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Scolicidal Potential of Tribulus terrestris L. Aqueous Fruit Extract: An In Vitro Study on Echinococcus granulosus Protoscolices

Mohammad M. Shakir[1], Sadia Sh. Hamad[2]
[1]Department of Biology, College of Education for Women, University of Kirkuk, Kirkuk, 36001, Iraq, mohamadaljuboory@uokirkuk.edu.iq
[2]Department of Biology, College of Science, University of Kirkuk, Kirkuk, 36001, Iraq,

Abstract
Cystic Echinococcosis (CE) is a global health and economic challenge, and its current treatments necessitate the development of safer and more effective therapeutic alternatives. This study aimed to evaluate the in vitro scolicidal efficacy of the aqueous fruit extract of Tribulus terrestris L. against protoscolices of Echinococcus granulosus sensu lato.A dry aqueous extract of T. terrestris fruits was prepared, and preliminary qualitative phytochemical screening for its major phytoconstituents was conducted. A separate hydro-ethanolic extract was also analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The efficacy of different concentrations of the extract dissolved in Phosphate Buffered Saline (PBS) (25, 50, 75, and 100 mg/mL) was evaluated against E. granulosus protoscolices at exposure times of 10, 20, 40, and 80 minutes, with estimation of the Lethal Concentration 50% (LC_{50}) and Lethal Time 50% (LT_{50}) values. Qualitative screening of the aqueous extract revealed the presence of important phytochemical groups such as flavonoids, saponins, phenolic compounds, tannins, and alkaloids. GC-MS analysis of the hydro-ethanolic extract primarily indicated the presence of fatty acids (such as linoleic acid and palmitic acid) and other compounds. The extract demonstrated significant concentration-dependent and time-dependent scolicidal activity ($P < 0.001$ for both, and for their interaction), with the final concentration of 100 mg/mL causing 100% mortality of protoscolices after 80 minutes. LC_{50} values decreased with increasing exposure time (the lowest estimated value of ~32.3 mg/mL at 80 minutes), and LT_{50} values decreased with increasing concentration (the lowest estimated value of 14.0 minutes at a final concentration of 100 mg/mL). The results of this study indicate that the aqueous fruit extract of T. terrestris possesses promising in vitro scolicidal efficacy against E. granulosus s.l. Protoscolices are likely attributable to the combined and synergistic effects of their diverse phytochemical constituents. These findings support the potential for exploring this extract as a natural source for new or complementary therapeutic agents against cystic echinococcosis and warrant further in vivo studies and advanced phytochemical characterization.

Keywords: *Echinococcus granulosus; Protoscolices; Tribulus terrestris L.; Scolicidal activity; Aqueous extract; Phytochemical screening; GC-MS; Cystic Echinococcosis.*

1. INTRODUCTION

Cystic Echinococcosis (CE), also known as Hydatid disease, is a zoonotic parasitic disease of significant global health and economic importance(1). This disease is caused by infection with the metacestode (larval stage) of the tapeworm *Echinococcus granulosus sensu lato*, which belongs to the phylum Platyhelminthes and class Cestoda(2). The parasite's life cycle is primarily completed in the intestines of canids, the definitive hosts, where infective eggs are shed in feces, contaminating the environment. Infection is transmitted to intermediate hosts, such as livestock and humans (considered accidental intermediate hosts), through the ingestion of these eggs via contaminated food, water, or hands(3). In the intermediate host, the eggs hatch, releasing oncospheres (hexacanth embryos) that penetrate the intestinal wall and migrate via the circulatory or lymphatic systems to typically settle in the liver (approximately 60-70%) or lungs (approximately 20-25%), forming a slowly growing hydatid cyst. Cysts can also develop in other organs such as the spleen, kidneys, brain, and bones, albeit less frequently(4).

The disease is particularly prevalent in pastoral and rural communities, and the Middle East and North Africa region is among those with high endemicity, where prevalence rates in some rural areas are estimated to range between 3% and 7%(5). Globally, over one million new human cases are reported annually, resulting in preventable deaths and substantial economic losses in the livestock sector, estimated at billions of dollars per year(6). Current therapeutic management of human cystic echinococcosis relies on options including the "watch

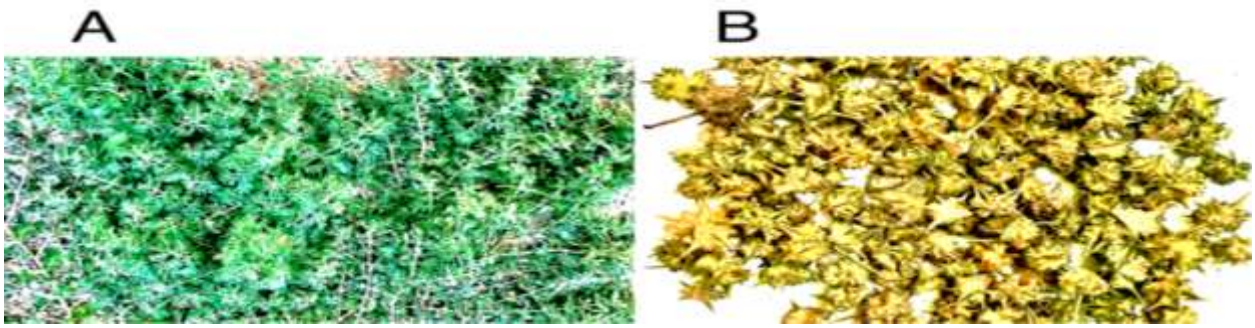
and wait" approach, chemotherapy with benzimidazole (BZD) compounds, minimally invasive techniques such as PAIR (Puncture, Aspiration, Injection, Re-aspiration), and conventional surgery(7). During these interventions, scolicidal agents, such as hypertonic saline solutions and ethanol, are commonly used to sterilize the cyst contents and prevent the dissemination of secondary echinococcosis (i.e., secondary disease). Despite their variable efficacy, the use of these conventional chemical agents is associated with serious complications such as sclerosing cholangitis and tissue irritation, with disease recurrence rates that can reach up to 25%, in addition to their potential cytotoxicity to healthy host tissues(8). Furthermore, long-term chemotherapy with benzimidazoles may be accompanied by side effects, and its efficacy is limited against certain cyst stages, with growing concerns about the emergence of drug-resistant strains of the parasite(9).Given these therapeutic challenges, there is increasing interest in searching for new, safe, and effective agents, especially from natural sources. Medicinal plants are characterized by a great diversity of biologically active phytochemicals, such as phenols, flavonoids, alkaloids, terpenes, and saponins, many of which have demonstrated antiparasitic properties through various mechanisms(10–12). For instance, garlic (*Allium sativum*) extract and barberry (*Berberis vulgaris*) extract have shown promising efficacy against *E. granulosus* protoscolices *in vitro* (13,14). *Tribulus terrestris* L. (Family: Zygophyllaceae), commonly known as puncture vine, is a medicinal plant with wide traditional uses and is renowned for its richness in biologically active compounds such as steroidal saponins (e.g., protodioscin) and flavonoids(15–17). Previous studies have shown that *T. terrestris* extracts possess diverse biological activities. Notably, some research has indicated promising efficacy against protozoan parasites such as *Trypanosoma* spp. and *Leishmania* spp., with proposed mechanisms including disruption of mitochondrial function and induction of oxidative stress in the parasite(18–20) . Due to the ease of preparing its aqueous extract, its local availability in Iraq, and its potential low cost, *T. terrestris* is considered an attractive candidate for study. However, to the best of our knowledge, its direct efficacy against *E. granulosus* protoscolices has not been evaluated. Therefore, this study aims to evaluate the *in vitro* scolicidal efficacy of the aqueous fruit extract of Iraqi *T. terrestris against E. granulosus* protoscolices. The research seeks to achieve this through preliminary characterization of the phytochemical constituents of the aqueous extract, chemical analysis of a hydro-ethanolic extract by Gas Chromatography-Mass Spectrometry (GC-MS), evaluation of the effect of different concentrations of the aqueous extract and varying exposure times on the viability of protoscolices, determination of the Lethal Concentration 50% (LC_{50}) and Lethal Time 50% (LT_{50}) of the extract, and documentation of any noticeable morphological changes in treated protoscolices. It is anticipated that the results of this study will provide a scientific basis for the potential use of *T. terrestris* fruit extract as a novel, effective, safe, and low-cost natural agent for sterilizing hydatid cysts during therapeutic interventions. This could help reduce recurrence rates and improve treatment outcomes for cystic echinococcosis, paving the way for further research to isolate and identify active compounds and elucidate their precise mechanisms of action.

