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Comparison of *in vivo* antiseptic and *in vitro* antimicrobial effects of *Peganum harmala* L. seeds ethanolic extract with Betadine

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PEER REVIEW

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Comments

The article has a good idea, and the authors worked to find a natural antibiotic from medicinal plant (*P. harmala*) instead of the chemical antibiotic. The authors discussed this point along the paper. The paper is well-written and the author used standard method and discussed the results in good manner.
Details on Page

ABSTRACT

Objective: To compare the antibacterial activity and antiseptic effect of ethanol extract of *Peganum harmala* L. (*P. harmala*) seeds and Betadine on eight reference strains of *Streptococcus pneumoniae*, *Salmonella typhimurium*, *Escherichia coli*, *Corynebacterium pseudotuberculosis*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Micrococcus luteus* which are known to cause different types of skin infections, and *Corynebacterium pseudotuberculosis* isolated from an abscess on horse's neck.

Methods: The antibacterial activity was assessed using a disc diffusion method. The minimum inhibitory concentration was tested by serial dilution method and the percentage of bacterial growth inhibition by absorbance microplate reader device. The minimum bactericidal concentration was then recorded. Clinical outcomes were obtained through washed up healing time of longitudinal and surface skin on the back of 16 rats with concentrations of 10, 50, 100, 150 and 500 mg/mL of *P. harmala* extract.

Results: The results of *in vitro* experiments showed that the lowest minimum inhibitory concentration (0.68-1.3 mg/mL) and minimum bactericidal concentration (1.3-5 mg/mL) values were observed in the ethanol extract of *P. harmala* seeds. Also the results of *in vivo* experiment showed that wound healing in the concentration of 50 mg/mL of this plant extract was better and quicker than Betadine.

Conclusions: This study confirmed that ethanol extract of *P. harmala* has appropriate effect on the microorganisms and the healing of skin wounds in comparison with Betadine.

KEYWORDS

Peganum harmala, Ethanolic extract, Antibacterial activity, Wound healing, Betadine, MIC, MBC, Microplate reader device

1. Introduction

Wounds are the result of injuries to the skin that disrupt the other soft tissue. Wound healing consists of an orderly progression of events that establish the integrity of damaged tissue. The healing involves different phases and processes including inflammation, wound contraction, reepithelialization, tissue remodeling and formation of granulation tissue with angiogenesis. Also it involves continued cell-cell interaction and cell-matrix interactions that allow to minimized tissue damage, debride nonviable tissue, and maximized tissue perfusion and oxygenation[1,2]. Various plant products have been used in treatment of wounds over the years. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms[3]. The medicinal value of these

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plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body[4]. Scientists have been interested to study on the herbal extracts for discovery of new effective drugs[5]. The plant extracts being more efficacious, free from undesirable side effects compared to their pure active principle revalidated the therapeutic benefits of herbs due to totality of constituents rather than the single molecule[6].

Peganum harmala L. (Zygophyllacea) (*P. harmala*), is known as Syrian rue, wild rue and harmal. This plant grows in uncultivated and steppes area from south of Spain and south-east semiarid and predesertic regions. *P. harmala* extracts are important for drug development, because they are reported to have numerous pharmacological activities in the Middle East, especially in Iran and Egypt[7,8]. For a long time *P. harmala* has been used in traditional medicines for relief of pain and as an antiseptic agent[9]. *P. harmala* also has antibacterial[10], antifungal[11], antiviral[12], antidiabetic[13], antitumor[14,15], antileishmanial[16], insecticidal[14] and cytotoxic activities[17]. Harmaline, harmine, harmalol, harman, quinazoline derivatives, vasicine, vasicinone, anthroquinons and fixed oils are reported from seeds and roots of this plant. This plant is used as a medicine in Turkey, Syria, Iran, Pakistan, India, Egypt and Spain[18].

Although the local traditional healers had medical knowledge on the value of this plant, any biological study on the wound healing activity of this plant was not done. Hence, the present study was undertaken to evaluate wound healing activity of the seeds ethanolic extract of this plant by excision, incision. Also a study with focusing on antibacterial activity of *P. harmala* seed extract on some different skin pathogens of human and animals has not been documented so far. The aim of the present study is assessment of the antibacterial and antiseptical effect (*in vitro* and *in vivo*) of *P. harmala* seed ethanolic extract against eight main skin pathogenic bacteria in comparison of Betadine.

2. Materials and methods

2.1. Extract preparation

The aerial parts *P. harmala* were collected in Fars Province, Iran, and identified by the botanists in Department of Biology, Shiraz University, Shiraz, Iran. A concentrated extract of this plant was prepared from the seed according to the method of Nayak *et al*[3]. The seeds of plant were shade dried and ground. Then the seeds powder was macerated by ethanol (into 1 g powder in 5 mL ethanol), filtered and dried at 35 ℃ using a rotary vacuum. The extract of sample was stored in the bottle and refrigerated at 4 ℃ prior to further analyses.

