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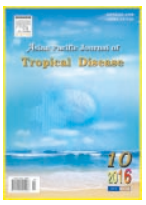

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Phytochemical and antimicrobial study of the seeds and leaves of *Peganum harmala* L. against urinary tract infection pathogens

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ABSTRACT

Objective: To investigate the antibacterial effect of *Peganum harmala* (*P. harmala*) extracts against urinary tract infection pathogens isolated from infected patients.
Methods: Agar diffusion technique was used for detecting the antibacterial activity. The minimum inhibitory concentration was tested by serial dilution methods. Alkaloids extract of seeds was fractioned using thin layer chromatography.
Results: Phytochemical screening in the leaves of *P. harmala* showed the presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids and the absence of anthraquinons, whereas the results in the seeds showed the absence of flavonoids and the presence of alkaloids, saponins, tannins, glycosides, anthraquinons, terpenoids and steroids. The results of thin layer chromatography revealed that the alkaloids profiles of the seeds extracts of *P. harmala* are different. The major alkaloids detected and quantified from the intensity of their fluorescence were harmine, harmaline, harmalol and harmol. The methanolic extract of the plant was effective against the four microorganism tested. It was observed that the minimum inhibitory concentration and minimum bactericidal concentration of flavonoids extracted from the leaves and seeds of *P. harmala* were bactericidal to *Staphylococcus aureus*.
Conclusions: Results of this study suggest that the flavonoids extract of *P. harmala* may be useful to treat uropathogenic bacteria.

1. Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans throughout their life span[1]. UTIs develop when bacteria get into the urinary system, a part of the body which normally has no bacteria. About 85% of these infections are caused by a normal intestinal bacterium named *Escherichia coli* (*E. coli*). However, other bacteria can cause an infection such as the Gram-negative species *Klebsiella, Proteus, Enterobacter, Pseudomonas* and *Serratia* and the Gram-positive bacterial cocci, *Enterococcus faecalis*, *Staphylococcus saprophyticus* and *Staphylococcus aureus* (*S. aureus*). In addition, fungi (*Candida* and *Cryptococcus* spp.) and some parasites (*Trichomonas* and *Schistosoma*) also may cause UTIs[2,3].

Antimicrobial resistance is known to be associated with the level of antibiotic use[4]. It is an increasingly serious threat to global public health that requires actions across all government sectors and societies, which might require using more expensive drugs in the near future.

Fortunately, a number of herbs have antimicrobial effects. Recently, searching for drugs and dietary supplements derived from plants have been accelerated. Plants are rich in a wide variety of secondary metabolites, such as flavonoids, terpenoids, tannins and alkaloids which have been found in many studies to have antimicrobial properties[5,6].

Peganum harmala L. (*P. harmala*) belongs to the family of Zygophyllaceae. It is a wild growing flowering plant. It is also called African rue, Syrian rue, wild rue and harmal in Algeria. The plant is widely distributed in predesertic regions of Southeast Morocco, North Africa and the Middle East[7]. *P. harmala* contains up to 4% total alkaloids[8]. Literature surveys revealed that *P. harmala* shows different pharmacological activities like antioxidant[9], anti-spasmodic, anti-histaminic, vasorelaxant effects[10], wound healing, immunomodulator properties, leukemia healing[11], antitumor[12], antibacterial and antitubercular activities[13]. Traditionally, the smoke of its seeds is used as a disinfectant[14].

The antimicrobial properties of various plants have been investigated by a number of researchers[15,16]. The antimicrobial activities of some medicinal plants against *Bacillus subtilis*, *E. coli, S. aureus, Pseudomonas aeruginosa* (*P. aeruginosa*), *Proteus*

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vulgaris and Klebsiella pneumonia were screened and showed that among these bacteria, *E. coli, Proteus vulgaris* and *S. aureus* were highly inhibited[16].

The aim of this study was to phytochemically study and the evaluate of antimicrobial activity of the seed and leave extracts of *P. harmala* against selected pathogens isolated from patients with UTIs.

2. Materials and methods

2.1. Plant material

The seeds and leaves of *P. harmala* were collected from south region of Tiaret, west of Algeria. The samples were harvested on the 7th day of April, 2007. The sample was identified and a voucher specimen (Al.T.P.h/4/2007) of the plant was kept at the Herbarium of Laboratory of Pharmacognosy and Api-Phytotherapy in University of Mostaganem, Algeria for future reference.