2. MATERIALS AND METHODS

2.1. Collection and Preparation of Plant Material

2.1.1. Plant Collection and Authentication

Mature fruits of *Tribulus terrestris* L. were collected from reliable medicinal herb suppliers in Kirkuk city, Iraq, during August 2024. The plant material was authenticated, and its taxonomic identity was confirmed by the Professor of Botany, Department of Biology, College of Science, University of Kirkuk. A voucher specimen (Voucher No KUK-BOT-TT-2024-01) was deposited in the herbarium of the department. The fruits were thoroughly cleaned with distilled water to remove dust and surface impurities, then air-dried in the shade at room temperature (25 ± 2 °C) away from direct sunlight to preserve the integrity of the active compounds (Figure 2-1).



(Figure 2-1) Fruits of Tribulus terrestris L. were used in the study. (A) The whole plant of T. terrestris L. in its natural habitat as collected. (B) Dried mature fruits of T. terrestris L., after cleaning and drying, were used for extract preparation

2.1.2. Preparation of Aqueous Dry Extract and Test Solutions

The dried fruits were finely ground using a sterile electric mill (e.g., IKA A11 basic analytical mill, IKA®-Werke GmbH & Co. KG, Germany) to create a fine powder, which was then sieved using standard sieves (Endecotts Ltd. sieve set, UK) to obtain particles ranging in size from 0.5 to 1 mm. To prepare the crude aqueous extract, 100 grams of the dried fruit powder were weighed and macerated in 1000 mL of sterile distilled water (1:10 w/v ratio) in a sterile glass flask. This mixture was heated to 60 °C with continuous stirring (300 rpm) using a magnetic stirrer with a hotplate (IKA C-MAG HS 7, Germany) for one hour, before being left to soak overnight (approximately 16-18 hours) at room temperature (25 ± 2 °C) to enhance extraction efficiency (21,22). After extraction, the extract was filtered through Whatman No. 1 filter paper (Cytiva, USA). Fine suspended particles were removed by centrifuging the filtrate (4000 rpm for 20 minutes at 4 °C) using a refrigerated centrifuge (Eppendorf™ Centrifuge 5810 R, Germany). The supernatant was carefully collected, and the solvent (water) was evaporated at 40 °C under reduced pressure using a rotary evaporator (BÜCHI™ Rotavapor® R-300, Switzerland) to yield a dry aqueous extract (powder). The dry extract was weighed and stored in an airtight container in a desiccator at 4 °C.For biological experiments, test solutions at concentrations of 25, 50, 75, and 100 mg/mL were prepared by dissolving appropriate amounts of the dry aqueous extract in sterile Phosphate Buffered Saline (PBS, pH 7.2) (prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g anhydrous Na_2HPO_4, and 0.24 g KH_2PO_4 (all analytical grade, Sigma-Aldrich) in 1000 mL distilled water, followed by pH adjustment and autoclaving (Hirayama HVE-50)). These solutions were stored in dark, sterile, airtight glass vials at 4 °C and used within 24 hours of preparation.

2.2. Preliminary Phytochemical Screening. Preliminary qualitative screening for the major groups of secondary phytochemical compounds in the dry aqueous extract (dissolved in distilled water at a reference concentration of 100 mg/mL for screening purposes) of *T. terrestris* fruits was performed using standard colorimetric chemical tests, according to methods described by(17,22–24) The presence of carbohydrates, resins, alkaloids, cardiac glycosides, phenolic compounds, tannins, flavonoids, saponins, triterpenoids and steroids, gums and mucilages, free amino acids, and organic acids was tested. The chemical tests employed and the expected positive results for each group of compounds are described in Table 2-1.

(Table 2-1) Qualitative phytochemical screening tests for major phytochemical groups in the aqueous fruit extract of Tribulus terrestris L

Phytochemical Group	Chemical Test Used	Expected Positive Result
Carbohydrates	Molisch's test	Appearance of a distinct violet ring at the interface between the solution and acid layers.
Resins	Water precipitation test	Appearance of turbidity or formation of a white precipitate upon addition of water to the alcoholic extract.
Alkaloids	Dragendorff's test	Formation of an orange to orange-red precipitate.
	Mayer's test	Formation of a creamy white or pale-yellow precipitate.

Phytochemical Group	Chemical Test Used	Expected Positive Result
Cardiac Glycosides	Keller-Kiliani test	Appearance of a reddish-brown ring at the interface, the upper layer (acetic acid) gradually turns bluish-green or blue.
Phenolic compounds	Ferric chloride test	Appearance of a dark olive-green, dark blue, violet, or black color, depending on the nature of the phenolic compound.
Tannins	Ferric chloride test	Appearance of a blue-black color (for hydrolysable tannins) or green-black (for condensed tannins).
Flavonoids	Shinoda test / Mg-HCl test	Appearance of a crimson red, pink, orange, or purple color, depending on the type of flavonoid.
Saponins	Froth test	Formation of a dense and persistent froth (height > 1 cm, lasting > 15 minutes) upon vigorous shaking of the aqueous extract.
Triterpenoids and Steroids	Liebermann-Burchard test	Appearance of coloration ranging from pink, red, or violet to blue or bluish-green, indicating the presence of steroids or triterpenoids.
Gums and Mucilages	Absolute alcohol precipitation test	Formation of a white gelatinous or filamentous precipitate upon the addition of absolute alcohol to the aqueous extract.
Free Amino Acids	Ninhydrin test	Appearance of a distinct violet (Ruhemann's purple) or blue-violet color after gentle heating.
Organic Acids	Sodium bicarbonate test	Strong and noticeable effervescence with the evolution of gas bubbles (carbon dioxide, CO_2) upon the addition of sodium bicarbonate solution.

