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ORIGINAL PAPER

Ruta graveolens, *Peganum harmala*, and *Citrullus colocynthis* methanolic extracts have in vitro protoscolocidal effects and act against bacteria isolated from echinococcal hydatid cyst fluid

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Abstract

Echinococcosis is a common and endemic disease that affects both humans and animals. In this study, the in vitro activities of methanolic extracts of *Ruta graveolens*, *Peganum harmala* aerial parts, and *Citrullus colocynthis* seeds against protoscolosis and isolated bacterial strains from hydatid cysts were assessed using disc diffusion methods and Minimum Inhibitory Concentration (MIC). The chemical composition of three methanolic extracts was studied using LC–MS. After 3 h of exposure to 40 mg/mL R. graveolens extract, a tenfold protoscolocidal effect was seen when compared to the convintional medication (ABZ) for the same duration ($P < 0.05$). The bacteria listed below were isolated from hydatid cyst fluid collected from a variety of sick locations, including the lung and liver. Micrococcus spp., *E. coli*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *Pseudomonas aeruginosa*, *Staphylococcus xylosus*, and *Achromobacter xylosoxidans* are among the bacteria that have been identified. The most effective extract was *R. graveolens*, followed by *P. harmala* and *C. colocynthis*, according to the results of antibacterial activity using the disc diffusion method. *R. graveolens* extract had the lowest MIC values (less than 2 mg/mL) against all microorganisms tested. This shows that the *R. graveolens* extract has additional properties, such as the ability to be both scolocidal and bactericidal. Because these bacteria are among the most prevalent pathogenic bacteria that increase the risk of secondary infection during hydatid cysts, the results of inhibitory zones and MICs of the *R. graveolens* methanol extract are considered highly promising.

Keywords Echinococcosis · Hydatid cyst · Antibacterial · *Ruta graveolens* · *Peganum harmala* aerial parts · *Citrullus colocynthis*

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Introduction

Cystic Echinococcosis (CE) is a parasitic disease that occurs in all mammals but mainly sheep and cattle. It also occurs in humans, caused by the larval stage of the tapeworm genus *Echinococcus* (Ali et al. 2012; Malekifard and Keramati 2018). Echinococcosis is a common and endemic health problem in humans and animals in most of the Mediterranean basin, including Jordan (Nasrieh et al. 2003). Canines serve as definitive hosts for the parasite, whereas herbivores serve as intermediate hosts for the hydatid cyst. After excretion by the definitive host, infection occurred due to intake of food or water contaminated with *Echinococcus spp*. eggs (Hijjawi et al. 2018).

The fertile hydatid cysts are typically filled with a clear fluid contains protoscolices which are mostly bacteriologically sterile. Sometimes, liver and lung hydatid cysts can be

infected with bacteria. Outside-released protoscolices have the ability to differentiate into secondary hydatid cysts in viscera. Cystic differentiation of protoscolices can probably be triggered by altered physiological conditions, such as bacterial diffusion into the cyst fluid causes the concerted effort between parasites and bacteria that cause some human and animal pathologies (Aitken et al. 1978). According to Boes and Helwigh (2000), there are two types of synergy between parasites and bacteria: first, indirect synergy, that causes an increase in the pathogenic effects of the bacteria and makes the host susceptible to the bacterial disease, especially when the bacteria and parasites occur in the same tissue or organ; and second, a direct synergy that occurs when bacteria transported into the host by the parasite after invading stages of the parasite present in the environment (Ziino et al. 2009).

In animals, the synergy between CE and bacteria results in significant economic losses due to decreased meat, wool, and milk production, as well as the condemnation of infected organs (Jahed et al. 2013), whereas in people, the economic issues are due to the amplified costs of therapy and surgery (Ahmed et al. 2021). Antibiotics, whether synthetic or natural, are important biochemicals produced by living organisms and widely employed in medical use. In spite of producing a large number of new antibiotics by the pharmaceutical industries within the last three decades, microbial resistance to these drugs has increased, as well (Al-Asoufi et al. 2017; dos Santos et al. 2001).

Uncontrolled use of commercial drugs by either patients or prescriptions that are made without susceptibility tests increases the resistance of bacteria and parasites (De Queiroz et al. 2014; Friedman et al. 2002). Therefore, more attention needs to be paid to increase the interest in plant extracts as antibacterial and anti-parasitic agents. Plant-based products are thought to account for 30% of all medicine sales worldwide. Jordan's check list of medicinal plants includes 2552 flowering plant species, and 363 of them are medicinal plants (Oran and Al-Eisawi 1998; Oran 2014), giving scientists encouragement to study and investigate their biological activities.

The plant *R. graveolens* belongs to the family *Rutaceae* and is commonly known as Rue (Oran 2014). It is a herbaceous perennial that was originally native to the Mediterranean region (Asgarpanah and Khoshkam, 2012). In Jordan, the plant *R. graveolens* is used as a spice (Oran and Al-Eisawi 1998). In folk medicine, it is used as an aphrodisiac and fertility-promoting agent (Asgarpanah and Khoshkam 2012), and is used to treat several diseases, including parasitic infections, inflammation, ulcers, hypotension, reproductive disorders, menstrual problems, and wounds. *R. graveolens* has anticancer and schistosomicidal activity (Amabye 2015; Asgarpanah and Khoshkam 2012; Carvalho et al. 2019; De Queiroz et al. 2014; Pathak et al. 2003). The *R. graveolens* extracts and essential oil showed good antibacterial and antifungal properties (Al-Shuneigat et al. 2015; Amabye 2015). According to Nabaei et al. (2014), no toxic effect was reported using different doses of hydroalcoholic extract of *R. graveolens* on the histopathology of the liver. The plant *P. harmala* is commonly known as Syrian Rue and has the Arabic names of Harmal and Harjal (Oran and Al-Eisawi 1998). It belongs to the family *Zygophyllaceae*, and is widely used in folk medicine. *P. harmala* alkaloids are used as anti-parasidal, antifungal, antibacterial, insecticidal, anti-leishmanial effects and anticancer by exhibiting a cytotoxic effect on leukemia cell lines (Mamedov et al. 2018; Moazeni et al. 2014, 2017; Moloudizargari et al 2013; Niroumand et al. 2015; Rezaee and Hajighasemi 2019; Sohrabi et al. 2018; Wink 2012).

