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ORIGINAL ARTICLE

Antiprotozoal potential of *Salvadora persica* against three virulent subtypes of *Blastocystis* sp.

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Abstract *Blastocystis* sp. is a group of anaerobic protozoa parasitizing the gastrointestinal tract of humans and a broad variety of animals. Evidences of *Blastocystis* parasites resistance development to antiprotozoal drugs urge the exploration of new therapeutics. Antiprotozoal potential of *Salvadora persica*, a medicinal plant traditionally used for oral hygiene, was evaluated in vitro against *Blastocystis* sp. human isolates. Until now, no study has described the effect of *S. persica* extracts on this parasitic protozoa. *Blastocystis* sp. positive stool samples collected from patients with gastrointestinal complaints and asymptomatic individuals diagnosed by microscopy were furthermore cultured in vitro and characterized by PCR and multiplex-PCR using sequence-tagged-site primers to determine their subtypes. Out of 21 *Blastocystis* sp. isolates, five were determined as ST1, 14 as ST3, and two as ST5 subtypes. Antiprotozoal activity of untreated and heat-treated *S. persica* roots aqueous extracts was evaluated in vitro by serial dilutions on three *Blastocystis* sp. subtypes; ST1, ST3, and ST5 isolated from symptomatic patients. A significant killing activity was observed with both, untreated and heat-treated aqueous extracts of *S. persica* at minimal concentration of 2.5 µl/ml compared to parasites' growth controls ($P < 0.05$). Maximal antiprotozoal effect was reached at a concentration of 20 µl/ml of *S. persica* aqueous extract. Means of growth inhibition effect obtained with untreated and heat-treated extracts at 40 µl/ml against the three subtypes of *Blastocystis* sp. were 80% (SD 2.3) and 82% (SD 1.1), respectively. No significant difference was observed in the inhibitory effect of *S. persica* extracts between the three *Blastocystis* sp. subtypes. Aqueous extract of *S. persica* roots contains therefore heat-stable components with significant antiprotozoal activity against *Blastocystis* sp. subtypes ST1, ST3, and ST5 in vitro. Further investigations are required to determine and characterize the active antiprotozoal components of *S. persica* roots and their evaluation in vivo.

Keywords *Blastocystis* sp. · *Salvadora persica* · STS sub-typing · Aqueous extract · Anti-protozoal activity

Introduction

Blastocystis sp. is a group of anaerobic protozoan parasites infecting the lower gastrointestinal tract of a wide variety of hosts including humans (Tan 2004). Its mode of transmission is not fully understood, however it is confirmed that the infection occurs after ingestion of a cyst form (Stenzel and Boreham 1996). Human infections have been recorded worldwide with wide-ranging prevalence, rising to levels as high as 60% in population groups with poor levels of hygiene or consumption of contaminated water and food and contact with animals (Pegelow et al. 1997; Tan 2008).

Blastocystis sp. infections have been reported in patients with various symptoms including weight loss, nausea, vomiting, abdominal pain, and diarrhea (Cirioni et al. 1999; Rossignol et al. 2005; Tasova et al. 2000), but *Blastocystis* parasites are also found in fecal samples from asymptomatic and apparently healthy persons (Dogruman et al. 2008; Leder et al. 2005; Mohamed et al. 2017).

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Accordingly, *Blastocystis* sp. pathogenicity remains arguable. Many reports suggested a direct relation between the density of *Blastocystis* sp. infection and pathogenesis (Giacometti et al. 1999; Kain et al. 1987; Sheehan et al. 1986; Zaki et al. 1991). Pathogenesis has also been directly related to the genotype and subtype of the infecting *Blastocystis* sp. parasites (Clark 1997; Hameed et al. 2011). It has been postulated that proteases activity in some *Blastocystis* sp. subtypes represents a significant virulence factor involved in protein and neutralizing mucosal antibodies degradation, and evasion from host immunity in vivo (Abdel-Hameed and Hassanin 2011; Puthia et al. 2005). Nevertheless, no virulence factor genes have been completely characterized (Tan 2008). Latest studies showed a large diversity in *Blastocystis* sp. subtypes based on the small subunit ribosomal DNA (SSU rDNA) gene sequences. PCR and multiplex-PCR methods using SSU rDNA sequence tagged-site (STS) primers are universally applied to determine the subtypes of *Blastocystis* sp. isolates (Stensvold et al. 2007; Yoshikawa et al. 1998). Among all known *Blastocystis* sp. subtypes, only nine (ST1-ST9) were isolated from humans so far (Parkar et al. 2010).