2.2. Preliminary phytochemical screening

2.2.1. Preparation of methanolic extract

Fifty grams of each sample dry powder (leaves and seeds) of *P. harmala* was mixed with 250 mL of methanol for 1 h. The extract was filtered by using Whatman filter paper. The filtrate was used for phytochemical screening.

2.2.2. Preparation of aqueous extract

A total of 250 mL of distilled water was added to 50 g of each sample dry powder (leaves and seeds) for 1 h. The extract was filtered by using Whatman filter paper. The filtrate was used for phytochemical screening.

2.2.3. Phytochemical screening

Chemical tests were carried out on the methanolic and aqueous extracts and on the powdered specimens using standard procedures to identify the constituents[17-20].

To detect the presence of tannins, 5 mL of aqueous extract solution and 1 mL of ferric chloride ($FeCl_3$, 1%) solution was mixed. In the presence of gallic tannins it develop blue color and green black for catecholic tannins. The presence of saponins is quantitatively determined by the calculation of the foam index. When the plant material (0.5 g) was soaked in 10 mL water and shaken, a foam produced persisted for 10 min indicated the presence of saponins. As for flavonoids, the plant powder was mixed with 2 mL of HCl and 1–2 drops of sodium hydroxide solution was added. It develops a yellow color and it becomes colourless in the presence of dilute acid indicated the presence of flavonoids. Alkaloids were determined by Dragendorff's reagents test using the methanolic extract. Few drops of acetic anhydride was mixed to the methanolic extract of the plant and 2 drops of pure sulphuric acid was added and red bluish color turning to green for steroids was noted. Salkowski test was performed using the methanolic extract to test terpenoids. After 0.5 g of plant powder extract was dissolved in 2.0 mL of glacial acetic acid along with one drop of ferric chloride solution and 1.0 mL of pure H_2SO_4 was added, brown ring developing at the interface indicated the presence of glycosides. To detect quinones, 1 g of powder of the plant with 1 mL of HCl was mixed after that 5 mL of chloroform was added and then left for several hours. The extracts were filtered, diluted ammonia (1/2) then were added to the filtrate. If it was observed that the aqueous phase did not stain, it indicated the absence of quinones. Finally, 0.5 g of the plant powder was macerated in water. After filtration, 1 mL of ammonia (10%) was added, the presence of anthraquinons is confirmed by the formation of red rose color. Quantitative analysis of the phytochemicals was done using standard methods[21].

2.2.4. Thin layer chromatography (TLC)

TLC was used for the conformation of the alkaloids on analytical plates. The prepared alkaloids extract of seeds were dissolved in methanol with a concentration of 1 mg/mL. Then, 10 µmL of the extract were loaded on the analytical plate (2.5 cm above from the bottom) and dried on air for 30 min. The spotted plates were kept in a previously saturated developing chambers containing mobile phase and allowed to run 3/4th of the height of the prepared plates[22]. The solvent system contained acetonitrile-alcohol isopropylic-water-(1:1:3) (v/v/v) as mobile phase. The different bands of chromatograms were observed under visible light and photographed. Dragendorffs reagent was used for the detection of alkaloids, The retention factor (Rf) values were calculated.

2.3. Tested microorganisms

Proteus mirabilis (*P. mirabilis*), *E. coli, P. aeruginosa* and *S. aureus* which are known to cause different types of UTIs isolated from infected patients in laboratory of isolated strains (Aintedless Hospital, Mostaganem, Algeria). The microorganisms were identified by standard biochemical reactions based on morphological and biochemical characters according to the methods described in Bergey's manual for systematic bacteriology[23,24]. Twenty-four-hour-old pure cultures were prepared for use each time.

2.3.1. Plant extract preparation for antibacterial test

2.3.1.1. Aqueous extract preparation (decoction)

Decoction involved boiling 1 g of each plant in 10 mL of distilled water for 5 min according to the traditional uses. We left the rest decoction successively for 15 min. After filtration, the crude extract was tested on bacteria of the urinary tract infection.

2.3.1.2. Alcoholic extract preparation for flavonoids

One gram of powder of the leaves of *P. harmala* was drenched in methanol for 24 h. The residues were recovered by evaporation to dryness in a water bath at 50 ˚C.