Citrullus colocynthis belongs to the family of *Cucurbitaceae* (Oran and Al-Eisawi 1998). It is distinguished by the occurrence of many constituents such as flavonoids, alkaloids, carbohydrates, tannins, gums, and mucilage. *C. colocynthis* has been used in the traditional medicine as anticancer, antibacterial, insecticidal, anti-diabetic, and anti-parasitic including *Leishmania*, *Plasmodium* and *Haemonchus contortus* (Ahmed et al. 2019; Dhakad et al. 2017; Uma and Sekar 2014).

The goal of this study was to find out how common bacterial infection is in hydatid cysts and to identify the most common bacterial species found in hydatid fluid. In addition, the effects of methanolic extracts of *P. harmala* aerial parts, *R. graveolens,* and seeds of *C. colocynthis* on the sustainability of bacterial strains and protoscolices isolated from hydatid cysts were studied in vitro.

Materials and methods

Microbial analysis for cyst fluid

An entire of 3725 animals (sheep and goats) including 1675 native and 2050 imported have been collected between 1/8/2020 to 1/10/2020, from slaughter houses in the area of Karak. The infected organs (liver or/and lung) were collected and transported to the laboratory within an hour of collection under refrigerated conditions. The infected organ surface was sterilized with 70% ethanol and washed with sterile distilled water. The hydatid fluid was aspirated by a sterile syringe, the protoscolices were isolated, and the hydatid fluid cultured for isolation and identification of bacteria.

Bacterial isolation and identification

Initially the hydatid fluid was inoculated on three different types of media: blood agar for the bacterial isolation of aerobic and facultative anaerobic Gram-positive, Eosin Methylene Blue (EMB) agar, and MacConkey agar for the isolation

of Gram-negative bacteria. Then, the grown colonies were picked and inoculated on tryptone soy agar and nutrient agar to get pure culture.

To identify the bacterial isolates, colonies and cells characteristics were determined microscopically. The Gram-positive isolates were further characterized using standard biochemical tests including oxidase, DNase, catalase, phosphatase, coagulase, and fermentation of mannitol, starch and sodium hippurate, pyrrolidonyl arylamidase (PYR) and Christie-Atkins, aesculin hydrolysis, Munch-Petersen (CAMP) tests, and novobiocin sensitivity. The Gram-negative isolates were further characterized using standard biochemical tests including motility, methyl red, urease, indole production, Voges-Proskauer, o-nitrophenyl-β-d-galactopyranoside (ONPG) potassium cyanide (KCN) and H_2S production, triple sugar iron agar (TSI), reactions of phenylalanine and lysine, lactose fermentation, and ornithine decarboxylase tests. In addition, the identification was confirmed using API 20E and API Staph diagnostic systems (Khleifat et al. 2008).

Plant materials

R. graveolens, *P. harmala,* and *C. colocynthis* were collected in June, 2020. *R. graveolens* was collected from Irbid, northern of Jordan. *P. harmala* and *C. colocynthis* were collected from AL Karak, southern of Jordan. The plants were identified to species level by Prof. Sawsan Al Oran, Biology Department, Faculty of Science, Jordan University. The freshly gathered materials were washed, air-dried in the shade at room temperature, and then ground into a reasonable powder using a mixer.

Extraction

A 100 g sample of both *R. graveolens* and *P. harmala* plants aerial parts, as well as *C. colocynthis* seeds, were steeped in 1000 mL methanol for 3 days at room temperature with continuous shaking. The solvent was extracted using a rotary evaporator at 45 °C with reduced pressure after filtration. The extracts were kept in sealed glass vessels at − 20 °C.

Determining the protoscolices' mortality

The mortality of protoscolices was determined by measuring cell motility while staining with 0.1 percent aqueous eosin solution. Dead protoscolices stained with eosin and appear in reddish color using a microscope, whereas alive protoscolices do not permeate the eosin and thus remain unchanged (Al-Arabi et al. 2019; Smyth and Barrett 1980). The rate of mortality was considered by taking the number of dead protoscolices divided by the number of predicted headings.

Extracted protoscolices were kept in a sterile Roswell Park Memorial Institute (RPMI) 1640 medium provided with fetal bovine serum (10%) under incubation of 37 °C. To control the contamination (Wang et al. 2015), penicillin (100 U/mL) and 100 µg/mL streptomycin sulfate were added to the medium (Malekifard and Keramati 2018; Monteiro et al. 2017). The impact of *P. harmala*, *R. graveolens*, and seeds of *C. colocynthis* methanol extracts on the percentage of mortality of protoscolices in vitro was conducted. Protoscolices were treated with 10–40 mg/ml with being 10 as intervals of the three plants by taking one milliliter of protoscolices suspension containing about 2×10^3 protoscolices/mL in test tubes. The length of exposure periods was 1, 3, 6, 12, 18, and 24 h. To test the viability of protoscolices, 100 µl of pooled protoscolices were mixed with 100 µl of 0.1 percent eosin on a slide for 15 min; dead protoscolices stained red, while surviving protoscolices remained colorless, as observed under a compound microscope. Only samples with 100 percent viable protoscolices were used for the in vitro studies (Yones et al. 2011). For comparison, stock solution of albendazole (ABZ) (protoscolicidal agent available for treatment of human hydatid disease) prepared by dissolving 0.5 g in 1 mL of 30% DMSO; the drug was filtered before using through a 0.22 µm filter. The efficacy of methanolic plants extract on the viability of protoscolices was compared with positive and negative control groups which received 20 mg/mL ABZ and normal saline, respectively (Blanton et al. 1998). Experiments were carried out in triplicates. For obtaining images, digital camera type (Pro-MicroScan, 8 M Pixels High-Speed) and light microscope (100x) model (OLYMPUS CX21FS1) were used.

Dual staining with acridine orange–ethidium bromide (AO/EB)

In seek to monitor the cellular and nuclear morphological changes, the protoscolices were treated with different concentrations of tested plants extracts and ABZ for 48 h, washed with PBS, and dual stained with an equal volume of AO (100 µg/ml) and EB (100 µg/ml) for 2 min (Durgadevi et al. 2019). The preparation was examined using a fluorescent microscope, in which green-colored cells were indicative of viable protoscolices, while those in red color are dead protoscolices.

Antibacterial assay

Bacterial strains

Methanolic extracts of aerial sections of *R. graveolens* and *P. harmala* plants, as well as seeds of *C. colocynthis*, were tested against eight bacterial species isolated from hydatid cysts. Two were Gram-positive bacteria (*Micrococcus* spp.

and *S. xylosus*) and six were Gram-negative bacteria (*P. aeruginosa, A. xylosoxidans, E. coli, E. amnigenus, E. aerogenes,* and *K. oxytoca*). In addition, strains of *S. aureus, B. cereus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* with known ATCC identity were employed.

