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Antimicrobial Activity of *Peganum harmala* Against Methicillin-Resistant *Staphylococcus aureus* Strains and Assessment of Its Cytotoxicity Effect on HEK-293 Cells

Mehdi Goudarzi,[1,2,*] and Hadi Azimi[3]

[1]Center for the Study of Religion and Health, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]English Language Teaching Department, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

[*]*Corresponding author*: Mehdi Goudarzi, Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tel: +98-123108104, Fax: +98-2122439972, E-mail: gudarzim@yahoo.com

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Abstract

Background: Medicinal plants have been playing important roles in the treatment of different diseases. *Peganum harmala* is a famous medicinal plant used in the Iranian traditional medicine, due to the antimicrobial compounds found in its seeds and roots.
Objectives: The main objective of the present study was to investigate the antibacterial activities of alcoholic extracts of *P. harmala* seeds on MRSA strains and cytotoxicity assessment of ethanolic extract of *P. harmala* seeds on HEK-293 cell line using MTT assay.
Methods: During an 11-month descriptive cross-sectional study, 90 MRSA strains isolated from hospitalized patients in ICU wards were investigated. Micro-broth dilution method was employed to evaluate the antimicrobial effects of the extract on MRSA strains. HEK-293 cells were exposed to different concentrations of ethanolic extract of *P. harmala* and cytotoxicity was evaluated using MTT assay.
Results: The minimum inhibitory concentration of *P. harmala* extract was observed in the range from 3.125 mg/ml to 25 mg/mL. The most antibacterial activity of the extract was found to be at 12.5 mg/ml concentration. MRSA strains were inhibited by *P. harmala* extract at MIC_{50} and MIC_{90} of 12.5 mg/mL and 25 mg/ml, respectively. MTT assay showed that the extract concentrations more than 0.5 mg/mL were toxic and caused more than 50% HEK-293 cell death.
Conclusions: The results revealed that the *P. harmala* extract was very effective against MRSA strains isolated from ICU patients and may be useful to treat some of the infections although further investigation is recommended to assess their toxicity prior to in vivo use.

Keywords: *Staphylococcus aureus*, ICU, MRSA, HEK-293

1. Background

Staphylococcus aureus (*S. aureus*), as a major cause of infection in either hospital or within the community, is responsible for a diverse spectrum of human infections ranging from mild skin infections to urinary tract infections (UTI), osteomyelitis, endocarditis, and life-threatening diseases such as pneumonia and bacteremia (1). This pathogen has an outstanding capability to acquire resistance, especially against methicillin, making it able to persist in the hospitals and the community. Soon after the introduction of penicillin in the 1960s, Methicillin-resistant *S. aureus* (MRSA) emerged and it has been endemic in hospitals around the world since 1980s (2). MRSA strains are shown to be able to rapidly develop multi-drug resistance (MDR) although a variety of therapeutic measures, including antibiotic therapy, have been introduced. The emergence and spread of MRSA harboring multi-resistance genes have increased academic burden, caused serious therapeutic problems, and worsened controlling infection in hospitals (3, 4).

Many *in vivo* and *in vitro* studies have shown antibacterial activities of medicinal plants in the past decades. World health organization (WHO) estimated that approximately 80% of the people in the developing countries trust traditional therapies, and their main medicinal source to treat infectious diseases is plant extracts (5). Oils or extracts of medicinal plants, which have antimicrobial and anti-inflammatory effects, have been recently used to treat many human infectious diseases (6). One of the most famous plants used in popular medicine is *Peganum harmala* L. (*P. harmala*), which belongs to *Zygophyllaceae*

family and widely grows in steppe areas and sandy soils in Mediterranean region, North Africa, and the Middle East (7). In Iran, this plant is called "Espand", in North Africa "Harmel", and in the United States "African Rue", "Mexican Rue", or "Turkish Rue". *P. harmala* is a perennial, bushy, wild-growing flowering plant, which has short creeping roots that can grow to 30 - 100 cm high (8). *Peganum. harmala* seeds contain 2% - 6% pharmacologically active alkaloids, which are mostly β-carbolines like harman, harmine, harmaline, and harmalol. Several therapeutic activities including antitumoral, antibacterial, antifungal, anti-parasitic, antinociceptive, anti-inflammation, antiproliferative, vasorelaxant, and antispasmodic activities have been attributed to *P. harmala*, in literature. Also, the smoke of harmala seeds is traditionally used as disinfectant (9).

