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Antioxidant, Antimicrobial and Antileishmanial Study of Different Parts of *Peganum harmala*

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Abstract

Peganum harmala is bright green, densely foliaged and herbaceous plant, used in folkloric medicine for the treatment of various diseases like asthma, jaundice and lumbago. It contains biologically active secondary metabolites of diverse chemical nature. Therefore present research was designed to determine antioxidant, antimicrobial and antileishmanial potential and phytochemical analysis of different parts of *P. harmala*. Two plant parts seeds and leaves were selected for comprehensive investigation. According to the results, *P. harmala* seeds extract showed potent antioxidant activity with IC_{50} values ranging between 40-129 µg/ml. In case of antibacterial assay, *P. harmala* seeds showed better inhibitory activity than leaves against both strains i.e. *Staphylococcus aureus* and *Pseudomonas aeruginosa* with values ranging between 70 to 100% while in case of antifungal assay water-acetone extract of seeds showed significant antifungal effect against *Aspergillus niger*. In terms of cytotoxic assay, hexane extract of seeds of were more cytotoxic against shrimp larvae (LD_{50} = 57.07 µg/ml). Aqueous extract of leaves of and acetone extract of seeds showed < 80% mortality in antileishmanial assay. GC-MS analysis revealed that leaves and seeds contain some important biological metabolites. It is concluded that selected plants could be a potential source of antileishmanial, antibacterial, antifungal and anticancer lead compound. Hence it is indicated to further investigate this plant *in vitro* as well as *in vivo* for new drug discovery.

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Introduction

Pakistan's flora is rich in medicinal plants due to many ecological regions and its diverse climatic and soil conditions. In Pakistan almost 6000 species of vegetative plants exists which are medicinally important (Nasir and Ali, 1972; Hamayun *et al.,* 2005). According to the World's history, natural products were utilized as a source of medicines over hundred thousand years ago (Krueger, 2005). Initially, they were used in raw form for the cure of many diseases, while numerous herbal dosage forms also made of these plants (Balick and Cox, 1997). Scientists discovered that active chemical constituents of plants impart variety of pharmacological activities. Drug discovery from these medicinally important plants has been started when the active substances was being isolated and developed in the form of drugs. Scientists are still researching on these plants for the purpose to isolate pharmacologically active metabolites.

Peganum harmala is known as Wild rue and Syrian rue. It is widely distributed in Middle East, Central Asia and North Africa consisting of 22 genera and more than 250 species belonging to belongs to a family *Zygophyllaceae* (Yousefi *et al.,* 2009). Certain pharmacological activities have been reported from this plant including antibacterial, antifungal, antiviral, hypoglycemic, analgesic, anti-inflammatory, antitumor, and cytotoxic activities (Khaled *et al.,* 2008; Goel *et al.,* 2009; Darabpour *et al.,* 2011). Different phytochemical compounds isolated and reported from *P. harmala* are anthraquinones, alkaloids (Harmaline, harmine, harmalol, harmol and tetrahydroharmine) and flavonoids (Bukhari *et al.,* 2008). Seeds and roots contain the highest levels of alkaloids as compared to stems, leaves, and flowers (Kamel *et al.,* 1970).

The current study is designed to evaluate the antimicrobial, antioxidant and cytotoxic potential of leaves and seeds of *P. harmala* by employing multiple solvent extraction system of different polarities. Also GC-MS analysis will be done of the extract possessing maximum biological activity.

Materials and methods

Collection of plants and identification

Fresh plants were collected from different areas of Peshawar, Khyber Pakhtunkhwa Pakistan in June 2014. Plant was identified and authenticated by Dr. Mushtaq Ahmad, Assistant Professor of plant sciences department, Quaid-i-azam University by comparing with the specimen stored in the Herbarium of Medicinal Plants, at Quaid-i-azam University Islamabad, Pakistan. The plant was identified as *Peganum harmala*. Two plant parts, leaves and seeds were collected in each case. These plant parts were then shade dried at room temperature. After adequate drying, plant material was then ground to obtain fine powder. The powdered material of each plant parts was weighed properly and stored in tight polythene bags for further usage.

Extract preparation

For extract preparation, different solvents and their combinations were used. All solvents were of analytical grade purchased from Sigma Aldrich. Total of 14 solvents includes single seven pure solvents and seven combinations were used. n-Hexane, Ethyl acetate, Chloroform, Acetone, Ethanol, Methanol, and Water were used as single solvents as well as combinations with 1:1 ratio for extraction purpose. 50 gram of powdered plant material of each part was taken and soaked in 150ml of the respective solvents. Soaked plant material was kept for 3 days at room temperature with regular shaking on daily basis.

After 3 days the samples were filtered by using whatmann's filter paper no.1 and next volume of solvent was added. Second volume of the solvent was filtered after 1 days and third volume was added. Third volume was also filtered after 2 days and all filtrates were pooled, concentrated by using rotary evaporator to obtain crude extract. All crude extracts were then stored in properly labeled 20ml vials at -20°C for further analysis.

Percent extract recovery

Percent recovery of the crude extracts of plants was calculated by weighing the dried extracts of pants.

Following formula was used for calculating percent extract recovery.