Disc diffusion method

The disc diffusion method was used to investigate the antibacterial activity of the various extracts (Alzoreky and Nakahara 2003). The cell number was adjusted to 2 10^6 CFU/mL through mixing the investigated bacteria's broth cultures with sterile nutritional agar that was cooled at 45–50 °C. The inoculated agar was then placed on sterilized Petri plates for 45–60 min to harden. Then, under aseptic conditions, a disc containing 1 mg, 2 mg of plant extracts, 10% DMSO (negative control), or tetracycline (positive control) was deposited on the surface of the agar plate. The growth inhibitory action was measured by measuring the diameter of the clear zone around the disc with a ruler after 24 h at 37 °C (Khleifat et al. 2006; Romero et al. 2005). Triplicates of each sample were evaluated.

Minimum inhibitory concentration (MIC)

Plant extracts' minimum inhibitory concentration (MIC) against bacterial growth was determined. A bacterial inoculum of 2×10^6 CFU/mL was inoculated into tubes containing a serial dilution of plant extract (0–2 mg/mL) in 5 mL Muller-Hinton broth. The MIC was considered as the lowest concentration of extracts that inhibited observable bacterial growth (Khleifat et al. 2010; Patel et al. 2011).

Liquid chromatography–mass spectrometry (LC–MS)

At a flow rate of 0.5 ml/min, HPLC separation was performed with the mobile phase containing solvents A and B in gradient, where A was 0.1 percent (v/v) formic acid in water and B was 0.1 percent (v/v) formic acid in acetonitrile, for the following gradient: 5 percent B for 5 min, 5–100 percent B in 15 min, and 100 percent for 5 min. The Agilent Zorbax Eclipse XDB-C18 column was used (2.1×150 mm $\times 3.5$ um). The sample injection volume was 1 l (18 mg/mL in methanol) and the oven temperature was 25 °C. The eluent was scanned from 100 to 1000 m/z and secondary scan from 50 to 100 m/z MRM mode using a Shimadzu LC–MS 8030 with an electrospray ion mass spectrometer (ESI–MS) in positive-ion mode. The ESI was performed with a 125 V fragment or and a 65 V skimmer. At a flow rate of 10 L/min, a nebulizer at 45 pressure, and a capillary temperature of 350 C, high-purity nitrogen (99.999 percent) was used as the drying gas. A blank of 0.1 percent formic acid was utilized in parallel. The Shimadzu CBM-20A system controller, the LC-30AD pump, the SIL-30AC autosampler with cooler, and the CTO-30 column oven were used to inject a sample into the mass detector.

Results

During inspections from January 8, 2020 to January 10, 2020, 3,725 butchered animals were checked. Hydatid cysts were found in 25 of the 3,725 animals killed.

Isolation of protoscolice-associated bacteria

A total of 8% of the cysts were found in the lung, 16% in the liver, and 76% in both the liver and the lung. Nineteen of the 25 hydatid cyst containing animals were infected with one or more bacterial species representing a bacterial infection rate of 76% (Table 1). Thirty-one bacterial isolates were collected from hydatid cysts fluid of lung, liver or lung and liver. Interestingly, 78.9% of the isolated bacteria were isolated from samples of bi-organs infected animals. Gram-negative bacteria were found in six of the isolates, while Gram-positive bacteria were found in two. *Staphylococcus xylosus, Achromobacter xylosoxidans*, and *Pseudomonas aeruginosa* have been the most prevalent bacteria detected in hydatid cysts fluid retrieved from the lungs, whereas *Micrococcus* spp. and *E. coli* were recovered from hepatic hydatid cysts fluid. *Klebsiella oxytoca*, *Enterobacter aerogenes, Pseudomonas aeruginosa,* and *Enterobacter amnigenus* were identified from the fluid of hydatid cysts in the lungs and livers. *Pseudomonas aeruginosa* was the most commonly isolated species, with 9 isolates; other species included 6, 5, 4, 2, and 2 isolates, respectively, for *K. oxytoca*, *E. amnigenus*, *E. aerogenes*, *A. xylosoxidans*, and *E. coli*. *Micrococcus* spp. was identified from two samples of fluid collected from the liver, whereas *S. xylosus* was recovered from one hydatid cyst fluid sample collected from the lung.

Treatment of protoscolices in vitro with methanolic extracts of *R. graveolens*, *P. harmala*, and *C. colocynths*

The hydatid cyst protoscolices were treated with methanolic extracts of *R. gravelens*, *P. harmala*, and *C. colocynths* for various periods of time (1, 3, 6, 12, 18, and 24 h). The extract concentrations used in this study were 10, 20, 30, and 40 mg/mL (Table 2 and Fig. 1a–c). When protoscolices were treated with *R. graveolens* methanol extract, the killing of protoscolices was dramatically increased. This study revealed that using different concentrations of methanol extracts from *R. graveolens*, *P. harmala*, and *C. colocynthis* resulted in a significant effect ($P < 0.05$), with mortality

Table 1 Bacteria isolated from hydatid cysts fluid in the lungs or/and livers of sheep and goats

Animal	Infected organs	Gram-negative bacteria						Gram-positive bacteria	
		P. aeruginosa	*K. oxytoca*	*E. amnigenus*	*E. aerogenes*	*A. xylosoxidans*	*E. coli*	*Micro-coccus spp.*	*S. xylosus*
Sheep	Lung and liver	*	*		*				
Sheep	Lung					*			*
Sheep	Lung and liver	*		*					
Sheep	Lung and liver		*						
Goats	Liver						*	*	
Sheep	Lung and liver			*					
Sheep	Lung and liver	*	*						
Sheep	Lung	*				*			
Sheep	Lung and liver				*				
Sheep	Lung and liver	*							
Sheep	Liver								
Sheep	Lung and liver	*							
Sheep	Lung and liver								
Sheep	Lung and liver	*		*					
Sheep	Lung and liver	*							
Sheep	Lung and liver	*	*						
Sheep	Lung and liver								
Sheep	Lung and liver								
Sheep	Lung and liver			*	*				
Sheep	Lung and liver								
Sheep	Lung and liver		*						
Sheep	Lung and liver			*					
Goats	Liver								
Goats	Liver						*	*	
Sheep	Lung and liver		*		*				
Total isolates		**9**	**6**	**5**	**4**	**2**	**2**	**2**	**1**

