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Screening for the antimicrobial activity of *Salvadora persica* extracts against *Enterococcus faecalis* and *Candida albicans*

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Abstract

Antimicrobial resistance of *Enterococcus faecalis* (*E. faecalis*) and *Candida albicans*(*C.albicans*), frequently implicated in dental infections, remains a challenge. Using a variety of solvents this study was performed to screen the antimicrobial activity of *Salvadora persica*(*S. persica*)extracts against these two organisms. Seven extracts of *S. Persica* were prepared using hexane, chloroform, ethyl acetate, methanol-soluble, methanol-insoluble, ethanol, and water. Antimicrobial activities against *E. faecalis* and *C.albicans* were assessed by colony forming unit (CFU) counts after 1, 3, 6 and 24 hours of exposure using doubling dilutions of the extracts ranging between 125 µg to 1mg/ml. Among the extracts of *S. persica* tested, hexane extract induced a steady and progressive reduction in CFUs of both the *E. faecalis* ($p \leq 0.001$)and *C. albicans*($p \leq 0.01$) at all concentrations beginning after 3 hours until 24 hours. Progressive inhibition of *E. faecalis* CFUs was also observed for ethanol beginning at 3 hours until 24 hours ($p \leq 0.001$) and chloroform only at 24 hours ($p \leq 0.001$) at all concentrations. Ethyl acetate extract of *S. persica* was effective against *C. albicans* at 250µg/mg after 6 hours ($p \leq 0.02$) and 24 hours ($p \leq 0.002$). No significant changes were observed in any of the other tested extracts of *S. persica.* Hexane extract of *S. persica* was found to exhibit maximum antimicrobial activity against *E. faecalis* and *C. albicans*. Further studies are recommended for evaluation of this extract as an effective anti-microbial agent.

Keywords: Antimicrobial, C. albicans, E. faecalis, *S. persica*

Introduction

The tooth brush tree, *Salvadorapersica* (*S. persica*), locally known as Miswak, is a member of the Salvadoraceae family. For centuries it has been used among Middle Eastern communities for maintenance of dental hygiene and has now been recognized as an effective agent for prevention of tooth decay [1].*S. persica* extract applied in high concentration is believed to be comparable to conventional oral disinfectants and anti-plaque agents, such as triclosan and chlorhexidinegluconate[2,3].

Enterococci are Gram positive microorganisms that normally reside in the human gastrointestinal tract, female genital tract and inside the oral cavity without harming the host [4,5].Clinical studies have shown that *Enterococci* make up a small proportion of the flora in untreated root canal infections [6,7]. Along with *Streptococci* and gram-positive rods, *Enterococci* have been frequently isolated from the root canals of teeth with failed endodontic treatment and persistent periapicallesions [8,9].*Enterococcus faecalis (E. faecalis)* strains have the ability to invade dentinal tubules, survive under harsh environments and resist the anti-microbial actions of a number of commonly used anti-microbial agents including calcium hydroxide [10,11].

Fungal infections in the oral cavity may present in a wide variety of clinical conditions such as: pseudomembranous candidiasis (oral thrush), chronic atrophic candidiasis (denture stomatitis), angular cheilitis, acute atrophic candidiasis and chronic hyperplasic candidiasis (*Candida leukoplakia*) [12]. In addition,*Candida* species have also been implicated in persistent cases of apical periodontitis [13].*Candidaalbicans*(*C. albicans*)is a common oral yeast species, followed by *C. glabrata, C. krusei, C. tropicalis, C. guilliermondii, C. kefyr,* and C*. parapsilosis* [14].A significant association between the presence of yeast in saliva and having a root canal infection has also been established[13].

S. persica is well known for its antibacterial [15]and an anti-fungal properties [16]. This study was performed to assess anti-microbial activities of *S. persica*against*E. faecalis* and *C.albicans* using *S. persica*extacts perpared by using a variety of solvents.

