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Research Article

Characterization of *Salvadora persica* and *Salvadora oleoides* Extracts Against Multidrug Resistant Bacterial Strains

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Abstract

The rapid emergence of drug-resistant bacteria is occurring worldwide, presenting urgent, serious, and concerning threats, many of which are already responsible for placing a substantial clinical and financial burden on the global healthcare system, patients, and their families. The use of plant-derived drug substances has a long tradition in medicine. Owing to the low cost and less toxicity, synthetic compounds and their derivatives are strong candidates to be used as potential drugs against multidrug-resistant bacterial strains. The current study aims to determine the potential antibacterial activity of two selected plant extracts. Two plants *Salvadora persica* and *Salvadora oleoides,* of Pakistani origin, were collected locally. The leaves and roots of both plants were dried, and extracts were prepared in four different solvents. The antibacterial activity against multidrug-resistant (MDR) strains of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* was evaluated by disc diffusion assay. Cytotoxicity of the extracts was tested by MTT assay. The plant extracts have shown varying antimicrobial activity against MDR bacterial strains with a zone of inhibition ranging from 16 mm ± to 6 mm ±. *In vitro* cytotoxicity evaluation showed that these extracts are non-toxic to cells even at high concentrations. The tested plant extracts have shown potent antibacterial activity against multidrug-resistant bacterial strains and thus can be further evaluated for extracting potent antibacterial compounds with broad therapeutic potentials.

Keywords: *Salvadora persica, Salvadora oleoides*, antibacterial, multidrug-resistant bacterial strains

1. Introduction

Natural products like plant extracts and compounds (sources of lead molecules) are often used as drugs; however, relatively few therapeutic plants have been scientifically examined. The vast expanse of different countries and their diverse soils, geography, and climate encourage the cultivation of various herbs, trees, and other plants. This sparked a burgeoning interest in locally cultivated plants, prompting both traditional healers and early formal physicians to conduct extensive research (Shikov et al. 2014). 25% of modern medicine is derived from plants; about the same percentage is the synthetic analog of phytochemicals(Kumari and Kotecha 2016). Antimicrobial, anti-cancer, anti-diabetic, age arresting, and many other biological activities of plants have been reported for decades (Pozharitskaya et al. 2007, Levy and Marshall 2004). Plants are proven to show a variety of antioxidant activities that help them treat chronic disorders(Wright and Sutherland 2007). A significant positive impact of plant extracts in terms of protein-based therapies and vaccines is also well established(Walsh 2014). Published data about medicinal plants is also increasing day by day.