2.3.1.3. Alcoholic extract preparation for alkaloids

Samples were justly dried and ground into fine particles and into crude powder. In the first step, 2 g of each sample was drenched in 50 mL of 95% methanol for 1 h at 50 ˚C in water bath. The residue was dissolved in 50 mL of HCl (2%) and filtered and then supplies were strained with Whatman No. 1 filter paper. In the next step, the filtrate was concentrated twice with 20 mL petroleum ether. Adding NH_4OH until the pH became 10.50 mL, chloroform was added to the basic solution. The chloroform layer was evaporated to dryness. The residues were solubilized in 0.2% dimethyl sulfoxide.

2.3.2. Antibacterial assay procedure

Zones of inhibition of various extracts of *P. harmala* were tested against urinary pathogens on agar disk diffusion method. Overnight cultures of bacteria were spread on Mueller-Hinton agar (Merck, Germany) and various extracts of *P. harmala* (aqueous, flavonoids and alkaloids extracts) of seeds and leaves were added to the 5 mm sterile disks (100 µg/disk) in the culture and incubated at 37 ˚C for 24 h. Then, the diameter of the inhibition zone was recorded.

2.3.3. Determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was the lowest concentration of a substance that preventsed visible growth of a bacterium[25]. In this test, the broth macrodilution method was used. Various concentrations of the stock (100.000, 50.000, 25.000, 12.500, 6.250 and 3.125 mg/mL) were tested against the four microorganisms.

The same volume of each extract with different concentrations was mixed with Mueller Hinton broth in tubes to make up 1 mL of solution than after 1 mL of McFarland standard of each bacteria suspension (10^6 colony-forming unit/mL) was added. All the tubes were incubated at 37 ˚C for 18–24 h. Two control tubes were prepared with one containing the extract without the organism and the second containing the growth medium with the inoculum. MBC was the concentration that resulted in microbial death. In other words, the highest dilution yielded no single bacterial colony. This was done on some extracts having a high antimicrobial activity.

3. Results

Preliminary phytochemical screening in the leaves of *P. harmala* showed the presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids and the absence of anthraquinons, whereas the results in the seeds showed the absence of flavonoids and the presence of alkaloids, saponins, tannins, glycosides, anthraquinons, terpenoids and steroids. Quantitative analysis from this study showed that the seeds of *P. harmala* contained high levels of alkaloids with low quantity in leaves, whereas the leaves contained the highest levels of flavonoids (Table 1).

Table 1

Phytochemicals screening of seeds and leaves of *P. harmala.*

Extract constituents	seeds	leaves	Concentraction (mg/g)	
			seeds	leaves
Flavonoids	-	+++	1	5
Alkaloids	+++	+	35	13
Glycosides	+	++	nd	nd
Tannins	+++	+++	nd	nd
Saponins	++	++	nd	nd
Steroids and terpenoids	+	+	nd	nd
Quinons	++	-	nd	nd
Anthraquinons	+	-	nd	nd

-: Absent; +: Present; +++: Present in high quantity; nd: Not determined.

Figure 1 and Table 2 show three spots (at 254 and 336 nm) with different Rf values or molecular weights in seed extracts of alkaloids of *P. harmala*. The seeds extracts of *P. harmala* had three different spots of alkaloids. From these spots, four alkaloids were identifying: the harmine appearing blue and purple with a Rf value of 0.82, harmaline having an emerald green fluorescence with a Rf of 0.25, harmol showing a violet blue fluorescence with its Rf of 0.71 and harmalol having a green fluorescence with a Rf of 0.21.