Material and Methods

Preparation of *S. persica* extracts

The roots of *S. persica* were collected in March 2010 from Almukwah, which lies in the Southern region of the Kingdom of Saudi Arabia. The plant was identified by ataxonomist and a voucher specimen (#1745) was deposited at the herbarium, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia for future reference. The fresh ground roots (100 g) were extracted three times by percolation for 24 hours for each extraction using hexane, chloroform, and ethyl acetate. The solvents were evaporated under vacuum, leaving hexane, chloroform and ethyl acetate residues. Another fresh sample of the root was extracted with methanol and evaporated under vacuum to obtain methanol-soluble and methanol-insoluble residues. The latter residue was treated with 10% water in ethanol yielding a dark brown residue. In addition, a fresh sample of the plant was also extracted with water to prepare an aqueous extract. All extracts were freeze-dried to ensure the complete removal of remaining solvents. *S. persica* extracts were suspended in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml. This stock preparation was kept in a freezer at -20 C. Working dilutions were made in physiological saline at pH 7.4.

Titration of microorganisms

E. faecalis (ATCC 29212) and *C. albicans* (ATCC 66027) cultures were retrieved from -40^0 freezer and were grown for 24hours at 37 C in 5ml of brain heart infusion (BHI) broth (Oxide Ltd. UK) and Sabouraud dextrose (SD) broth (Oxide Ltd. UK) respectively. Tubes with microbial cultures were centrifuged at 2000 *g* for 10 min and the pellets were resuspended in original volume of respective broths. Log dilutions were made in sterile physiological saline, BHI agar and SD agar plates were inoculated in triplicate with 0.1ml of inoculum in each plate. Plates were incubated for 48-72 hours and microbial colonies were counted.

Colony forming unit (CFU) counts

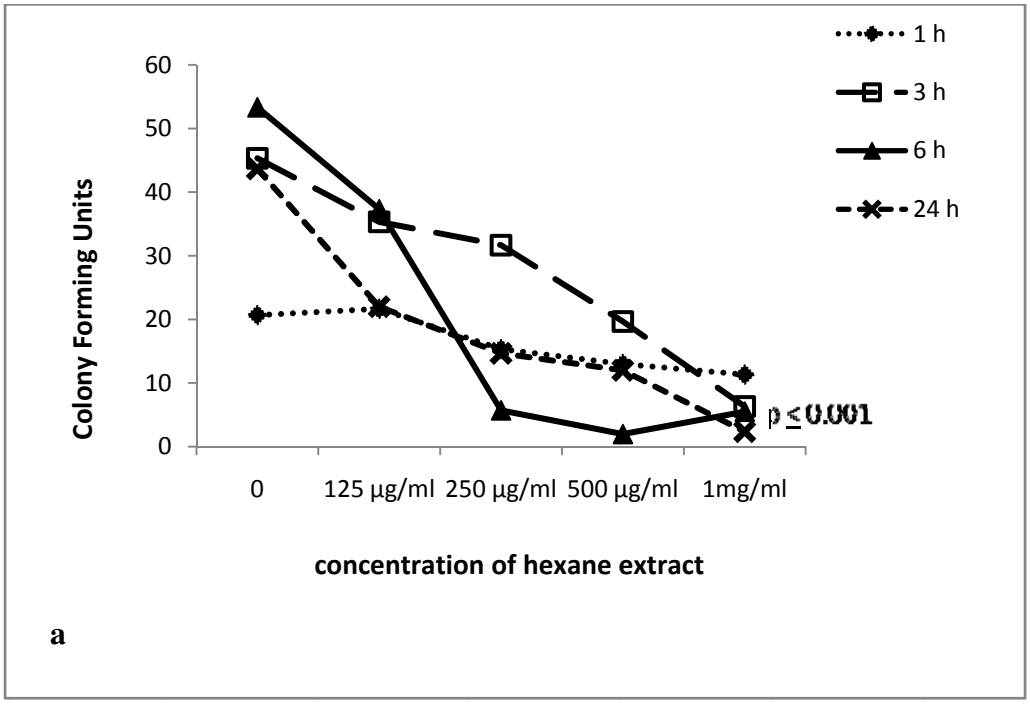
The stock culture of *C. albicans* was grown in 5ml of SD broth for 24 hours and after centrifugation at 2000xg, the pellet was resuspended in original volume of the same broth. Culture was diluted in PBS to 0.5 McFarland standard. Similarly, the *E. faecalis* culture was grown in 5 ml of BHI broth for 24 hours and was processed in a similar fashion as C. albicans culture was handled. Double dilutions of *S. persica* extracts ranging between 125 µg to 1mg/ml were prepared in triplicates in sterile Eppendorf tubes in a volume of 0.1ml and equal volume of microbial cultures were added in each tube. Control tubes contained the microbial culture with 2%DMSO in physiological saline. All tests and controls were incubated at 37^{o}C for a maximum period of 24 hours in sets of four aliquots each for all concentrations. A single aliquot was assessed periodically at 1, 3, 6 and 24 hours by preparing log dilutions in physiological saline. These dilutions were added to BHI agar plates in triplicate and the inoculum was spread with the help of a sterile glass rod using a plate rotator. Plates were incubated in anaerobic jars (BBL, CA, USA) with gas generating pouches and colonies were counted after 72 hours of incubation using a Colony counter (Quebec Dark Field Colony Counter, Cambridge Instruments Inc., Buffalo, NY, USA). The bacterial count in control tubes was always more than six logs although log dilutions were employed ranging between10^{-1} to 10^{-8} for each sample, where no CFU were observed. A gradual decline in CFU counts was noted with the increasing concentrations of *S. persica* extract in the test samples