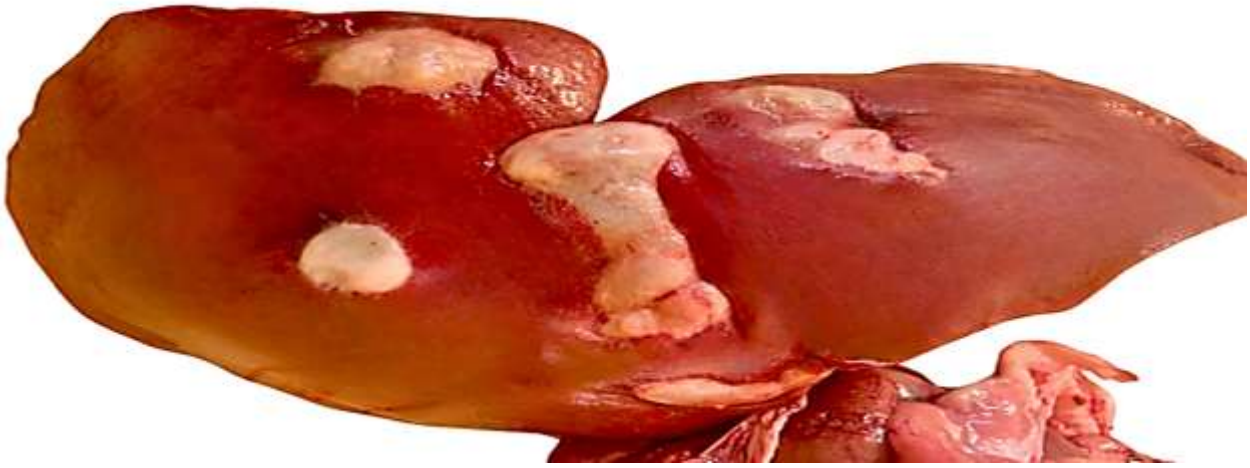
2.3. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

To complement the qualitative screening results and to identify a broader fingerprint of chemical compounds, especially semi-polar and volatile compounds, a hydro-ethanolic extract was separately prepared from *T. terrestris* L. fruits for GC-MS analysis. Ten grams (10 g) of the dried fruit powder were macerated in one hundred milliliters (100 mL) of aqueous ethanol solution (70% ethanol (HPLC grade, ≥ 99.9%, Sigma-Aldrich) v/v in distilled water) for 24 hours at room temperature (25 ± 2 °C) with continuous magnetic stirring. After filtration (Whatman No. 1 paper), the filtrate was dried under reduced pressure using a rotary evaporator (BÜCHI™ Rotavapor® R-210, Switzerland) at a temperature ≤ 40 °C. The resulting dry material was redissolved in two milliliters (2 mL) of methanol (GC grade, ≥ 99.9%, Sigma-Aldrich), and the solution was filtered through a syringe filter (0.22 µm PTFE, Millipore Millex®, Merck KGaA, Germany) before injection.GC-MS analysis was performed using an Agilent 7890B GC system coupled to an Agilent 5977A MSD (Agilent Technologies, USA). One microliter (1 µL) of the extract was injected into the injection port (280 °C, Split ratio 10:1). A DB-5MS column (30m × 0.25mm × 0.25µm; Agilent J&W GC Columns, USA) was used with helium (purity ≥ 99.999%) as the carrier gas (1.46 mL/min). The oven temperature program was: 80 °C (2 min), then ramped at 10 °C/min to 300 °C (6 min). The mass spectrometer was operated in electron impact (EI) ionization mode at 70 eV (ion source temperature 200 °C, transfer line 250 °C), scanning a mass range of m/z 40-800 after a solvent delay of 2 minutes. Tentative identification of compounds was performed by comparing their mass spectra with the NIST20 Mass Spectral Library (Similarity Index, SI ≥ 70%) and retention indices (RI) with reference values.

2.4. Parasitological Studies

2.4.1. Collection and Preparation of Protoscolices

Hydatid cysts were collected from the livers and lungs of naturally infected sheep with *Echinococcus granulosus* from the local Kirkuk abattoir under veterinary supervision (Figure 2-2). The cysts were immediately transported in sterile, refrigerated containers to the Parasitology Laboratory, Department of Biology, College of Science, University of Kirkuk, and processed on the same day of collection under aseptic conditions (Biological Safety Cabinet, Thermo Scientific™ 1300 Series A2).



(Figure 2-2) Liver of a sheep naturally infected with cystic echinococcosis, showing hydatid cysts of Echinococcus granulosus.

Intact and non-calcified hydatid cysts were superficially washed with 70% ethanol solution and then with Phosphate Buffered Saline (PBS, pH 7.2). The hydatid fluid content was aspirated from each cyst using a sterile syringe fitted with a needle (23G gauge). After carefully opening the cyst using sterile surgical instruments (Fine Science Tools), the remaining hydatid fluid, along with protoscolices and hydatid sand, was collected into sterile conical centrifuge tubes (Falcon® 50 mL). The fluid was allowed to sediment the protoscolices by gravity for 30-60 minutes at room temperature. The supernatant was carefully removed, and the pellet containing protoscolices underwent three to five successive washes with sterile PBS, with centrifugation at 2000 rpm for 5 minutes at 4 °C after each wash (Eppendorf™ Centrifuge 5430 R). After the second wash, an antibiotic mixture, consisting of Penicillin (Penicillin G sodium salt, Sigma-Aldrich, at a final concentration of 100-200 IU/mL) and Streptomycin (Streptomycin sulfate, Sigma-Aldrich, at a final concentration of 100-200 µg/mL), was added to the protoscolex suspension to prevent bacterial contamination. The final pure protoscolex pellet was resuspended in an appropriate volume of physiological saline solution (0.9% NaCl, prepared from analytical grade NaCl, Sigma-Aldrich, in sterile distilled water), and the suspension concentration was adjusted to 5×10^3 protoscolices/mL using a counting chamber (Neubauer improved).