2.2. Bacterial strains

Streptococcus pneumoniae (*S. pneumoniae*), *Salmonella typhimurium* (*S. typhimurium*), *Escherichia coli* (*E. coli*), *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), *Micrococcus luteus* (*M. luteus*) which are known to cause different types of skin infections and *Corynebacterium pseudotuberculosis* spp. (*C. pseudotuberculosis* spp.) (isolated from an abscess on horse's neck) strains were prepared from Microbiology Unit of Department of Veterinary, Shiraz University, Shiraz, Iran. The isolated strains were identified by standard biochemical reactions based on morphological and biochemical characters according to the methods described in Bergey's manual for systematic bacteriology[19-22].

Mueller-Hinton broth (Merck) cultures of *S. pneumoniae*, *S. typhimurium*, *E. coli*, *C. pseudotuberculosis*, *K. pneumoniae*, *S. aureus* and *C. pseudotuberculosis* spp at 37 ℃ and *M. luteus* at 25 ℃ were prepared. An initial bacterial suspension containing 10^7 CFU/mL was made from the flask broth cultures. Subsequent dilutions were made from the above suspensions, which were then used in tests.

2.3. Minimal inhibitory concentration (MIC) assay by Serial dilution method

MICs were determined by serial dilution method in tubes[23], with some modification. In the tube dilution assay, the extract was initially prepared at 50 mg/mL. Then standard bacterial suspension and different concentrations of extract (0.34, 0.68, 1.3, 2.56, 5 and 9.75 mg/mL) or Betadine were added to tubes containing 1.9 mL Muller-Hinton Broth (Merck). Each tube was inoculated with 0.1 mL of suspension containing 10^7 CFU/mL of each bacterium and incubated. These tubes were incubated at 25 ℃ (for *M. luteus*) and at 37 ℃ (the other strains) for 24 h. The negative control tube received no antimicrobial agent, and the positive control tube received no concentration of extract. The tubes were examined for visible growth or lack of growth for each dilution of test bacteria. Turbidity indicated growth of the microorganism and the MIC was the lowest concentration where no growth was visually observed[23].

2.4. Minimum bactericidal concentration (MBC) assay

The MBC values of the extract or Betadine were determined by the drop plate method from the tubes, where apparently no visible growth was found according to Kowser and Fatema[24]. Some modifications were made to the method. The MBC assay was performed as an adjunct to the MIC and was used to determine the concentration of extract that was lethal to the target bacteria. About 0.1 mL of the broth from each MIC broth tube without visible growth was poured onto across the entire surface of the nutrient agar (NA, Merck) (for *M. luteus*) and Muller-Hinton agar (MHA) (for the other strains) plates. Then the dilution of the sub cultured MIC tube was recorded on each plate and incubated 25 ℃ (for *M. luteus*) and at 37 ℃ (the others strains) overnight. The MBC plates were examined for colony growth or lack of growth for each dilution subcultured. No growth indicated that the extract or Betadine was bactericidal

at that dilution. Growth indicated that the extract or Betadine was bacteriostatic but not bactericidal at that dilution.

2.5. The growth inhibition assay by microplate method

The microplate assay of Stubbings *et al*. was used with some modification to determine the growth inhibition percentage of extract against all of the test bacteria[25]. Sterile 96-well microplates were used for the assay. The stock extract (40 mg/mL) was dissolved in sterile distilled water. All wells (two rows for each microorganism) were filled with 0.1 mL tryptic soy broth (TSB, Merck). Test extract (0.1 mL) or Betadine was added to the first well of each row and serial two-fold dilutions (0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 mg/mL) were made down to the desired minimum concentration. The wells (two rows for each microorganism) were inoculated with 0.1 mL suspension of each test bacteria (0.5 McFarland) and incubated at 25 ˚C (for *M. luteus*) and at 37 ˚C (the other strains) overnight. The growth of each microorganism in the different dilutions of extract or Betadine was determined by measuring the optical density at 570 nm with a spectrophotometer. The wells filled with TSB medium and the suspension of each test bacteria were included as a positive control in each assay. The wells filled with TSB medium and extract were used as a negative control. All assays were carried out in triplicate. The inhibition demonstrated by the extract is expressed by the following equation[26]:

$$\text{Growth inhibition } (\%) = \frac{OD_c - OD_t}{OD_c} \times 100$$

Where OD_c is the optical density 600 for the negative control (containing no extract) and OD_t is the optical density 600 for the sample treated with the antimicrobial compounds dilution[23].

