Original Article

Antioxidant, Antimicrobial, Phytochemical and FTIR Analysis of *Peganum harmala* (Fruit) Ethanolic Extract From Cholistan Desert, Pakistan

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Abstract

The aim of this study was to evaluate antioxidant and antimicrobial potential of *Peganum harmala* fruit. Ethanolic extract was prepared and phytochemical screening showed the presence of a lot of chemical compounds. Fourier transform infrared spectroscopy (FTIR) spectra indicated the presence of organic acids, hydroxyl and phenolic compounds, amino groups, aliphatic compounds, and functional groups such as amide, ketone, aldehyde, aromatics, and halogen compounds. Antioxidant activity of the ethanolic extract of *P. harmala* by the DPPH method showed 71.4% inhibition, whereas IC_{50} ± SEM (μg/mL) was .406 ± .11. Antibacterial activity was performed against *Escherichia coli, Bacillus subtilis, Bacillus pumilus, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis and Bordetella bronchiseptica.* Maximum antibacterial activity was exhibited by *Bacillus subtilis (24.33 ± 2 mm) and Bacillus pumilus (23.33 ± 2 mm). Zone of inhibition was 19 ± 2 mm by P. aeruginosa*, and it was *18.33 ± 2 mm by Bordetella bronchiseptica. Staphylococcus aureus and Staphylococcus epidermidis had inhibitory effect in the range of 12.33 ± 2 mm and 13.66 ± 3 mm, respectively. 11.66 ± 2 mm and 10 ± 2 mm was zone of inhibition by Micrococcus luteus and E. coli, respectively*. Antifungal activity was performed against *Aspergillus terreus, Aspergillus fumigatus, Aspergillus flavus and Candida albicans.* Ethanolic extract of *P. harmala* showed antifungal activity against *Aspergillus flavus (5* ± 1 mm) *and Candida albicans* (4 ± 1 mm). *Mild antifungal activity was reported by* Aspergillus *fumigatus* (*3* ± 1 mm), whereas no activity was exhibited by *Aspergillus terreus*. Further research is needed in order to evaluate the cytotoxic effects of *P. harmala* as well.

Keywords

antioxidant, antibacterial, antifungal, phytochemical

Introduction

Medicinal plants have been used for treating illnesses from centuries.[1] Careful observations of the efficacy and use of traditional medicinal plants markedly contribute to its healing properties.[2] Such medicinal plants are frequently used even if their chemical composition is not known.[3] All over the world especially in third world countries, indigenous medicinal plants are of great importance especially in health care system.[4] About 250 to 500 thousand of plant species are present on the earth, and just about 1 and 10% are utilized by humans and animals as food.[5] Pakistan is blessed with a diversified wealth of medicinal flora and a number of plant species are still unknown.[6] That's the reason treatment with medicinal plants is in vogue as it is cheaper

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and no strict quality control or standardization methods are in practice by the local healers.[7] In developing countries, infectious diseases are important risk factors for increased morbidity and mortality of the general population.[8] Pharmaceutical companies are trying their best to develop such antibacterial and antifungal preparations that can address microbial resistance.[9] Bacteria have the ability to develop resistance against the antibacterial drugs, so there is need of such medications that can cope with such genetic and structural changes/adaptations of bacteria.[10]

Peganum harmala is also known as wild rue or Syrian rue.[11] It is also known as "Harmal" by locals.[12] It belongs to Zygophyllaceae family.[13] Peganum species are distributed widely in Mediterranean, North Africa, Middle East, India, southern parts of Iran and Pakistan.[14] *P. harmala* is also called as "Espand" in Iran.[15] Conventionally *P. harmala* is propagated from the seeds.[16] *P. harmala* fruit is frequently used for the treatment of various ailments in traditional system of medicine in areas where it is present naturally. Its fruit is used as antiseptic and analgesic in conventional medicine.[17] It also possess numerous pharmacological properties such as narcotic,[18] alterative,[19] anthelmintic,[20] antiperiodic,[21] emetic[14] and antispasmodic.[22] *P. harmala* has been utilized for the management of many diseases such as jaundice,[17] emmenagogue, lumbago,[23] colic,[24] asthma[25] and as a stimulant.[26] Seeds have hallucinogenic and hypothermic properties.[14] Recent pharmacological studies indicate anti-histaminic,[27] hypoglycemic,[16] vasorelaxant,[28] immunomodulator,[29] antitumor,[30] hepatoprotective,[31] analgesic,[32] cytotoxic,[33] antioxidant,[34] anti-spasmodic[35] and insecticidal effect.[36] *P. harmala* also has reported antimutagenic[37] and antioxidant potential. *P. harmala* fruits yield a red dye and oil.[12] Ripe fruits have more alkaloid content as compared to unripe fruits.[38] Anti-inflammatory and analgesic effects of its fruit are because of the presence of alkaloids.[39] It is also effective in peripheral as well as central nervous system for its analgesic properties.[40] *P. harmala* is also used in the form of smoke to kill various parasites like molds and algae as well as various bacterial strains.[41] The aim of current research is to evaluate phytochemical as well as antioxidant and antimicrobial (both antibacterial and antifungal) potential of *P. harmala* as a potent traditional medicinal plant.