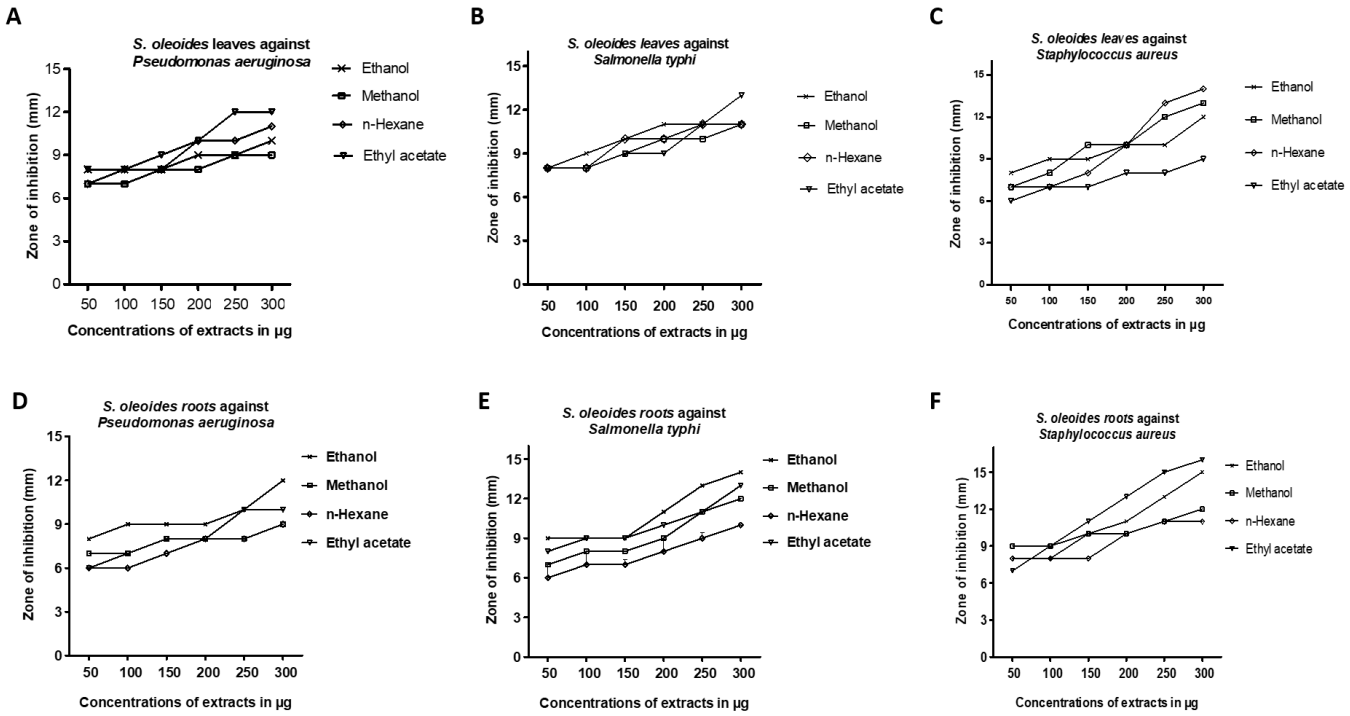

Figure 1. Antibacterial Activity of *S. oleoides* extracts (A), (B), and (C) *S. oleoides* leaf extracts against *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus,* respectively. (D), (E) and (F) *S. oleoides* root extracts against *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus,* respectively.

Drug resistance in bacteria goes back to the 1930s when Sulfonamide resistant *Streptococcus pyogenes* appeared in military hospitals. In 1950s and 1960s, multidrug-resistant (MDR) *Escherichia coli, Shigella,* and *Salmonella* were detected(Lee et al. 2006). Drug resistance is one of the foremost challenges in curing infectious diseases. Combinatorial administration of antibiotics has resulted in the accumulation of multidrug-resistant (MDR) phenotypes of bacteria(Lewis and Ausubel 2006). Multidrug resistance is leading to an increasing economic concern globally. In the USA, it is causing an annual cost of about 150 million to 30 billion US dollars(Arora et al. 2015). A project commissioned by the British government has surprisingly released estimates of the near-future global toll of antibiotic resistance that scale 10 million deaths per year due to superbugs, which is more than cancer(Khatak et al. 2010). Genetic and physiological factors cause antibiotic resistance in bacteria, and it is increasing remarkably due to the propensity of bacteria to horizontally exchange genetic material among species and genera ("Antibiotics," 2005, p. 4). The major drug resistance mechanism includes enzymatic drug transformation or sequestration, physiological barrier development, or active efflux of drugs through membrane-bound pumps(Taha 2008). There is a dire need for the discovery of new drugs effective against resistant bacteria. These drugs are explored in bacteria and plants and are sometimes designed and synthesized chemically. Even genetically engineered plants can be used as a source for new drugs(Ahmed et al. 2008).

In the current study, two plants, *Salvadora persica* and *Salvadora oleoides* of Pakistani origin, have been evaluated for their antibacterial activity against multidrug-resistant bacteria activity. *Salvadora* belongs to the *Salvadoraceae* family. *Salvadora oleoides*, a bushy tree found in Pakistan

and India, is reported for antiinflammatory and antimicrobial activities(Shikov et al. 2008). *Salvadora persica* is a popular medicinal plant throughout the Indian subcontinent and is commonly known as "Miswak". *S. persica* is traditionally used in the treatment of leprosy, ulcers, gonorrhea, rheumatism, scurvy, dental diseases, and tumors(Al-Otaibi et al. 2004). Many studies prove that the extract of *Salvadora persica* has many biological characteristics, including antibacterial, antifungal, antiplaque effect, anticaries, and antiinflammatory, and it also tends to reduce gingivitis and gingival bleeding. It contains a large number of medicinal compounds, for example, salvadoricine, salvadourea, di-benzyl thiourea, rutin, trimethyl amine and thioglucoside(Al-Sabawi, Al Sheikh Abdal, and Taha 2007). Antiviral activities of *S. persica* extracts have also been reported(Mann 2005). Table 1 describes the ethnobotanical & ethnopharmacological significance of *S.oleoids* & *S.oleoids* and includes some biologically active compounds derived from both plants. The main objective of this study is to evaluate the antibacterial activities of *S. persica* and *S. oleoides* against multidrug-resistant (MDR) bacterial species.