There are strong evidences of resistance development in *Blastocystis* sp. parasites against antiprotozoal drugs in use, namely metronidazole as first line and cotrimoxazole as second line therapy (Haresh et al. 1999; Moghaddam et al. 2005; Rajamanikam et al. 2019; Wu et al. 2014). Hence, is becoming necessary to investigate new treatments for *Blastocystis* sp. infections, especially against virulent subtypes. Various medicinal plants have been explored as alternative sources to synthetic anti-protozoal drugs against *Blastocystis* sp. parasites; such as *Brucea javanica* (Yang et al. 1996), *Artemisia judaica* (Mokhtar et al. 2019), *Quercus infectoria* (Sawangjaroen and Sawangjaroen 2005), and *Nigella sativa* (Wakil 2007). Yet, no previous studies had evaluated the antiprotozoal activity of *Salvadora persica* (Salvadoraceae) plant against *Blastocystis* sp. parasites. *S. persica* is a perennial tree or shrub with a large geographic distribution including wide regions of Asia and Africa that has been traditionally used in many countries for its diverse biological activities (Sher et al. 2010). Sticks harvested from *S. persica* roots, twigs, and stems are popularly used as tooth brushing sticks and for oral hygiene (Hattab 1997). Reports from previous investigations showed that it has important anti-bacterial, anti-fungal, and anti-inflammatory action (Abeer et al. 2011; Noumi et al. 2010; Sofrata et al. 2008). In the present study we explored in vitro susceptibility of *Blastocystis* sp. isolates from symptomatic patients to *S. persica* components collected in aqueous extract.

Materials and methods

Samples collection, examination, and in vitro cultures

This study included fecal specimens received for analysis in the central laboratory of a tertiary health care center of Makkah city in 2019 between January and June. A part of samples were from patients with gastrointestinal complaints and another part from asymptomatic persons who underwent a regular or mandatory health exam. All samples were examined by direct microscopy for parasites. Samples from patients with GIT symptoms, if negative by direct wet mount, were further examined after formalin ethyl acetate concentration. *Blastocystis* sp. parasites were recognized by morphological features as vacuolar, granular, ameboid, or cystic and with very variable sizes (Mohamed et al. 2017). Two parts of about 0.5 g of each *Blastocystis* sp. positive sample were inoculated in two separate 15 ml tubes containing 3 ml of culture media consisting of Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, USA) containing 15% inactivated horse serum (Gibco), 12 mg/ml ampicillin, and 4 mg/ml streptomycin. The prepared DMEM media was sterilized by filtration.

Subtyping of *Blastocystis* sp. isolates

First, genomic DNAs were extracted from parasites subcultures using QIAmp DNA extraction kit (QIAmp, QIAGEN Inc, Germany) following the manufacturer's instructions. Molecular subtyping of *Blastocystis* isolates was performed by PCR and multiplex-PCR using sequence-tagged site (STS) primers under the conditions reported by (Chandrasekaran et al. 2014; Yoshikawa et al. 2004) (Table 1). PCR reactions were carried out in duplicate for each isolate's genomic DNA and each primer set as described by Bakri et al. 2019. PCR and multiplex-PCR PCR products were separated in 1.5% agarose gels, stained with ethidium bromide, and photographed.

Preparation of *Salvadora persica* roots extracts

Freshly harvested roots of *S. persica* were kindly provided by an expert botanist after a field tour to its natural habitats in Jazan area in the south west region of Saudi Arabia. Immediately, 100 g of fresh roots were chopped in tiny pieces and soaked into 300 ml of distilled water under agitation at 4 °C for 24 h. The mixture was thoroughly blended, sieved under pressure, and centrifuged at 5000 rpm for 15 mn. The supernatant was then concentrated in a rotary vacuum evaporator at 50 °C to a final

Table 1 SSUrDNA-based primer pairs for sequence-tagged-site PCR for *Blastocystis* sp. subtyping (Chandrasekaran et al. 2014; Yoshikawa et al. 2004)