Statistical Analysis

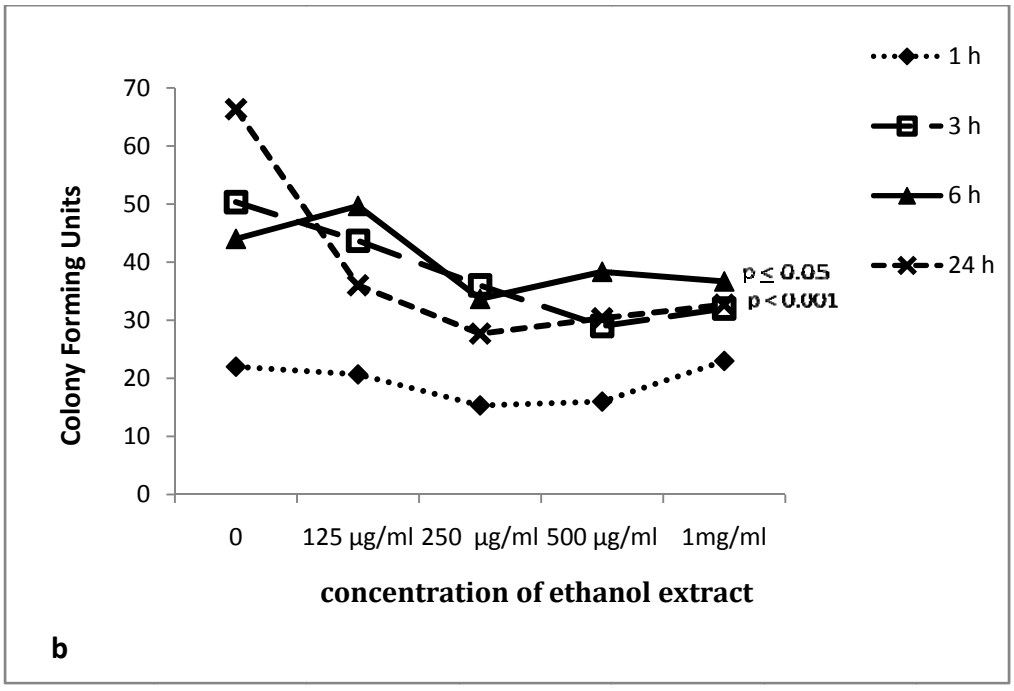
The data were recorded in Microsoft Excel computer software and analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were used to summarize the continuous outcome variable. One-way analysis of variance was used to compare the mean values of the outcome variable, followed by Tukey'spost-hoc test. A *p*-value of either equal to or less than 0.05 was considered statistically significant.