Table 2 Protoscolosidal effect of various concentrations of *R. graveolens, C. colocynthis,* and *P. harmala* methanolic extracts and ABZ.in vitro

Plant extract	Con. mg/mL	Mortality rate (%)					
		1 h	3 h	6 h	12 h	18 h	24 h
R. graveolens	10	65.3 ± 4.6	87.5 ± 0.3	96.0 ± 2.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	20	75 ± 2.0	97.5 ± 1.3	98.5 ± 1.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	30	84.5 ± 1.8	98 ± 1.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
	40	98 ± 1.2	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
P. harmala	10	2.9 ± 1.5	4.0 ± 0.4	7.7 ± 1.0	9.7 ± 1.2	11.6 ± 1.4	15.3 ± 2.0
	20	4.8 ± 0.9	9.1 ± 2.2	18.4 ± 1.4	28.3 ± 1.7	31.5 ± 0.8	34.7 ± 1.0
	30	8.4 ± 0.6	16.6 ± 1.5	22.2 ± 2.3	27.9 ± 2.7	40.3 ± 4.7	45.7 ± 1.4
	40	14.5 ± 2.3	19.2 ± 2.4	26.3 ± 3.5	33.7 ± 2.1	42.7 ± 5.6	50.0 ± 1.7
C. colocynths	10	2.7 ± 0.6	4.7 ± 1.5	6.0 ± 2.6	7.8 ± 2.3	9.7 ± 2.1	10.3 ± 2.3
	20	1.7 ± 0.6	4.0 ± 1.0	4.3 ± 0.6	6.3 ± 1.5	8.0 ± 1.7	9.7 ± 1.5
	30	4.0 ± 1.0	5.3 ± 1.2	7.3 ± 1.5	9.3 ± 1.5	10.3 ± 1.2	12.7 ± 1.5
	40	5.3 ± 0.6	7.3 ± 1.2	8.3 ± 0.6	10.3 ± 0.6	12.3 ± 1.5	15.3 ± 2.1
− ve Control		1.7 ± 0.6	1.7 ± 0.6	2 ± 1.2	2.5 ± 0.6	3 ± 0.6	4.5 ± 0.0
+ Ve Control (ABZ)	20	3 ± 2.0	9.7 ± 1.5	22.7 ± 4.5	47.5 ± 4.6	71.7 ± 2.5	96.3 ± 3.1

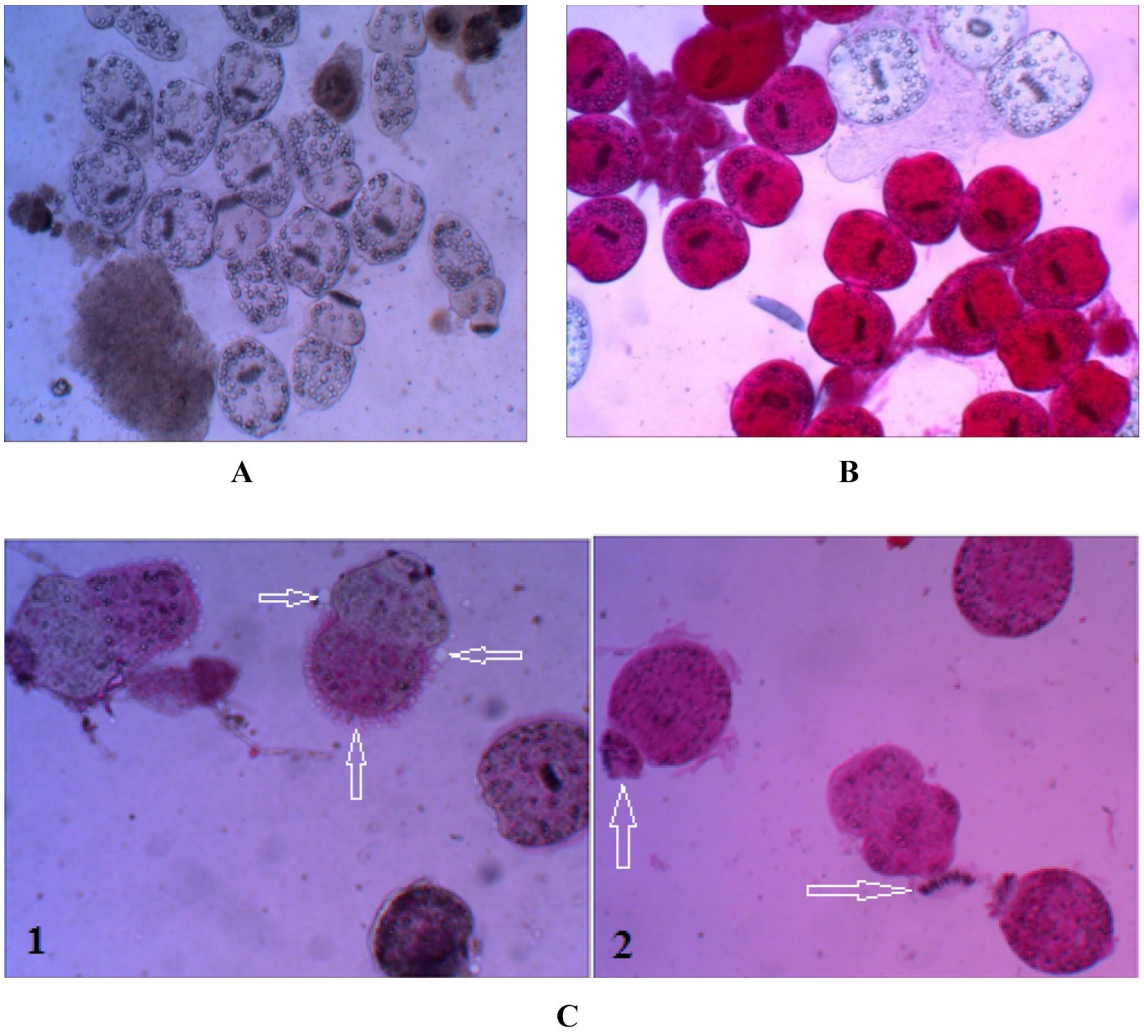

Fig. 1 Images of living, dead, and partially dead E. granulosus protoscolices following staining with 0.1 percent eosin; (**a**), protoscolices untreated with plant extract. (**b**), effect of 20 mg/mL ABZ on protoscolices after 24 h of exposure (**c**), total mortality of protoscolices after 1.25 h (75 min) of exposure to 40 mg/mL R. graveolens extract 1c. Blebs formation in tegument, 2c. Rostellar disorganization and loss of hooks and microtriches (Total magnification 100X)