2.6. Antibacterial assay by disc diffusion method

The disc diffusion assays of Lennette were used with some modification to determine the extract antibacterial effect against all of the test bacteria[27]. NA and MHA were used for the other strains to prepare the culture medium and autoclaved at 121 ˚C for 15 min. Briefly, plates (8 cm diameter) were prepared with 1.9 mL MHA and inoculated with 0.1 mL of bacterial suspension (0.5 McFarland). Sterile paper discs (5 mm in diameter) were impregnated with 0.02 mL of different concentrations of extract (50, 100, 200, 300, 400 and 500 mg/mL) placed onto NA (for *M. luteus*) and MHA (for the other strains). The plates were incubated at 25 ˚C (for *M. luteus*) and at 37 ˚C (for the other strains) overnight. Negative controls were prepared using the same solvent employed to dissolve the plant extract. Tetracycline and chloramphenicol (30 μg) antibiotic discs were prepared from Difco, and were tested in the same conditions as positive controls[28]. Inhibition zones in mm (without disc paper diameter) around discs were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extract against the test microorganisms. The experiment was repeated in triplicate and the mean of the inhibition zones diameter was calculated.

2.7. In vivo wound healing assay by excision and incision methods

Sixteen Sprague-Dawley rats, weighting 200-250 g were housed in metal cages under controlled condition (12 h light-dark cycle, 25 ˚C, 50% humidity) and pastured with standard food and sterile water. The rats were anaesthetized intramuscularly before and during creation of the experimental wounds. Excision and incision wound models were used to evaluate the wound healing activity of *P. harmala*.

2.8. Excision wound model

The rats were anaesthetized with 50 mg drug/kg body weight ketamine plus 5 mg drug/kg body weight xylazine combination prior to creation of the wounds. The dorsal fur of the rats was shaved with electric clipper. Back of the animals in the thoraco-lumbar region was surgically prepared for aseptic surgery. On the left side a full thickness of the excision wound of 10 mm (circular area=100 mm^2) in length and 10 mm depth was created using a sterile surgical scalpel. The entire wound was left open. The rats were divided into eight groups of two each. Groups 1 to 6 were treated with the ethanolic extract of *P. harmala* at doses of 5, 10, 50, 100, 150 and 500 mg/mL respectively. Group 7 was treated with Betadine (10%) and the control group was treated with normal saline by sterile cotton tip swabs once daily till complete epithelialization. Wound contraction was assessed by tracing the wound area on polythene paper first and subsequently transferred to 1 mm^2 paper sheet from which the wound surface area was evaluated on Days 1, 3, 7, 14, 20 and 22 post wounding. The percentage of wound contraction (taking the initial size of the wound, 100 mm^2, as 100%) by using the formula[18,28]:

$$\text{Wound contraction } (\%) = \frac{\text{Initial wound size-Specific day wound size}}{\text{Initial wound size}} \times 100$$

2.9. Incision wound model

After shaving dorsal fur of the rats, on the right side a longitudinal paravertebral incision, 15 mm in length was made through the skin on the back as described by Barua *et al*[29]. The wounds were left undressed. The rats were treated with the *P. harmala* ethanolic extract at doses of 5, 10, 50, 100, 150, 500 mg/mL respectively (Groups 1 to 6). Group 7 was treated with Betadine (10 %) and the control group was treated with normal saline by sterile cotton tip swabs once daily. The skin breaking strength was measured according to method of Cole *et al.* at 1, 3, 7, 14, 20 d after creation of wound[30].

2.10. Ethical statement

All animal experiments were conducted in compliance with

the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals (Pub. No. 85-23 Revised 1985). Handling of animals was in accordance with relevant institutional and ethical guidelines as approved for scientific study and all efforts were made to minimize the number of animals involved and potential of sufferings.

2.11. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean±SD. A comparison of MIC and MBC values of the extract against all test bacteria with Betadine was evaluated by applying a two tailed-unpaired *t*-test. Bacteria dependent variation in different concentrations both the *P. harmala* extract and Betadine were detected by analysis of variance (ANOVA) followed by all pairwise multiple comparisons by Duncan test. Concentration dependent variation for each bacteria was studied by repeated measures analysis of variance (general linear model) followed by a student-Newman-Keuls test, and was considered significantly at $P<0.05$. All statistics were performed using SPSS for windows version 16, Chicago, USA.

3. Results

Results of MIC and MBC determination (as shown in Figure 1) showed that MIC and MBC values for the seed extract of *P. harmala* against the two Gram-negative strains (*E. coli* and *K. pneumoniae*) were the same (1.3 mg/mL). While for Betadine were different. Moreover, MIC and MBC values for the extract against tested microorganisms were significantly lower than Betadine ($P<0.05$). The MIC value range for the extract was 0.68-1.3 mg/mL and the MBC value range for the extract was 1.3-5 mg/mL. The MIC and MBC values range for Betadine were respectively 1.3-5 mg/mL and 2.56-5 mg/mL.

