic areas. In this study, the in vitro efficacy of aqueous and ethanolic extracts of garlic (Allium sativum) and pineapple (Ananas comosus) against viable protoscoleces isolated from fertile hydatid cysts collected from naturally infected slaughtered livestock in Kirkuk Province, Iraq, was investigated. Protoscoleces of hydatid cysts that were viable (>95%) were exposed to four concentrations of the extract (10%, 25%, 50% and 75%) for 15, 30 and 60 min. The mortality rates were determined by the eosin exclusion test and median lethal concentrations (LC50) were calculated for comparison of the potency of the extracts. All the treatments showed marked protoscolicidal activity, which was time- and concentration-dependent. Among the preparations tested, the ethanolic garlic extract was the most effective, with the lowest LC 50 value (5.9 mg/mL) and 92.33 ± 2.52% mortality at 75% concentration after 60 min. The activity of aqueous garlic extract was found to be remarkable with 86.33 ± 2.1% mortality and LC50 of 8.8 mg/mL. Pineapple extracts had relatively lower but significant activity; ethanolic pineapple extract caused 79.33 ± 1.53% mortality with an LC50 of 14.4 mg/mL, while aqueous pineapple extract caused the least effect with 73.00 ± 2.65% mortality with an LC50 of 45.1 mg/mL. Overall ethanolic extracts were consistently better than aqueous extracts, suggesting better extraction of biologically active antiparasitic constituents. The results showed that both A. sativum and A. comosus have significant protoscolicidal effects, the garlic extracts being much more powerful than the pineapple extracts. The effectiveness of ethanolic garlic extract was higher which indicates that sulfur-containing phytochemicals could be promising for natural scolicidal formulations. These findings offer experimental support to investigate plant-derived bioactive compounds as alternative or supplementary agents for hydatid disease control and require additional phytochemical characterization, toxicity evaluation and in vivo validation.

Keywords: Cystic Echinococcosis, Echinococcus Granulosus Protoscoleces, Garlic Extract, Pineapple Extract, Protoscolicidal Activity

Introduction

From an evolutionary and historical perspective, cystic echinococcosis is one of the oldest known zoonoses. Hydatid cysts have been described in antiquity with distinct clinical descriptions identified in the Babylonian Talmud as well as in the medical treatises of Hippocrates in the 4th century B.C. and Galen in the 2nd century A.D. But the discovery of the biological nature of the parasite was not made until the 17th century, when Francesco Redi first suggested the parasitic origin of these fluid-filled structures. Then, in 1766, Pierre Simon Pallas proposed the first hypothesis that these cysts were the larval, intermediate forms of tapeworms, the foundation of modern helminthology [1].

From a taxonomic perspective, the causative organism is classified within the Domain Eukaryota, Kingdom Animalia, Phylum Platyhelminthes, Class Cestoda, Order Cyclophyllidea, and Family Taeniidae. The genus Echinococcus comprises several closely related species and genotypes. Among these, Echinococcus granulosus sensu stricto is recognized as the principal species responsible for sustaining domestic transmission cycles in most endemic regions worldwide [2].

Cystic echinococcosis (CE), also called hydatid disease, is a chronic zoonotic parasitic disease caused by the larval stage of Echinococcus granulosus sensu lato [3]. It is one of the most important neglected zoonotic parasitic diseases in the world; it is preventable but still a public health and animal production challenge. The disease is especially common in endemic areas where domestic dogs are kept in close contact with livestock. Humans become infected through accidental ingestion of parasite eggs excreted in the feces of infected canids. The main intermediate hosts responsible for maintaining the life cycle of the parasite and the continuous transmission of the infection are livestock, especially sheep and cattle [4;5].

After ingestion, parasite eggs release oncospheres that penetrate the intestinal mucosa and enter the bloodstream. These larvae are carried through the circulatory system to the various organs, the liver and lungs being the most common sites of settlement. The larvae develop into hydatid cysts over time. When these cysts are fertile, they contain viable protoscoleces, which are the infective stage for definitive hosts and play a fundamental role in maintaining the parasite's life cycle. Viable protoscoleces persistence is also associated with continued transmission, reinfection and emergence of secondary hydatidosis when cyst fluid is accidentally spilled during surgical removal or percutaneous treatment procedures [4-6].

Cystic echinococcosis is usually treated by surgery, percutaneous interventions and chemotherapy with benzimidazole drugs such as albendazole and mebendazole. While these treatment options are the standard of care, they each have significant limitations. Surgical procedures can cause the release of viable protoscoleces into the surrounding tissues causing a secondary infection. Long-term use of antiparasitic medications can lead to adverse effects and does not always lead to a consistent therapeutic success [7]. Thus, the search for effective protoscolicidal agents that can kill viable protoscoleces in a short time with good safety has been the focus of studies in recent decades [4].

In the last few years, natural products have been increasingly investigated as possible alternatives to conventional antiparasitic treatments. Medicinal plants are rich sources of bioactive compounds which can act against parasites through different mechanisms such as disruption of cellular architecture, inhibition of essential metabolic pathways and induction of irreversible cellular damage. These properties have resulted in numerous studies of plant-derived extracts for possible protoscolicidal activity against protoscoleces of Echinococcus granulosus [8].

The garlic (Allium sativum L.) has been used due to its different pharmacological properties among all other medicinal plants studied. The main biological activity of this plant is provided by its constituents, such as allicin, and other organosulfur compounds, which have demonstrated their high antimicrobial and antiparasitic activity in experiments. The compounds are believed to act by disrupting the membranes of parasites and impairing their essential biochemical processes needed for survival; therefore, garlic is considered a viable natural source to control the parasites [9].

Pineapple (Ananas comosus L.) has also been studied as a rich source of bioactive compounds of natural origin. In particular, the presence of bromelain, an enzymatic complex of mainly cysteine proteases and several other associated enzymes, makes it a viable candidate for study. Bromelain has attracted the attention of many researchers due to its wide range of biological activities, such as anti-inflammatory, antimicrobial, antioxidant, and therapeutic properties. The enzymes' proteolytic

capability is responsible for degrading a variety of protein substrates and biological tissues and is believed to be one of the mechanisms that may contribute to the antiparasitic activity of the enzyme [10]. Pineapple is also rich in phenolic compounds and other phytochemicals that could further boost its biological activity in a complementary and synergistic manner [10].