Results

Among all the extracts of *S. persica* tested for antibacterial and antifungal activity, significant inhibitory effects were observed for hexane, ethanol, ethyl acetate and chloroform. Hexane extract was able to induce significant inhibition of both the *E. faecalis* and *C. albicans* at varying concentration over different time intervals. Although no inhibition was evident against *E. faecalis* after one hour at any of the concentrations, it was however evident after 3 hours which increased progressively after 6 hours particularly at 500 µg/ml concentration attaining a maximum inhibition ($p \leq 0.001$) at all concentrations after 24 hours (Figure.1a). A similar trend of hexane induced inhibition was observed against *C. albicans* where inhibition was however more pronounced after 24 hours at all concentrations (Figure. 2a). Ethanol extract of *S. persica* after one hour also failed to show any notable inhibition of *E. Faecalis* however inhibition was observed after 6 hours at 250µg/ml concentration achieving a maximum inhibitory effect ($p \leq 0.001$) after 24 hours at all concentrations (Figure. Ib).Chloroform extract of *S. persica* also inhibited *E. faecalis,* but the effect was only evident after 24 hours($p \leq 0.001$) at all concentrations (Figure.1c).


1 h
3 h
6 h
24 h
Colony Forming Units
60
50
40
30
20
10
0
p ≤ 0.001
0
125 µg/ml
250 µg/ml
500 µg/ml
1mg/ml
concentration of hexane extract

a


1 h
3 h
6 h
24 h
Colony Forming Units
70
60
50
40
30
20
10
0
p ≤ 0.05
p < 0.001
0
125 µg/ml
250 µg/ml
500 µg/ml
1mg/ml
concentration of ethanol extract