Based on the results of the different concentrations percentage of both *P. harmala* seeds ethanolic extract (as shown in Table 1) and Betadine (as shown in Table 2) against tested microorganisms, the percentage of growth inhibition increased with a rise in the concentrations. In the different concentrations, efficacy of the extract was better compared to Betadine against tested microorganisms.

Table 3 presents diameters of inhibition zones exerted by the different concentration of the *P. harmala* seeds ethanolic extract and the two standards (tetracycline and chloramphenicol) towards tested microorganisms. *P. harmala* seeds extract was more effective against the four Gram-positive strains (*S. pneumoniae*, *C. pseudotuberculosis*, *M. luteus* and *C. pseudotuberculosis* spp.) and a Gram-negative strain (*S. typhimurium*) than antibiotics. The extract showed lower activity against the Gram-negative strain (*K. pneumoniae*). In the case of *S. aureus*, the extract showed the maximum inhibitory zone of 15 mm at 200 mg/mL. For *S. typhimurium*, *M. luteus*, *C. pseudotuberculosis*, and *C. pseudotuberculosis* spp. strains, the extract exhibited the maximum

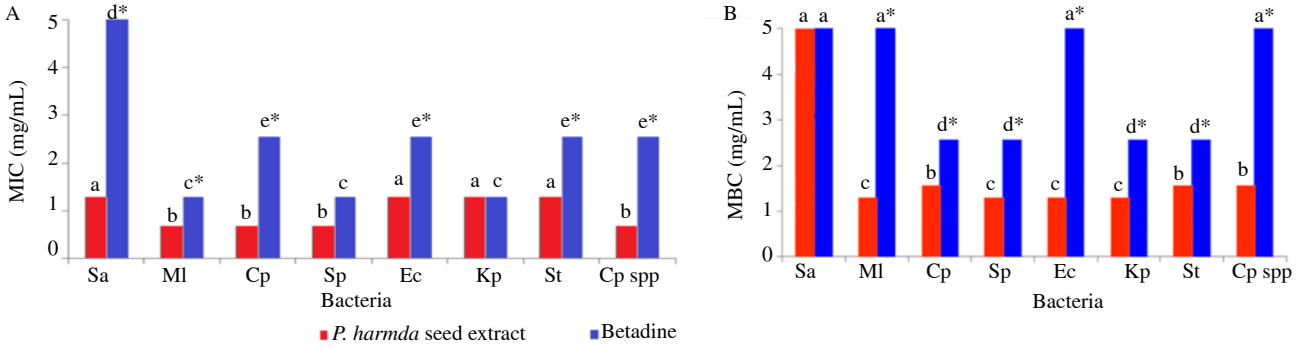

Figure 1. Determination of the MIC (A) and the MBC (B) from eight test bacteria exposed with the ethanolic extract of *P. harmala* seeds and Betadine. Similar values among bacteria are identified by the same. Sa: *S. aureus*; Ml: *M. luteus*; Cp: *C. pseudotuberculosis*; Sp: *S. pneumoniae*; Ec: *E. coli*; Kp: *K. pneumoniae*; St: *S. typhimurium*; Cp spp.: *C. pseudotuberculosis* spp. The values where are similar ($P>0.05$) in columns are identified by the same letter a, b, c, d, e ($P>0.05$). *: Significant statistical difference between effect of extract and Betadin on bacteria.

Table 1

The growth inhibition values (%) in the different concentrations of ethanolic extract of *P. harmala* seeds for test bacteria.

Bacteria	Concentration (mg/mL)									
	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	0.019
S. aureus	76.0±0.9^{ab}	71.0±0.1^{a}	66.0±1.5^{a}	45.0±5.5^{a}	37.0±5.8$^{ae/a}$	36.0±9.3$^{a/a}$	27.0±5.3^{a}	20.0±2.8^{a}	15.0±0.6^{a}	6.0±1.5^{ab}
M. luteus	79.0±0.3^{b}	72.0±2.1$^{a/a}$	72.0±5.1$^{b/a}$	63.0±1.7^{b}	67.0±32.7^{b}	35.0±1.7^{a}	27.0±3.0^{a}	20.0±0.6^{a}	13.0±0.0^{ad}	-
C. pseudotuberculosis	95.0±2.9^{c}	87.0±2.4^{b}	80.0±4.0^{c}	73.0±4.7^{c}	50.0±7.1^{c}	42.0±0.6^{b}	33.0±1.0^{b}	24.0±0.6^{b}	14.0±0.1^{a}	5.0±0.7^{ab}
S. pneumoniae	75.0±1.2^{ad}	71.0±1.5^{a}	66.0±6.1$^{a/a}$	63.0±0.1$^{b/a}$	43.0±3.0^{d}	35.0±2.6^{a}	27.0±2.3^{a}	19.0±0.9^{ac}	12.0±1.3^{ad}	7.0±2.5^{a}
E. coli	64.0±3.4$^{e/a}$	67.0±4.3$^{c/a}$	57.0±1.5^{d}	38.0±1.0^{d}	34.0±1.8^{ef}	27.0±3.5^{c}	20.0±3.6^{c}	14.0±0.2^{d}	10.0±1.7^{bd}	4.0±2.4^{b}
K. pneumoniae	71.0±4.2^{f}	67.0±2.8^{c}	63.0±4.7^{ae}	43.0±4.4^{a}	40.0±10.8^{ad}	31.0±6.7^{d}	24.0±7.1^{ad}	16.0±6.9^{cd}	8.0±6.6^{bc}	
S. typhimurium	72.0±6.6$^{df/a}$	69.0±5.8$^{ac/a}$	63.0±4.9^{ae}	39.0±10.6^{d}	32.0±7.9^{f}	26.0±6.1$^{c/b}$	26.0±3.4$^{ad/b}$	21.0±0.7^{ab}	13.0±0.1^{ad}	5.0±0.1^{ab}
C. pseudotuberculosis spp.	72.0±3.9^{df}	67.0±3.9^{c}	60.0±4.0^{de}	55.0±4.0^{e}	35.0±4.2^{ef}	28.0±5.1^{cd}	23.0±0.2^{cd}	15.0±0.8^{d}	6.0±0.8^{c}	-