% Extract recovery = (a / b) × 100

Where; a = Total weight of dried extract obtained after drying, and b = Total weight of ground plant material taken for each extraction.

Phytochemical analysis

Determination of total phenolic contents (TPC)

Plant samples were studied for total phenolic contents by using previously described method (Clarke *et al.,* 2013). Stock solutions of plants crude extracts (4 mg/ml) were prepared in DMSO. 20 µl of each extract was transferred to each well of 96 wells plate. The solutions were then mixed with 90 µl of F-C (Folin–Ciocalteu) reagent which was freshly 10 times diluted with double distilled water. After five minutes, the reaction mixture was mixed with 90 µl of Na_2CO_3 solution (7.5%). The reaction mixtures were incubated for 1 hr. and absorbance was measured at 650 nm by using microplate reader. Blank (DMSO) and standard (Gallic acid in DMSO) were run simultaneously. The resultant TPC was calculated as µg Gallic acid equivalent per mg of extract (µg GAE/mg extract).

Determination of total flavonoid content (TFC)

Total flavonoid contents of the extracts were determined according to the method previously described by Haq *et al.,* (2012). Aliquots of each sample solution (20 µl, 4 mg/ml DMSO) were mixed with 10 µl of aluminum chloride (10%) and 10 µl of potassium acetate (1 M) solutions.

Consequently distilled water was added to get a final volume of 200 µl. After 30 min of incubation, absorbance was measured by using microplate reader at 415 nm at 37°C. Calibration curve was made by using Quercetin as standard at concentrations of 0, 2.5, 5, 10, 20, 40 µg/ml and the flavonoid content was established in microgram quercetin equivalent per mg of extract (µg QE/mg extract). Quercetin was used as standard flavonoid and assay was performed in triplicate.

Biological evaluation

DPPH free radical scavenging assay

DPPH reagent employed for the determination of scavenging activity of the given samples described previously by Clarke *et al.,* (2013) after slight modification. Stock solutions (4 mg/ml) of crude extracts were prepared in DMSO. Aliquot of 10 µl of each test sample was mixed with 190 µl of DPPH in methanol. The reaction mixture was incubated in dark for 1 hour. The optical density of the test samples were measured at 515 nm using microplate reader. Ascorbic acid was employed as positive standard while DMSO as negative control. Test samples were first screened at final concentration of 200 µg/ml and those exhibiting good quenching activity (≥ 50%) were tested at lower concentration to find IC_{50} values. Percent inhibition was calculated by the following formula:

%inhibition of the test sample (% scavenging activity) = $(1 - Ab_s / Ab_c) \times 100$

Where; Ab_s is the absorbance of DPPH solution with sample, Ab_c is the absorbance of negative control (containing the reagent except the sample).

The IC_{50} was calculated by using 2D Table curve software Ver. 4.

Antimicrobial assays

Antibacterial activity

The antibacterial activity of crude fractions was determined by 96 well microplate reader method which was previously illustrated, along with some modifications (Marasini *et al.,* 2015). Two bacterial strains were used i.e. *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Antifungal activity

The antifungal potential of extracts was evaluated by disc diffusion method (Sharma *et al.,* 2009). Aliquot of 100 µl of each fungal strain was swabbed on plates containing sterile Sabouraud Dextrose agar (20 ml). Sterile filter paper discs impregnated with 5 µl of each test extract were placed on the seeded plates.

DMSO impregnated disc was used as negative control while those with clotrimazole (standard antifungal) served as positive control. Following incubation at 28°C for 24-48 hours, the average diameter (mm) of the zone of growth inhibition around the samples as well as control treated discs was measured and recorded.

Cytotoxicity assay

Brine shrimp lethality assay

The degree of lethality to brine shrimps was determined by following previously described protocol (Chang *et al.,* 1991). Stock solutions (100 mg/ml) of each sample were prepared in DMSO. *Artemia salina* (brine shrimp) eggs were hatched in a bi-partitioned tank filled with artificial sea water (3.8% sea salt solution supplemented with 6 mg dried yeast, pH 7). After 24-48 hours. Incubation period the phototropic nauplii were harvested by using micro pipette and transferred to 96 wells plate. Various sub dilutions of 100 mg/ml DMSO stock solution of each extract were tested (200, 100, 50 and 25 µg/ml).

The corresponding micro liter of each dilution was transferred to each well containing 10 nauplii and 300 µl sea water supplemented with dried yeast (6 mg). Negative control vial included DMSO, nauplii and sea water, but no sample, whereas positive control included 4 mg/ml standard drug doxorubicin, nauplii and sea water. After 24 hrs incubation period, dead nauplii were counted in each well and LD_{50} was determined accordingly by comparing percentage mortality with standard drug using table curve software 2D Ver. 4.