2.4.2. Viability Assessment of Protoscolices

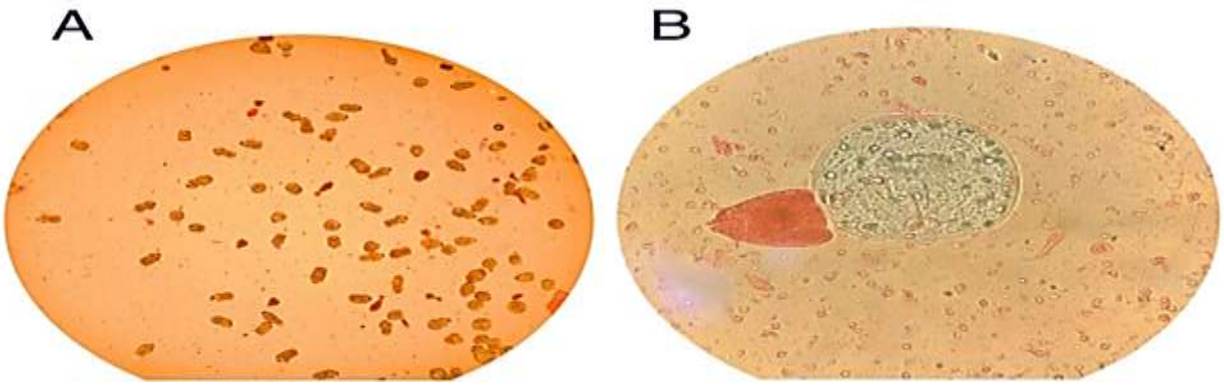
The viability of protoscolices was assessed before each experiment using two methods:

2.4.2.1. Direct Microscopic Examination: A drop (approximately 10 µL) of the protoscolex suspension (prepared in 0.9% NaCl) was placed on a clean glass microscope slide and covered with a coverslip. The sample was examined under a light microscope (Olympus BX53, at 100× and 400× magnification). Protoscolices were considered viable if they exhibited one or more of the following criteria: normal muscular contractions of the body, active flickering movement of flame cells, or the phenomenon of scolex invagination and evagination.

2.4.2.2. Eosin Staining Method: Ten microliters (10 µL) of the protoscolex suspension were mixed with 10 µL of eosin Y stain solution (0.1% w/v, prepared by dissolving 0.1 g of Eosin Y powder (Eosin Y disodium salt, high purity ≥ 99%, Sigma-Aldrich) in 100 mL distilled water and stored in a dark bottle at 4 °C) on a glass slide. After gentle mixing, the mixture was left for 1-2 minutes at room temperature to allow the stain to react. The mixture was covered with a coverslip and examined under a light microscope. Protoscolices that did not absorb the stain and remained transparent or pale green were considered viable, while those stained red or pink were considered dead, due to loss of their cell membrane integrity (Figure 2-3). The percentage of viability was calculated by counting at least 100-200 protoscolices randomly in at least three microscopic fields for each batch of protoscolices (in three independent replicates), using the following formula:

Viability (%) = (Number of viable protoscolices / Total number of protoscolices counted) × 100 (Equation 1).

Protoscolex batches were used in subsequent experiments only if their initial viability was not less than 95%.



(Figure 2-3) Viability assessment of Echinococcus granulosus s.l. protoscolices using the eosin staining method (0.1%). (A) Viable (unstained) and dead (stained red/pink) protoscolices under 100× magnification. (B) The field under 400× magnification, illustrating the difference in stain uptake and morphological details.

2.4.3. *In Vitro* Scolicidal Activity Assessment

To evaluate the scolicidal activity, 1.0 mL of the protoscolex suspension (prepared in 0.9% NaCl at a concentration of 5×10^3 protoscolices/mL) was added to sterile glass test tubes (Pyrex®) each containing 1.0 mL of one of the previously prepared stock extract solutions (at concentrations of 25, 50, 75, and 100 mg/mL in PBS). The tubes were incubated at 37 °C in a shaking water bath (Grant OLS200, UK) for varying exposure periods of 10, 20, 40, and 80 minutes, with three replicates for each concentration and time point. The experiment included a negative control group consisting of 1.0 mL of protoscolex suspension (in 0.9% NaCl) added to 1.0 mL of sterile PBS (pH 7.2), and a positive control group consisting of 1.0 mL of protoscolex suspension (in 0.9% NaCl) added to 1.0 mL of hypertonic saline solution (20% NaCl w/v, prepared from analytical grade NaCl, Sigma-Aldrich, in sterile distilled water). After each exposure period, 3 mL of PBS (pH 7.2) was added to stop the reaction, followed by centrifugation (2000 rpm, for 5 minutes). The pelleted protoscolices were resuspended in 100 µL of PBS, and their viability was then assessed using the eosin staining method as described in section 2.4.2.2. The percentage of protoscolex mortality was calculated using the following formula:

Mortality (%) = (Number of dead protoscolices / Total number of protoscolices counted) × 100 (Equation 2)

2.5. Statistical Analysis

All quantitative data were expressed as Mean ± Standard Deviation (SD) of three independent replicates and analyzed using SPSS Statistics for Windows, Version 29.0 (IBM Corp., Armonk, NY, USA). Two-way Analysis of Variance (ANOVA), followed by Tukey's HSD (Honestly Significant Difference) test for multiple comparisons, was used to evaluate the effects of extract concentrations (25, 50, 75, and 100 mg/mL) and varying exposure times, as well as their interaction effect, on the percentage of protoscolex mortality. A **P**-value < 0.05 was considered indicative of statistically significant differences.Lethal Concentration 50% (LC_{50}) values for each exposure time, and Lethal Time 50% (LT_{50}) values for each extract concentration, were estimated using linear interpolation from the respective dose/time-response curves.

2.6. Ethical Considerations and Quality Control

Approval was obtained from the Research Ethics Committee of the Department of Biology, College of Science, University of Kirkuk (Approval No: 3/7/2516, Date: 26/03/2024). Cysts were collected from sheep slaughtered for human consumption. Experiments (n≥3) were conducted under standardized conditions, with controls. Protoscolex viability was checked (≥95%) before commencing experiments. Instruments were periodically calibrated according to approved standards to ensure measurement accuracy.

3. RESULTS

3.1. Qualitative Phytochemical Screening of the Extract of *T. terrestris* L. Fruits

The results of the preliminary qualitative phytochemical screening of the dry aqueous extract of *T. terrestris* L. fruits revealed a broad spectrum of biologically active compounds. The presence of carbohydrates, alkaloids, phenolic

compounds, tannins, flavonoids, saponins, triterpenoids and steroids, gums and mucilages, free amino acids, and organic acids was confirmed. Conversely, the tests for resins and cardiac glycosides were negative, indicating the absence of these two groups of compounds or their presence at concentrations below the Limit of Detection (LOD) by the colorimetric chemical methods used in this study. Detailed results are summarized in Table 3-1.

(Table 3-1) Results of qualitative phytochemical screening for major phytochemical groups in the aqueous fruit extract of Tribulus terrestris L.

Phytochemical Group	Test Result (+/-)
Carbohydrates	+
Resins	-
Alkaloids	+
Cardiac Glycosides	-
Phenolic compounds	+
Tannins	+
Flavonoids	+
Saponins	+
Triterpenoids and Steroids	+
Gums and Mucilages	+
Free Amino Acids	+
Organic Acids	+
+: Positive result (indicates the presence of the phytochemical group); -: Negative result (indicates the absence of the group or its presence in very trace amounts undetectable by the methods used).	