The biological activity of plant extracts is highly dependent on the method of extraction, particularly on the solvent used. The polarity of the solvent determines the recovery of bioactive constituents. Ethanol generally extracts many important secondary metabolites of pharmacological interest better than water and often gives extracts with higher biological and antiparasitic activity. Thus, the evaluation of both aqueous and ethanolic extracts is important to know which extraction strategy is the most effective and to maximize the recovery of compounds responsible for the protoscolicidal activity [10].

The rationale for choosing garlic and pineapple in this study was based on differences of their major bioactive constituents and mechanisms of biological effects. Garlic is well known for its sulfur containing compounds and pineapple for its proteolytic enzyme complex, especially bromelain. Comparison of these two medicinal plants, differing both in botanical origin and in chemical composition, may provide valuable insight into the relative protoscolicidal potential of sulfur-based phytochemicals and proteolytic enzymes as natural antiparasitic agents [11].

On this basis, the present study aimed to evaluate and compare the in vitro protoscolicidal activity of aqueous and ethanolic extracts of garlic (Allium sativum) and pineapple (Ananas comosus). Viable protoscoleces were collected from fertile hydatid cysts of slaughtered livestock in Kirkuk province, Iraq. The protoscolex mortality and the median lethal concentration (LC50) were used to assess the efficacy of each extract, thus allowing a comparative assessment of their protoscolicidal efficacy.

Materials and Methods

Ethics Statement and Sample Collection

The hydatid cysts were recovered from naturally infected cattle and sheep at the Kirkuk Central Slaughterhouse (Municipal Abattoir) from the lungs and liver tissues of the animals during the routine postmortem examination of their abattoir carcases. Importantly, no animal has been killed on the premises of this research; all animals had already died, and the waste materials were taken from animal remains that were being processed for more common commercial purposes under the official regulatory framework.

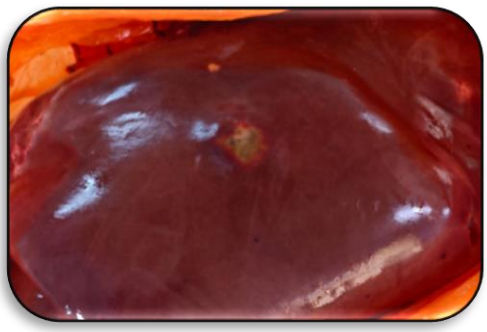


Figure 1. Hydatid cyst recovered from an infected livestock organ at Kirkuk Central Slaughterhouse.

When recognized infected organs, intact hydatid cysts were carefully dissected under aseptic conditions and transferred to sterile containers filled with physiological saline solution (0.9% NaCl). These specimens were kept at 4°C and transported using insulated ice containers to the Parasitology Laboratory, College of Veterinary Medicine, University of Kirkuk. All materials were processed within 2 hours after harvest and ensured that the sample was viable and intact.

Upon arrival in the laboratory, all downstream procedures were done under sterile conditions. The aspiration of hydatid cyst fluid was carried out using sterile disposable syringes, and it was then placed in sterile test tubes. The cyst that was filled with this protoscolex-rich fluid was immediately

treated to assess the fertility of the cysts, the degree of viability of the protoscoleces, and the activity that the fluid exerted on the protoscoleces. These steps were performed quickly to reduce the contamination risk and maintain sample quality, helping to maintain baseline reliability of the biological material during all of the subsequent parasitological assays [12;13].



Figure 2. Representative hydatid cyst specimens collected from naturally infected livestock.

Aseptic Processing and Determination of Cyst Fertility and Viability

The outer surface of each hydatid cyst was swabbed with 70% ethanol before puncturing in order to overcome the risk of cross-contamination. The sterile laminar airflow working area was used to withdraw the fluid containing the protoscolecia and place it in sterile centrifuge tubes. Concentration of the fluid was made by sedimentation or by centrifugation at 3000 rpm for 5 minutes and careful decanting of the supernatant. The one remaining pellet was washed with sterile phosphate-buffered saline (PBS; pH 7.2–7.4) three times to remove the cellular debris and fluids from the pellet. These were then purified targets that were stored under refrigerated conditions awaiting experimental use.

Cysts were microscopically screened, and the number of free or invaginated protoscoleces along with brood capsules was used to determine the cyst fertility. Cysts with alive protoscoleces were considered fertile, while all those without protoscoleces or with only necrotic remains were rejected as sterile. A 0.1% aqueous eosin solution was used for eosin exclusion to assess the baseline viability. The dye was mixed with 10 μL aliquots of the suspension of protoscolex (equal volume in both samples) on a clean glass slide. The mixture was set aside for 5 minutes and then looked at under a light microscope at 400x magnification. The dye was not taken up by live protoscoleces, which have intact and selectively permeable membranes and differentiated based on the normal morphology and the active kinetics of the flame cells. The dead protoscoleces, however, took the stain up because of membrane rupture, they were pink or red. The percentage of total viability was determined by the ratio between the number of viable organisms and the total number that were counted.

$$\text{Viability } (\%) = \frac{\text{Number of viable protoscolices}}{\text{Total number of protoscolices}} \times 100$$

The principle of this assay is based on the integrity of cell membranes, which is a well-established and reliable measure of protoscolex viability. Living cells have intact membranes that actively exclude penetration of eosin into the cytoplasm. On the other hand, any disruption to the stability of the membranes, be it due to senescence or caused by protoscolicidal treatment, will allow the entry of the dye, which will then enter the cell to bind to the mitochondria. There is, therefore, uniform staining of non-viable protoscoleces, which is clearly visible for the separation of viable from non-viable parasites. Only fertile cysts with a base value protoscolex viability rate ≥ 95% were selected for the following experimental stages to guarantee the experimental stringency [14].