Materials and Methods

Plant Collection and Identification

P. harmala (Harmal) fruit was collected from Cholistan Desert near to Bahawalpur, South Punjab, Pakistan. The fruit was authenticated and identified by Dr Ghulam Serwar, Assistant Professor, Department of Botany, The Islamia University of Bahawalpur, and voucher numbers were obtained for Harmal, 34/Botany.

Extract Preparation

Dried fruit was ground to get coarse powder that was soaked into 70% ethanol for 15-days proceeded by the filtration initially with the muslin cloth and subsequently with filter paper. At that point, the acquired filtrate was instilled for solvent evaporation into the revolving evaporator to obtain the unrefined concentrate. Within the unrefined system, the acquired plant separates were then placed away in the completely closed-off container for further use.[24]

Phytochemical Screening

Various chemical tests were performed using hydro-ethanolic extract of *P. harmala* to detect the presence of various phytoconstituents like terpenes, flavonoids, saponins, steroids, cardiac glycosides, proteins, carbohydrates, alkaloids, tannins and phenolic compounds.[22]

Fourier transformed infrared spectroscopic analysis. The plant extract was checked utilizing Fourier transform infrared spectrometer in the scope of 4000–400 cm^{-1}. The resultant spectral information contrasted with the reference graph to recognize the presence of functional group in the extract.[42]

Antioxidant Activity

Antioxidant activity was performed by using the DPPH method as mentioned by Ratshilivha et al in 2014 with some modifications. Ascorbic acid was the standard control. 100 µL was the total volume of assay comprising of 90 µL of DPPH solution (.1 mM) and 10 µL of the ethanolic extract (5 mg/mL) in every well of 96-well plate. The prepared well plate was incubated for 30 minutes at 37°C. The absorbance was taken at 517 nm with Synergy HT Bio Tek® USA microplate reader.[19] The experiment was done in triplicate. Radical scavenging activity was determined by the following formula:

$$(\%)\ \text{Inhibition} = 100 - (\text{O.D. of test solution/O.D. of control}) \times 100$$

Antimicrobial Activity

Bacterial and fungal strains (test organisms). Bacterial strains that is, *Escherichia coli, Bacillus subtilis, B. pumilus* and *Micrococcus luteus* were availed from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS) Lahore, University of Punjab, in the form of stock culture. *Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis and Bordetella bronchiseptica* were isolated from microbiologic Inc. Bacterial strains for antibacterial assay are given in Table 1

Fungal strains were obtained from the First FCBP, IAGS Lahore, The University of Punjab. Fungal strains used for the antifungal assay are shown in Table 2.

Inoculums preparation. **Bacterial Inoculums** were prepared from cultures that were 24 hours old. The turbidity was altered to .5 McFarland turbidity level, which was comparable to 1-5x

Table 1. Bacterial Strains Used for the Antibacterial Assay.

Serial No.	Name	Type	Voucher Number
1.	*Bacillus subtilis*	Gram +ve	45
2.	*Bacillus pumilus*	Gram +ve	074
3.	*Staphylococcus aureus*	Gram +ve	ATcc 6539
4.	*Staphylococcus epidermidis*	Gram +ve	ATcc 9027
5.	*Micrococcus luteus*	Gram −ve	072
6.	*E. coli*	Gram −ve	088
7.	*Pseudomonas aeruginosa*	Gram −ve	147
8.	*Bordetella bronchiseptica*	Gram −ve	100

Table 2. Fungal Strains Used for the Antifungal Assay.

Serial No.	Name	Voucher No.
1.	*Aspergillus terreus*	002
2.	*Aspergillus fumigatus*	013
3.	*Aspergillus flavus*	005
4.	*Candida albicans*	007

108 CFU/ml cell densities, by taking a few colonies of the particular bacteria and shifting them to 5 mL of standard sterile saline solution.