Antileishmanial assay

Strain of *L.tropica*kwh23 was incubated for 6-7 days and cultured in Medium-199 supplemented with 10% fetal bovine serum (Khan *et al.,* 2012). About 195 µl of harvested *L. tropica* cells were transferred onto 96-wells microtiter plate and incubated at 36.5°C in humidified CO_2 (5%) incubator for 24 hours. To each well of this plate, 5 µl of sample solution containing 1% DMSO in PBS (pH 7.4) was added. Stock solution of the sample was serially diluted in 96 wells plate. Samples were tested at 1000, 100 and 10 µg/ml. Amphotericin-B was used as positive control while 1% DMSO in PBS served as negative control. Plates containing reaction mixture were incubated for 3-5 days at 25°C in humidified CO_2 incubator. After 72 hours incubation period, 15 µl of the test culture was visualized under light microscope for surviving promastigotes and enumerated using the improved Neubauer chamber and percent mortality was determined.

Results

Effect of extraction solvent and extract yields

Highest yield of 19.57 ± 0.31 % was obtained when leaves were extracted with methanol. In case of seeds samples W and CM extracts were considered of high amounts with values of 19.77 ± 0.16 and 18.38 ± 0.44 % respectively (Table 1).

Table 1. Percent Recovery and Respective Solvents used for Extraction of *P. harmala*.

Percent Extract Recovery (g/50 g of DW)				
S.no	Extraction solvents	Sample	Leaves	Seeds
1	n-hexane	H	6.11 ± 0.63	9.45 ± 0.39
2	Chloroform	C	6.26 ± 0.19	8.50 ± 0.35
3	Acetone	A	7.56 ± 0.31	8.45 ± 0.39
4	Ethyl acetate-acetone	EaA	8.37 ± 0.45	10.63 ± 0.26
5	Ethyl acetate	Ea	14.57 ± 0.31	9.85 ± 0.81
6	Chloroform-Ethanol	CE	10.50 ± 0.35	16.95 ± 0.04
7	Chloroform-Methanol	CM	11.72 ± 0.20	18.38 ± 0.44
8	Ethyl acetate-Ethanol	EaE	7.44 ± 0.40	12.71 ± 0.21
9	Ethyl acetate-Methanol	EaM	8.10 ± 0.64	14.55 ± 0.32
10	Water-Acetone	WA	17.73 ± 0.90	16.57 ± 0.02
11	Ethanol	E	8.63 ± 0.26	12.45 ± 0.39
12	Methanol	M	19.57 ± 0.31	4.51 ± 0.35
13	Water-Methanol	WM	14.84 ± 0.11	15.84 ± 0.11
14	Water	W	19.44 ± 0.40	19.77 ± 0.16

Each sample was analyzed individually in triplicate and mean ± SD was determined. DW= dry weight.

Total phenolic and flavonoid contents

The TPC were estimated by the method as described previously using FC reagent. Gallic acid was used as standard. Results were expressed as Gallic acid equivalent phenols (GAE) in each sample. A, E, EaM and WA extracts of seeds showed significant amount of phenolic contents with values of 116.01, 80.52, 73.53 and 70.96 µg GAE/mg respectively (Table 2). In case of leaves extracts total of 4 samples showed phenolic contents more than 70 µg GAE/mg. The total flavonoid contents were determined and Quercetin was used as a standard flavonoid (Table 2).

Table 2. Total Phenolic and Flavonoid Contents of *P. harmala*.

Total Phenolic Contents (µg GAE/mg extract)			Total flavonoid contents (µg QE/mg extract)	
Samples	Leaves	Seeds	Leaves	Seeds
H	50.36	27.72	11.24	13.23
C	75.89	55.21	23.88	10.76
A	67.01	116.01	27.33	31.28
EaA	83.67	69.51	39.08	20.01
Ea	70.86	47.92	25.5	42.94
CE	73.66	64.39	21.48	33.25
CM	70.56	61.86	13.69	28.69
EaE	66.84	60.5	28.73	38.6
EaM	52.42	73.53	13.04	35.63
WA	34.67	70.96	12.51	42.44
E	52.2	80.52	23.9	41.56
M	59.93	72.08	23.54	31.91
WM	32.84	30.46	25.84	16.63
W	29.36	46.52	26.26	24.84

*GAE=Gallic acid equivalent, QE=Quercetin equivalent.

Ea extract of seeds showed highest amount of flavonoid contents with value of 42.94 µg QE/mg followed by WA and E with values of 42.44 and 41.56 µg QE/mg respectively. In case of leaves samples EaA extract showed highest flavonoid content i.e. 39.08 µg QE/mg followed by EaE (28.73 µg QE/mg) and A (27.33 µg QE/mg).

DPPH free radical scavenging assay

Free radical scavenging activity was determined by using DPPH percent scavenging assay and IC_{50} values were determined by using table curve software (Table 3).

Table 3. DPPH Free Radical Scavenging Assay of *P. harmala*.