2. Objectives

The present study was carried out to determine the antibacterial activities of alcoholic extracts of *P. harmala* seeds on MRSA strains. In addition, the cytotoxicity of this extract was evaluated on human embryonic kidney 293 (HEK-293) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

3. Methods

3.1. Bacterial Strains

In the present cross-sectional study, 90 MRSA strains were collected from hospitalized patients in ICUs of 5 medical centers in Tehran, Iran, from March 2015 to January 2016. The research was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Code #6766). The inclusion criterion was existence of MRSA isolated from hospitalized patients in ICUs. The exclusion criterion was *S. aureus* isolated from hospitalized patients, outpatients, community-acquired, and other wards of hospitals. The samples obtained from patients' specimens, including wound (n = 40; 44.4%), blood (n = 30; 33.3%), pus (n = 8; 8.9%), body fluids (n = 7; 7.8%), catheter (n = 3; 3.4%), and urine (n = 2; 2.2%), were transported to the laboratory within 4 hours of collection and were processed according to the standard microbiological procedures, such as colony morphology, Gram staining, growth on mannitol salt agar, and production of catalase, coagulase, and DNase. All the isolates were confirmed using polymerase chain reaction (PCR) for *fem*A and *nuc*A genes (10).

3.2. MRSA Screening

Methicillin resistance was detected using a cefoxitin disc (30 μg) and an oxacillin disc (1 μg) on Mueller Hinton agar plates supplemented by 4% NaCl in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline (11). Isolates with phenotypic resistance to oxacillin were also tested for the presence of *mec*A gene using PCR.

3.3. Identifications and Preparation of P. harmala Seeds

Peganum. harmala was collected from a local farm in the South of Iran in May 2015. Herbarium of the plant was confirmed by research center of medicinal plants. The plant was dried at room temperature and then, the seeds were separated from the other parts and grounded into fine powder. The powder was preserved for the extraction procedure.

3.4. Preparation of Ethanolic Extract

The ethanolic extract of the dry powdered seeds was prepared by soaking 10 g of powdered seeds in 60 mL of 95% ethanol for one day (shaking occasionally with a shaker). After one day, suspensions were filtered (Whatman no. 1 filter paper). Then, ethanol was removed by evaporation using a rotary evaporator (6).

3.5. Minimum Inhibitory Concentration (MIC) Assay

MIC values were determined for the bacterial strains based on a broth microdilution method. In brief, bacterial suspension was adjusted equivalent to the 0.5 McFarland standard (approximately 1.0×10^8 CFU/mL) in Brain Heart Infusion broth (BHI) medium. This suspension was further diluted 1:100 (10^6 CFU/mL) in the broth medium. MIC values ranged between 1.56 mg/mL and 50 mg/mL. To each well, 100 μL of BHI was added. Then, 100 μL of each concentration of extract was added to the wells. The wells with BHI medium (sterility control) and BHI and bacterial suspension (drug-free control) were also used as controls. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated; they were then examined to determine MIC after incubation at 37°C overnight. To estimate MIC of P. harmala seeds, the absorbance of each well was measured at 595 nm. MIC level was defined as the lowest concentration at which no growth was observed.

3.6. Cell Culture and MTT Assay

To determine the toxicity of ethanolic extract of *P. harmala*, HEK-293 cell line was used. HEK-293 cell line was cultured in DMEM medium (Dulbecco's modified eagle medium) supplemented with 14% bicarbonate sodium, 7% fetal bovine serum, 100 unit/mL penicillin, 100 μg/mL

streptomycin sulfate, and 0.25 μg/mL amphotericin B. The cultures were maintained in a 5% CO_2 incubator at 37°C. The cell count was adjusted to 2×10^5 cells in 1 mL of fresh medium. To determine viability of HEK-293 cell line, MTT assay was used, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase of viable cells (12).