Fungal Inoculums were prepared by transferring a few colonies of fungus to 5 mL of sterile saline solution from 3-day-old crop plates and adjusting the turbidity to .5 McFarland turbidity levels. To reach an inoculum density of 1-5x103 CFU/ml, this 1:10 solution was diluted 3 times using a growth medium.

Preparation of agar. 28 g of nutritional agar was dissolved in clean water and sterilized in an autoclave for 15 minutes at 121°C and 15 lbs pressure.

Antibacterial activity. Petri dishes were sterilized in a heated air oven at 180°C for 30 minutes before being installed in an aseptic laminar flow hood. At 35°C, sterilized nutritional agar was cooled. In petri plates, 20 mL of sterilized nutrient agar was transferred and left to solidify at room temperature. 60 µL inoculums were placed in a petri plate and disseminated using a sterile L-shaped rod. On the 6 mm filter paper disc, 30 µL of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.5 mg/mL and 3.125 mg/mL concentrate was put. After drying, the inoculated petri plates were mounted on them. Petri dishes were placed in the refrigerator for diffusion after filtration of the filter paper bacterial culture and then transported to the incubator for 24 hours of incubation at 37°C. To compare antibacterial impacities, ciprofloxacin was used as a positive control of 30 µL per filter paper disk and DMSO as a negative control. Following that, the inhibitory region was measured in millimeters (mm). Both experiments were carried out in triplicate, and mean data was calculated.[43]

Antifungal activity. Petri dishes were sterilized in a warm air furnace at 180°C for 30 minutes before being put in an aseptic setting in a laminar flow hood. The autoclave was used to sterilize Sabouraud's dextrose agar medium, which was then cooled to room temperature. At room temperature, 20 mL of Sabouraud's dextrose agar was flowed into petri plates, causing it to solidify. The sterile paper disc was then produced. 60 µL inoculums were placed in a petri plate and disseminated using a sterile L-shaped rod. The filter paper disc was soaked in 30 µL of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.5 mg/mL and 3.125 mg/mL ethanolic extract and put on petri plates. After drying, the inoculated petri plates were mounted on them. Petri dishes were placed in the refrigerator for diffusion after filtration of the filter paper fungal culture and then transported to the incubator for 24 hours of incubation at 37°C. To compare antifungal effects, terbinafine (5 mg/mL) was utilized as a positive control while DMSO was used as a negative control. After that, the inhibitory region was measured in millimeters. Both processes were repeated 3 times, with the average values calculated.[44]

Results

Phytochemical Screening

Phytochemical screening of ethanolic extract of *P. harmala* revealed the presence of various chemical components. *P. harmala* extract was found to be enriched with secondary metabolites such as carbohydrates, phenols, flavonoids, cardiac glycosides, steroids, alkaloids *(turbidity formation)*, proteins and tannins. However, saponins and terpenoids were found to be absent on standard test performed on *P. harmala* extract (Table 3).

Fourier Transform Infrared Spectroscopy Analysis of P. harmala

The peaks at 3922.22 and 3861.26 cm^{-1} showed the presence organic acids (-COOH). The peaks 3750.49 and 3719.81 indicated hydroxyl and phenolic compounds. The peaks at 3516.82–3152.48 cm^{-1} refer to the presence of amino groups. The peaks at 2952.79, 2846.44, and 2704.63 cm^{-1}

corresponds the presence of aliphatic compounds (C–H bend). The peak at 1615.48 cm^{-1} is an ester peak. The peaks from 1267.10 cm^{-1} to 1001.45 cm^{-1} indicated the presence of functional groups such as amide, ketone, aldehyde, aromatics (C–C and C–O) stretch (in–ring), and aliphatic amines (C–N stretch). The peaks from 921.74 cm^{-1} to 615.19 cm^{-1} revealed the presence of halogen compounds (C-Cl, C-F, C-Br). Spectra of *P. harmala* are shown in Figure 1.

Antioxidant Activity

The ethanolic extract of *P. harmala* exhibited antioxidant potential with DPPH. % inhibition of the extract was 71.4%, whereas $IC_{50} \pm SEM$ (μg/mL) was .406 ± .11. Results showed that ethanolic extract had a significant free radical reduction capability by changing the color of DPPH from purple to yellow[45] (Table 4 and Figure 2).