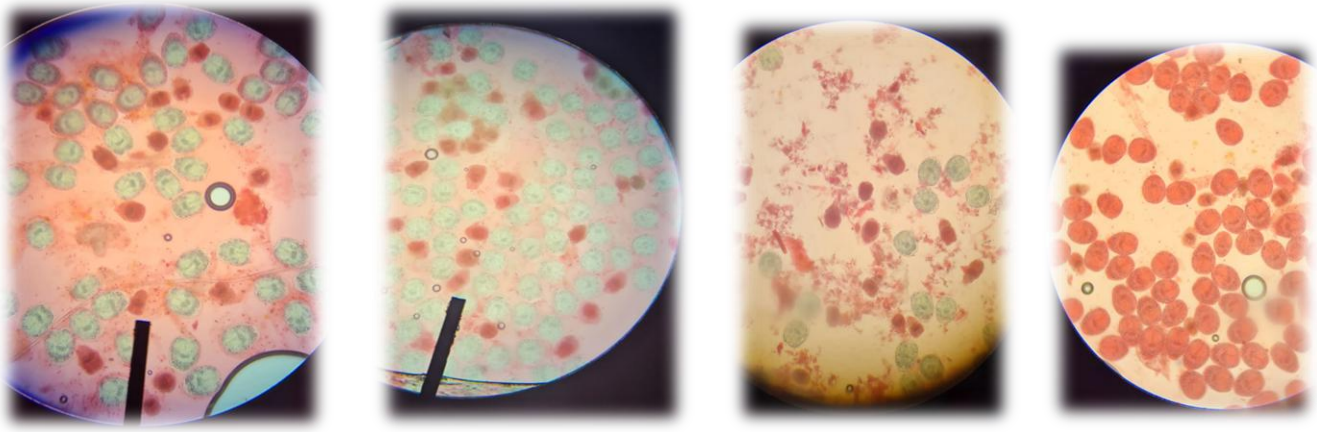


Figure 3. Microscopic appearance of hydatid cyst protoscoleces during viability assessment.

Preparation of Plant Extracts

Preparation of Aqueous Garlic Extract (Allium sativum)

In order to perform experimental processing, fresh garlic (Allium sativum) bulbs were previously botanically authenticated before arriving in the laboratory from local markets in Kirkuk Province. Peeled cloves (around 150g) were carefully washed with distilled water to remove surface impurities and homogenized in a sterile mechanical blender. The mixture was filtered through four layers of sterile muslin cloth and then centrifuged at 4500 rpm for 20 minutes to sediment any other impurities. The supernatant after the original extraction was filtered through Whatman No. 1 filter paper to remove any remaining particulate matter and obtain a completely clear crude extract. The recovered filtrate was the 100% crude aqueous garlic extract and kept at 4°C up to the time of experimental application [14].

Preparation of Ethanolic Garlic Extract (Allium sativum)

Garlic cloves (peeled) were finely sliced and then put into a hot-air oven set at 60°C and dehydrated for 48 hrs. After complete drying, the botanical material was ground into a fine powder and then used in the ethanolic extraction process using 96% ethanol. Two hundred milliliters of ethanol were added to a Soxhlet apparatus containing 10 g of the garlic powder, which was processed at 60°C, and then the solvent was removed post-extraction in a Soxhlet extract evaporator using ethanol as an evaporating agent at reduced pressure at 45-50°C temperature, selected taking into account the thermal stability of the garlic extract and to prevent any damage that may happen to the biologically active organosulfur derivatives present in the extracts. The concentrated mass left over was then evaporated to dryness and redissolved in 1% dimethyl sulfoxide (DMSO) for greater solubility and ease in compounding the stock doses to be used in the experiments [15].

Preparation of Aqueous Pineapple Extract (Ananas comosus)

Fruits of the pineapple (Ananas comosus) were collected from local markets, washed with distilled water, and cut into small pieces. Fruit material was homogenized using sterile distilled water at a 20:200 ratio (20 g of tissues/200 mL of sterile distilled water). Homogenate was stored under refrigeration (4°C) for 24 hours to retain bromelain activity and other heat-sensitive bioactive compounds. It was then passed through sterile gauze and centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was then filtered through Whatman No. 1 filter paper till a clear filtrate was obtained. The filtrate was served as a crude aqueous extract of pineapple (100%) and stored in the refrigerator at 4°C for further use [16].

Preparation of Ethanolic Pineapple Extract (Ananas comosus)

Fresh pineapple pulp was homogenized with a mechanical blender and mixed with 96% ethanol at a 1:1 (w/v) ratio. Pineapple (Ananas comosus) fruits were carefully washed with distilled water and then sliced into pieces to get the fresh and mature ones from local commercial markets. This plant tissue was homogenized with sterile distilled water in a fixed ratio of 20 g of plant tissue: 200 mL of sterile distilled water. To reduce possible degradation of thermolabile bioactive components such as bromelain, the homogenate was stored for 24 hours at 4°C. This incubation was followed by sterile

gauze passing through and centrifuged at 3000 rpm for 10 minutes. Subsequently, the isolated supernatant was filtered through Whatman NO. 1 filter paper for obtaining a completely clear filtrate. The last preparation, which is 100% crude aqueous pineapple extract, was stored at 4°C prior to the experimental deployment ([17].

Preparation of Experimental Concentrations

The stock solutions of 100% crude ethanolic and aqueous solutions of A. sativum and A. comosus were used as a baseline. These stocks were then serially diluted to obtain four specific working concentrations, 10%, 25%, 50%, and 75%, which were used for the experiments immediately. The aqueous cohorts were administered in sterile distilled water, while the ethanolic cohorts were diluted with 1% dimethyl sulfoxide (DMSO) for optimal compound solubility and bioactive metabolite uniformity. All working dilutions were freshly prepared before every experimental run to ensure that degradation does not occur and maximum biological potency of the compound remains.

Extensive control systems were developed and included in the design to validate the experimental results. A positive reference standard, albendazole, was chosen. The negative control arm, on the other hand, had two different groups: physiological saline and 1% DMSO in physiological saline. This is because these baseline groups eliminate all possible confounding biological effects due to the vehicle or solvents and ensure that the observed protoscolicidal activity is solely attributed to the botanical extracts being tested [14;17].



Figure 4. Preparation of aqueous and ethanolic plant extracts under laboratory conditions.