In brief, cell lines were seeded (8000 cells/well) in a 96-well plate and incubated for 24 hours at 37°C. Different concentrations of *P. harmala*'s ethanolic extract (ranging from 1.56 mg/mL to 50 mg/mL) were added to each well and the plate was re-incubated for 48 hours. After incubation, 20 μL MTT (5 mg/mL) was added to each well and the wells were re-incubated for an additional 5 hours. Then, cell culture media and MTT solution were removed and formazan crystals, formed in the last incubation step, were dissolved in 200 μL of dimethylsulfoxide. Absorbance was read at wavelength of 570 nm to calculate the percentage of viable cells using ELISA plate reader (12, 13).

4. Results

In the present study, all the phenotypically MRSA isolates were found to carry *mecA* gene, too. The mean age of patients was 41 years (median 40.8 years, ranging from 5 months to 65 years). Wound (44.4%) and blood (33.3%) samples were the most prevalent samples in the present study. The level of MIC of seed extract of *P. harmala* was observed in a range from 1.56 mg/ml to 50 mg/mL. The results of antibacterial activities of *P. harmala* ethanolic extract showed that the growth of 22 MRSA isolates (24.4%) was inhibited at 25 mg/mL, 45 isolates (50%) at 12.5 mg/ml, 18 isolates (20%) at 6.26 mg/mL, and 15 isolates (5.6%) at 3.125 mg/mL concentration of the extract. The results showed that 12.5 mg/mL concentration of the extract had the most antibacterial activity on MRSA strains. All the isolates were inhibited by the *P. harmala* extract at MIC_{50} and MIC_{90} of 12.5 mg/mL and 25 mg/mL, respectively.

HEK 293 cell lines were exposed to 12 concentrations of *P. harmala* extract (0.025, 0.05, 0.01, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 mg/ml). The results of the MTT assay showed that concentrations more than 0.5 mg/mL of the extract caused cell death over 50%.

5. Discussion

The widespread emergence of drug resistance among MRSA strains is becoming a great challenge for public health. This problem leads to the restriction of therapeutic options and exacerbation of disease in hospitalized patients (1). Medicinal plants, as potential sources of natural pharmaceutical products, are playing an important role in the treatment of different diseases (6). Thanks to their fewer side effects, easy access, rational price, and no bacterial resistance, medicinal plants are suitable alternatives for chemical antimicrobial agents (14).

Several studies confirmed antimicrobial properties of *P. harmala* against different microorganisms (7, 15, 16). The present study showed a good antibacterial activity for *P. harmala* seeds extract in different concentrations against MRSA clinical strains, which is in agreement with the results of other studies from Iran by Mazandarani et al (17) and Mohsenipour et al (18). The antibacterial activity of *P. harmala* observed in the present study may be due to the existence of high quantity of polyphenols, known to possess efficient antibacterial activity.

In the present study, the MIC of *P. harmala* extract ranged from 3.125 to 25 mg/mL against the MRSA strains. Our findings indicated that 12.5 mg/mL concentration of the extract had the most antibacterial activity against MRSA strains. The value is lower than the earlier value reported by Amel et al. who showed that the concentration of 100 mg/ml of *P. harmala* seeds extract inhibits the growth of *Staphylococcus aureus* and *Staphylococcus saprophyticus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Serratia spp*,. They also showed a higher antibacterial activity for the seed extract against gram-positive strains, especially *S. aureus*, compared to gram negative tested strains (19). In a study conducted by Darabpour et al., it was reported that the alcoholic extract of *P. harmala* at 50 - 400 mg/mL concentrations had inhibitory effects on gram-positive germs, like Bacillus cereus, *S. epidermidis*, *Streptococcus pyogenes*, *S. aureus*, as well as gram-negative germs, like *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeroginosa*, and *Klebsiella pneumonia* (20).