Table 3. Phytochemical Screening Extracts of *P. harmala.*

Sr. #	Tests	*Present*	*Not present*
1	Carbohydrates	+ve	−ve
2	Phenols	+ve	−ve
3	Flavonoid	+ve	−ve
4	Saponins	−ve	+ve
5	Cardiac glycosides	+ve	−ve
6	Steroid	+ve	−ve
7	Terpenoids	−ve	+ve
8	Alkaloids	+ve	−ve
9	Protein	+ve	−ve
10	Tannin	+ve	−ve

Table 4. Antioxidant Activity of *P. harmala* Extracts at .5 mg/Well.

Sr. No.	% Inhibition	$IC_{50} \pm SEM$ (μg/mL)
1	71.4	.406 ± .11

Antibacterial Activity

Ethanolic extract of *P. harmala* showed good susceptibility to almost all pathogens. Maximum antibacterial activity was exhibited by *Bacillus subtilis (24.33 ± 2 mm) and Bacillus pumilus (23.33 ± 2 mm). Zone of inhibition was 19 ± 2 mm by Pseudomonas aeruginosa* and it was *18.33 ± 2 mm by Bordetella bronchiseptica. Staphylococcus aureus and Staphylococcus epidermidis had inhibitory effect in the range of 12.33 ± 2 mm and 13.66 ± 3 mm, respectively. 11.66 ± 2 mm and 10 ± 2 mm was zone of inhibition by Micrococcus luteus and E. coli, respectively. P. harmala* showed very good antibacterial action against all bacterial strains; results are shown in Table 5.

Antifungal Activity

Ethanolic extract of *P. harmala* showed antifungal activity against *A. flavus (5* ± 1 mm) *and C. albicans (*4 ± 1 mm). *Mild antifungal activity was reported by A. fumigatus* (*3* ± 1 mm), whereas no activity was exhibited by *A. terreus*. Results are shown in Table 6.

Discussion

Effect of *P. harmala* fruit extract on different bacterial and fungal strains was tested. Numbers of therapeutic and medicinal effects of *P. harmala* are known to the world.[16] Presence of large concentration of alkaloidal content is said to be the reason of these affects. These compounds are produced by the plant in order to provide protection against many