Blastocystis subtype	Primer sets	GenBank accession nos.	Primer sequences (5′-3′)	Product size (bp)
ST 1	SB83	AF166086	F: GAAGGACTCTCTGACGATGA	351
			R:GTCCAAATGAAAGGCAGC	
ST 2	SB155	AF166087	F:ATCAGCCTACAATCTCCTC	650
			R: ATCGCCACTTCTCCAAT	
ST 3	SB227	AF166088	F:TAGGATTTGGTGTTTGGAGA	526
			R:TTAGAAGTGAAGGAGATGGAAG	
	SB228	AF166089	F: GACTCCAGAAACTCGCAGAC	473
			R: TCTTGTTTCCCCAGTTATCC	
	SB229	AF166090	F: CACTGTGTCGTCATTGTTTTG	631
			R: AGGGCTGCATAATAGAGTGG	
ST4	SB332	AF166091	F: GCATCCAGACTACTATCAACATT	338
			R:CCATTTTCAGACAACCACTTA	
ST5	SB340	AY048752	F: TGTTCTTGTGTCTTCTCAGCTC	704
			R:TTCTTTCACACTCCCGTCAT	
ST6	SB336	AY048751	F:GTGGGTAGAGGAAGGAAAACA	317
			R:AGAACAAGTCGATGAAGTGAGAT	
ST7	SB337	AY048750	F: GTCTTTCCCTGTCTATTCTTGCA	487
			R:AATTCGGTCTGCTTCTTCTG	

volume of 100 ml, filtered through 0.45 µm pore-size syringe filters (Durapore PVDF membrane, Millipore), and stored at 4 °C for posterior use (Fig. 1). Each µl in the final extract solution was corresponding then to the aqueous material present in 1 µg of fresh roots. Heat-treatment of extracts was performed at 100 °C for 10 mn before use.

In vitro antiprotozoal activity assays

Antiprotozoal activity of untreated and heat-treated aqueous extract of *S. persica* roots was evaluated on three *Blastocystis* sp. isolates of different genotypes, molecularly characterized as ST1, ST3, and ST5 subtypes. Untreated and heat-treated aqueous extracts were tested using serial dilutions from 40 to 2.5 µl/ml of culture media. Each assay was carried out in triplicate in 3 ml culture media seeded with 10^5 parasites/ml and incubated at 37 °C for 48 h in presence of a gas pack (BD gas pack-Becton, USA) to create an anaerobic environment as described by (Zaman and Zaki 1996). Metronidazole was used at a concentration of 0.2 mg/ml as antiprotozoal control. Three cultures without additions were also used in parallel as parasites

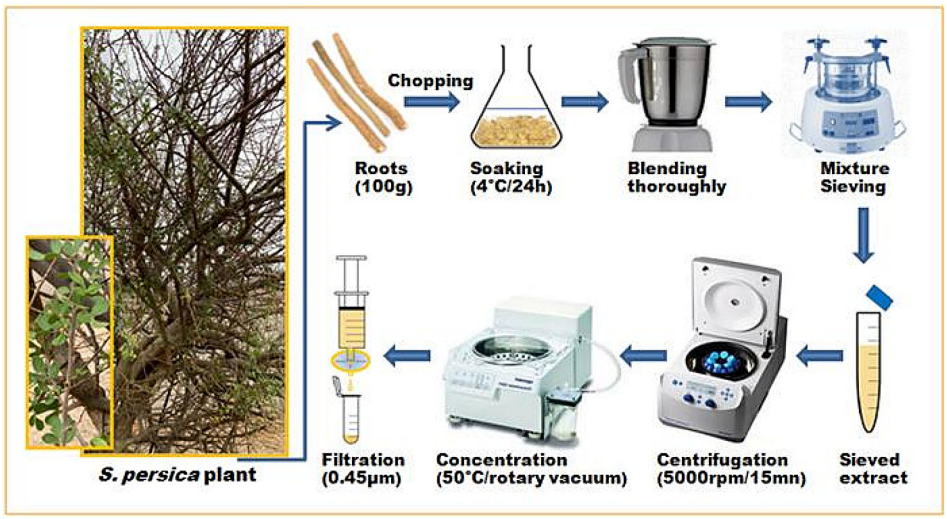

Fig. 1 Representation of the process of *Salvadora persica* roots aqueous extract preparation

growth controls. After 48 h incubation time, parasites were precipitated by centrifugation at 500 rpm/5 min, 2.5 ml of supernatant were discarded, and the sediment was fully resuspended in the remaining 0.5 ml of media. Trypan blue (Sigma-Aldrich Corp. USA) was then supplemented at a concentration of 0.4% as viability indicator before counting living parasites in haemocytometer chambers (Improved Neubauer, Hausser Scientific).