b

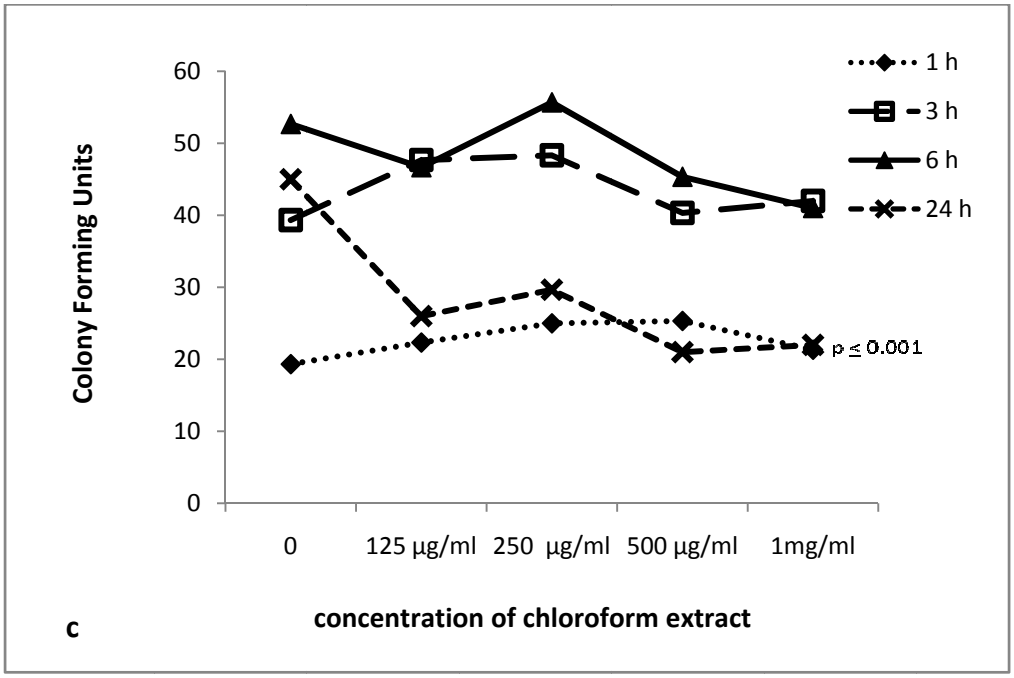
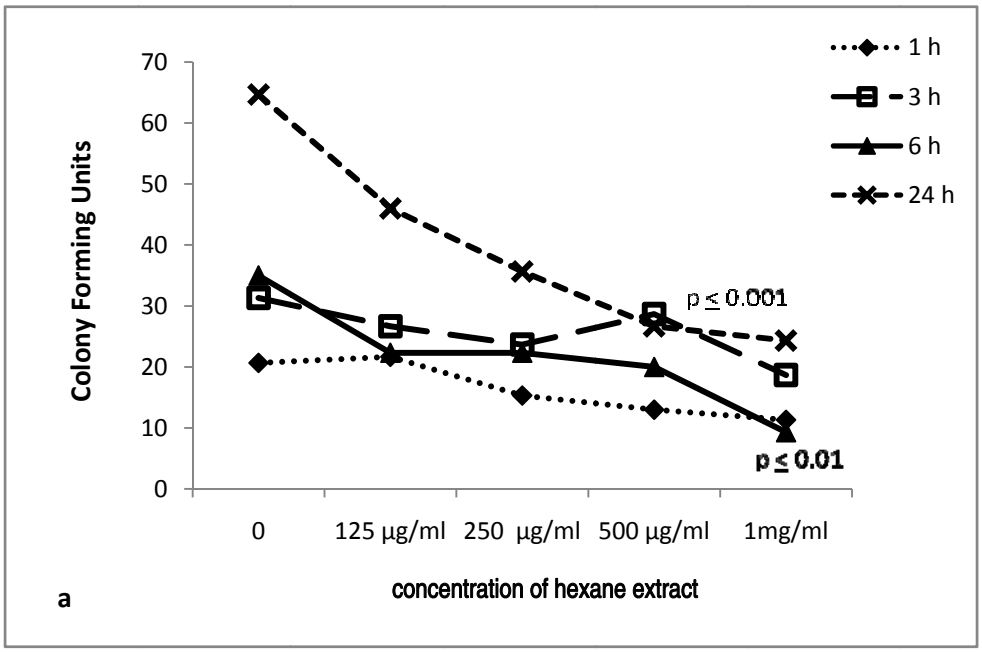


Figure 1: Hexane(a), Ethanol(b) and Chloroform(c) extracts of *S. persica* showing inhibition of *E. faecalis* at varying concentrations and time points.

Ethyl acetate extract of *S. persica* was also able to induce inhibition of *C. albicans*. Significant decrease in CFUs of *C. albicans* was observed at 250µg/ml after 6 hours ($p \leq 0.002$) and at 1mg/ml ($p \leq 0.02$) after 24 hoursof exposure to ethyl acetate extract (Figure. 2b). No significant changes were observed in any of the other extracts of *S. persica* tested against *E. faecalis* and *C. albicans* (data not shown).