Scientific evidence has proven that ethanolic extract of *P. harmala* seeds has strong antibacterial effects on MRSA strains (21). In the study by Hassan Ali et al. in 2011, which was carried out to evaluate the effectiveness of some medicinal plant extracts against clinical isolates, *P. harmala* was shown to be effective on *S. aureus*, *Acinetobacter calcoaceticus*, and *Candida albicans* (22).

However, there are contradictory results about inhibitory effects of various extracts of *Peganum harmala*. Edziri et al conducted a study to compare the antibacterial, antiviral, and antioxidant activities of various extracts of *P. harmala*. They showed that chloroform extract had the best antibacterial activity and methanol extract had the best antiviral activity. The chloroform extract may be a significant form of antibacterial compounds against gram-positive bacteria (23). Investigation of *P. harmala* biological activities in Hayat study clearly demonstrated that

chloroformic, ethyl acetate, butanolic, and methanolic extracts of *P. harmala* leaves had satisfactory antifungal activities. The chloroformic and methanolic extracts represented a more significant antibacterial activity on gram-positive bacteria than gram negative bacteria (24). Also, the results of a study by Moghadam et al. showed that ethanolic *P. harmala* extract had a high antibacterial activity against *MRSA* (25). In another study, Mohsenipour et al. showed that the inhibitory effect of ethanolic extracts was more than that of methanolic extract. They also suggested the best inhibitory effect for ethanolic extract of *P. harmala* against *S. aureus* and the lowest inhibitory effect for methanolic extract against *S. pneumoniae* and *K. pneumonia* (18). Moreover, in another study, it was demonstrated that the antibacterial and antifungal activities of the acetone extract were more effective than those of the ethanol and aqueous extracts (26). The difference in the reported antimicrobial effects of different extracts of *P. harmala* may be due to the different solubility of various compounds found in *P. harmala*, particularly when some solvents with specific antifungal or antimicrobial activities are used.

Several studies confirmed that the extracts from seeds and roots of *P. harmala* have more inhibitory effects compared to the extracts from other parts of *P. harmala* (18). Based on literature, the constituents of roots and seeds are distinct from each other. Amel et al. believed that the seed is more active than the root. They also showed that the concentration of 100 mg/ml of crude extract of seeds inhibits the growth of all bacterial strains studied, while the same concentration of the crude extract of roots inhibited the growth of 85.7% of bacterial strains tested (19).

Although this plant has numerous therapeutic properties, it has a great cytotoxicity, as well. When systemically used in high concentrations on animals, *P. harmala* caused several severe side-effects, including cardiovascular, nervous, hepatic, and gastrointestinal complications. The toxicity of this herb is known to be related to its inhibitory effect on Monoamine Oxidase, and the ability to intercalate into DNA causing frame shift mutation (27). Previous studies have demonstrated cytotoxicity effects of ethanolic extract of *P. harmala* against tumor cells and other cell lines, such as human embryonic skin fibroblast, epithelial carcinoma of uterus cervix, and oral epithelial carcinoma by MTT assay (28). In contrast to other reports which emphasized the antibacterial properties of *P. harmala* in high concentrations, the result of MTT assay in the present study indicated that up to 50% of cultured HEK 293 cell lines could survive only in concentrations less than 0.5 mg/mL of this extract (20, 29, 30). Although high concentrations of *P. harmala* extract have more inhibitory effects on different bacteria especially gram-positive bacteria, the cytotoxic effect of *P. harmala* extract should be considered, as well.

Considering the antibacterial activity of the seed extract of *P. harmala* against MRSA clinical isolates, it can be concluded that this extract could be exploited as an affordable and available source of therapeutic agents as well as an alternative approach to resistance management. Hence, it can be suggested for the treatment of MRSA infections although it is recommended that more studies be carried out to elucidate the precise bioactive natural compounds that lead to cytotoxicity against HEK 293 cell line.

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Footnotes

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