Statistical analysis

Statistical analysis of data was performed using Chi square test considering P values < 0.05 as statistically significant. A Persons correlation study was conducted. Statistical analysis was done using SPSS software version 21.

Results

During the period between January and June of 2019, microscopic examination in a tertiary health care center of 643 fecal samples revealed 27 *Blastocystis* sp. positive ones. In vitro cultures of positive stools were successful for 21 specimens, among which 15 (isolates 1s–15s) were from patients with gastrointestinal complaints and 6 (isolates 1a–6a) from asymptomatic and apparently healthy individuals. Molecular subtyping of the 21 isolates by specific sequence-tagged-site (STS) primers identified five isolates as ST1, 14 as ST3, and two as ST5 subtypes (Fig. 2).

Susceptibility assays to *S. persica* aqueous extract were carried out on the three isolates labeled 5s, 1s, and 7s representing the three different subtypes ST1, ST3, and ST5. A significant killing activity was observed with both, untreated and heat-treated *S. persica* aqueous extracts starting from the minimal concentration of 2.5 µl/ml compared to growth control cultures ($P < 0.05$). Maximal antiprotozoal effect was reached at a concentration of 20 µl/ml. *Blastocystis* sp. Means of growth inhibition effect obtained with untreated and heat-treated extracts at 40 µl/ml against the three subtypes of *Blastocystis* sp. were 80% (SD 2.3) and 82% (SD 1.1), respectively. No significant difference of killing effects of both, untreated and heat-treated *S. persica* extract, were noticed between *Blastocystis* sp. subtypes ST1, ST3 and ST5 tested in this study (Fig. 3).

To explore the antiprotozoal effect of both untreated and heat-treated extracts on each *Blastocystis* sp. subtypes (ST1, ST3 and ST5) tested in this study, Pearson's correlation coefficient was determined between the concentration (volume) of each extract and parasites counts (growth inhibition) of each *Blastocystis* sp. isolate. A statistically significant association was observed between concentrations of both extracts and the counts of parasites after 48 h incubation for each of the three *Blastocystis* sp. (Table 2).

Discussion

Salvadora persica has been used by humans since ancient times as a safe medicinal plant, particularly for oral hygiene (Hattab 1997). Since past several years, therapeutic potential of different parts of this plant has been

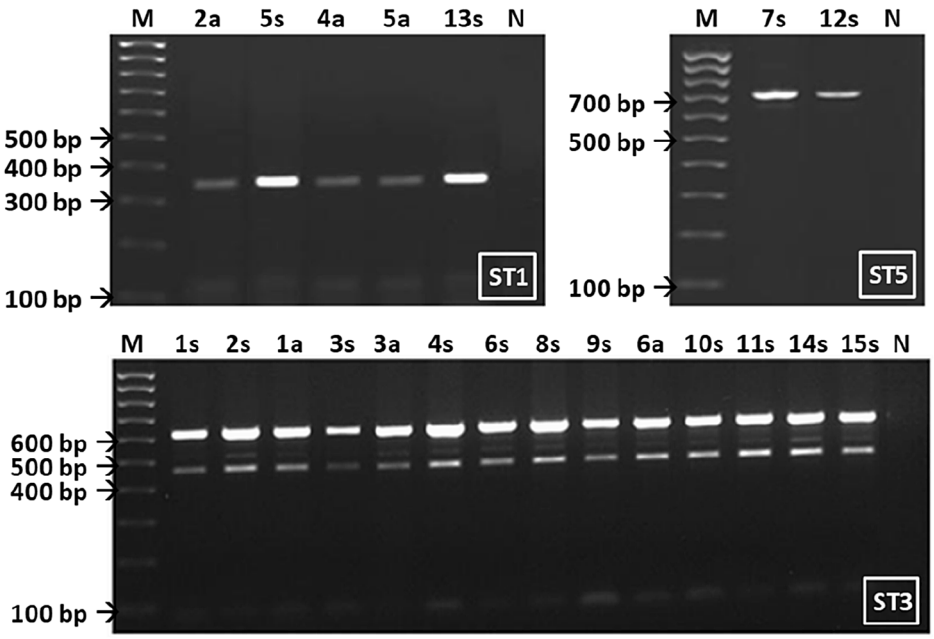

Fig. 2 Analytical gels of sequence-tagged-site PCR products of *Blastocystis* sp. ST1, ST3, ST5 subtypes collected from symptomatic patients (N°s) and asymptomatic individuals (N°a). Negative controls (lanes N) and 100 bp molecular size marker (lanes M) were separated in parallel