2. Materials & Methods

2.1. Plant Material

S. Oleoides was collected from CIDS (Cholistan Institute of Desert Studies), the Islamia University of Bahawalpur (IUB), and verified by Dr. Shazia Anjum (director CIDS). *S. persica* was collected from a village "Goth Bahar" District Lodhran near Bahawalpur City and verified by the taxonomist Dr. Qasim Hayat (ASAB, NUST). Both the plants were verified by comparing them with the voucher specimens available at Royal Botanical Garden Kew (RBGK, UK) and Royal botanical garden Edinburgh (RBGE, UK) Herbarium catalogs, respectively. The details are *Salvadora persica* Linn., Voucher/barcode ID No. K001110836 (http://specimens.kew.org/herbarium/K001110836); *Salvadora oleoides* Decne., Voucher/barcode ID No. E00448636 (http://data.rbge.org.uk/herb/ E00448636). The local botanical descriptions of *Salvadora persica* and *Salvadora oleoides* are also available at flora of Pakistan (*Salvadora persica*, http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=220011923; *Salvadora oleoides*, http://www.efloras.org/florataxon.aspx?flora_id=5&taxon_id=250063241) and original plant material was kept at MPRL (Medicinal Plant Research Laboratory), ASAB, NUST for future references. Both plant materials were collected in May 2015. After drying under shade at room temperature, the roots and leaves of the plants were ground to a fine powder.

2.2. Extraction

Different solvents, namely ethanol, methanol, n-hexane and ethyl acetate (Sigma Aldrich, USA) prepared extracts of plant material. In short, 10 g of powdered plant material was soaked in 100 ml of the solvent for 24 hours. After filtration, extracts were evaporated and concentrated by a rotary evaporator under pressure. The temperature of the rotary evaporator was set below the boiling point (around 50°C) of the respective solvent. After drying in an incubator (Mermet GmbH, Germany) at 37°C for 72 hours(Suffredini et al. 2004), the extracts were stored at -20°C until further use. For cytotoxic and antibacterial activity assays, the extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50mg/ml, filter-sterilized using 0.2 µm syringe filter, and stored at 4°C till further use.

2.3. Antibacterial Activity

Three clinical drug-resistant bacterial strains of *Salmonella typhi, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were used in the study. The strains were generously provided by the Phage Research Group of the Department of Industrial Biotechnology, Atta ur Rahman School of Applied Biosciences (ASAB, NUST). Single colonies of bacteria were picked from agar and further treated for evaluation of their antibiotic