	Leaves		Seeds	
Samples	% scav (at 200 µg/ml)	IC_{50}	% scav (at 200 µg/ml)	IC_{50}
H	34.89 ± 0.07	>200	22.77 ± 0.17	>200
C	42.57 ± 0.26	>200	32.69 ± 0.22	>200
A	35.50 ± 0.31	>200	54.79 ± 0.15	172.28
EaA	37.63 ± 0.22	>200	61.63 ± 0.26	170.56
Ea	33.92 ± 0.05	>200	67.57 ± 0.30	129.4
CE	30.72 ± 0.17	>200	60.76 ± 0.17	155.46
CM	42.44 ± 0.34	>200	74.29 ± 0.21	108.74
EaE	22.52 ± 0.30	>200	76.76 ± 0.17	40.2
EaM	20.90 ± 0.06	>200	32.77 ± 0.18	>200
WA	50.51 ± 0.30	>200	79.35 ± 0.46	125.42
E	26.56 ± 0.27	>200	66.76 ± 0.17	141.21
M	28.90 ± 0.06	>200	63.90 ± 0.07	153.51
WM	34.70 ± 0.19	>200	68.78 ± 0.16	122.1
W	23.70 ± 0.18	>200	55.71± 0.21	144.11

Each sample was analyze individually in triplicate and mean ± SD was determined. % scav = percent scavenging

Ascorbic acid was used as standard drug. Total of 5 samples of seeds EaE, CM, WM, WA and Ea showed significant activities with IC_{50} values of 40.2, 108.74, 122.1, 125.42 and 129.4 µg/ml respectively. Leaves samples were found inactive in DPPH scavenging assay.

Antibacterial Assay

Antibacterial assay was performed by using two strains, *Staphylococcus aureus* (Gram +ve) and *Pseudomonas aeruginosa* (Gram -ve). Results were expressed in percentage inhibition of each strain. Seeds showed more significant and better inhibitory activity than leaves in case of both strains with values ranging between 70 to 100%. Total of five samples of seeds CE, EaE, EaM, EaA and A showed significant activities with values of 99.94 ± 0.05, 99.44 ± 0.04, 97.07 ± 0.04, 88.46 ± 0.15 and 88.30 ± 0.05% respectively against *P. aeruginosa*. Two leaves samples A and CE also showed potent effect against *P. aeruginosa* with values of 79.38 ± 0.03 and 78.37 ± 0.45 % inhibition respectively. All samples of leaves showed non-significant results with % inhibition below 70% against both bacterial strains (Table 4).

Table 4. Antibacterial Assay of *P. harmala*.

	% inhibition at 200ug/ml			
	S. aureus		*P. aeruginosa*	
Samples	Leaves	Seeds	Leaves	Seeds
H	10.35± 0.25	----	62.06 ± 0.02	26.35 ± 0.01
C	----	16.52 ± 0.34	8.16 ± 0.01	57.79 ± 0.11
A	2.18 ± 0.18	79.69 ± 0.93	79.38 ± 0.03	88.30 ± 0.05
EaA	2.50 ± 0.08	16.89 ± 0.08	31.71 ± 0.21	88.46 ± 0.15
Ea	2.35 ± 0.39	10.78 ± 0.86	68.35 ± 0.46	39.66 ± 0.12
CE	23.75 ± 0.18	79.85 ± 0.10	78.37 ± 0.45	99.94 ± 0.05
CM	14.43 ± 0.41	50.35 ± 0.46	36.51 ± 0.35	64.53 ± 0.28
EaE	17.70 ± 0.21	50.77 ± 0.17	29.16 ± 0.12	99.44 ± 0.02
EaM	10.44 ± 0.40	62.41 ± 0.41	34.83 ± 0.12	97.07 ± 0.04
WA	2.38 ± 0.44	10.90 ± 0.08	32.83 ± 0.13	51.13 ± 0.13
E	1.24 ± 0.17	61.66 ± 0.24	21.75 ± 0.18	83.58 ± 0.08
M	----	54.82 ± 0.13	19.30 ± 0.21	78.75 ± 0.25
WM	----	12.44 ± 0.40	40.97 ± 0.02	28.13 ± 0.14
W	----	6.36 ± 0.46	44.62 ± 0.27	57.76 ± 0.13

Each sample was analyzed individually in triplicate and mean ± SD was determined.

Antifungal activity

Antifungal potential was assessed against three strains of filamentous fungi i.e. *A. niger, A. flavus* and *F. solani*. Maximum zone of inhibition was shown by WA extract of seeds with value of 12.38 ± 0.44 mm against *A. niger*. Significant zones of inhibition were also observed in some leaves samples against *A. niger*. All extracts of leaves and seeds showed zone of inhibition below 10 mm against *A. flavus*. When samples were studied against *F. solani* only extract C of leaves showed significant inhibition with value of zone of inhibition of 11.97 ± 0.02 mm, while all other samples of leaves and seeds were inactive (Table 5).

Table 5. Antifungal Assay of *P. harmala*.