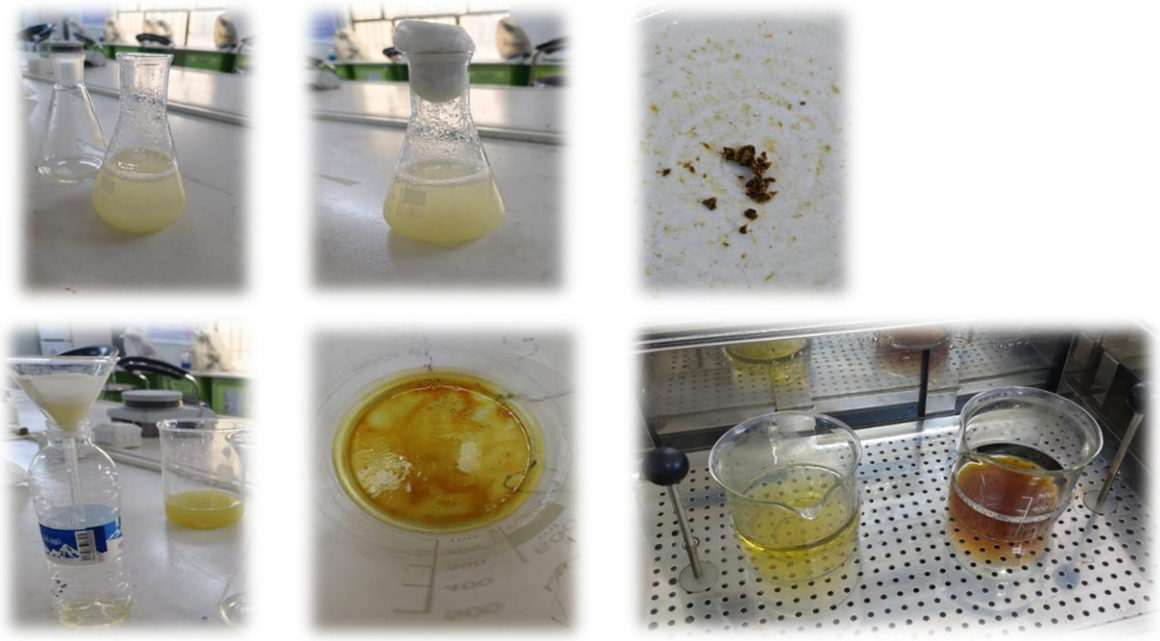


Figure 5. Laboratory extraction, filtration, and preparation of the plant formulations.

In Vitro Protoscolicidal Assay

The ability of the botanical ingredients to kill protoscoleces was tested in vitro against viable Echinococcus granulosus protoscoleces. A standardized suspension (around 1,000 viable organisms/mL) was prepared and divided into sterile test tubes and used under strictly sterile conditions. An equal volume (0.5 mL) of this parasite suspension was added to each of the designated extract dilutions (10%, 25%, 50%, and 75%), giving a final reaction volume of 1.0 mL per test tube. All experimental matrices used involved freshly compounded solutions in order to preserve stability and reproducibility of the active secondary metabolites.

The reaction tubes were incubated in total darkness at 37° in conditions most favorable to the occurrence of photolytic degradation of the phytochemical constituents and were exposed to daylight at regular intervals, 15, 30, and 60 min. The time was optimized within 60 minutes, which allowed the determination of the median lethal concentration (LC50) of different bioactive compounds and was appropriate for the destructive interaction between the bioactive compounds of the plant and the parasite's membrane architecture in line with the already established in vitro helminthological screening models.

$$\text{Mortality (\%)} = \frac{\text{Number of dead protoscolices}}{\text{Total number of protoscolices}} \times 100$$

At the end of each exposure interval, the reaction suspensions were either briefly centrifuged or allowed to naturally settle to allow for careful decanting of the supernatant. The isolated protoscoleces were then washed twice with sterile phosphate-buffered saline (PBS) to remove any extract that may have been left over from the process, which would interfere with the following viability tracking. The survival and mortality dynamics that occurred were quantified using the 0.1% eosin exclusion protocol, where an aliquot of the parasite suspension was mixed with eosin stain before being examined by light microscopy. For each technical replicate, a minimum of 100 individual protoscoleces from randomly selected microscopic fields were scored to ensure the statistical stringency.

Live protoscoleces were excluded from the dye and did not take up the dye, confirming intact cellular membrane integrity. Dead parasites, however, absorbed the eosin after breaking down the membrane and showed up as a red color. The total mortality percentage (scolicidal efficiency) was determined as the ratio of dead organisms to the total number counted in the population, using a quantitative viability formula that was traditionally applied.

Data reliability and trends that are reproducible were achieved by performing each assay a minimum of three times in three separate experimental trials (N=3). The overall mortality data generated by the tested extracts were plotted, and each extract's mortality dose- and time-dependent curve was mapped by comparing the mortality percentages across the different concentration matrices and the different time intervals to map the extracts [12;13].

Mathematical Estimation of Median Lethal Concentration (LC50)

The median lethal concentration (LC50) was then determined for each botanical treatment to produce a quantitative measure of the treatment's toxicity that could be compared to the others. This is the specific concentration of the extract needed to kill 50% of the E. granulosus protoscoleces after 60 minutes of exposure is used as the benchmark of the most effective time for the protoscoleicidal activity. The empirical dose–response mortality percentages observed at the four experimental doses for the LC50 thresholds were used in a standard linear interpolation model. This could be demonstrated as shown below:

$$LC_{50} = C_1 + \frac{(50 - M_1)(C_2 - C_1)}{(M_2 - M_1)}$$

Where:

- C_1: the highest concentration producing mortality below 50%

- C_2: the lowest concentration producing mortality above 50%
- M_1: the observed mortality percentage at C_1
- M_2: the observed mortality percentage at C_2

Using this interpolation model assures a reliable assessment of LC50 values, which will provide a consistent pharmacological background for the comparison of the relative protoscolicidal ability of aqueous and ethanolic preparations of A. sativum and A. comosus. Finally, such calculated LC50 values allowed for a direct and stringent comparison of the anti-parasitic activity among the different tested formulations in full uniformity of the experimental parameters [13].