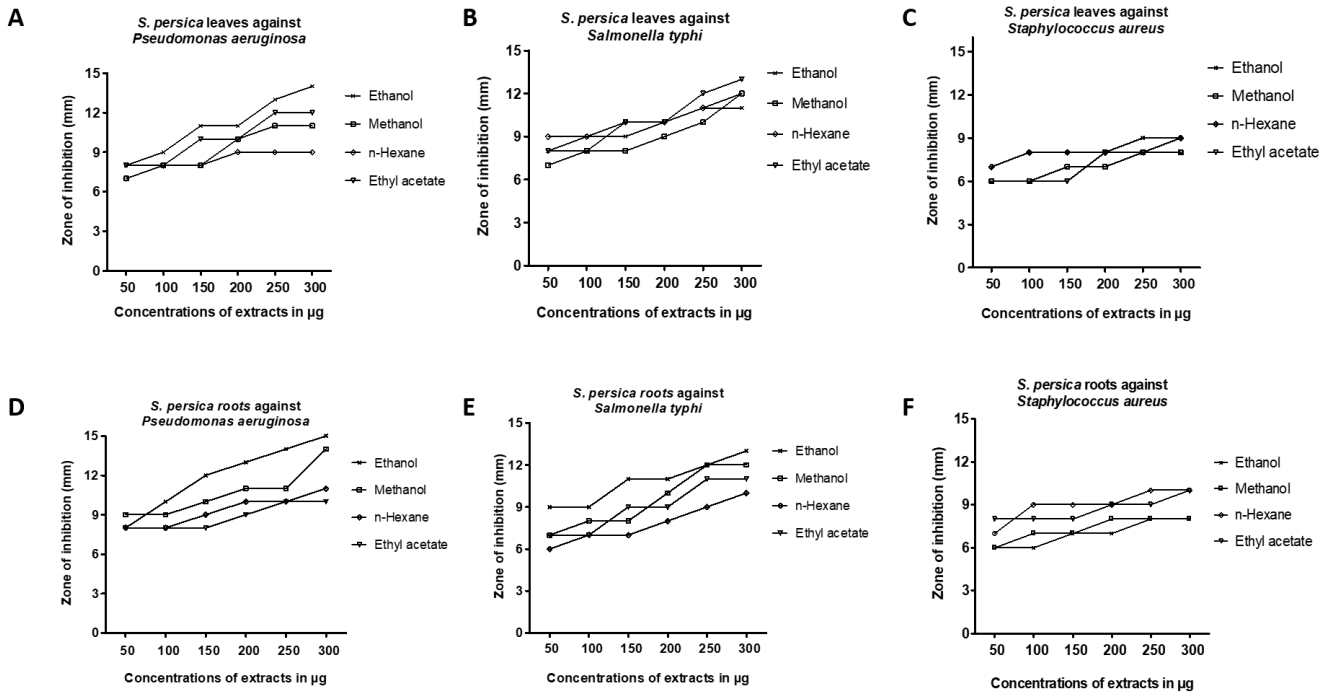

Figure 2. Antibacterial Activity of *S. persica* extracts (A), (B), and (C) *S. persica* leaf extracts against *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus,* respectively. (D), (E) and (F) *S. persica* root extracts against *Pseudomonas aeruginosa*, *Salmonella typhi,* and *Staphylococcus aureus,* respectively.

resistance profile against some commonly used antibiotics, including Erythromycin, Penicillin, Doxycycline, Ciprofloxacin, and Fusidic acid. Pure bacterial strains were then collected in 87% glycerol stock solutions and stored at -80°C. Inoculums of pure colonies were prepared in solutions of 0.9% normal saline.

As previously described, the disc diffusion method was used for antibacterial activity (Das et al., 2012). The density was adjusted by spectrophotometer at a wavelength of 530 nm to yield a stock suspension of 1-5 x 10^6 cells per mL. The bacterial inoculum was swabbed on nutrient agar (Merk) plates using sterile cotton swabs. Sterile filter paper discs of 6 mm diameter were prepared from Whatman Filter Paper Grade 4 and then placed on inoculated plates by using a sterile syringe (BD 5 mL syringe, Becton and Dickinson, Pakistan) at points that were already marked for each dilution of extract. Stock solution (50 mg/ml) was diluted to required concentrations (50, 100, 150, 200, 250, and 300 µg/ml), and 10 µl of each dilution of the extract was then dispensed and allowed for absorption by filter paper discs. Deionized water was used as a negative control for the disc diffusion assay. After setting up plant extract-impregnated discs for disc diffusion assay, these plates were incubated overnight at 37 °C, and each experiment was repeated thrice to ensure validation of the assay. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the extracts.

2.4. In-vitro Cytotoxic Assay

The Human Hepatocellular Carcinoma cell line (Huh-7) was used to evaluate the cytotoxic effect of plant extracts by MTT Assay as previously described (Mosmann, 1983). The cell lines were grown and maintained in Dulbecco's Modified Eagle Media (High Glucose) (Sigma Aldrich, USA) supplemented with 10% FBS (PAA laboratories) and 1% pen-strep antibiotic (Sigma Aldrich, USA). 1 x 10^4 cells were plated into each of 96 well plates

and incubated for 24 hours before incubating them with varying concentrations of root and leaf extracts of both plants. DMSO was used as solvent control. 20 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) reagent was added to each well, including controls. The plate was incubated for 3 hours until a purple precipitate was visible. After adding 100 µl of DMSO, the plate was left covered in the dark for 1 hour at room temperature, and then the absorbance was recorded at 570 nm in a microplate reader. Percentage viability was calculated as;

$$\% \text{ cell viability} = (As-Ab) / (Ac-Ab) \times 100$$

As corresponds to the absorbance value of the sample, Ab corresponds to the absorbance value of the blank, and Ac corresponds to the absorbance value of the control.