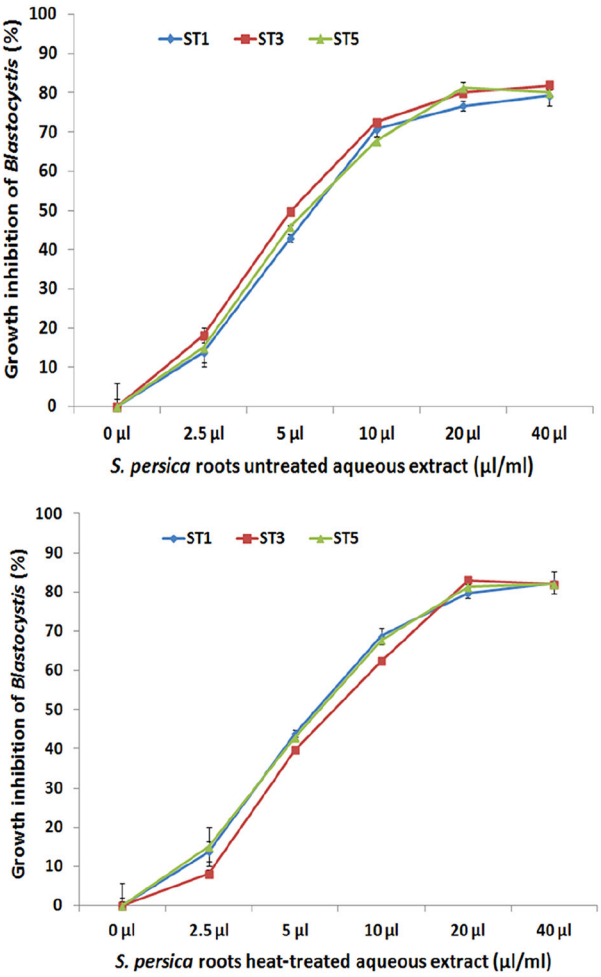

Fig. 3 *In vitro* antiparasitic activity of untreated (left) and heat-treated aqueous extracts of *S. persica* roots serially diluted (40–2.5 µl/ml) against *Blastocystis* sp. subtypes ST1, ST3 and ST5 collected from symptomatic patients

investigated against several pathogens including bacteria, fungi, and parasites (Sher et al. 2010). Nevertheless, until now, no study has described the effect of *S. persica* extracts on *Blastocystis* sp., a very common gastrointestinal protozoan parasite existing under different subtypes with controversial degrees of pathogenecity (Tan 2008). The aim of the present study was to explore in vitro antiprotozoal potential of *S. persica* against the different subtypes of *Blastocystis* sp. found in Saudi patients with gastrointestinal complaints. Among 21 *Blastocystis* sp. isolates collected in a Saudi tertiary health care center and successfully cultivated in vitro, five were identified as ST1, 14 as ST3, and two as ST5 subtypes. Worldwide, nine genetic subtypes of *Blastocystis* sp. have been isolated from humans and the four most common ones are ST1, ST2, ST3 and ST4. ST5 subtype has rarely been described in humans, is rather a common zoonotic subtype (Tan 2008). Among the 21 isolated parasites, two ST1, eleven ST3 and two ST5 were collected from symptomatic patients. Antiprotozoal effect of *S. persica* roots extract was evaluated against the three *Blastocystis* sp. subtypes (ST1, ST3 and ST5) isolated from symptomatic patients in this study for a wider applicability.

Salvadora persica roots are traditionally used as sticks for teeth brushing; a practice during which sticks are partly chewed and the chewed extract is naturally swallowed with the saliva without harmful consequences on the gastrointestinal tract (Khoory 1983; Wu et al. 2001). Thus, we investigated the aqueous extract of *S. persica* roots as a potential anti-*Blastocystis* sp. natural remedy against ST1, ST3 and ST5 isolated strains. Different types of *S. persica* extracts have been investigated for their antibacterial, antifungal, and antiparasitic potential. Ethanol and hexane extracts of *S. persica* were found to exhibit significant antimicrobial activity against Gram-positive bacteria species most commonly associated with dental caries such as *Streptococcus mutans*, *S. sanguis* and *S. salivarius* (Balto et al. 2017). Methanolic extract inhibited growth of both Gram positive and negative bacterial strains, although it was more effective on Gram positive bacteria than Gram negative ones (Amir et al. 2014). Aqueous extract of *S. persica* has also shown to have significant antibacterial properties against several bacterial strains including *E. coli,*