rates of 100%, 50%, and 15% after treatment for periods of 1.25 h, 24 h, and 24 h, respectively, and using the maximum concentration (40 mg/mL). The survivability testing findings were in line with the morphological changes and structural damages seen in protoscolices. As shown in structural and morphological investigations involving SEM, there was a positive association between the intensity of injury and the extract concentration (Fig. 2a–d). Alterations in protoscolices included loss of motility, paralysis, tegument bleb formation, contraction of the soma area, rostellar disarray, and loss of hooks and microtriches. Normal protoscolices showed green fluorescence as a result of acridine orange penetrating the normal cell membrane, but apoptotic protoscolices showed orange colored apoptotic bodies occurring as a result of nuclear shrinkage, damage, and blebbing (Fig. 3). When studied under a fluorescent microscope, dead protoscolices showed red hue fluorescence due to their loss of membrane integrity. These structural and morphological changes were identical to those seen in protoscolices that had been treated with ABZ in vitro.

Antibacterial activity

The disc diffusion method was used to test the antibacterial activity of methanolic extracts of *R. graveolens*, *P. harmala*, and *C. colocynthis* at two different concentrations. In general, all of the extracts examined had varying antibacterial activity that was dose-dependent. *R. graveolens* was the most effective extract, followed by *P. harmala* and *C. colocynthis*. The Gram-negative *A. xylosoxidans* strains were the most sensitive, with the largest inhibition zone (18.3 mm). All bacterial strains were inhibited

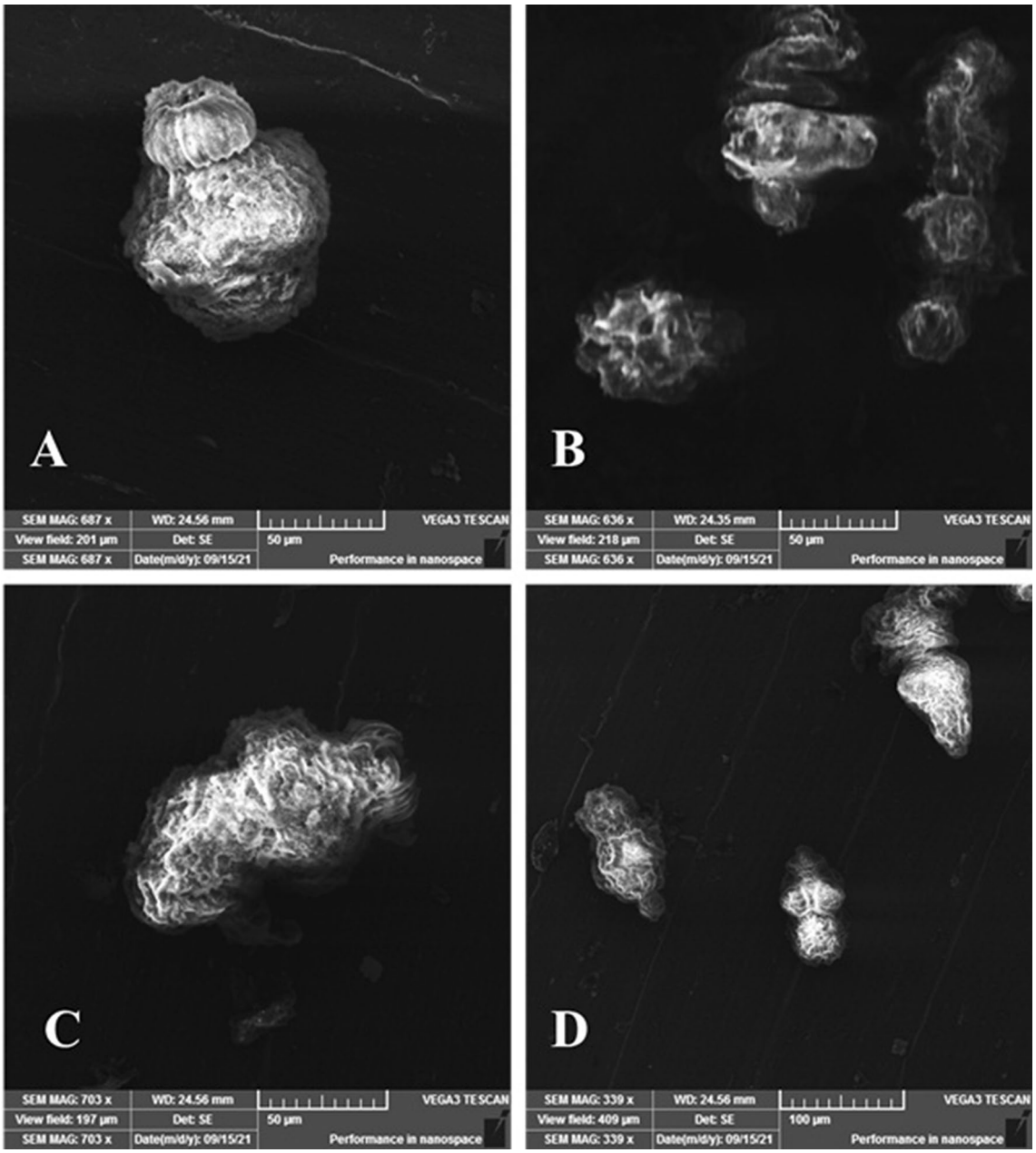

Fig. 2 The ultrastructural damages observed with scanning electron microscopy when treated with R. graveolens extract. (**A**) Evaginated control protoscolex, (**B**) Invaginated protoscolices, clearly altered after culture in the presence of R. graveolens extract, collapse of soma region, disorganization in the rosteller cone is visible with damage in scolex and sucker region. (**C**) Loosening of hooks, disorganization of hooks was also observed at rosteller cone (**D**) Collapse of the soma region, shedding of microtriches of the scolex region and damage at the surface teguments is also observed. (Magnification A, B, C 686X, D, 340X)

by *R. graveolens* methanolic extract at both concentrations tested, with the exception of *S. xylosus* at 1 mg/disc. Strong antibacterial activity of *R. graveolens* extract against *A. xylosoxidans*, *E. amnigenus*, *S. aureus* ATCC 43,300, *B. subtilis* ATCC 6633, *E. aerogenes,* and *B. cereus* ATCC 11,778 was seen at the maximum dosage tested (2 mg), with inhibition zones ranging from 14.0 to 18.3 mm.