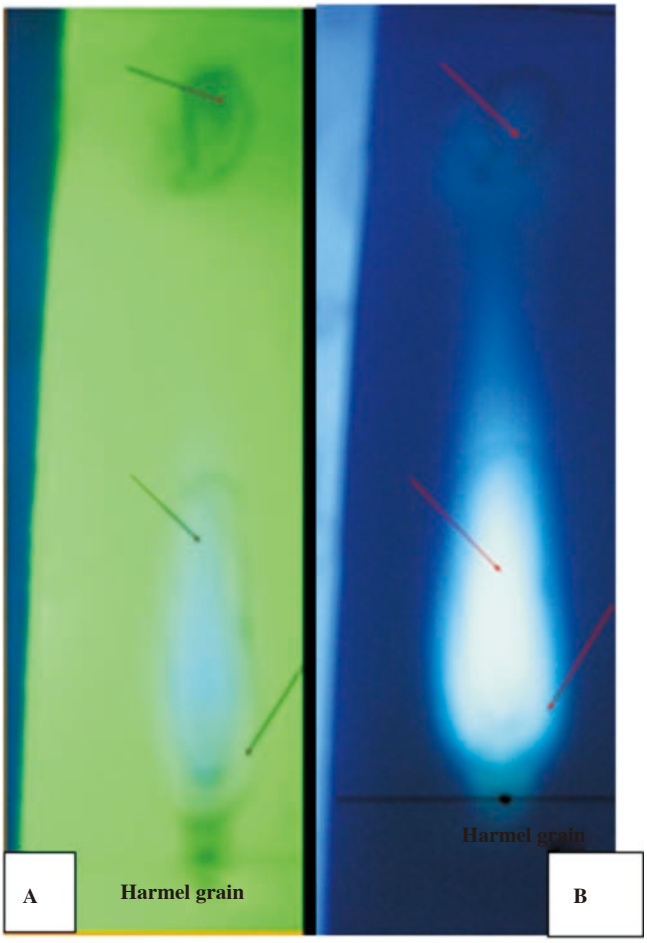

Figure 1. TLC profiles of alkaloids extract of *P. harmala* seeds. A: UV 254 nm; B: UV 336 nm.

Table 2

Effect of aqueous and methanolic extracts of leaves and seeds of *P. harmlaon* urinary tract infection pathogens.

Microorganisms	Leaves			Seeds		
	AQ	F	AL	AQ	F	AL
P. mirabilis	11.0 ± 0.8	5.0 ± 0.5	10.0 ± 0.5	5.0 ± 0.7	5.0 ± 0.5	5.0 ± 0.5
E. coli	10.0 ± 1.0	15.0 ± 0.4	10.0 ± 0.2	5.0 ± 0.4	8.0 ± 0.2	10.0 ± 0.3
P. aeruginosa	5.0 ± 0.2	8.0 ± 0.7	5.0 ± 0.3	5.0 ± 0.6	15.0 ± 0.6	10.0 ± 0.7
S. aureus	5.0 ± 0.2	15.0 ± 0.7	12.0 ± 0.5	5.0 ± 0.6	12.0 ± 0.4	5.0 ± 0.2

Disc diameter: 5mm; AQ: Aqueous extract; F: Flavonoids extract; AL: Alkaloids extract.

Based on the inhibitory zone diameters (Table 3), the methanol extract of leaves of *P. harmala* had a greater inhibitory effect on the four microorganisms tested than the aqueous extract. However, the seeds extracts of *P. harmala* did not inhibit the growth of *P. mirabilis* and had a slight inhibitory effect on the other microorganisms tested except for flavonoids extracts on *P. aerugenosa* and *S. aureus*.

Table 3

MIC (mg/mL) and MBC (mg/mL) of the flavonoids extracts.

Microorganisms	Leaves		seeds	
	MIC	MBC	MIC	MBC
P. mirabilis	3.125	100.000	12.500	100.000
E. coli	50.000	50.000	3.125	100.000
P. aeruginosa	6.250	12.500	12.500	25.000
S. aureus	50.000	50.000	50.000	50.000

The flavonoids extracts in seeds and leaves showed greater antibacterial effects from 8 to 15 mm.

Results of MIC and MBC determination and the rapport of MBC/MIC showed that *S. aureus* (Gram-positive strain) was more sensitive to the seeds and leaves of flavonoids extract of *P. harmala*, while *E coli* was sensitive to the leaf extract but was resistant to the seed extract (Table 4).

Table 4
The rapport of CMB/CMI.

Microorganisms	Leaves		Seeds	
	MBC/MIC	BA	MBC/MIC	BA
P. mirabilis	32	Resistant	8	Bacteriostatic
E. coli	1	Bactericidal	32	Resistant
P. aeruginosa	2	Bacteriostatic	2	Bacteriostatic
S. aureus	1	Bactericidal	1	Bactericidal

BA: Bactericidal activity; MBC/MIC < 2: Bactericidal; MBC/MIC > 2: Bacteriostatic; MBC/MIC ⩾ 32: Resistant.

4. Discussion

Uurinary tract infection is one of the commonest encountered infections. Recently, there has been an increase in the incidence of resistant organisms causing urinary tract infection (uropathogens). *E. coli* is the predominant uropathogen responsible for approximately 80% of UTIs, followed by *Staphylococcus*, *Klebsiella*, *Enterobacter* and *Enterococci* species[26].