Value are expressed as mean±SD, $n=3$. Data are identified by Duncan's test. The growth inhibition values where are similar ($P>0.05$) in columns are identified by the same letter before slash. The growth inhibition values where are similar ($P>0.05$) in rows are identified by the same letter after slash.

Table 2

The growth inhibition values (%) in the different concentrations of Betadine for test bacteria.

Bacteria	Concentration (mg/mL)									
	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	0.019
S. aureus	60.0±1.1$^{a/a}$	58.0±0.4$^{a/a}$	47.0±0.7$^{a/b}$	46.0±3.7$^{a/b}$	41.0±3.4^{a}	36.0±4.3^{a}	31.0±4.0^{a}	25.0±6.1^{a}	21.0±4.6^{a}	8.00±0.50^{a}
M. luteus	42.0±8.0$^{b/a}$	44.0±7.5$^{b/a}$	42.0±9.3$^{b/a}$	32.0±14.2$^{b/b}$	31.0±19.5$^{b/b}$	25.0±19.6^{b}	22.0±20.5^{bc}	18.0±20.8$^{b/c}$	16.0±22.0$^{b/c}$	-
C. pseudotuberculosis	50.0±0.3^{c}	47.0±0.4^{b}	39.0±0.3$^{b/a}$	38.0±0.8^{cd}	36.0±0.7^{cd}	33.0±1.8^{ac}	28.0±0.9^{ae}	24.0±1.7^{a}	14.0±7.8^{bd}	-
S. pneumoniae	55.0±1.9$^{d/a}$	53.0±0.3$^{c/a}$	49.0±1.1^{a}	37.0±1.2^{c}	32.0±0.5^{b}	24.0±0.9^{b}	19.0±1.6^{b}	13.0±1.6^{c}	6.0±1.5^{c}	0.52±0.70^{b}
E. coli	62.0±1.7^{ae}	59.0±1.1^{ad}	46.0±2.3^{a}	41.0±1.7^{de}	38.0±2.8^{ad}	31.0±1.1^{c}	27.0±0.0^{de}	13.0±4.6^{c}	-	-
K. pneumoniae	63.0±1.1^{ae}	58.0±2.6^{a}	53.0±3.6^{c}	38.0±3.3^{cd}	34.0±6.1^{bc}	30.0±5.8^{c}	24.0±6.1^{cd}	19.0±7.1^{db}	-	-
S. typhimurium	60.0±1.4$^{a/a}$	59.0±4.7$^{ad/a}$	47.0±1.0^{a}	41.0±3.1^{de}	36.0±3.8^{cd}	30.0±3.3^{c}	24.0±3.4^{cd}	19.0±4.2^{db}	12.0±3.0^{d}	-
C. pseudotuberculosis spp	64.0±0.3$^{e/a}$	62.0±0.3$^{d/a}$	48.0±0.7^{a}	43.0±0.2^{ae}	39.0±1.6^{ad}	33.0±0.4^{ac}	27.0±0.1^{de}	22.0±1.9^{ad}	13.0±6.4^{d}	-

Value are expressed as mean±SD, n=3. Data are identified by Duncan's test. The growth inhibition values where are similar (P>0.05) in columns are identified by the same letter before slash. The growth inhibition values where are similar (P>0.05) in rows are identified by the same letter after slash.

Table 3

The inhibition zones around the discs (mm) produced by antibacterial activity of different concentrations of *P. harmala* (mg/mL) and standard antibiotics against test bacteria.