2.5. Statistical Analysis

For Data Compilation and statistical evaluation, the Statistical Package GraphPad PRISM Ver. 5.01 Software was used. One-way ANOVA was the statistical analysis of choice with posthoc analysis using the Bonferroni posttest, where comparisons with control and other groups were required. Descriptive statistics using cross-tabulation was used to evaluate data, and T-tests were used for group-group significance and testing of hypotheses. A 95% Confidence interval was used throughout, and all values having a p-value showing $p < 0.05$ were taken to be statistically significant.

3. Results

3.1. Antibacterial Activity

The antibacterial activity of extracts was measured by the disc diffusion method. Observations were recorded in the form of millimeter (mm) clearing zone (Supplementary files 1 and 2). Figure 1 demonstrates the antibacterial activity of different extracts of *S. oleoides* leaves (A – C) and roots (D-F), whereas Figure 2 shows the antibacterial activity of *S.persica* leaves (A – C) and roots (D-F) against three multidrug-resistant strains of *Salmonella typhi*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa*. These strains were already evaluated by the partner research group for their antibiotic susceptibility and were found to be totally resistant to multiple drugs. All the extracts showed antibacterial activity against these drug-resistant strains in a dose-dependent manner. The negative control containing deionized water did not show any zone of inhibition.

The *S. oleoides* leaf extracts showed variable antibacterial activity against all three strains with different solvents ranging from 6 to 14mm zone of inhibition. The maximum diameter of the zone of inhibition (14mm) for *S. oleoides* leaves was observed with n-Hexane extract against *Staphylococcus aureus* (Figure 1C).

S. oleoides root extracts also showed good antibacterial activity against all three bacterial strains ranging from 6- to 16mm zone of inhibition (Figure 1D-F). Maximum inhibition (16mm) was observed with ethyl acetate extract against *Staphylococcus aureus,* signifying the efficacy of extracts against antibiotic-resistant strains of bacteria.

The *S. persica* roots and leaves extract showed better antibacterial activity against *Pseudomonas aeruginosa* and *Salmonella typhi* as compared to *Staphylococcus aureus* (Figure 2). The minimum and maximum diameters for the zone of inhibition ranged from 6mm to 14mm (Figure 2 A-F).

3.2. Cytotoxicity Evaluation of Plants Extracts

The cytotoxic activity of the extracts was tested on Huh-7 cells using various concentrations (5, 15, 30, 60, 125, 250, 500, and 1000 µg/ml) of each extract. None of the extracts from leaves and roots of *S. oleoides* showed a 50% reduction in cell viability except ethanolic leaf extract, which reduced the cell viability up to 48%, 47%, and 44% at the concentration of 250 µg, 500 µg, and 1000 µg respectively (Figures 3A and 3B).

Root extracts of *S. persica* showed more than 50% cell viability; however, different leaf extracts reduced the cell viability below 50% at higher concentrations. The ethanolic extract reduced the cell viability up to 43% at the concentration of 1000