The extracts of medicinal plants are used for their antibacterial, antifungal and antiviral properties in many parts of the world[27,28]. The antibacterial action of the plants is poorly understood and remains in debate[29].

In this study, *P. harmala* was chosen. It is used to treat gastrointestinal problems, infertility and urinary tract infection in Algeria. *P. harmala* has some alkaloids and flavonoids derivatives that provide it's pharmacological activities[30].

The phytochemical study of *P. harmala* extracts has shown that the seeds of this herb contains alkaloids, saponins, tannins, quinones, anthraquinones, terpenoids and steroids.

P. harmala seed extracts are reported to contain alkaloids, flavonoids and anthraquinones[31]. The study of quantitative evaluation of alkaloids has shown that the highest percentage was with the seeds, which can be considered as storage areas of the plant alkaloids studied[32]. According to this study, the TLC revealed that the alkaloids profiles of the seeds extracts of *P. harmala* are different. The major alkaloids detected and quantified from the intensity of their fluorescence are harmine, harmaline, harmalol and harmol.

Phytochemical studies of *P. harmala* led to the isolation of different types of chemical ingredients. The isolated alkaloids presenting in *P. harmala* seeds extracts are identified as harmaline, harmalolpeganine, harmine, β-carboline alkaloids, anthraquinons and fixed oils[33].

The results revealed that the extracts of *P. harmala* showed antibacterial activity with varying magnitudes depending on the method of extraction (aqueous, alkaloids and flavonoids extracts).

The flavonoids extract of leaves of *P. harmala* had a greater inhibitory effect on *S. aureus* (Gram-positive). The extract showed lower activity against the Gram-negative strains (*P. mirabilis* and *E. coli*).

These results are not in agreement with the finding of Amel *et al.*[34] who reported that the seed alkaloid extract of *P. harmala* has an inhibitory effect on Gram-positive bacterial strains such as *S. aureus* and staphylococcus, saprophyticus and Gram-negative such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis* and *Serratia* spp. Our results showed that alkaloids extract in leaves and seeds of *P. harmala* have moderate effect on different microorganisms tested as compared to flavonoids extract in leaves.

The antibacterial activity of flavonoids extract of *P. harmala* in this study might be linked to the high quantity of different flavonoids in the leaves of this plant. The antibacterial effect of flavonoids may have multiple cellular targets rather than one specific site of action. One of their molecular actions is the formation of complexes with the proteins by non-specific forces such as hydrogen bonding and hydrophobic effects and by forming a covalent bond. Thus, their antimicrobial effect can be connected to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins. Lipophilic flavonoids may also disrupt microbial membranes[35,36].

The MIC and MBC ranges of the flavonoids extract of *P. harmala* leaves. In this study, a substance was called bactericidal when the ration MBC/MIC < 2; when the ration MBC/MIC > 2, the substance was called bacteriostatic and when the ration MBC/MIC ⩾ 32, the microorganisms were resistant to this substance. It can be concluded that the leaf flavonoids extract of *P. harmala* has a bactericidal effect on *S. aureus* and *E. coli* and bacteriostatic effect on *P. aeruginosa*. The seed flavonoids extract has a bactericidal effect on *S. aureus* and bacteriostatic effect on *P. aeruginosa* and *P. mirabilis*.

The results of this study are comparable with the findings of Khademalhosseini *et al.*[37] and Darabpour *et al.*[38] who reported that MIC and MBC values of seed and the root extracts of *P. harmala* against *S. aureus* were resistant to methicillin and seed extracts against *E. coli* and *Salmonella typhi* were equal.

This study will be continued by other studies to confirm the exact mechanism of action of flavonoids and elucidate the structure of bioactive principle for the claimed antibacterial mechanism of *P. harmala*.

The results of this study showed *P. harmala* as a potential source of antimicrobial drug against the four urinary pathogens tested. This is particularly important in the fight against the recent resistant organisms with multiple drugs. These results suggest that future researches should be done to investigate the *in vivo* activity of this plant, toxicity and bioavailability and thus to determine the pharmalogical activity and to identify the polyphenols in the leaves of this plant that have the antibacterial effect.

Conflict of interest statement

We declare that we have no conflict of interest.

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