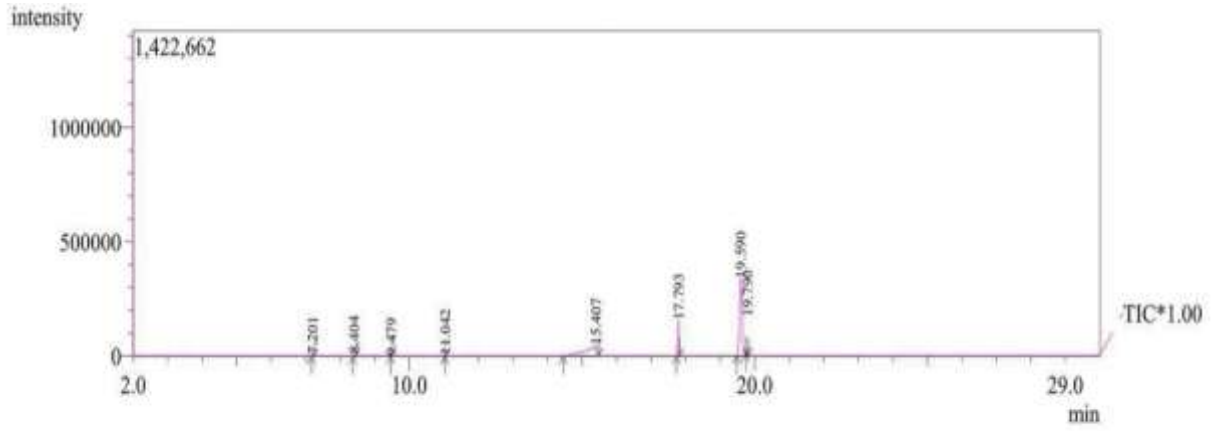
3.2. GC-MS Chemical Analysis of the Extract of *T. terrestris* L. Fruits

To gain a broader insight into the chemical composition of *T. terrestris* fruits and to identify semi-polar and volatile compounds, the prepared hydro-ethanolic extract was analyzed by GC-MS. The Total Ion Chromatogram (TIC) (Figure 3-1) showed several chromatographic peaks, from which eight major compounds were tentatively identified by comparing their mass spectra with the NIST20 Mass Spectral Library. (Table 3-2) Details of these identified compounds. Overall, fatty acids dominated the chemical profile of the extract, with *Linoleic acid* being the most abundant component (53.79%), followed by Ethanol, 2-(1-methylethoxy)-, acetate (32.31%), and then *Palmitic acid* (11.40%). Other compounds were also identified in smaller amounts, including *Linoleylalcohol*, *Isovanillin* (4-hydroxy-2-methoxybenzaldehyde), and a pyrazoline derivative.

(Table 3-2) Chemical compounds were tentatively identified in the hydro-ethanolic extract of T. terrestris L. fruits using GC-MS.

Peak #	RT (min)	Compound Name (Common/Known Name)	Molecular Formula	MW (g/mol)	Area %	SI (%)
1	7.201	2-Tetrazene, 1,1-diethyl-4,4-dimethyl-	$C_6H_{16}N_4$	144	0.46	86
2	8.404	1H-Pyrazole, 4,5-dihydro-3-methyl-1-propyl- (Pyrazoline derivative)	$C_7H_{14}N_2$	126	0.77	89
3	9.479	4-Pentenenitrile (Allylacetonitrile)	C_5H_7N	81	0.03	97
4	11.042	4-Hydroxy-2-methoxybenzaldehyde (Isovanillin)	$C_8H_8O_3$	152	0.13	97
5	15.407	Ethanol, 2-(1-methylethoxy)-, acetate (Isopropyl cellosolve acetate)	$C_7H_{14}O_3$	146	32.31	79

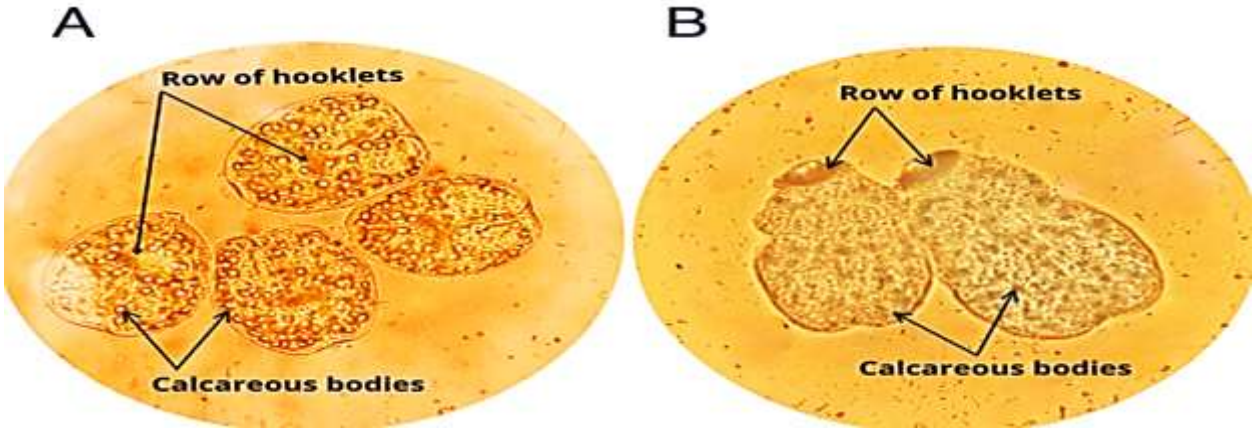
Peak #	RT (min)	Compound Name (Common/Known Name)	Molecular Formula	MW (g/mol)	Area %	SI (%)
6	17.793	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256	11.40	73
7	19.590	9,12-Octadecadienoic acid, (9Z,12Z)-(Linoleic acid)	$C_{18}H_{32}O_2$	280	53.79	79
8	19.790	(9Z,12Z)-9,12-Octadecadien-1-ol (Linoleyl alcohol)	$C_{18}H_{34}O$	266	1.11	76



(Figure 3-1) Total Ion Chromatogram (TIC) of the GC-MS analysis of the hydro-ethanolic extract of Tribulus terrestris L. fruits, showing the major peaks (numbered 1-8) of the compounds identified in Table 3-2.