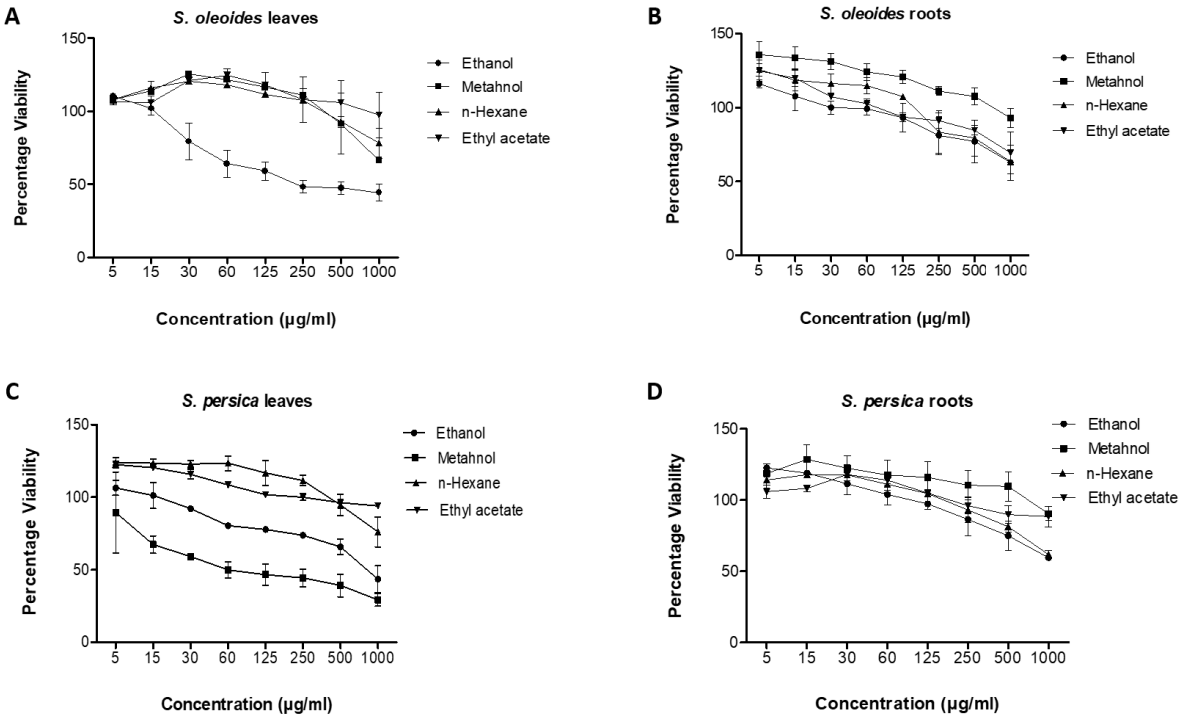

Figure 3. In-Vitro Cytotoxicity of plant extracts in four different solvents on HuH-7 Cell line (A) *S. oleoides* leaves and (B) Roots. None of the *S. oleoides* extracts showed a 50% reduction in cell viability except ethanolic leaf extract (C). Different leaf extracts of *S. persica* reduced the cell viability below 50% at higher concentrations. The methanolic extract reduced the cell viability up to 28% at 1000 µg. (D) no root extract of *S. persica* showed a 50% reduction in cell viability.

µg. Methanolic extract of the *S. persica* leaves reduced the cell viability up to 28%, 39%, 44%, and 46% at 1000 µg, 500 µg, 250 µg and 125 µg concentrations, respectively (Figure 3C and 3D).

4. Discussion

The discovery of antibiotic agents has marked the twentieth century as one of the triumphant periods in history as it revolutionized the treatment of bacterial infections. Nonetheless, the excessive use, misuse, and abuse of these antimicrobial agents have eventually resulted in the emergence of antibiotic-resistant bacteria, causing a major public health crisis of global proportions. The antibiotic resistance crisis is now mainly attributed to a global reliance on antibiotic agents and a lack of new drug development by pharmaceutical industries due to reduced economic incentives. The need of the hour is to explore more natural antibiotic compounds against the resistant strains to combat the antibiotic resistance problem.

Medicinal plants are rich sources of biologically active compounds. Methanol, ethanol, and ethyl acetate extracts of *Cassia angustifolia* have flavonoids that showed significant antibacterial and antioxidant activities. Similarly, flavonoids like amurensin and cosmosiin extracted from *Trigonella foenum graecum* showed antioxidant and neuroprotective properties(Alali et al. 2005, Al-Bayati and Sulaiman 2008). An oil extract of Chamomilla recutita flowers was also found to have promising preventive effects against H. pylori when tested in vitro(Arshad et al. 2010).

In the current study, two plants, i.e., *S. persica* and *S. oleoides* of genus *Salvadora* were studied for their potential biological activities. Both these plants contain a number of phytochemicals that have

therapeutic potential, e.g., salvadorin, beta sitisterol, salvadricine, salvado-urea, beta-isothiocyanate etc. (Atassi 2002, Das, Patro, and Dinda 2012).