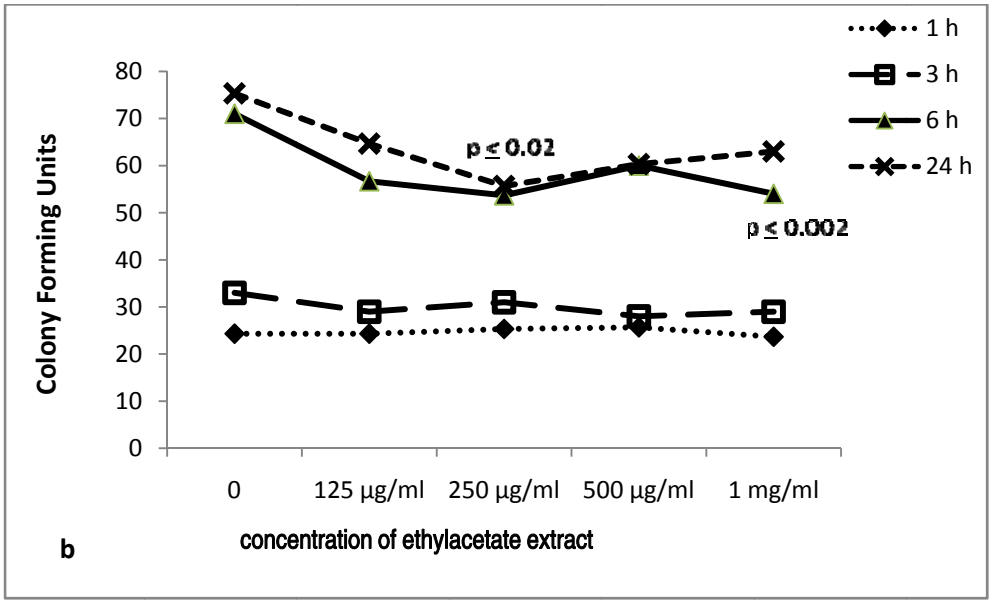


Figure 2: Hexane (a) and Ethylacetate (b) extracts of *S. persica* showing inhibition of *C.albicans* at varying concentrations and time points.

Discussion

Hexane extract was shown to exhibit a potent antimicrobial activity against *E. faecalis* and *C. albicans* in the present study. Although hexane as solvent has been used for preparation of extracts but to the best of our knowledge this was the first time ever that hexane extract of *S. persica* was tested for its antibacterial activity against *E. faecalis*. Hexane extract of *Urticadioica* leaves has recently been shown to be an effective antibacterial agent against five clinical isolates of Gram positive and Gram negative bacteria [17].These findings were further confirmed by Elzaawely et al. [18] usinghexane extract of *Urticadioica* leaves in a separate study. Hexane extract of the sea urchin, *Temnopleurusalexandri* performs well as a potent antibacterial agent against a vast majority of gram-positive and gram-negative bacteria except *K.pneumoniae* [19].Moreover hexane extract of Schinusterebinthifolius (Anacardiaceae) has also been shown to exhibit strong anti-microbial activity against the isolates of Paracoccidioidesbrasiliensis. Collectively these observations and the findings of the present study indicate that hexane performs well as a solvent in preparation of potent plant extracts [20].

The findings of this study demonstrated that hexane extract of *S. persica* had a significant inhibitory activity after six hours at the concentration of 500µg/ml against *E. faecalis* and at 1mg/ml against *C. albicans*. Such variation in the effective concentrations could be due to differences in the structure of cell wall of the tested microorganisms. Gram-positive bacteria have peptidoglycans in their cell wall, while the Candida cell wall comprises mainly of glucan [21].In addition hexane has been shown to be a very effective solvent for the outer coat Mycobacterium tuberculosis [22]. It is therefore highly likely that better anti-microbial activity associated with hexane extracts could possibly due to hexane being a better solvent a property that may support a better extraction of active ingredients.