R. graveolens methanolic extract had a moderate antibacterial activity against E. *coli, P. aeruginosa* ATCC 27,853*, Micrococcus spp*, *S. xylosus, P. aeruginosa*, and *K. oxytoca*, with an inhibition zone ranging from 10.3 to 13.3 mm (Table 3). *S. aureus* was the most susceptible strain to *P. harmala* extract, with inhibition zones of 12.0 and 14.3 mm at dosages of 1 and 2 mg/disc, respectively. *P. harmala* extract inhibited *B. cereus* ATCC 11,778, *B. subtilis* ATCC 6633,

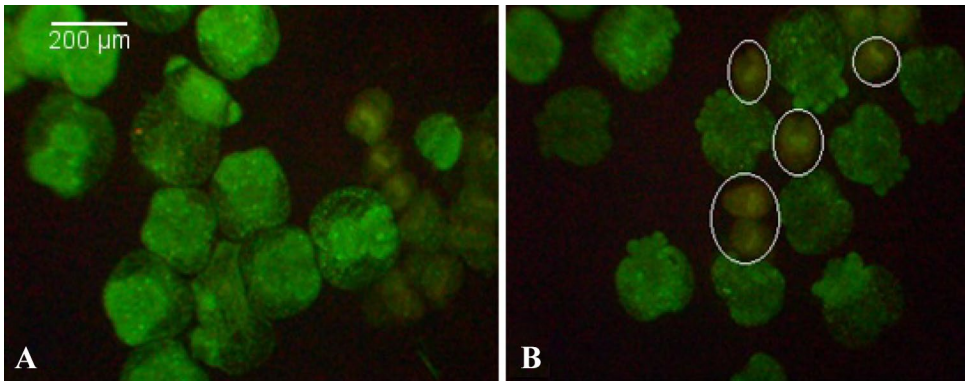

Fig. 3 Viable and dead protoscolices stained by acridine orange and ethidium bromide (**a**) viable protoscolices appeared in bright green color and (**b**) dead protoscolices with red color when treated with R. graveolens

P. aeruginosa ATCC 27,853, *P. aeruginosa*, *E. coli* ATCC 25,922, *Micrococcus* spp, *K. oxytoca,* and *A. xylosoxidans* at the maximum dose tested, with inhibition zones ranging from 13.0 to 9.7 mm. Because no inhibitory zones were found, *E. amnigenus, E. aerogenes,* and *S. xylosus* appear to be resistant to *P. harmala* extract. The disc diffusion method also revealed that the extract of *C. colocynthis* has antibacterial activity against Gram-positive bacteria. *S. aureus* ATCC 43,300, *B. cereus* ATCC11778, *B. subtilis* ATCC 6633, and *Micrococcus* spp. were inhibited by *C. colocynthis* extracts in inhibition zones ranging from 13.3 to 8.7 mm. Against all Gram-negative bacteria tested, no inhibition zones were found.

Table 4 shows the MIC of MeOH extracts of *R. graveolens, P. harmala,* and *C. colocynthis*. In general, the MIC results were consistent with the inhibitory zones seen, *with R. graveolens* being the most potent extract, followed by *P. harmala*, and then, *C. colocynthis*. *B. cereus* ATCC 11,778 was the most sensitive bacterial strain. All of the extracts studied are still more efficient against Gram-positive bacteria than Gram-negative bacteria. All of the tested bacteria showed MIC values of less than 2 mg/mL against *R. graveolens* extract. The extract MIC value for *B. cereus* ATCC 11,778 (0.25 mg/mL) was the lowest, followed by *S. aureus* ATCC43300 (0.5 mg/mL), *B. subtilis* ATCC 6633 (0.5 mg/mL), *A. xylosoxidans* (0.5 mg/mL), and *K. oxytoca* (1.5 mg/mL). *P. harmala* extract had MIC values of 0.25 mg/mL against *B. cereus* ATCC 11,778 and *B. subtilis* ATCC 6633, respectively. *P. harmala* extract had MIC values of 1 mg/mL against *Micrococcus* spp., *S. aureus* ATCC 43,300, *E. coli, E. coli* ATCC 25,922, and *P. aeruginosa* ATCC 27,853, but 1.5 mg/mL against *P. aeruginosa* ATCC 27,853. *S. xylosus E. amnigenus, E. aerogenes,* and *K. oxytoca* were resistant to *P. harmala* extract with MIC values less than 2 mg/mL, according to disc diffusion technique data. *C. colocynthis* extract was more efficient against Gram-positive bacteria

Table 3 Antibacterial activity of methanolic extracts of *R. graveolens*, *P. harmala* and *C. colocynthis* using disc diffusion method

Bacterial species	Zone of inhibition (mm)					
	R. graveolens		*P. harmala*		*C. colocynthis*	
	1 mg/disc	2 mg/disc	1 mg/disc	2 mg/disc	1 mg/disc	2 mg/disc
Gram-positive bacteria						
Micrococcus spp.	8.7 ± 0.57	13.0 ± 1.0	9.0 ± 1.0	11.3 ± 0.57	− ve *	8.7 ± 0.57
S. xylosus	− ve	10.3 ± 1.5	− ve	− ve	− ve	− ve
S. aureus ATCC 43,300	12.0 ± 1.0	15.4 ± 1.2	12.0 ± 1.0	14.3 ± 1.15	11.0 ± 1.0	13.3 ± 0.58
B.cereus ATCC11778	10.0 ± 1.3	14.0 ± 1.0	9.7 ± 0.58	13.0 ± 1.0	9.3 ± 0.58	12.4 ± 0.55
*B. subtilis*ATCC6633	10.0 ± 1.0	14.7 ± 0.58	10.0 ± 1.0	13.0 ± 1.0	9.0 ± 1.0	12.3 ± 0.58
Gram-negative bacteria						
P. aeruginosa	10.0 ± 1.3	13.0 ± 1.0	10.0 ± 1.0	12.7 ± 0.6	− ve	− ve
A. xylosoxidans	14.0 ± 1.5	18.3 ± 1.5	0.0 ± 0.0	9.7 ± 0.6	− ve	− ve
E. coli	10.3 ± 1.5	13.3 ± 1.5	8.7 ± 0.57	11.66 ± 0.57	− ve	− ve
E. amnigenus	11.0 ± 1.0	16.0 ± 0.8	− ve	− ve	− ve	− ve
E. aerogenes	10.7 ± 0.6	14.7 ± 1.15	− ve	− ve	− ve	− ve
K. oxytoca	10.3 ± 1.5	12.7 ± 2.0	− ve	10.7 ± 0.6	− ve	− ve
*E. coli*ATCC 25,922	10.0 ± 1.0	12.4 ± 0.55	9.0 ± 1.0	11.4 ± 0.53	− ve	− ve
P. aeruginosa ATCC27853	9.3 ± 0.58	13.0 ± 1.0	10.3 ± 0.58	13.0 ± 1.0	− ve	− ve