Statistical Analysis

Analytical treatment and evaluation of all the empirical data obtained from the protoscolicidal bioassays were performed by the use of a Statistical Package for the Social Sciences (SPSS), version 26.0. Quantitative results were obtained and expressed as mean ± standard deviation (SD) of the average of three independent experiment trials. Before initiating the hypothesis testing, it was determined that all of the metrics being used for the percentage mortality and viability fulfill the necessary statistical assumptions and stabilize variance.

Analysis of variance (ANOVA) models were used to identify the differences between treatment groups and to calculate the gradients and intervals of exposure. Either one-way or two-way ANOVA was used for interaction mapping in various variables depending upon the design matrix. If significant differences are identified among the groups, Tukey's Honestly Significant Difference (HSD) post hoc analysis is used to pinpoint differences between specific groups. $P < 0.05$ was used as the criterion for statistical significance.

Diagnostic imaging is the primary diagnostic and clinical aspect of a diagnosis of CE. The World Health Organization [5], recommended standard guidelines for ultrasonography (US) use, which have provided the accepted gold standard in epidemiological screening and field classification, especially when conducted within these guidelines. Magnetic Resonance Imaging (MRI) can provide higher diagnostic resolution than Computed Tomography (CT) with superior visualization of the various elements of internal cysts, namely, the daughter cysts, the integrity of the membrane, and the characteristics of the hydatid fluid for more detailed preoperative evaluation. Such diagnostic accuracy facilitates pre-surgical planning and a fast clinical decision-making process. Finally, these imaging modalities gave clinical context to the characterization of cystic echinococcosis [18], while rigorous statistical modeling provided a reliable interpretation of the in vitro data.

Results

Distribution of Hydatid Cysts

Sheep and cattle were slaughtered in the Kirkuk slaughterhouse, and then the hydatid cysts were obtained. Most cysts were found in the liver and lungs, which were the most infected organs. Samples collected comprised fertile, sterile, and calcified cysts, with differences in the distribution of the cysts among the examined animals. The liver was the most frequently affected organ, and the largest number of hydatid cysts was obtained from it. Table 1 gives the distribution of cysts by species of animal, organ affected, and cyst status as follows:



Figure 6. Hydatid cysts observed in the affected organs of slaughtered livestock.

Table 1. Distribution of hydatid cysts collected from infected livestock

Sample No.	Date	Species	Age	Sex	Infected organ	Total cysts	Fertile cysts	Infertile cysts	Calcified cysts
1	1\29	caw	3y	Female	Liver	4	Zero	3	**1**
2	2\3	Caw	2.5y	Female	Liver	84	60	10	**14**
3	2\8	Caw	3y	Female	Lung	7	Zero	7	**Zero**
4	2\16	Caw	2y	Female	Liver	16	Zero	9	**7**
5	2\22	Sheep	2y	male	Liver	32	20	7	**5**
6	3\15	Caw	1.9y	Female	Liver	18	Zero	12	**6**
7	4\19	caw	4y	Female	Liver	15	Zero	5	**10**
8	4\21	sheep	1.5y	Female	Liver	38	18	11	**9**

Protozoological Activity of Aqueous Garlic Extract

Applied as an aqueous garlic extract, the protoscolicidal activity was really high and was enhanced with the increasing concentration and exposure time. In the present context, the lowest mortality was seen at the concentration of 10%, while higher concentrations resulted in a progressive rise in the mortality of protoscolex. The lowest level was noted at 75% concentration at 60 minutes after the treatment, with a mortality of 86.33 ± 2.1%. Furthermore, garlic extract aqueous proved to be a potent inhibitor of the viable protoscoleces of hydatid cysts, with the LC50 value being 8.8 mg/mL (Illustration in Table 2).

Table 2. Protoscolicidal activity of aqueous garlic extract at different concentrations and exposure times (LC50 = 8.8 mg/mL)

Con	15 min	30 min	60 min
10	20.33±1.53	32.33±2.52	**51.33 ±1.53**
25	32±1.73	52.67 ±2.31	**71.33 ±1.53**
50	48.67±1.53	66±2	**81±2.64**
75	60.33±2.52	80.67 ±1.53	**86.33 ±2.1**

Data were analyzed using a two-way analysis of variance (ANOVA) to evaluate the main effects of aqueous garlic extract concentration, exposure time, and their interaction on the mortality rate of protosciences . The results revealed a statistically significant interaction between concentration and time $(F_{(6, 24)} = 10.62, P < 0.0001)$, indicating that the concentration dependent lethal efficacy against protosciences was significantly influenced by the duration of exposure .

Protozoological Activity of Ethanolic Garlic Extract

The ethanolic garlic extract completely eradicated protoscoleces at all concentrations and exposure times compared to the aqueous garlic extract. Overall, the more concentrated the extract was and the longer the incubation time, the higher the mortality was. The 92.33 ± 2.52% highest mortality was obtained at 60 minutes using the 75% extract. A similar finding was observed with the ethanolic extract of the plant, where it gave the lowest LC50 (5.9 mg/mL), which is an indication of its strong protoscolicidal effect (as presented in Table 3).

Table 3. Protoscolicidal activity of ethanolic garlic extract at different concentrations and exposure times (LC50 = 5.9 mg/mL)

Con	15 min	30 min	60 min
10%	30±1	48.33 ±2.52	**56.67 ±1.15**
25%	47±1.73	65.67 ±2.52	**71.33 ±1.53**
50%	56.33 ±1.53	70.33 ±2.52	**79.67 ±1.53**
75%	73.33 ±2.52	82.67 ±2.1	**92.33 ±2.52**

Statistical evaluation using "two-way ANOVA revealed a significant interaction between concentration and time ($F_{(6, 24)} = 4.44$, $P = 0.0037$) , confirming that prolonged exposure significantly enhances sporicidal efficacy when higher concentrations of the extract are used.

Protozoological Activity of Pineapple Extracts

The concentration and exposure time increased in both extracts from pineapples, along with an increase in the mortality rate of viable protoscoleces. Higher concentrations and longer exposure times lead to a higher mortality rate of viable protoscoleces in both the aqueous and ethanolic extracts of pineapples. The activity of the aqueous extract was moderate with a maximum mortality of 73.00 ± 2.65% after 60 minutes of treatment at 75% concentration and an LC50 value of 45.1 mg/mL.