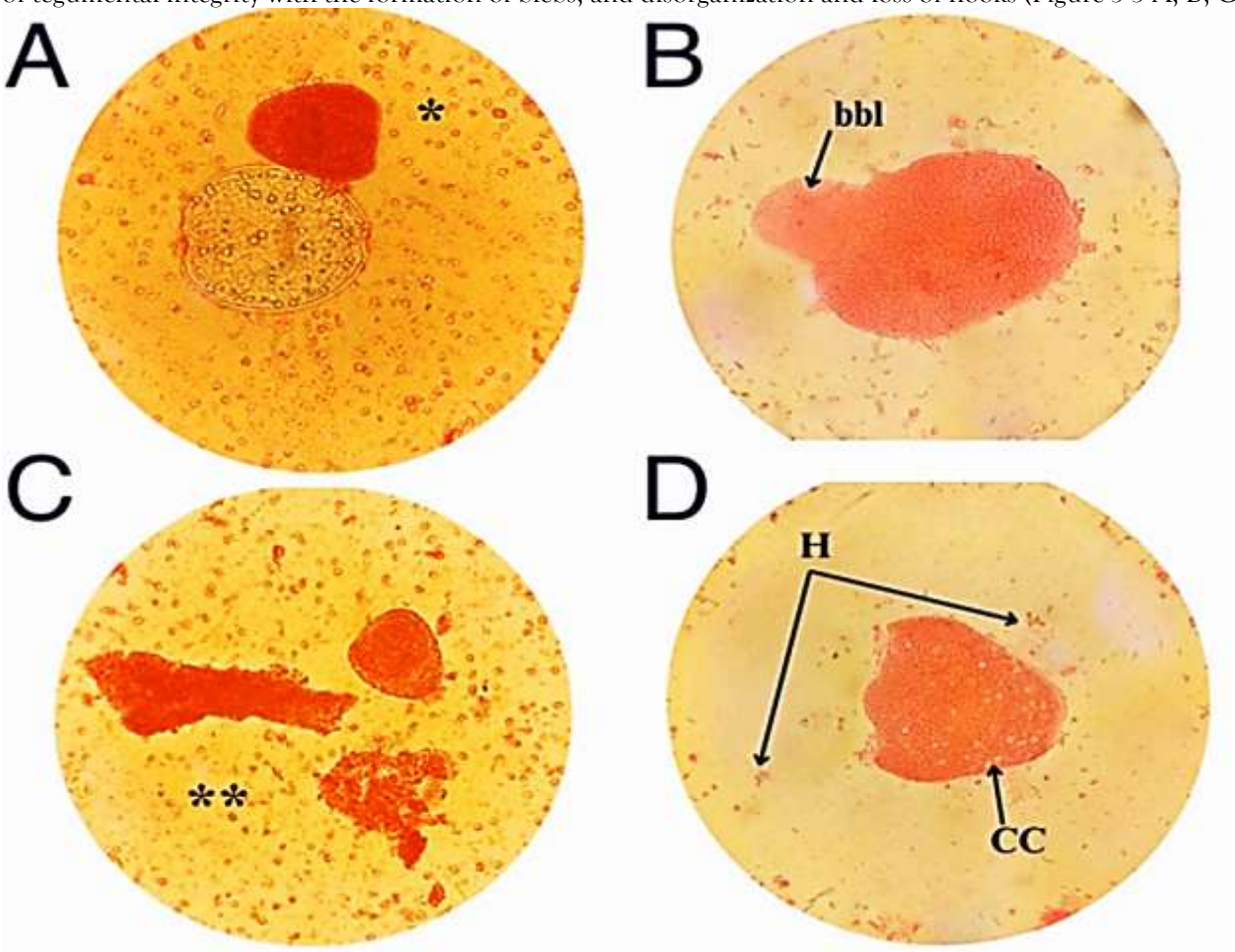
3.3. Morphological Changes in *E. granulosus* Protoscolices After Treatment

Microscopic examination of *E. granulosus* protoscolices showed a clear contrast in morphological characteristics between the negative control group and protoscolices treated with the aqueous fruit extract of *T. terrestris* (at a concentration of 100 mg/mL). In the negative control group, protoscolices maintained their viability and typical morphological features. Spontaneous vermicular movement and regular body wall contractions were observed, along with a clearly defined scolex region with intact structure, and naturally distributed calcareous corpuscles. These protoscolices were also characterized by an intact and cohesive outer tegument and a regular arrangement of hooks on the muscular rostellum (Figure 3-2 A, B).



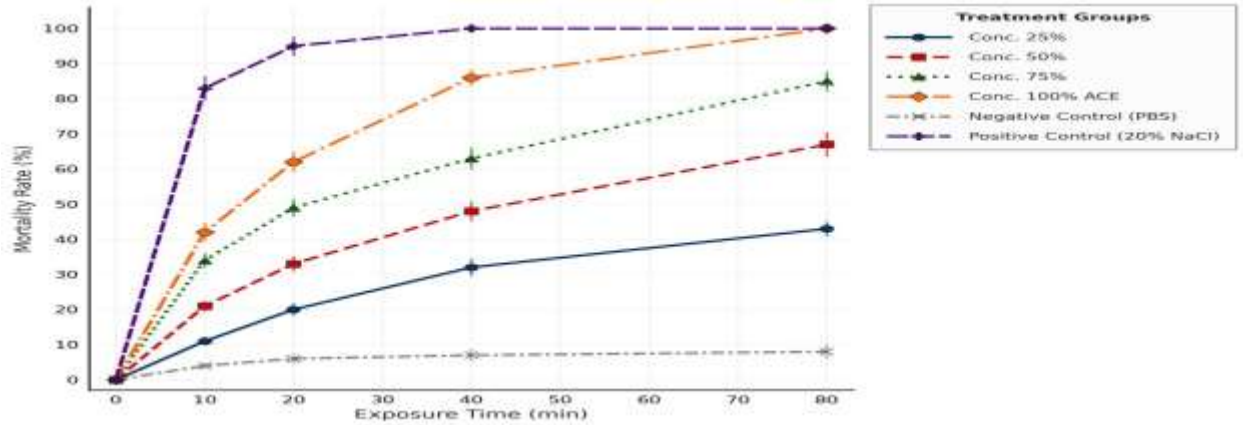
(Figure 3-2) Light micrographs illustrating the morphological characteristics of Echinococcus granulosus protoscolices in the negative control group after 80 minutes of incubation at 37 °C (400× magnification). (A) An invaginated protoscolex showing intact anatomical structure. (B) An evaginated protoscolex with a clear scolex region and normally arranged hooks.

In contrast, protoscolices treated with 100 mg/mL of *T. terrestris* aqueous fruit extract exhibited severe morphological alterations after 80 minutes of exposure. These changes included: complete cessation of movement, loss of normal shape with body shrinkage, scolex invagination, appearance of cytoplasmic vacuoles, deterioration of tegumental integrity with the formation of blebs, and disorganization and loss of hooks (Figure 3-3 A, B, C, D).



(Figure 3-3) Light micrographs showing noticeable morphological changes in Echinococcus granulosus protoscolices after treatment with 100 mg/mL of T. terrestris L. aqueous fruit extract for 80 minutes at 37 °C (400× magnification). (A) Deformation, shrinkage, and scolex invagination (*). (B) Tegumental deterioration and bleb formation (bbl). (C) Partial tissue disintegration (**). (D) Disorganization and loss of hooks (H). 3.4. In Vitro Scolicidal Efficacy of T. terrestris Aqueous Fruit Extract Against Protoscolices.