Table 4 The MIC of MeOH extract of *R. graveolens*, *P. harmala* and *C. colocynthis* plants on different bacterial strains [mg/ ml]

	MIC (mg/mL)		
	R. graveolens	*P. harmala*	*C. colocynthis*
Gram-positive bacteria			
Micrococcus spp.	1.0	1.0	2.0
S. xylosus	2.0	> 2.0	> 2.0
S. aureus ATCC 43,300	0.5	1.0	2.0
B. cereus ATCC 11,778	0.25	0.25	0.5
*B. subtilis*ATCC 6633	0.5	0.25	0.5
Gram-negative bacteria			
P. aeruginosa	1.0	1.0	> 2.0
A. xylosoxidans	0.5	2.0	> 2.0
E. coli	1.0	1.0	> 2.0
E. amnigenus	1.0	> 2.0	> 2.0
E. aerogenes	1.0	> 2.0	> 2.0
K. oxytoca	1.0	> 2.0	> 2.0
*E. coli*ATCC 25,922	1.0	1.0	> 2.0
P. aeruginosa ATCC 27,853	1.0	1.0	> 2.0

than Gram-negative bacteria, similar to the results of the disc diffusion approach. *C. colocynthis* extract has an MIC of less than 2 mg/mL against all Gram-negative bacteria tested. *B. cereus* ATCC 11,778 and *B. subtilis* ATCC 6633 had the lowest MIC values (0.5 mg/mL and 2.0 mg/mL, respectively), followed by Micrococcus spp. (1.5 mg/mL) and *S. xylosus* and *S. aureus* ATCC 43,300 (2.0 mg/mL, respectively).

Chemical composition of *R. graveolens*, *P. harmala*, and *C. colocynthis* using LC–MS

LC–MS was used to examine the chemical composition of methanolic extracts of *R. graveolens*, *P. harmala* aerial parts and the *C. colocynthis* seeds (data not shown). In the methanolic extract of the aerial portion of *R. graveolens*, a total of 26 compounds were identified. The main components of *R. graveolens* methanolic extract were rutin (13.7%), quercetin (9.3%), isoquinoline (6.9%), methoxypsoralen (6.8%), procyanidin (6.3%), and tropane (6.3%). (5.5 percent). In the aerial component of *P. harmala* methanolic extract, 31 compounds were identified using LC–MS. The primary components were discovered as harmaline (10.6 percent), harmine (6.3 percent), and pinene (6.3 percent). Linalool (5.9%), squalene (5.8%), terpineol (5.5%), catechin (5.4%), limonene (5.3%), terpinene (5.35), flavan (4.9%), and anthraquinone (4.9%) were found abundant in the extract (4.7%). The methanolic extract of *C. colocynthis* seeds comprised 32 compounds. Lactic acid was found in the highest concentration (10.2%), followed by xylitol (7.4%), glycerol (7.2%), proline (6.2%), inositol (5.4%), glucitol (5.4%), lauric acid (5.4%), linoleic acid (5.3%), phytol (5.3%), and campesterol (5.3%).

Discussion

E. granulosus causes cystic echinococcosis, a parasitic cestode illness. It causes a medically and veterinary-important persistent infection (Ahmed et al. 2021; Zeghir-Bouteldja et al. 2009). After three hours of treatment, *R. graveolens* extract produced a result that was incomparable to conventional ABZ treatment, which produced a tenfold extra deadly effect than ABZ. The viability test results corroborate morphological and structural changes detected using a compound, fluorescence, and scanning electron microscopy. Tissue injury was assessed at the ultrastructural level using a scanning electron microscope (SEM). After culture in the presence of *R. graveolens* extract and ABZ, invaginated protoscolices showed evident changes, including the collapse of the soma region, disarray in the rosteller cone, damage to the scolex and sucker region and damage to the surface teguments. In comparison to the control group, the damage was decreased but still significant at the highest concentration of *C. colocynthis* extract.

The percentage of bacterial infection of hydatid cysts in this study was 76 percent. Several previous studies found a high incidence (88 percent) of bacterial infection in hydatid cysts isolated from cattle, goats, and sheep (Hadadi et al. 2020; Khleifat et al. 2010; Ziino et al. 2009). Furthermore, *P. aeruginosa*, *K. oxytoca*, *E. amnigenus*, and *E. aerogenes* were the most common bacterial isolates, indicating that Gram-negative bacteria are the most common bacterial invaders in hydatid cysts. According to similar studies, Gram-negative bacteria were found to be prevalent in hepatic and lung hydatid cyst fluids in sheep, with *E. coli* and *K. pneumoniae* being the most common isolates (Abdullah et al. 2021; Fallah et al. 2014; Khleifat et al. 2010). In contrast, *S. aureus* was recovered from hepatic hydatid cyst fluids in a recent test, demonstrating that the types of bacteria isolated from the cyst fluids are highly varied (Najim et al. 2020). This could be due to the infective stages ability to live in the outside environment, as well as the life cycle of *Echinococcus* spp., which entails tissue translocation into the intermediate host.

Despite the idea that hydatid cyst fluid is a sterile fluid, bacterial pathogens from the respiratory and gastrointestinal tract were found in high numbers in bi-organ cyst fluid samples in this study. In addition to being harmful, these isolates are naturally widespread in the environment and are part of the usual flora of warm-blooded animals. According to one theory, intermediate animals such as sheep and goats swallow bacteria-infected *Echinococcus* spp. eggs (Ahmed et al.

2021; Fallah et al. 2014). When these eggs reach the colon, they hatch, and the bacterially tainted oncosphere embryo penetrates the mucosa, eventually forming hydatid cysts in the liver and lungs. According to certain views, the infection may have entered by the bile duct or enterohepatic circulation. Although only to a limited extent, hydatid cysts can be infected through the bronchial tree or a hematogenous pathway (Ahmed et al. 2021; Wani et al. 2010; Ziino et al. 2009).