Table 2 Correlation coefficient between concentrations of untreated and heat-treated aqueous extract of *Salvadora persica* roots and *Blastocystis* sp. subtypes ST1, ST3 and ST5 parasites' counts during in vitro susceptibility assays

S. persica aqueous extract	*Blastocystis* sp. subtypes	Pearson correlation coefficient (r) and *P* value
Untreated	ST1	r = -0.804 $P < 0.001$
	ST3	r = -0.809 $P < 0.001$
	ST5	r = -0.828 $P < 0.001$
Heat-treated	ST1	r = -0.799 $P < 0.001$
	ST3	r = -0.800 $P < 0.001$
	ST5	r = -0.816 $P < 0.001$

Streptococcus pyogenes, methicillin-resistant Staphylococcus aureus (MRSA), Acinetobacter baumannii, and *Stenotrophomonas maltophilia* (Al-Ayed et al. 2016). Antifungal effect of methanol, ethyl acetate, and acetone extracts of *S. persica* were confirmed against different *Candida* species, with ethyl acetate extract being the most potent one (Noumi et al. 2010). Aqueous extract of *S. persica* roots has also shown in vitro high fungicidal effect against isolated *Candida* species (Al-Bagieh et al. 1994), and *Aspergillus* niger, *A. flavus*, and *A. fumigatus* pathogenic species (Saddiq and Alkinani 2019).

In our study, *S. persica* aqueous extract showed significant concentration-dependent in vitro antiprotozoal activity against *Blastocystis* sp. ST1, ST3 and ST5 subtypes reaching 80% (SD 2.3) parasites growth inhibition mean at 40 µl/ml after 48 h incubation. Antiprotozoal effects of different extracts of *S. persica* were reported against other human parasites; stems and leaves extracts showed antiplasmodial activity against *Plasmodium falciparum* NF54 strain at 0.6 mg/ml and 0.7 mg/ml, respectively (Ali et al. 2002). A significant in vitro killing activity of *S. persica* extracts against erythrocytic schizonts of *P. falciparum*, intracellular amastigotes of *Leishmania infantum* and *Trypanosoma cruzi*, and free trypomastigotes of *T. brucei* have been reported (Al-Musayeib et al. 2012). Roots extract of *S. persica* has also been reported to have effective anticoccidial activity against *Eimeria paillata* induced infection in mice jejunum (Dkhil et al. 2019). Antihelmintic activity of *S. persica* roots extract has been proved in vitro against protoscolices from hydatid cysts of *Echinococcus granulosus* at a concentration of 50 mg/ml, killing 81.4% then 100% of protoscolices after 10 and 20 min, respectively (Abdel-Baki et al. 2016).

Heat treatment at 100 °C for 10 min of the aqueous extract did not affect its killing effect on the three tested subtypes of *Blastocystis* sp. showing 82% (SD 1.1) parasites growth inhibition mean at 40 µl/ml after 48 h incubation. These findings reveal for the first time that the *S. persica* roots extract have a potential anti-*Blastocystis* property which is stable even in extreme temperature conditions. Results of this study open an opportunity to further investigate the prevalence of *Blastocystis* sp. infections among regular users of *S. persica* as natural toothbrushes and also on the potential of *S. persica* roots extract mixed food in controlling *Blastocystis* sp. infections among farm animals.

Conclusion

Aqueous extract of *S. persica* roots contains heat-stable components with significant antiprotozoal activity against *Blastocystis* sp. in vitro. *S. persica* roots aqueous extract showed similar growth inhibition effect on parasites of three different subtypes; ST1, ST3, and ST5 collected from patients with gastrointestinal complaints. Further investigations are required to determine and characterize the active antiprotozoal components of *S. persica* roots and their evaluation in vivo.

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Author contributions MAE-B coordinated the study and wrote the first draft of the manuscript. AA and RTM performed in vitro cultures and susceptibility assays. MAEL-M shared in microscopic examination and genotyping experiments, RAB and SAA shared in molecular experiments and statistical analysis. All authors read and approved the final manuscript.

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Compliance with Ethical standards

Conflict of interest Authors have declared that no conflict of interest exist.

Ethical approval Ethical permission for this research study and informed consent form were officially agreed by Medical Research Ethics Committee of Umm-Alqura University, Saudi Arabia (Reference Number #43409049).

Informed consent All participants whose samples were included in this study had given their consent and signed the informed consent form.

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