	Zone of inhibition (mm) at 400µg per disc					
	Leaves			Seeds		
Samples	*A. niger*	*A.flavus*	*F. solani*	*A.niger*	*A.flavus*	*F. solani*
H	7.44 ± 0.02	9.94 ± 0.16	7.95 ± 0.06	8.90 ± 0.26	----	7.83 ± 0.12
C	10.91 ± 0.05	8.89 ± 0.08	11.97 ± 0.02	9.36 ± 0.45	----	7.90 ± 0.07
A	9.39 ± 0.50	7.91 ± 0.07	----	7.90 ± 0.07	----	6.87 ± 0.10
EaA	8.79 ± 0.08	8.81 ± 0.13	----	4.35 ± 0.46	----	6.91 ± 0.09
Ea	9.71 ± 0.30	7.89 ± 0.08	6.97 ± 0.02	7.95 ± 0.03	8.42 ± 0.41	7.94 ± 0.07
CE	10.73 ± 0.18	7.85 ± 0.11	8.82 ± 0.13	7.83 ± 0.12	8.97 ± 0.02	7.97 ± 0.02
CM	9.84 ± 0.06	8.84 ± 0.12	6.97 ± 0.02	9.82 ± 0.12	8.96 ± 0.03	7.98 ± 0.04
EaE	8.69 ± 0.08	8.45 ± 0.39	7.83 ± 0.12	4.37 ± 0.44	----	8.70 ± 0.31
EaM	7.91 ± 0.02	----	6.99 ± 0.01	6.89 ± 0.08	9.89 ± 0.08	7.67 ± 0.26
WA	9.97 ± 0.33	7.89 ± 0.08	8.89 ± 0.08	12.38 ± 0.44	----	7.90 ± 0.07
E	7.90 ± 0.07	7.82 ± 0.13	----	----	----	8.92 ± 0.06
M	8.75 ± 0.02	8.91 ± 0.06	----	----	----	7.95 ± 0.03
WM	8.61 ± 0.17	8.35 ± 0.46	7.91 ± 0.06	7.95 ± 0.04	----	----
W	6.89 ± 0.08	8.83 ± 0.12	8.77 ± 0.17	9.90 ± 0.07	6.98 ± 0.02	8.84 ± 0.18

Each sample was analyzed individually in triplicate and mean ± SD was determined.

Brine shrimp lethality bioassay

Cytotoxicity potential of extracts of both plants was determined against *Artemia salina larvae (*brine shrimps*)*. Only two H and C extracts of seeds showed significant activity with LD_{50} value at 57.07 and 60.17 µg/ml respectively while 6 samples (EaA, Ea, CE, CM, EaM, WA) showed moderate activity. Only two extracts of leaves showed moderate cytotoxicity activity (Table 6).

Table 6. Brine Shrimp Lethality Assay of *P. harmala* Showing % Mortality and LD_{50}.

	Leaves					Seeds				
Samples	200 (µg/ml)	100 (µg/ml)	50 (µg/ml)	25 (µg/ml)	LD_{50}	200 (µg/ml)	100 (µg/ml)	50 (µg/ml)	25 (µg/ml)	LD_{50}
H	36.67	30	10	0	>200	90	66.67	46.67	0	57.07
C	60	26.67	20	0	165.33	60	56.67	46.67	40	60.17
A	40	16.67	6.67	0	>200	0	0	0	0	>200
EaA	10	6.67	0	0	>200	60	46.67	40	16.67	102.34
Ea	50	13.33	10	0	200	50	36.67	16.67	6.67	200
CE	56.67	26.67	20	0	174.5	50	30	23.33	10	158.3
CM	10	0	0	0	>200	66.67	30	10	6.67	158.3
EaE	30	10	0	0	>200	30	23.33	20	6.67	>200
EaM	30	0	0	0	>200	70	26.67	16.67	13.33	163.22
WA	30	16.67	0	0	>200	60	30	26.67	30	187.92
E	36.67	20	10	0	>200	40	30	6.67	0	>200
M	20	0	0	0	>200	30	16.67	10	0	>200
WM	40	16.67	6.67	0	>200	40	23.33	10	0	>200
W	20	6.67	0	0	>200	43.33	26.67	6.67	0	>200

Each sample was analyzed individually in triplicate and mean ± SD was determined.

Antileishmanial Assay

Antileishmanial capability of different extracts is evaluated for the first time against *L. tropica* kwh 23 strain in Pakistan. Antileishmanial activity was described as % mortality and amphotericin B was used as standard drug. Plant extracts of both plant parts showed significant results. Total of 4 samples (W, WM, E and A) of leaves showed more than 75% mortality and highest value of 85% mortality was observed in case of aqueous extract. Among seeds samples A showed highest % moartality with value of 84% followed by EaE (76), CE and C with value of 72% each (Table 7).

Table 7. Antileishmanial Assay of *P. harmala*.

	Percent mortality at 50 µg/ml	
Samples	Leaves	Seeds
H	52.36 ± 0.47	48.08 ± 0.01
C	43.06 ± 0.02	72.02± 0.04
A	75.12 ± 0.07	84.05 ± 0.02
EaA	28.03 ± 0.01	51.08 ± 0.09
Ea	38.16 ± 0.13	43.08 ± 0.03
CE	42.05 ± 0.02	72 .04± 0.02
CM	55.17 ± 0.18	46.07 ± 0.05
EaE	54.04 ± 0.01	76.05 ± 0.02
EaM	53.08 ± 0.09	65.27 ± 0.35
WA	49.06 ± 0.02	48.05 ± 0.03
E	82.18 ± 0.18	48.28 ± 0.34
M	52.09 ± 0.09	47.11 ± 0.11
WM	83.22 ± 0.23	53.19 ± 0.19
W	85.08 ± 0.01	45.04 ± 0.02

Each sample was analyzed individually in triplicate and mean ± SD was determined.