The aqueous fruit extract of *T. terrestris* L. exhibited significant scolicidal activity against *E. granulosus* protoscolices, and this efficacy was highly dependent on the final extract concentration and exposure time (Two-way ANOVA: Concentration effect: $F(3, 32) = 112.53$, $P < 0.001$; Time effect: $F(3, 32) = 95.78$, $P < 0.001$; Concentration × Time interaction: $F(9, 32) = 42.91$, $P < 0.001$) (Figure 3-4; Table 3-3). The concentration of 100 mg/mL resulted in 100.0 ± 0.00% mortality of protoscolices after 80 minutes, while the mortality rate was 42.0 ± 2.80% after only 10 minutes at this same final concentration. In the positive control group (20% NaCl), a high protoscolex mortality rate of 83.0 ± 3.50% was observed at 10 minutes, reaching 100% after 40 and 80 minutes of exposure. In the negative control group (PBS), protoscolex mortality was very low, ranging from 4.0 ± 1.00% to 8.0 ± 2.10% during the different exposure periods. Multiple comparisons using Tukey's test showed significant differences ($P < 0.05$) in protoscolex mortality rates among most of the tested concentrations and time intervals.



(Figure 3-4) Effect of different final concentrations (25, 50, 75, and 100 mg/mL) of Tribulus terrestris L. aqueous fruit extract on the percentage mortality of Echinococcus granulosus protoscolices over varying exposure periods (10, 20, 40, and 80 minutes) at 37 °C. Each point represents the mean ± standard deviation of three independent replicates.

(Table 3-3) Percentage mortality of Echinococcus granulosus protoscolices (Mean ± SD, n=3) after treatment with different final concentrations (25, 50, 75, and 100 mg/mL) of Tribulus terrestris L. aqueous fruit extract and control groups at varying exposure times.

Concentration (mg/mL)	Mortality Percentage (Mean ± SD, n=3) after Exposure Time (minutes)			
	10 min	20 min	40 min	80 min
25	11.0 ± 1.20aA	20.0 ± 1.80aB	32.0 ± 2.50aC	43.0 ± 2.10aD
50	21.0 ± 1.50bA	33.0 ± 2.20bB	48.0 ± 2.80bC	67.0 ± 3.50bD
75	34.0 ± 2.00cA	49.0 ± 2.50cB	63.0 ± 3.10cC	85.0 ± 2.80cD
100	42.0 ± 2.80dA	62.0 ± 3.00dB	86.0 ± 2.50dC	100.0 ± 0.00dD
Negative Control (PBS)	4.0 ± 1.00eA	6.0 ± 1.50eA	7.0 ± 1.80eA	8.0 ± 2.10eA
Positive Control (20% NaCl)	83.0 ± 3.50fA	95.0 ± 2.80fB	100.0 ± 0.00fC	100.0 ± 0.00fC

Values represent Mean ± Standard Deviation of three replicates (n=3). Different lowercase letters (a-f) in the same column indicate statistically significant differences ($P < 0.05$) between different concentrations at the same exposure time. Different uppercase letters (A-D) in the same row indicate statistically significant differences ($P < 0.05$) between different exposure times at the same concentration (based on Tukey's test).

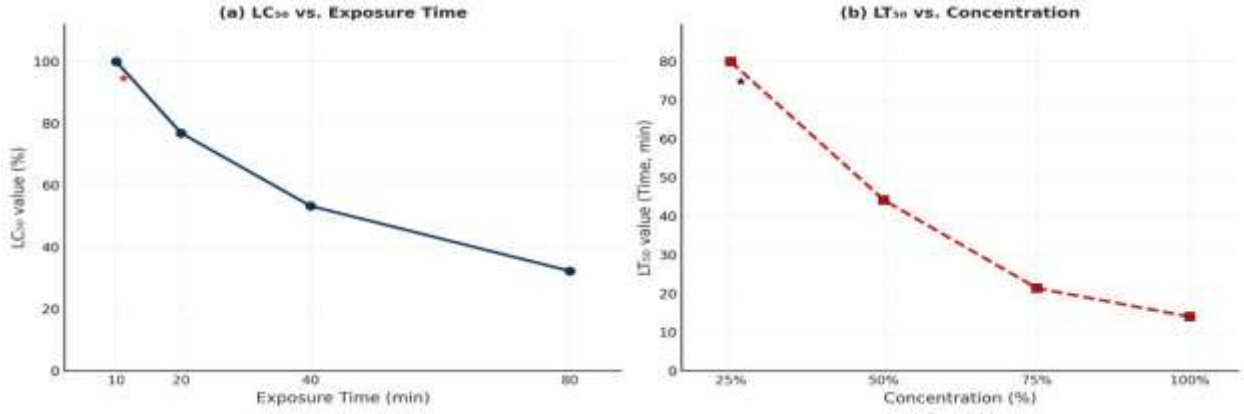
3.5. Estimation of Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) Values of the Extract

The Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) values for the aqueous fruit extract of *T. terrestris* were estimated using linear interpolation based on the mean mortality data at the actual extract concentrations (25, 50, 75, and 100 mg/mL). The results showed that the LC_{50} value clearly decreased with increasing exposure period, being > 100 mg/mL at 10 minutes, then gradually decreasing to its lowest value of approximately 32.3 mg/mL after 80 minutes of exposure. Similarly, the LT_{50} value markedly decreased with increasing extract concentration; while it exceeded 80 minutes (> 80 minutes) at a concentration of 25 mg/mL, it reached only 14.0 minutes at a final concentration of 100 mg/mL. These results, illustrated in Table 3-4 and Figure 3-5, confirm the concentration- and time-dependent nature of the extract's scolicidal effect.

(Table 3-4) Estimated Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) values (using linear interpolation) for *T. terrestris* aqueous fruit extract against *E. granulosus* protocolizes.

Median Lethal Concentration (LC_{50})	Exposure Time (min)	Estimated Value (mg/mL)
	10	> 100.0
	20	76.9
	40	53.3
	80	32.3
Median Lethal Time (LT_{50})	**Concentration (mg/mL)**	**Estimated Value (min)**
	25	> 80.0
	50	44.2
	75	21.4
	100	14.0

Note: The ">" symbol indicates that the corresponding value exceeds the highest concentration or longest period tested experimentally.



(Figure 3-5) Relationship between Median Lethal Concentration (LC_{50}) and exposure time (a), and Median Lethal Time (LT_{50}) and extract concentration (b) for Tribulus terrestris aqueous fruit extract against Echinococcus granulosus protoscolices.

4. DISCUSSION

The present study aimed to evaluate the *in vitro* scolicidal efficacy of the aqueous fruit extract of *Tribulus terrestris L.* against protoscolices of *Echinococcus granulosus*. The results confirmed that the aqueous extract possesses significant concentration-dependent and time-dependent efficacy, with the highest extract concentration (100 mg/mL) leading to complete (100%) mortality of protoscolices after 80 minutes. This efficacy, although potentially requiring a longer time to achieve complete killing compared to the positive control (20% NaCl), is considered promising when compared to results from previous studies on other plant extracts(25–27) . However, caution should be exercised when making direct comparisons due to potential differences in extract preparation methods, effective concentrations, plant origin, and parasite strain susceptibility. The ease of aqueous extract preparation, its potential low cost, and the local availability of *T. terrestris* enhance the significance of these findings as a step towards developing natural agents for combating cystic echinococcosis.