A recent study investigating the antibacterial activity of acetone and hexane extracts derived from the root, stem, and leaf of *Raphanussativus* against a number of resistant pathogens found that the antibacterial component in the extract was isothiocyanate. Among the various isothiocynates investigated 4-(methylthio)-3-butenyl isothiocyanate was shown to exhibit maximum antibacterial effect against *E. faecalis* [23].Furthermore, a study attempting to identifythe antibacterial agent of *S. persica* in extracts prepared from the roots found benzyisothiocyanate to be the most effective antibacterial agent [24,25]. These observations suggest that, although antibacterial activity of a variety of plants may reside in isothiocyanates, various forms of isothiocyanates with antimicrobial properties might vary in different species. Whether the higher potency of hexane against *E. faecalis* and *C. albicans* observed in the present study compared to other solvents was due to differences in the isothiocyanate contents of the extracts requires further investigation.

Chloroform and ethanol extracts of *S. persica* inhibited *E. faecalis* significantly after 24 hours at all concentrations tested. Similar observations have been reported in the past using ethanolicextract of *S.persica* against *S.fecalis* and *S. mutans* [26,27].Chloroform and ethanol extracts of *S. percsica* have also been tested with regards to their anti-biofilm activity against carcinogenic *S.mutans* strains. Both the extracts were shown to inhibit biofilm formation of *Streptococcus mutans* strains by 85.75% and 72.44% respectively [28].Data regarding the efficacy of chloroform and ethanol extracts of *S.persica* against *C. albicans* are limited(26). There is however evidence that ethanol, chloroform and hexane extracts of algae perform remarkably well as an antifungal agent when tested against *C. albicans* (29). Apart from the hexane extract both

chloroform and ethanol extracts of *S. persica* failed toshow significant antifungal activity in the present study. However, ethyl acetate extract of *S. persica* performed well as an antifungal agent in the present study. This observation was in contrast to a recent study reporting a poor performance of ethyl acetate extract of *S. persica* as an antifungal agent [30].Less than optimal antifungal activities of *S. persica* have also been reported in a number of studies [31,32].On the other hand, several investigators have demonstrated that the aqueous extract of *S. persica* performs well as an antifungal agent[16, 33,34]. Collectively these data indicate that because of the prevailing discrepancies further investigations are needed for better elucidation of role of *S. persica* extracts as anti-bacterial and anti-fungal agents.

Despite this study reporting hexane extract of *S. persica*as the most effective agent against *E. faecalis* and *C.albicans* controversies still prevail with regards to the most effective *S. persica* extract preparation. Al-Bayati and Sulaiman[34] and Sher et al. [35]have suggested that aqueous extract is better than the alcohol extract, whereas Abd EL Rahman et al. [29] have demonstrated a higher efficacy of ethanol extract. Similarly data regarding the anti-microbial spectrum of *S. persica* are also inconclusive. Al-Bayati and Sulaiman[34] have shown that *S. persica* extract is highly effective against *E. faecalis*, where as Sheret al.[35] demonstrated that *S. persica* aqueous extract was active against all oral pathogens particularly against *Staphylococcus aureus* (*S. aureus*). On the other hand, AbdELRahmanet al. [27] have reported *S. persica* as the most effective anti-microbial agent against *Streptococcus mutans* (*S. mutans*), where as Almas [31] has failed to demonstrate *S. persica* activity against *S. mutans*, *S. aureus*, or *C. albicans*. This contradiction in the antimicrobial activities may be attributed to differences in the timing and location of plant collection, extraction methods, variability in the tested bacterial species and the evaluation techniques [34,31,27].It is therefore imperative to establish a well-defined source and extraction method for production of an effective *S. persica* extract with a high degree of reproducibility.

Conclusion

Hexane extract of *S. persica* was found to exhibit maximum antimicrobial activity against *E. faecalis* and *C. albicans*. Further studies are recommended for evaluation of this extract as an effective anti-microbial agent.

Authors' contributions

HB: Participated in the conception and design of the study and manuscript writing.
TH: Participated in the collection of the plant (*S. persica*) and the extraction procedures.
YS & AS: Carried out the technical part of the study and acquisition of data.
ZS, HH, SH: Participated in the conception and design of the study, analysis and interpretation of data.
ZS: has been involved in revising the manuscript for important intellectual content and have given final approval of the version to be published.

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