GC-MS analysis of acetone extracts of P. harmala

Acetone sample of leaves was analyzed through GC-MS and different peaks were observed in its chromatogram with variable retention time ranging from 3.02 – 29.05 min (Fig. 1a). Peaks with retention time of 23.822 and 25.718 showed highly concentration of compounds because of the greater area under curve. Mass spectra (Fig. 1 b &c) of these two peaks compared with compounds existing in library and showed the presence two compounds 1-2, dicarboxylic acid, mono(2-EthylHexyl) Ester **(1)** and 2,6,10,14,18,22 tetracosahexane,2,6,10,15,19,23-HeaxaMethyl-,(ALL-E) **(2)** respectively.

Similarly different peaks were also observed in GC-MS chromatogram of acetone extract of seeds. Three prominent peaks with retention times of 20.715, 23.777 and 25.763 (Fig. 2a) were analyzed for their mass spectrum. Three compounds 9, 17-octadecadienal **(1),** 1-hexyl-2-nitrocyclohexane **(2)** and 9-octadecenamide, (z)- **(3)** were identified corresponding to these peaks respectively (Fig. 2 b-d).

Discussion

Phytochemical and biological Study

Percent extract recovery of different parts of were determined through maceration technique. Maximum extract yield was found significant with methanol in case of leaves with value of 19.36 ± 0.01% while in case of seeds samples percent recovery of extract was highest when water and chloroform-methanol were used. The variations were observed in extract yields with reference to plant parts as well as polarity of extraction solvents. These results indicated that diversity of chemical components, their availability for extractable solvents is the basic factor which influence the extraction yield (Hassim *et al.,* 2015). The biological activities and extraction efficiency are strongly dependent upon the nature of extraction solvents polarity, due to the presence of diverse compounds of varied chemical characteristics that may or may not be soluble in a particular solvent. It has also been suggested that maceration method along with combinations of different solvents could be a better choice for extraction of secondary metabolites from plant parts (Tatiya *et al.,* 2011).

In most of the medicinal plants, phenolic and polyphenolic compounds such as flavonoids, phenolic acids and tannins are the major contributors to the antioxidant activity. Therefore determination of plant phenolic contents is considered an important parameter to establish its medicinal value (Dai and Mumper 2010). A, E, EaM and WA extracts of seeds showed significant amount of phenolic contents with values of 116.01, 80.52, 73.53 and 70.96 µg GAE/mg respectively. In case of leaves, EaE, C, CE and Ea extracts showed significant results. These results are correlated with previous reports that seeds contain a measurable quantity of total phenolic and total flavonoid contents and hence can be used as a potent antioxidant plant (Chabir *et al.,* 2014). The aqueous extract can be used as natural source of antioxidant (Baghiani *et al.,* 2012; Kaskoos *et al.,* 2014).

Total flavonoid contents of extracts were also determined by using Quercitin as standard. Ethyl acetate extract of seeds showed highest amount of flavonoid contents with value of 42.94 µg QE/mg followed by WA and E while EaA extract of leaves showed highest flavonoid content i.e. 39.08 µg QE/mg. these findings are correlated with previous reports that seeds of *P. harmala* contains flavonoids (Djarmouni *et al.,* 2012; Baghiani *et al.,* 2012; Kaskoos *et al.,* 2014).

Antioxidant activity was determined by using DPPH scavenging assay and IC_{50} values were determined by using table curve software. Total of 5 samples of seeds EaE, CM, WM, WA and Ea of seeds showed significant activities with IC_{50} values ranging between 40 -129 µg/ml. Leaves samples were not found to be active in DPPH scavenging assay. These results are in agreement that seeds of plant contain sufficient amount of phenolic and flavonoids which could be the source of potent antioxidant effects of extracts (Baghiani *et al.,* 2012; Kaskoos *et al.,* 2014). In another study it is reported that petroleum ether, chloroform, ethyl acetate, ethanol and water extracts from seeds of *P. harmala* exhibit antioxidant activity (Chabir *et al.,* 2015).

Antibacterial potential of samples was determined by using *S. aureus* and *P. aeruginosa*. According to our findings seeds showed better inhibitory activity than leaves against both strains i.e. *S. aureus* and *P. aeruginosa* with values ranging between 70 to 100%. All leaves samples were not active. These results are correlated with previous results that *P. harmala* showed significant antibacterial activity (Arshad *et al.,* 2008; Mashreghi *et al.,* 2012; Irshaid *et al.,* 2014). It is reported that extracts seed and leaves of *P. harmala* showed the significant antibacterial activities against both gram positive and negative bacteria (Nehaah, 2010; Benyounes *et al.,* 2013).