Bacteria	Concentration (mg/mL)						Standard	
	50	100	200	300	400	500	Tetracycline	Chloramphenicol
S. aureus	0.0±0.0$^{a/a}$	13.0±1.4$^{/b}$	15.0±1.4$^{ab/b}$	15.0±1.4$^{a/b}$	15.0±1.4$^{a/b}$	15.0±1.4$^{a/b}$	17.5±0.7^{a}	15.5±0.7^{a}
M. luteus	11.5±0.7^{b}	13.5±0.7	16.0±1.4^{ab}	19.5±0.7^{bc}	21.0±1.4^{bc}	21.5±0.7^{b}	23.5±0.7^{b}	18.0±1.4^{a}
C. pseudotuberculosis	12.0±1.4$^{bd/a}$	14.0±1.4$^{/a}$	17.5±0.7$^{bc/b}$	20.0±1.4$^{b/bc}$	20.5±0.7$^{bc/bc}$	21.0±0.0$^{b/c}$	20.0±0.0^{c}	21.5±2.1^{b}
S. pneumoniae	10.5±0.7$^{bc/a}$	12.5±0.7$^{/a}$	15.5±0.7$^{ab/b}$	17.5±0.7$^{ac/b}$	18.0±0.0$^{ab/b}$	18.0±0.0$^{a/b}$	0.0±0.0^{d}	17.5±0.7^{a}
E. coli	11.5±0.7$^{b/a}$	13.0±0.0$^{/a}$	16.5±0.7$^{ab/b}$	16.5±0.7$^{a/b}$	17.0±0.0$^{a/b}$	17.0±0.0$^{a/b}$	17.0±1.4^{a}	17.0±1.4^{a}
K. pneumoniae	9.5±0.7$^{c/a}$	11.5±0.7$^{/ab}$	14.0±0.0$^{a/bc}$	15.5±0.7$^{a/c}$	16.0±0.0$^{a/c}$	16.0±0.0$^{a/c}$	16.5±0.7^{a}	20.5±0.7^{b}
S. typhimurium	10.5±0.7$^{bc/a}$	13.0±1.4$^{/ab}$	15.5±0.7$^{ab/bc}$	16.0±0.0$^{a/c}$	16.0±0.0$^{a/c}$	16.5±0.7$^{a/c}$	0.0±0.0^{d}	11.0±0.0^{c}
C. pseudotuberculosis spp	13.5±0.7$^{d/a}$	16.0±1.4$^{/a}$	20.0±1.4$^{c/b}$	22.5±0.7$^{d/bc}$	23.0±0.0$^{c/bc}$	24.0±0.0$^{b/c}$	17.5±0.7^{a}	20.5±0.7^{b}

Values are expressed as mean±SD, n=3. Data are identified by Duncan's test. The growth inhibition values where are similar (P>0.05) in columns are identified by the same letter before slash. The growth inhibition values where are similar (P>0.05) in rows are identified by the same letter after slash.

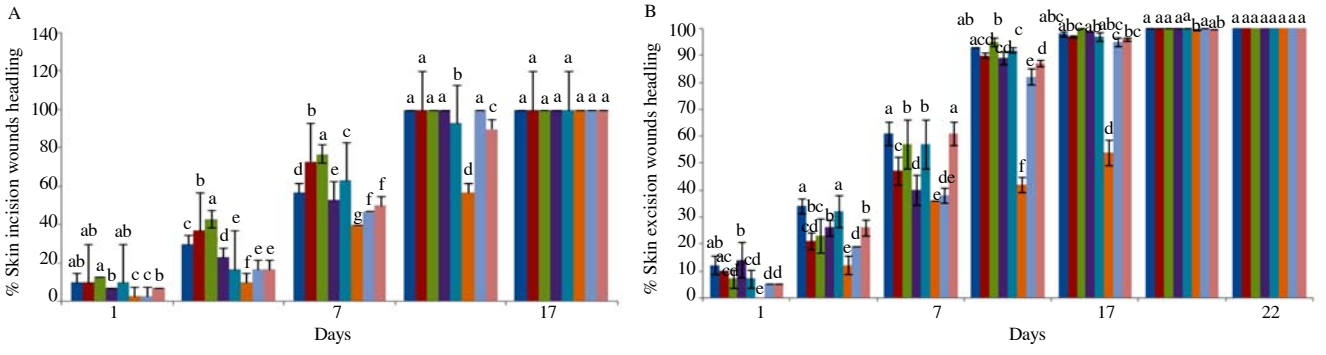

Figure 2. Percentage of skin incision (A) and excision (B) wounds healing in different concentrations of *P. harmala*, Betadine and control in 1, 3, 7, 14, 17, 20 and 22 d after surgical.

Values are expressed as mean±SD, n=3. The values where are similar (P>0.05) in columns are identified by the same letter (P>0.05). There was significant difference within different days in each group (P<0.05)

inhibitory zone of 16.5, 21.5, 21.0 and 24.0 mm at 500 mg/mL. In the case *E. coli*, the extract showed to some extent similar inhibition zone [(17.0±0.0) mm] compared to antibiotics [(17.0±1.4) mm].