The ethanolic extract was more effective under all the tested conditions as compared to the other extract. Also, it had the lowest exposure time of 79.33 ± 1.53%, which was the highest mortality rate of all the treatments and the lowest LC50 value of 14.4 mg/mL. The results indicate that the use of ethanol in pineapple extraction yields superior overall efficacy (Tables 4 and 5) compared to the other solvents due to the extraction of more active ingredients with this solvent, which provides the protoscolicidal effect.

Table 4. Protoscolicidal activity of aqueous pineapple extract at different concentrations and exposure times (LC50 = 45.1 mg/mL)

Con	15 min	30 min	60 min
10%	13.33 ±2.1	19.33 ±1.53	**27.67 ±2.1**
25%	22.33 ±2.52	31±2	**40.33 ±1.53**
50%	36.67 ±2.52	44.67 ±3.512	**52.33 ±1.53**
75%	51.33 ±1.53	64±2.65	**73.00±2.65**

Two-way ANOVA analysis showed that both concentration ($F_{(3, 24)} = 535.63$, $P < 0.0001$) and exposure time ($F_{(2, 24)} = 160.60$, $P < 0.0001$) significantly affected the scolicidal activity of the aqueous pineapple extract, However, no statistically significant interaction was observed between concentration and time ($F_{(6, 24)} = 1.65$, $P = 0.1772$) indicating an independent additive effect of both concentration and duration .

Table 5. Protoscolicidal activity of ethanolic pineapple extract at different concentrations and exposure times (LC50 = 14.4 mg/mL)

Con	15 min	30 min	60 min
10%	23.33 ±1.53	32.67 ±2.1	**45.33 ±1.53**
25%	38.67 ±2.52	54.33 ±2.1	**61.33 ±1.53**
50%	46.33 ±1.15	59.00±2	**66.67 ±2.52**
75%	48.33 ±1.53	62.33 ±2.52	**79.33 ±1.53**

For the ethanolic pineapple extract two-way ANOVA revealed highly significant main effects for both concentration and exposure time , a significant interaction between concentration and exposure time was also observed ($F_{(6, 24)} = 6.58$, $P = 0.0003$) highlighting a synergistic enhancement of protoscolicidal efficacy over time at higher concentrations.

Comparison of LC50 Values

A comparison of LC50 values revealed that there was a significant difference among the potencies of the plant extracts in terms of their protoscolicidal contents. The ethanolic garlic extract, however, proved to be the most effective with the lowest LC50 of 5.9 mg/mL, followed by the aqueous garlic extract with an LC50 value of 8.8 mg/mL. This ethanolic pineapple extract exhibited moderate activity, as indicated by the LC50 value of 14.4 mg/mL, while the aqueous pineapple extract is the least effective, with the highest LC50 value of 45.1 mg/mL. Overall these results indicate the potency of garlic extracts as compared to pineapple and that ethanolic extract is more active than aqueous extract in terms of protoscolicidal activity (Table 6).

Table 6. Median lethal concentrations (LC50) of tested garlic and pineapple extracts

Extract	LC50 (mg/mL)
Ethanolic garlic extract	5.9
Aqueous garlic extract	8.8
Ethanolic pineapple extract	14.4
Aqueous pineapple extract	45.1

Effect of Albendazole

Albendazole was very effective in destroying the protoscoleces, and its activity enhanced as the concentration and exposure time increased. Adverse effects were consistently seen with higher concentrations and with longer treatment times. The results of detailed mortality analysis following albendazole treatment are provided below.

Table 7. Protoscolicidal activity of albendazole at different concentrations and exposure times

con	15 min	30 min	60 min
10 %	16±2.65	21±2.65	**27.67 ±1.53**
25%	23.67 ±1.53	28.67 ±2.1	**45.33 ±0.58**
50%	59±2	88.33 ±2.1	**94.33 ±1.53**
75%	45.33 ±2.1	48.33 ±1.53	**69.33 ±1.53**

The effect seen in the positive control group is consistent and in line with the well-established pharmacological action of albendazole. Albendazole is a benzimidazole agent that interferes with the ability of parasites to form microtubules through binding to parasite β-tubulin. The interference also interferes with important cell functions, particularly in glucose uptake, rendering the protoscoleces' glycogen stores depleted. It leaves a person unable to make use of their energy and ultimately leads to cell deterioration and the death of parasites.

The comparable scolicidal activity of the extracts from the plants used in this study indicates that these plant extracts contain different but probably synergistic active compounds that affect the parasites. The impairments can be at several biochemical targets that influence key metabolic pathways.

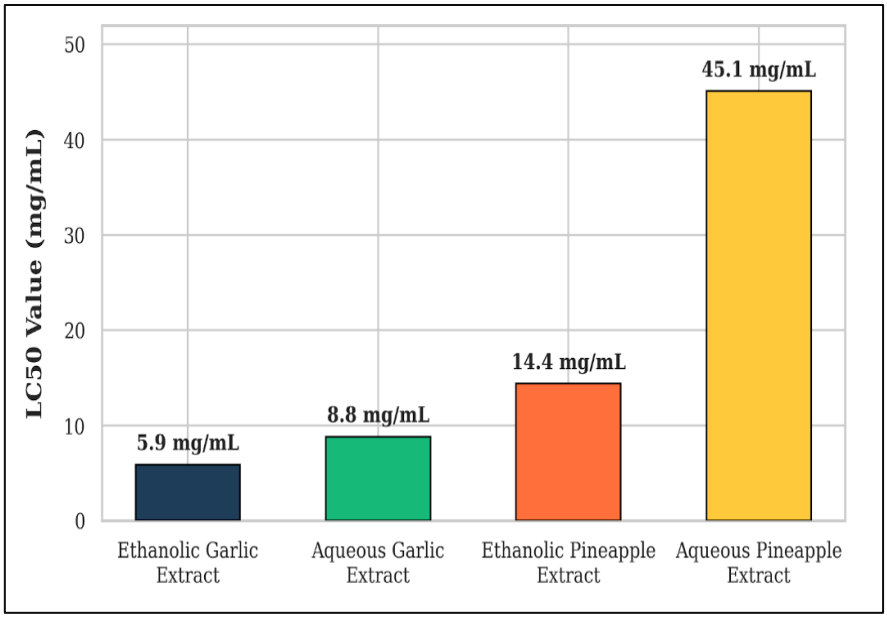


Figure 7. Median lethal concentration (LC50) of garlic and pineapple extracts against Echinococcus granulosus protoscoleces after 60 minutes.