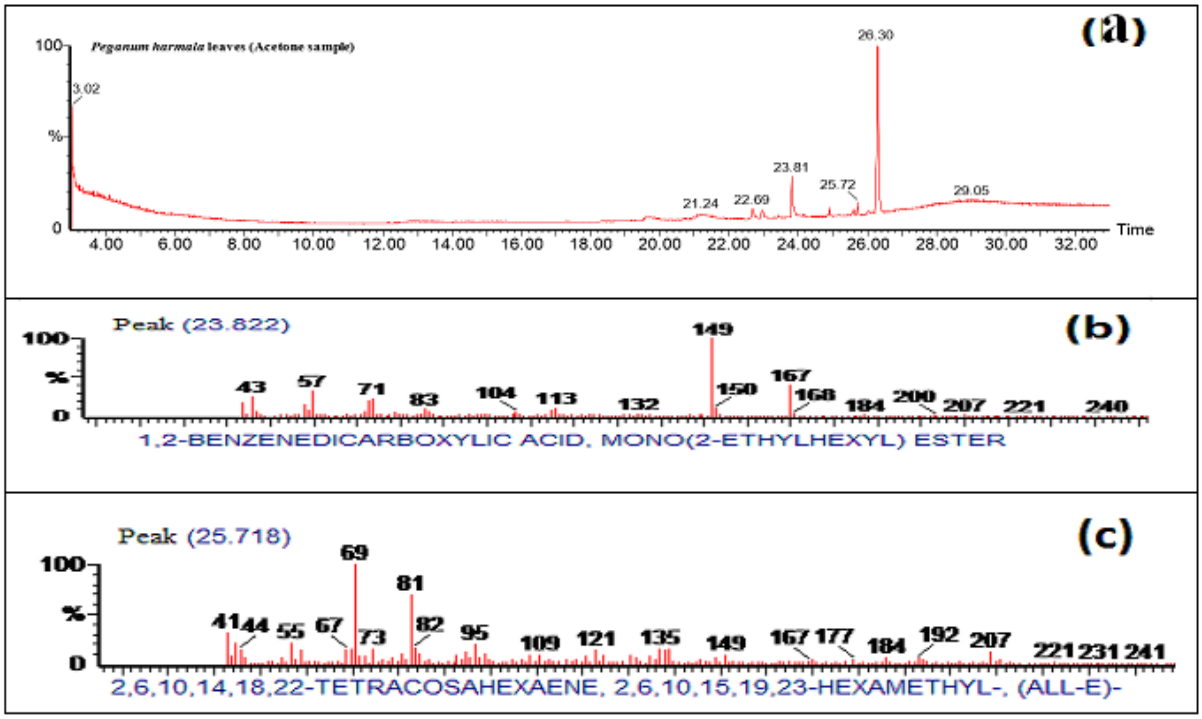

Fig. 1. GC-MS chromatogram of acetone sample of *P. harmala* leaves.

Antifungal potential of samples was assessed against *A. niger, A.s flavus* and *F. solani*. It was observed that WA extract of seeds exhibit maximum zone of inhibition with value of 12.07 ± 0.02 mm and significant zones of inhibition were also observed in leaves samples against *A. niger*. These samples were moderately active against *A. flavus*. Only chloroform extract of leaves showed significant activity against the *F. solani* with value of 11.95 ± 0.01 mm while all other samples of leaves and seeds were not active. These findings are correlated with studies that alkaloids isolated from *P. harmala* showed antifungal and antibacterial activities (Arshad *et al.,* 2008; Nenaah, 2010; Akhtar *et al.,* 2015). In another study Xiaojin isolated antifungal protein from seeds of *P. harmala* (Xiaojin *et al.,* 2013). Antifungal activity of alcoholic extract of *P. harmala* was also reported against *Candida* spp., *A. niger and A. fumigatus* (Sarpeleh *et al.,* 2009; Diba *et al.,* 2011).

Cytotoxicity potential of samples was tested against *Artemia salina* larvae (brine shrimps) so that lethality profile should be revealed. Only H and C extracts of seeds showed significant activity with LD_{50} value at 57.07 and 60.17 µg/ml respectively. Some samples of leaves also showed moderate cytotoxicity activity. These findings were correlated with previous report by Dastagir and Hussain, about cytotoxic activities of hexane and methanolic extracts of *P. harmala* plant with LD_{50} values below 100 µg/ml (Dastagir and Hussain 2014). There are also reports about cytotoxic activities of alkaloids extracted from seeds of this plant against cancer cell lines (Wang *et al.,* 2008; Lamchouri *et al.,* 2013). Reza and his colleagues reported cytotoxic activity of harman alkaloids against brine shrimp larvae (Reza *et al.,* 2007).

Leishmaniasis is one of the six most common parasitic infections in the tropical regions. There are different therapeutic modalities, however therapeutic resistance is developed and resulted in numerous problems. Both plant parts showed significant results. Highest value of 85% mortality was observed in aqueous extract of leaves. Among seeds extracts EaE showed highest % mortality with value of 84% followed by A with value of 76% mortality. Our results are correlated with previous reports that hydroalcoholic extract of seeds of this plant showed significant antileishmainial activity because of presence of potent alkaloids (e.g peganine) (Moghaddam *et al.,* 2011). In another study it is also reported that ethanolic extract of this plant showed in vivo antileishmaninal activity in mice model (Mirzaie *et al.,* 2007). So according to our findings it is suggested that this plant have potential role in the search for novel antilieshmaneal drugs so further analysis about the search of novel antileishmanial drugs should be conducted in future.