The study's objective was to evaluate both plants for their antibacterial potential. In the case of *S. persica*, no root extract showed a 50% reduction in cell viability; however, different leaf extracts reduced the cell viability below 50% at higher concentrations. The ethanolic extract reduced the cell viability up to 43% at the concentration of 1000 µg. Methanolic extract of the *S. persica* leaves was the most lethal. It reduced the cell viability up to 28%, 39%, 44%, and 46% at 1000 µg, 500 µg, 125 µg and 500 µg concentrations respectively (Figure 3). Our results are in line with previous studies where polyamides isolated from *S. oleoides* showed weak activity against breast and colon cancer cell lines (Daxenbichler et al. 1991, Dhankhar et al. 2012, Farag et al. 2017). Freshly cut *S. persica* showed no cytotoxic effects (Garg et al. 2013, Halawany 2012, Khalil 2006, Khalil et al. 2019, Krishnaraju et al. 2005)

Antibacterial activity of different extracts of *S.persica* and *S. oleoides* plants against three multidrug-resistant (MDR) strains of bacteria named *Salmonella typhi*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* was evaluated. The current study indicated dose-dependent bactericidal activity against these bacteria by showing zones of inhibition of increasing diameter with increasing concentration of extracts. The maximum diameter of the zone of inhibition was found to be approximately 16 mm signifying the efficacy of extracts against antibiotic-resistant strains of bacteria. The minimum diameter of the zone of inhibition was observed to be 6mm (Figures 1 and 2). There was no zone of inhibition around filter paper discs that were treated with deionized water, acting as a negative control, indicating that the zones of inhibition observed in discs treated with extracts were actually due to the presence of extracts. *S. oleoides* has been reported effective against *E. coli* and *S. aureus* (Mosdam 1983, Rajesh et al. 2010, Khumar and Arya , Sumitra, Vijay, and Sharma 2013). *S. persica* has been reported for antimicrobial activity against *Streptococcus mutans, P. aeruginosa, S. pyrogenis,* and *S. faecalis (SORATHIA 2013, Tare and Sharma 1991, Alali et al. 2005).*

Miswak extracts have been shown to reduce bacterial mouth plaques. However, antimicrobial activities against multidrug-resistant strains of these plants have not been reported so far. The current study has provided the first evidence of the bactericidal effects of these plants, indicating potent efficacy against (MDR) *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi*.

Extracts were evaluated for their cytotoxicity against human hepatocellular carcinoma (Huh-7) cells by using MTT assays in a dose-dependent manner. Root extracts of studied plants showed very less inhibition in cell growth. However, some leaf extracts showed cytotoxicity at concentrations approaching 250 µg and higher (Figure 3).

In conclusion, extracts of leaves and roots of *Salvadora persica* and *Salvadora oleoides* were obtained by using four different solvents. Extracts were observed to have bactericidal ability against MDR *Staphylococcus aureus, Salmonella typhi,* and *Pseudomonas aeruginosa*, implying their applications as antimicrobial agents and potential disinfectants. These extracts showed less cytotoxicity against the HuH-7 cell line. After analyzing data and results depicted in this study, it is recommended to purify and characterize these extracts by using comprehensive techniques, e.g., HPLC, GC-MS, NMR etc. and use *in vitro* techniques to find out the mechanisms underlying the bactericidal activities of *Salvadora persica* and *Salvadora oleoides* which might help in discovering new drugs with broad therapeutic potentials.

5. Conclusions

This study primarily reports the presence of potent compounds in plant extracts derived from *Salvadora persica* and *Salvadora oleoides* and their efficacy against multidrug-resistant (MDR) strains of *Staphylococcus aureus, Pseudomonas aeruginosa,*

and *Salmonella typhi*. Moreover, we report low cytotoxicity of these extracts against human cell lines. We therefore strongly recommend a more detailed investigation to characterize these plant extracts using comprehensive techniques. Evaluating these plant extracts' activities may help discover new drugs with broad therapeutic potential.

Conflict of Interest

The authors, who contributed to this original article, do not have any conflict of interest.

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Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Consent Forms

NA.

Data Availability

The authors possess all the raw data related to this manuscript.

Author's Contributions

AJ and SA conceived the study. MS performed the experiments. AJ and MQH analyzed and interpreted the data. MS and AJ were major contributors to writing the manuscript. AZ made changes to the manuscript. AJ, MQH, SA, and HN critically reviewed and approved the final manuscript.

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