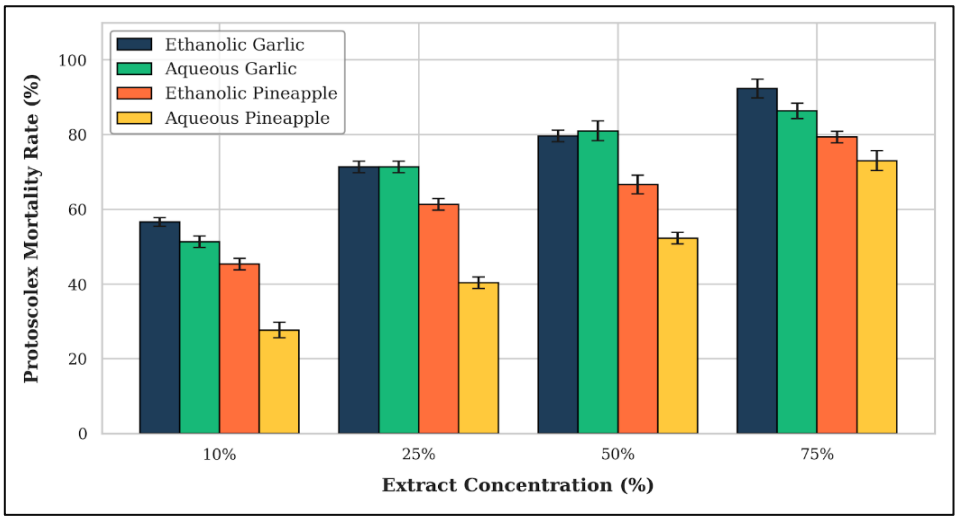


Figure 8. Protoscolicidal mortality rates of garlic and pineapple extracts at different concentrations after 60 minutes.

Discussion

The present study demonstrates the measurement of the protoscolicidal activity of both Allium sativum (garlic) and Ananas comosus (pineapple) extracts on viable hydatid cysts' protoscoleces in vitro. For example, the mortality was shown to increase with the increase in dose of extract and exposure time in all the tested groups, which is a clear dose- and time-dependent effect. This implies that increased exposure could increase the effect of the protoscoleces and the compound derived from the plants, leading to the progressive damage of the structures and functions of the parasites, eventually causing their death.

Perhaps most important of all, garlic extracts had a definite therapeutic advantage over the pineapple alternatives. The increased protoscolicidal efficacy was observed to occur with both aqueous and ethanolic garlic, as both yielded a higher mortality rate and lower LC50 values. This bioactivity is mainly due to the large amount of organosulfur derivative compounds in garlic, such as allicin. Very active molecules, which easily bind with enzymatic sulfhydryl groups, thus destroying the membrane and causing metabolic collapse. As a result, the marked loss of viability of protoscoleces reported here is due to irreversible changes in their structure and the alteration of biochemical pathways that play a fundamental role in the survival of the parasite.

Of the formulated extracts evaluated, the ethanolic extract of garlic proved to be the most effective protoscolicidal extract with the lowest LC50 (5.9 mg/mL). The high efficiency is indicative that ethanol is a better solvent for the extraction of the bioactive compounds in charge of the anti-parasitic activity. Ethanol, unlike water, can effectively dissolve a wider range of phytochemicals, including those that are of intermediate polarity, which are not normally recovered in water. This finding means it is likely that the ethanolic preparation contains a higher concentration and a wider variety of sulfur-containing metabolites, which is why it is likely to have the greatest effect. Furthermore, its robustness in all exposure periods illustrates the critical importance of the type of extraction method used for the botanical antiparasitics.

At the same time, aqueous garlic extract showed very promising anti-parasitic activity with an LC50 value of 8.8 mg/mL. Although the ethanolic extract exhibited lesser potency, it still demonstrated significant bioactivity, and the water-soluble components remain an important factor in suppressing parasites. The different performance of the two solvents highlights the importance of solvent choice in extracting phytochemicals from garlic and underscores the need for this garlic to be broadly effective, with an array of bioactive classes acting synergistically.

Extracts of pineapple still showed satisfactory inhibitory activity against viable protoscoleces but were not as effective as garlic. Bromelain is a complicated system of proteolytic enzymes that has a strong effect against parasites. This seems to be the main effect of this product. Bromelain is a complex proteolytic enzyme system that has the ability to hydrolyze a variety of protein substrates and is

thought to account for much of the antiparasitic activity of this product. The gradual loss of viability of protoscoleces is probably caused by the degradation of necessary cellular targets such as proteins, which are important for the structure and metabolism of the parasite. In addition to this enzymatic component, pineapple's secondary metabolites (phenolic compounds) seem to provide additional bioactivity. Both increasing doses and longer exposure times resulted in the increased mortality of parasites, suggesting a cumulative effect of these components with continued exposure and decreasing viability.

Both the pineapple formulations showed advantages when compared to each other, but the ethanolic extract proved to be more advantageous than the aqueous extract. This excellent protoscolicidal activity shown by the ethanolic preparation was also reflected in a significantly lower threshold (LC50 = 14.4 mg/mL) than the aqueous one (LC50 = 45.1 mg/mL). This discrepancy is probably due to the ability of ethanol to sequester phenolics, flavonoids, and other bioactive metabolites. Although water has partial dissolution of bromelain, ethanolic extraction of the plant seems to have the optimum recovery of complementary components. The combined effects of these secondary constituents in tandem with the action of the proteolytic enzymes can then further enhance the overall anti-parasitic activity, demonstrating that the processing conditions are key in the biological expression of botanical products.