To explain this observed efficacy, an integrated approach was adopted, including qualitative screening for major chemical groups in the dry aqueous extract (used for preparing test solutions, Table 3-1), in addition to Gas Chromatography-Mass Spectrometry (GC-MS) analysis of a separate hydro-ethanolic extract (Table 3-2) to obtain a broader compound fingerprint and identify some less polar components such as fatty acids and their derivatives. Qualitative screening of the aqueous extract clearly showed the presence of important groups of polar and water-soluble compounds, including alkaloids, phenolic compounds, tannins, flavonoids, and saponins, in addition to triterpenoids and steroids. These compounds, particularly saponins, flavonoids, and phenolic compounds, are

widely known for their potent biological activities, including antiparasitic effects(15,17,28–30) . *T. terrestris* is known for its richness in these classes of compounds, as documented by numerous studies(23,28,31,32) .
Concurrently, GC-MS analysis of the hydro-ethanolic extract (which may differ in its precise composition from the aqueous extract but indicates some plant components) revealed the presence of fatty acids as major compounds, notably *Linoleic acid* (53.79%) and *Palmitic acid* (11.40%). These fatty acids, especially unsaturated ones like linoleic acid, are also known for their biological activities, including antimicrobial and antiparasitic effects(17,29,33). Their mechanism of action is thought to involve disrupting the integrity of parasite cell membranes and increasing their permeability (34). This is consistent with the observed morphological changes in treated protoscolices (Figure 3-3). GC-MS analysis also revealed the presence of the compound Ethanol, 2-(1-methylethoxy)-, acetate at a high percentage (32.31%). The nature of this compound as an authentic natural component of *T. terrestris* and its role in antiparasitic activity require further investigation, as it has not been widely documented in this context and might be related to the extraction process, a previously used solvent, or even a degradation product of a larger compound. Additionally, other compounds such as linoleyl alcohol, isovanillin, and a pyrazoline derivative were identified in smaller amounts, which may contribute secondarily or synergistically to the overall efficacy.Therefore, it is highly probable that the observed scolicidal efficacy of the aqueous extract is largely attributable to the combined and integrated effect of multiple polar compounds (revealed by the qualitative analysis of the aqueous extract, such as saponins, flavonoids, and phenols), which are likely the main contributors to the efficacy of this type of extract. A potential contribution from some fatty acids or other compounds similar to those identified in the hydro-ethanolic extract, which might be present in smaller amounts or different forms (like salts) in the aqueous extract, cannot be excluded. Saponins can affect cell membranes by forming complexes with membrane sterols, leading to disruption of membrane permeability and cell lysis(29,33) . Flavonoids and phenols may act as antioxidants, enzyme inhibitors, metal chelators, or may interfere with vital bioenergetic pathways in the parasite(21,35) . This diversity of compounds and their potential mechanisms of action supports the idea of a synergistic effect. The morphological changes observed in treated protoscolices (such as body shrinkage, loss of membrane integrity, and appearance of vacuoles, as shown in Figure 3-3) may be a result of the combined effect of these diverse compounds on the integrity and functions of the parasite cell.
Statistical analysis of the efficacy results (Two-way ANOVA) supports the concentration-dependent and time-dependent nature of the extract's effect ($\mathbf{P} < 0.001$ for both), with a significant interaction between them ($\mathbf{P} < 0.001$), indicating that the effect of one factor is influenced by the level of the other. This dynamic behavior was also confirmed by the Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) values estimated using linear interpolation (Table 3-4). The LC_{50} values showed a clear decrease with increasing exposure period (from > 100 mg/mL at 10 minutes to approximately 32.3 mg/mL at 80 minutes), while LT_{50} values decreased with increasing extract concentration (from >80 minutes at a final concentration of 25 mg/mL to 14.0 minutes at a final concentration of 100 mg/mL). This suggests that higher concentrations accelerate the protoscolex killing process and that the extract becomes more effective with longer contact time, which is consistent with a mechanism of action that may require sufficient time for active compounds to accumulate within the protoscolices or interact with their molecular targets.Despite these promising results, it is important to acknowledge the limitations of the current study. The restriction of GC-MS chemical analysis to a separate hydro-ethanolic extract did not fully reflect the chemical composition of the aqueous extract used in the efficacy tests, especially concerning the polar compounds indicated by qualitative analysis. Also, the estimation of LC_{50} and LT_{50} values was done using linear interpolation, which is considered a preliminary estimation. Therefore, future studies are strongly recommended to conduct a more comprehensive chemical analysis of the aqueous extract using appropriate techniques such as High-Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS) (LC-MS) to identify and quantify the major polar compounds. Furthermore, this study was limited to *in vitro* evaluation and does not necessarily reflect efficacy under *in vivo* conditions. This calls for *in vivo* studies on appropriate animal models to assess the extract's efficacy, determine potential therapeutic doses, and fully study its pharmacokinetics and toxicological safety profile. Exploring the possibility of synergistic effects between *T. terrestris extract* and currently used standard chemical drugs (such as albendazole) may also open new avenues for improving treatment protocols.

In conclusion, the results of this study indicate that the aqueous fruit extract of *Tribulus terrestris* L. possesses significant scolicidal activity against *Echinococcus granulosus* protoscolices *in vitro*, and this efficacy is concentration- and time-dependent. Qualitative screening supports the presence of important groups of polar compounds (such as flavonoids, saponins, and phenols) in this extract, which are likely to play a major role in this activity, possibly in synergy with other compounds to a lesser extent, such as fatty acids. These preliminary findings are encouraging and support the potential for exploring *T. terrestris* as a natural source of new or complementary therapeutic agents against cystic echinococcosis. However, there remains an urgent need for more in-depth phytochemical, pharmacological, and toxicological research, including *in vivo* studies, before considering any potential clinical applications for this promising extract.

5. CONCLUSION

This study demonstrated that the aqueous fruit extract of *Tribulus terrestris* L. possesses significant concentration- and time-dependent efficacy in killing *Echinococcus granulosus s.l.* protoscolices under *in vitro* conditions. The highest final extract concentration (100 mg/mL) led to the complete elimination of protoscolices after 80 minutes of exposure. Preliminary estimates of Median Lethal Concentration (LC_{50}) and Median Lethal Time (LT_{50}) values also supported this dose- and time-dependent nature of the observed efficacy. Qualitative screening of the crude aqueous extract indicated its content of diverse phytochemical groups, notably alkaloids, phenolic compounds, tannins, flavonoids, and saponins. It is probable that these polar compounds, either individually or synergistically, play the major role in the observed activity against protoscolices. Although GC-MS analysis of a separate hydro-ethanolic extract primarily indicated the presence of fatty acids, their relative contribution to the overall efficacy of the aqueous extract used in biological activity tests requires further clarification and study. These findings provide preliminary scientific evidence supporting the potential exploration of aqueous *T. terrestris* extract as a promising source of novel natural anti-echinococcal agents, especially considering its ease of preparation, potential low cost, and local availability. However, the study strongly recommends further research, including comprehensive chemical analysis of the aqueous extract to accurately identify and quantify active compounds, as well as *in vivo* studies to comprehensively evaluate efficacy and toxicity, and to explore potential synergistic effects with standard drugs, before considering any promising clinical applications for this extract.

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