According to the current study's findings, the plants studied have varying levels of antibacterial activity. The inhibitory zone results matched the MIC values for the different plant extracts. The most effective extract was *R. graveolens*, followed by P. harmala and C. colocynthis. The most sensitive strains of *A. xylosoxidans* were Gram-negative, with the largest inhibition zone (18.3 mm). The methanolic extract of *R. graveolens* inhibited all of the bacterial strains tested. *S. xylosus* demonstrated an inhibiting effect only at a concentration of 2 mg/disc. At the highest dose tested (2 mg), *R. graveolens* extract demonstrated strong antibacterial activity against *A. xylosoxidans*, *E. amnigenus*, *S. aureus* ATCC 43,300, *B. subtilis* ATCC 6633, *E. aerogenes,* and *B. cereus* ATCC 11,778, with inhibition zones ranging from 14.0 to 18.3 mm. With inhibition zones ranging from 10.3 to 13.3 mm, *R. graveolens* methanolic extract had moderate antibacterial activity against *E. coli*, *P. aeruginosa* ATCC 27,853, *Micrococcus spp, S. xylosus, P. aeruginosa, and K. oxytoca.* The inhibitory zone results matched the MIC values for *R. graveolens* extract. This demonstrates that the *R. graveolens* extract has both scolocidal and bactericidal properties. Because these bacteria are among the most common pathogenic bacteria that increase the risk of secondary infection during hydatid cysts, the results of the *R. graveolens* extract inhibition zones and MICs are deemed highly promising. These findings are consistent with those of (Pavić et al. 2019), who found that *R. graveolens* extract had excellent antibacterial activity against gram-positive bacteria such *Staphylococcus aureus*, *Streptococcus pyogenes, Listeria monocytogenes,* and *Bacillus subtilis.* Molnar et al. (Molnar et al. 2018) found that *R. graveolens* methanolic extracts had good antibacterial activity against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*, which is consistent with our findings. Rutin, quercetin, isoquinoline, tropane, procyanidin, and hydroxyl benzoic acids are phenolic chemicals found in the aerial portions of *R. graveolens* that have antibacterial and antifungal properties (Wolters and Eilert 1981). Acridone alkaloids and coumarin, phytochemical substances found in *R. graveolens* aerial portions, showed the strongest antibacterial action against Gram-positive and Gram-negative bacteria (Ivanova et al. 2005). Flavonoids such as rutinand quercetin, phenolic compounds, alkaloids, and terpenoides isolated from *R. graveolens* showed antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Amabye 2015), the high antimicrobial activity of this plant may be due to presence of these compounds. The MIC values of *R. graveolens* extract against all tested bacteria were less than 2 mg/mL, with *B. cereus* and *K. oxytoca* having the lowest (0.25 mg/mL) and highest (1.5 mg/mL) MIC values, respectively.

In this study, *P. harmala* extract was more effective against Gram-positive bacteria than Gram-negative bacteria, with the latter being moderately sensitive to *P. harmala* extract (Sohrabi et al. 2018; El-Zayat et al. 2021). According to another study, *P. harmala* chloroform extract has strong antibacterial activity against *P. aeruginosa* and *S. aureus*, with an MIC value of 1.56 ug/mL (Hadadi et al. 2020). A flavonoid-rich extract of *P. harmala* leaves had excellent antibacterial activity against *E. coli* but not against S. aureus (Fatma et al. 2016). The methanolic extract of *P. harmala* aerial parts was shown to be high in beta-carboline, harmaline, and harmine in this study. The beta-carboline molecules are one of *P. harmala*'s most potent components (Moloudizargari et al. 2013). Harmane, harmine, harmaline, and harmalol have been found to be bacteriostatic against *E. coli*, *Proteus vulgaris, S. aureus,* and *B. subtilis* (Nenaah 2010). In vivo and in vitro, these core chemicals may be responsible for antibacterial, anti-parasitic, and other biological actions (Doskaliyev et al. 2021). Other chemicals identified from *P. harmala* extracts, such as catechin, apigenin, rutin, anthraquinone, and flavan, may have diverse biological actions, as demonstrated in various investigations (Allaq et al. 2021; Darabpour et al. 2011; Elansary et al. 2020; Mounira et al. 2021). Most bacteria isolated from hydatid cysts were resistant to *P. harmala* extract at high concentrations (MIC > 2 mg/ml), while MIC results for different ATCC bacteria ranged between 0.25 and 1.5 mg/mL. The varying quantities of antibiotics provided to afflicted sheep may have contributed to the high resistance of bacteria isolated from hydatid cysts.

C. colocynthis seed extract has been shown to have weak antibacterial activity. However, against Gram-positive bacteria, *C. colocynthis* seed extract was more efficient than against gram negative bacteria. It was mentioned that all Gram-negative bacteria, as well as *S. aureus* was resistant to *C. colocynthis* seed extracts (Bourhia et al. 2021). Gram-positive and Gram-negative bacteria were inhibited by *C. colocynthis* extracted using high polarity solvents such as water and acetone. Cucurbitacin B, E, and I, among the most potent components of *C. colocynthis* seed, have been shown to have antibacterial action against *Staphylococcus aureus, Bacillus cereus*, and *K. pneumonia* (Ali et al. 2013). Cucurbitacin E has also been shown to have antibacterial properties against M. tuberculosis H37Rvat (Bourhia et al. 2020). The biological activity of plant extracts is dependent on the solvent and extraction method used, and the antimicrobial effectiveness of plant extracts is dependent on the active substances, selected bacterial strains, and plant parts tested, so

antimicrobial activity may differ from one bacterium Gram-negative to another Gram-positive (Qaralleh et al. 2019). This low activity could be due to acquired resistance through mutations, or to infected animals' failure to respond to all applicable treatments. The findings of variances in antibacterial outcomes could be related to differences in plant collecting time or phytochemical concentration during season growth (Kumar et al. 2006; Esiyok et al. 2004).

Conclusion

The current study demonstrated the antibacterial activity of *R. graveolens, P. harmala* and *C. colocynthis* against pathogenic bacteria isolated from hydatid cysts. The results of this study provide evidence to use and develop naturally occurring agents such as *R. graveolens* and *P. harmala* aerial part extracts as antibacterial agents. Scolocidal and antibacterial properties of methanolic extracts of *R. graveolens* may have the ability to reduce the in vivo appearance of secondary infection in hydatid cysts. This can be applied to protecting and treating humans as well as animals. However, further investigation is required, including studying their toxicity effects of these extracts.

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Declarations

Competing Interests The authors declare no competing interests.

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