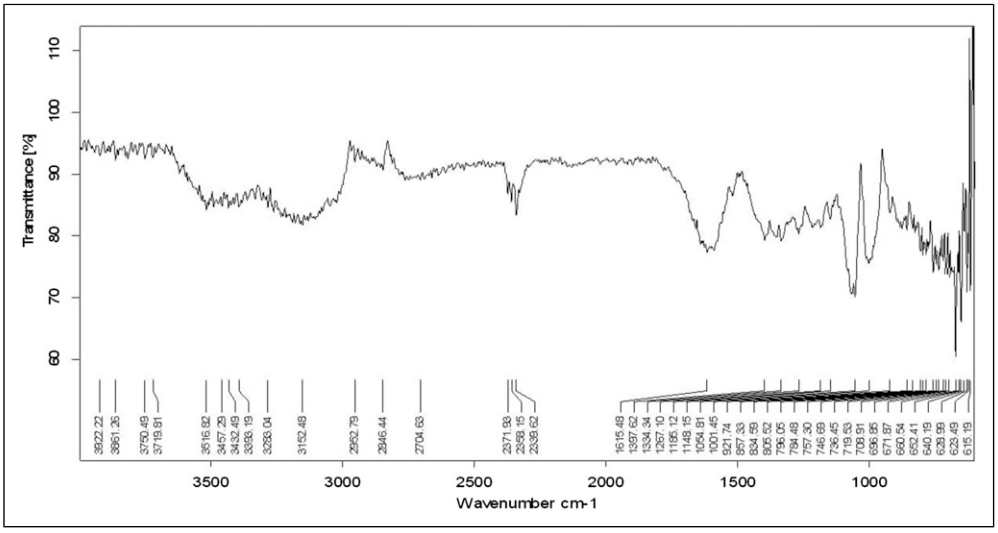

Figure 1. FTIR spectra of *P. harmala.*

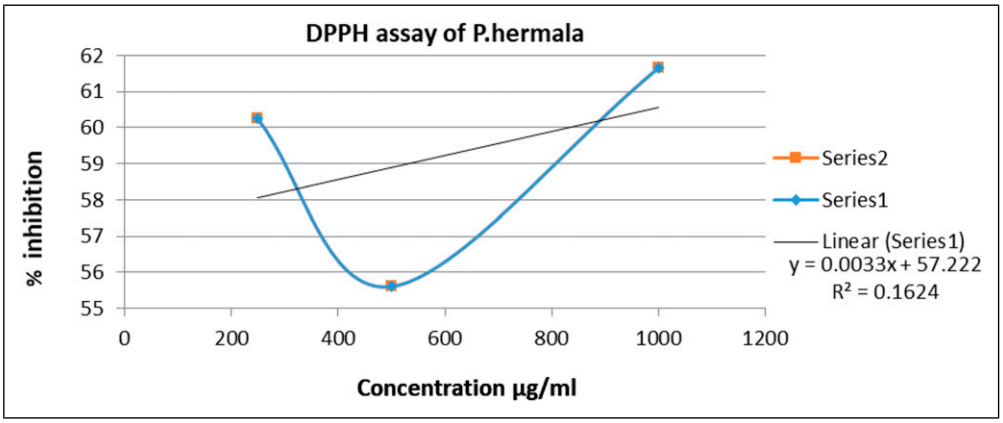

Figure 2. Graphical representation of DPPH assay of *P. harmala*.

Table 5. Antibacterial Activity of *P. harmala* Ethanolic Extract.

Zone of Inhibition Mean + Standard Deviation (mm)									
Extract Concentration (mg/mL)	*B. subtilis*	*B. pumilus*	*S. aureus*	*S. epidermidis*	*M. luteus*	*E. coli*	*P. aeruginosa*	*B. bronchiseptica*	Ciprofloxacin
50	24.33 ± 2	23.33 ± 2	12.33 ± 2	13.66 ± 3	11.66 ± 2	10 ± 2	19 ± 2	18.33 ± 2	45 ± 3
25	17.66 ± 2	18 ± 3	5 ± 2	6 ± 2	4 ± 3	3 ± 2	11 ± 2	9 ± 2	20 ± 2
12.5	5 ± 3	7 ± 4	0 ± 0	1 ± 1	0 ± 0	0 ± 0	2 ± 1	1 ± 1	9 ± 2
6.25	2 ± 1	2 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 1
3.125	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2 ± 1

Table 6. Antifungal Activity of *P. harmala* Ethanolic Extracts.

	Zone of Inhibition Mean + Standard Deviation (mm)				
Extract Concentration (mg/mL)	*Aspergillus flavus*	*Candida albicans*	*Aspergillus fumigatus*	*Aspergillus terreus*	Terbinafine
50	5 ± 1	4 ± 1	3 ± 1	00 ± 00	8 ± 2
25	1 ± 1	1 ± 1	00 ± 00	00 ± 00	5 ± 2

insects, microorganisms and herbivores.[46] As main components of *P. harmala* are fat soluble and aromatic, that's why extraction with ethanol derives more chemical constituents of the plant.[47] That's why 70% ethanol was used for extraction of this plant.[48]

The objective of this paper was evaluation of antimicrobial efficacy of *P. harmala* in order to know a potential therapeutic agent. With the help of recent findings, it is clear *P. harmala* has a lot of noticeable properties as antioxidant, antibacterial, and mild antifungal activities.[49] A number of secondary metabolites present in *P. harmala* can have their role in attributing the above mentioned activities.[50] Therapeutic properties of *P. harmala* are mostly due to high concentration of alkaloidal content in its various parts.[51] Beta-carbonyl derivations such as harmalol, harmaline, deoxyisopeganine, isopeganine and peganine are the most important alkaloids.[52] On the other hand, quinazoline derivations deoxyvasicinone, vasicine and vasicinone are also present.[53] The most important alkaloid that was studied in many researches is harmaline.[54] *P. harmala* also has reported cytotoxicity despite the numerous therapeutic properties.[16] Many severe side effects such as nervous, gastrointestinal, hepatic and cardiovascular complications have also been observed by the systemic use of high concentration of *P. harmala*.[55] Toxicity is mainly attributed due to ability to intercalate into DNA and inhibitory effect on monoamine oxidase (MAO).[56] Although *P. harmala* extract had noticeable inhibitory effect on various bacterial strains as compared to positive controls,[57] cytotoxic effects of *P. harmala* ethanolic extract on epithelial carcinoma of uterus cervix, human embryonic skin fibroblast and oral epithelial carcinoma are also reported.[58] But toxicological studies are still needed to further investigate its toxicity level and its mechanism of action.

Conclusion

P. harmala ethanolic extract has the capacity to inhibit the growth of many bacteria. Although ciprofloxacin had reported antibacterial activity, *P. harmala* extract also has comparable results. It also exhibited good free radical scavenging activity and has the capability to be effective against many illnesses. Further investigations are needed in order to get an effective remedy against the resistant bacterial strains.

Declaration of Conflicting Interests

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