The LC50 measurements were quantitatively assessed, and then a clear hierarchy was created for the relative toxicities of each of the treatments evaluated. The efficacy was consistently in the order: ethanolic garlic extract > aqueous garlic extract > ethanolic pineapple extract > aqueous pineapple extract. Both types of plants have obvious protoscolicidal activities, but garlic, under these laboratory conditions, proved to be a much more effective plant. The lower LC50 values for the garlic formulations show much lower doses required to kill parasites compared to the pineapple formulations, indicating a higher intrinsic anti-parasitic activity of garlic formulations in killing hydatid cyst protoscoleces.

The outcomes are dose-dependent and are practically relevant when developing drugs. A side benefit of an optimal protoscolicidal candidate is that it should be able to kill parasites quickly at a low concentration with excellent safety. The rather high mortality rate induced by garlic, particularly ethanolic, makes it a promising natural extract for developing its further uses in the field of hydatid disease management. Importantly, however, these trends were observed under in vitro control conditions and should be considered in the context of some early in vitro testing.

It is important the limitations in the study be recognized. The results obtained in this study were in an in vitro environment and could vary from those in the complex in vivo environment. In addition, there is no in-depth phytochemical analysis that allows the exact isolation of the metabolites responsible for this antiparasitic success. Future research studies should thus focus on detailed chemical profiling, ultrastructural evaluation of treated parasites, and in vivo studies for the understanding of the mechanisms of development and evaluation of true clinical or veterinary viability.

To sum up, both garlic and pineapple showed significant bioactivity on the hydatid cysts' protoscoleces, with the ethanol extract of garlic being the most effective extract with the lowest LC50 and highest mortality rate. In summary, these findings further support the wealth of literature that supports the use of plant compounds as an alternative or supplement to current protoscolicidal medications.

These findings are similar to those of [19;5], analyzed a methanolic extract of A. sativum against E. granulosus. They killed 100% of the cells within 10 minutes at a concentration of 50 mg/mL; they also completely cleared the cells within 60 minutes at a lower concentration of 25 mg/mL, with a high concentration effect. The ethanolic garlic extract in this study was able to achieve a 92.33% ± 2.52% mortality rate after 60 minutes, while its calculated LC50 is 5.9 mg/mL.

The different killing velocities and effectiveness of the two data sets are probably due to differences in extraction methods and solvents used. The high polarity and the high dielectric constant of methanol could perhaps be better for the isolation of low-molecular-weight organosulfur elements and for organole polarity-related phytochemicals, leading to a rapid disruption of the protoscolex membrane. In contrast, [19] used a high-concentration, rapid-acting model formulated in a methanolic solution for immediate surgical application; this study expands the niche of investigation to ethanolic solution models with lower LC50 values and yet is highly effective. Moreover, the incorporation of

Ananas comosus (bromelain-based) data results in a broader comparative matrix for botanical anti-Echinococcus screening.

These findings also are in line with the findings of [20], by observing the protoscolicidal activity of Satureja khuzestanica and Olea europaea in vitro. Their studies found that both plants had scolicidal activity, but in S. khuzestanica, the activity was more effective than in O. europaea, and the time of exposure was one of the factors that led to the death of parasites. In the present study, time- and concentration-dependent dynamics were also noted in all the test groups.

The main difference is the relative potency, as S. khuzestanica was the most effective in the study conducted by [21]; this one series revealed that ethanolic Allium sativum is the most effective formulation (LC50 of 5.9 mg/mL), while pineapple formulations showed less comparative strength. In conclusion, despite the different plant species and processing, both studies highlight the potential of medicinal flora and the importance of the parameters of extraction and exposure to obtain an anti-parasitic effect.

Conclusion

The results of the above indicate that extracts prepared from both Allium sativum (garlic) and Ananas comosus (pineapple) have significant in vitro activity in killing the protoscolecia from the viable fertile hydatids. For all the evaluated cohorts, biocidal efficiency increased with increasing levels and longer exposure times, creating a clear dose-time relationship.

Among the formulations analyzed, garlic ethanolic extract proved to be the strongest antiparasitic compound with the lowest LC50 value (5.9 mg/mL) and mortality percentages that were always very high. The aqueous extract of pineapple, however, showed the least response, followed by the ethanolic extract. Overall, the ethanolic processing yielded better results in both plant species compared to aqueous extraction conditions, thus emphasizing the influence of the extractant over the extraction of bioactive compounds endowed with parasiticidal properties. The trends mentioned in this study indicate that garlic—especially when isolated through its ethanolic extract—could be considered a botanical repository of agents with hydatidicidal activity that provides a baseline for other therapeutic approaches for the treatment of hydatid disease or other supportive treatment approaches. However, these initial findings would need to be taken through several additional steps of elaborate and rigorous work before they could be translated to practical clinical/vet applications, such as a thorough phytochemical screening, mapping of the mechanisms, toxicity testing, and in vivo profiling.

Aside from allicin, the first organosulfur compound to be discovered, the therapeutic potential of Allium sativum includes a wide range of other organosulfur compounds. The phytochemical composition of garlic suggests that it has a rich profile of secondary constituents, including alliin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, and S-allyl cysteine, along with B-complex vitamins, vitamin C, and vital micronutrients. Garlic also has anti-inflammatory effects, which have been documented to modulate key intracellular signaling cascades like nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) and not only help with destroying parasites but also help reduce inflammation. Blocking these particular pathways results in decreased production of pro-inflammatory cytokines, which has the effect of changing host–parasite dynamics.

Similarly, the medicinal properties of Ananas comosus are largely on the basis of bromelain, a complex enzymatic mixture consisting for the most part of thiol endopeptidases that are also accompanied by phosphatases, glucosidases, peroxidases, cellulases, and custom proteinase inhibitors. Each toxicological assessment confirms the excellent safety profile of bromelain: high biocompatibility and no systemic toxicity were observed in animal testing. The median lethal dose (LD50) of it in rats and mice exceeds 10 g/kg in rodents and rabbits. Moreover, rats consuming high doses of up to 1500 mg/kg per day for a long time exhibited no signs of mutation, teratogenesis, or cancer, which confirms its safety, even at such high therapeutic doses.

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