The significant rise in the wound healing activity was observed in the rats treated with ethanolic extract of *P. harmala* seed in comparison with Betadine or normal saline (control). Figure 2A shows the effects of this extract at different concentrations for 17 d on wound healing activity in rats inflicted with incision wound. In the incision wound model, a significant rise in the wound breaking strength was observed compared with control. In excision wound model (as shown in Figure 2B), area of the wound decreased from 3-22 d in all of the groups. Except 500 mg/mL, the other concentrations showed significant differences in comparison with Betadine. In the group treated with 50 mg/mL concentration of extract, the wound contraction was significantly lower than Betadine and control (P<0.05).

4. Discussion

Multi-drug resistant strains of bacteria are imposing the need for new drugs. Reliable natural sources with minor side effects are needed to control anti-human and anti-animal pathogenic invaders specially bacteria. Given the demands for natural products that are inherently safe and environmentally compatible, the advancement in antimicrobial potential has provided a better alternative to synthetic resistance antibiotics[31].

In the present study, ethanolic extract of *P. harmala* seeds was investigated for its antimicrobial and antiseptic potential. This extract was tested against three microorganisms including four Gram-negative bacterial strains *E. coli*, *K. pneumoniae* and *S. typhimurium*, and five Gram-positive bacterial strains *S. aureus*, *M. luteus*, *C. pseudotuberculosis*, *S. pneumoniae* and *C. pseudotuberculosis* spp. The MIC and MBC ranges of the ethanolic extract of *P. harmala* seed and Betadine against tested microorganisms in this study were 0.68-1.3 mg/mL and 1.3-5 mg/mL for extract, and 1.3-5 mg/mL and 2.56-5 mg/mL for Betadine respectively. The MIC value range for the extract was 0.68-1.3 mg/mL and the MBC value range for the extract was 1.3-5 mg/mL. The MIC and MBC values range for Betadine were respectively 1.3-5 mg/mL and 2.56-5 mg/mL. Aligiannis *et al.* have proposed a classification of plant extracts on the basis of their MIC values: strong inhibition (MIC<500 μg/mL), moderate inhibition (600 μg/mL<MIC<1 500 μg/mL) and low inhibition (MIC>1 600 μg/mL) [32]. On the basis of this classification, the *P. harmala* seed extract exerts a better inhibitory activity than Betadine on all of tested bacteria. The comparison of MICs and MBCs values allows a better evaluation of antibacterial effect of bioactive compounds. According to Boulekbache-Makhlouf *et al.*[33], a substance is bactericidal when the ratio MBC/MIC<2, and bacteriostatic if the ratio MBC/MIC>2. The MIC and MBC are often near or equal values. So, in this study, it can be concluded that seed extract of *P. harmala* has a bactericidal effect on *S. pneumoniae*, *S. typhimurium*, *E. coli*, *C. pseudotuberculosis*, *K. pneumoniae* and *M. luteus* and a bacteriostatic effect on *S. aureus* and *C. pseudotuberculosis* spp. Also the MIC and MBC values for the seed extract of *P. harmala* against the two Gram-negative strains (*E. coli* and *K. pneumoniae*) were the same (1.3 mg/mL). It is generally held that for bactericidal agents, the MIC and MBC are often near or equal values. So, it can be concluded that these extracts of *P. harmala* have a bactericidal effect on the mentioned bacteria[34]. The present results are comparable with the finding of Darabpour *et al*[34]. They reported that MIC and MBC values for the seed and root extract of *P. harmala* against methicillin resistant *S. aureus* and for seed extract against *E. coli* and *Salmonella typhi* were equal (0.625 mg/mL).

In the present study, enhancement of growth inhibition with an increase in the concentrations suggested concentration dependent activity. This may be attributed to the increased levels of the principle alkaloids, while increasing the extract concentrations, and these alkaloids (which are heterocyclic nitrogen compounds) have the ability to intercalate with DNA of the microorganisms[34]. Some of these alkaloids present in *P. harmala* seeds extracts are: harmaline, harmine, harmalol peganine, β-carboline alkaloids, anthraquinones and fixed oils[35]. According to this study, in the different concentrations, the efficacy of extract was better against tested microorganisms compared to Betadine. It shows that *P. harmala* extract as natural and environmentally friendly compound could be an important source of antibacterial agent and active principles may be useful in the topical treatment of superficial skin infections of tested bacteria. Amel *et al*. have reported an inhibitory effect of seed alkaloid extract of *P. harmala* against some Gram-positive bacterial strains such as *S. aureus* and *Staphylococcus saprophyticus* and Gram-negative such as *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Serratia* spp., which is in agreement with the present results[36].