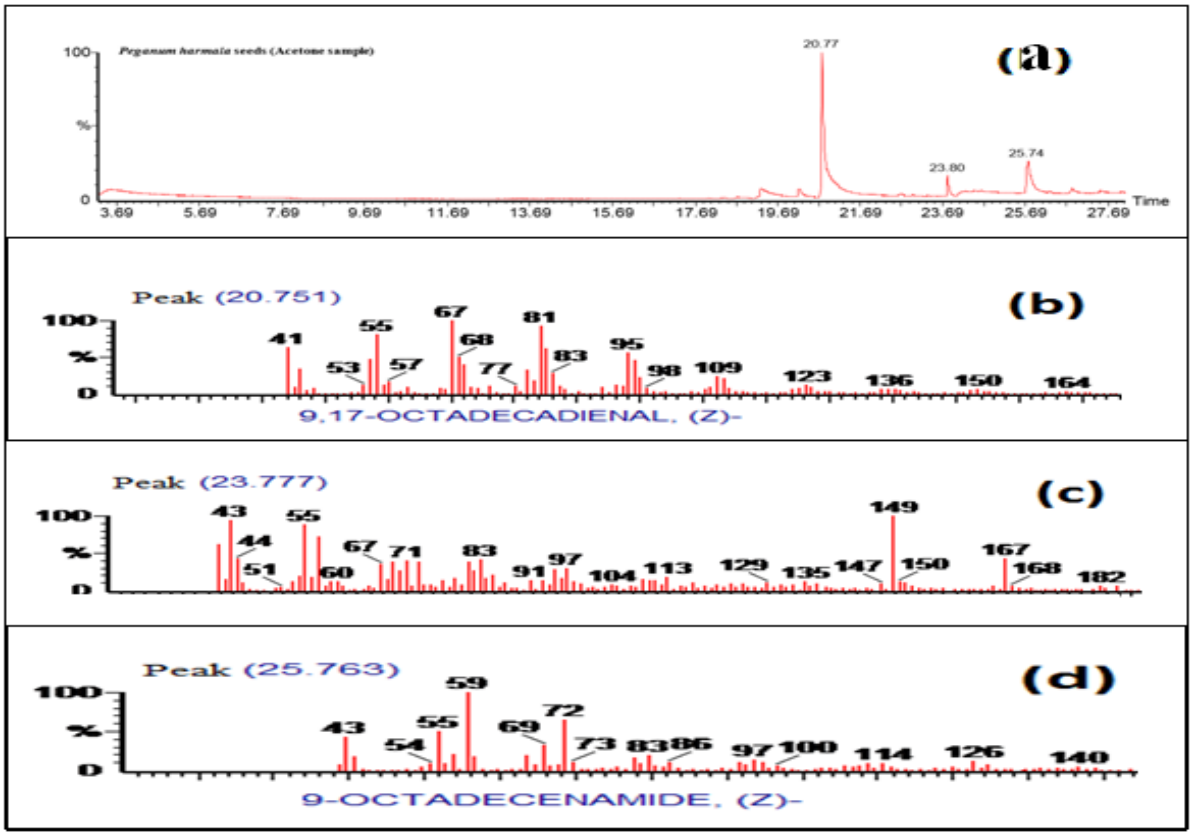

Fig. 2. GC-MS chromatograms of acetone sample of *P. harmala* seeds.

GC-MS analysis

Acetone sample of leaves was analyzed through GC-MS and two prominent peaks when analyzed for mass spectrum. These peaks corresponds to two compounds 12, dicarboxylic acid, mono (2-EthylHexyl) Ester (1) and 2,6,10,14,18,22tetracosahexane,2,6,10,15,19,23Heaxa Methyl-, (ALL-E) (2). Both compounds were reported previously for their biological potential (Veerabahu *et al.,* 2013; Krishnan *et al.,* 2014). Krishnan and his colleagues reported that compound 1 has significant cytotoxic activity (Krishnan *et al.,* 2014), while compound 2 was reported for its antioxidant, anticancer, pesticide, sunscreen and chemo preventive potential (Veerabahu *et al.,* 2013). Three different peaks were observed in GC-MS chromatogram of acetone extract of seeds which were identified as 9, 17-octadecadienal (1), 1-hexyl-2-nitrocyclohexane (2) and 9-octadecenamide, (z)- (3). Compound 1 was reported for its anti-microbial and anti-inflammatory activities while compounds 2 and 3 were previously reported anti-oxidant activity (Subashri *et al.,* 2014).

Conclusion

The antimicrobial, antioxidant and cytotoxic activities of leaves and seeds of *P. harmala* reported in this study may explain some of the medicinal uses of this plant. Different combinations of organic solvents were used for the extraction which helped us in profiling the wide area of secondary metabolites that are biologically and chemically important. From the current study it is inferred that solvents of medium polarity (A, Ea, EaA, EaE & WA) may be a potential source of biological active compounds. Acetone, Ethyl acetate-ethanol and aqueous extracts of the both parts showed significant activity against leishmaniasis. The present study thus suggests to further investigation aimed at isolation the biological active compounds responsible for the activities observed as well as this plant should be further investigated in future on different human cancer cell lines to obtain its complete spectrum of cytotoxic potential.

Competing interests

Participating authors declare that they have no competing interests.

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