In present study, all of the different concentrations tested for antibacterial potential showed varying degree of antibacterial activities against the Gram-positive, Gram-negative bacterial species. Ethanolic extract of *P. harmala* showed 15.0 mm inhibition zone against *S. aureus* at 200 mg/mL concentration, and 21.5 mm, 21.0 mm, 16.5 mm and 24.0 mm against *M. luteus*, *C. pseudotuberculosis*, *S. typhimurium* and *C. pseudotuberculosis* spp at 500 mg/mL. *P. harmala* seeds extract was more effective against the four Gram-positive strains (*S. pneumoniae*, *C. pseudotuberculosis*, *M. luteus* and *C. pseudotuberculosis* spp) and a Gram-negative strain (*S. typhimurium*) compared to the antibiotics. The extract showed lower activity against the Gram-negative strain (*K. pneumoniae*). This could be attributed to the concentration of the active substance causing the inhibitory effect which could have been higher in this part of plants[34]. Previously, Prashanth and John evaluated the antibacterial activity of *P. harmala* seeds ethanolic extract against the same bacteria and exhibited positive results[37]. Similar antimicrobial activity was also reported by Mashreghi and Niknia[38].The antimicrobial activities of *P. harmala* are evaluated by different workers. According to the study of Darabpour *et al*. among the evaluated different parts of *P. harmala*, the seed and root extracts showed the best antibacterial activity against Gram-positive bacterial species, including *Bacillus anthracis*, *Bacillus cereus, Bacillus pumilus, S. aureus, Staphylococcus epidermidis, Listeria monocytogenes* and *Streptococcus pyogenes*, and Gram-negative bacterial species, including *Pseudomonas aeruginosa, Brucella melitensis, Proteus mirabilis, Salmonella typhi, E. coli* and *K. pneumoniae*[34].

Wounds are the result of injuries to skin that disrupt other soft tissue. Healing of a wound is a complex and protracted process of tissue repair and remodeling in response to injury. Various plant products have been used in treatment of wounds over the years. Wound healing herbal extracts promote blood clotting, fight infection, and accelerate the healing of wounds. Also *in vitro* assays are useful, quick, and relatively inexpensive. Meanwhile small animals provide a multitude of model choices for various human wound conditions[3]. Therefore in the present study, wound healing efficacy of ethanolic extract of *P. harmala* seed was evaluated in excision, incision on rats. At Day 7 extract-treated animals exhibited 77% reduction in the wound area compared with controls (50%) at 50 mg/mL concentration. The skin breaking strength was significantly higher in extract-treated animals compared with controls. These observations support the use of *P. harmala* in the management of wound healing. These results are in agreement with other study by Derakhshanfar *et al*[9]. They showed that *P. harmala* extract significantly increased the number of fibroblasts and capillary buds, and also decreased the epithelial gap which showed the better healing in treatment group.

In conclusion, the low values of MIC and MBC for ethanolic extract of *P. harmala* seeds against the most important of tested bacterial pathogens especially *M. luteus* and *S. pneumoniae* was valuable.

The present study showed that *P. harmala* seeds extract was more effective against the four Gram-positive strains (*S. pneumoniae*, *C. pseudotuberculosis*, *M. luteus* and *C. pseudotuberculosis* spp) and a Gram-negative strain (*S. typhimurium*) compared with antibiotics. Enhancement of growth inhibition with an increase in the concentrations suggested concentration dependent activity. Also, ethanolic extract of *P. harmala* seed had properties that rendered it capable of promoting accelerated wound healing activity compared with Betadine and controls. Further studies are needed to perform *in vitro* and *in vivo* with specified chemical constituents of *P. harmala* extract especially with antiseptic and antimicrobial actions and be tested separately for the antimicrobial and antiseptic activities.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Multi-drug resistant bacteria made a serious problem all over the world. So, searching on new compounds became very important. The plant extract especially the wild medicinal plants contains many active compounds, which could be used as antibacterial against the multidrug resistant bacterium. I see that the authors went in this direction to control many bacterial strains using the plant extract as antibacterial and they succeeded to get good results.

Research frontiers

The cutting edge of this work is new and this kind of work has great attention from many scientists.

Related reports

There are many reports related to this work and this actually appeared in the introduction, materials and methods and in discussion as well. Prashanth and John evaluated the antibacterial activity of *P. harmala* seeds ethanolic extract against the same bacteria and exhibited positive results. Similar antimicrobial activity was also reported by Mashreghi and Niknia.

Applications

The application in this work is the usage of the medicinal plant extract as antibacterial against the multidrug resistant bacteria.

Peer review

The article has a good idea, and the authors worked to find a natural antibiotic from medicinal plant (*P. harmala*) instead of the chemical antibiotic. The authors discussed this point along the paper. The paper is well-written and the author used standard method and discussed the results in good manner.

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