Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(2):32-39

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Phytochemical Screening, Total Phenolics and Biological Activities of Tunisian *Peganum harmala* Seed Extracts

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ABSTRACT

Peganum harmala, belonging to Zygophyllaceae family, is a herb with medicinal properties. This study evaluates the antioxidant activity of Tunisian Peganum harmala seed extracts obtained by two methods of extraction. The antimicrobial activity was also evaluated. The antioxidant activity of the extracts of Peganum harmala was studied by three different methods, including scavenging activity by DPPH, phosphomolybdenum method and reducing power of iron. The antimicrobial activity was evaluated using the broth microdilution assay. Results show that extract concentration was directly proportional to the antioxidant activity and the extracts of the maceration extraction showed a higher antioxidant activity than that in extracts of the soxhlet extraction. At all concentrations compared with BHT, the hexane extract have a higher antioxidant activity than those of all extracts of Peganum harmala seeds. For the phosphomolybdenum method, all the extracts showed an increase in antioxidant capacity with an increase in dose. A quantitative analysis of polyphenols, flavonoids and tannins was also performed. For the antibacterial activity, all the extracts tested shown an important antibacterial effect expect for the hexanic extract obtained by soxhlet extraction. Indeed, the MIC values of the different extracts ranged from 0.097 to 0.78 % (v/v) against studied strains.

Keywords: Antioxidant; Phenolics; Tannins; Flavonoid; Antimicrobial activity

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INTRODUCTION

Natural products play an important roles of drug discovery process include provide basic compounds affording less toxic and more effective drug molecules, serve as extremely useful natural drugs, exploration of biologically active prototypes towards newer and better synthetic drugs and modification of inactive natural products by suitable biological or chemical means into potent drugs [1]. Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [2]. Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years [3]. Medicinal plants are valuable natural resources and regarded as potentially safe drugs and have been tested for biological, antimicrobial and hypoglycemic activity also play an important role in the modern medicine [4]. Therapeutic benefits can be traced to specific plant compounds; many herbs contain dozens of active constituents that, together, combine to give the plant its therapeutic value [5]. Phytochemical screening of various plants has been reported by many workers [6]. These studies have revealed the presence of numerous chemicals including alkaloids, flavonoids, steroids, phenols, glycosides and saponins. The

phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites [7]. A number of studies have focused on the biological activities of phenolic compounds which are antioxidants and free radical scavengers [8,9]. Reactive Oxygen species (ROS), including free radicals such as superoxide anion radical (O_2), hydroxyl radical species (OH.), singlet oxygen ($^1O_2$) and hydrogen peroxide(H_2O_2) are active oxygen species that are often generated by biological oxidation reactions of exogenous factors [10]. These oxidative mediators can lead to the damage of biological structures such as nucleic acids, proteins and lipids. Many free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, ageing and Alzheimer's disease [11]. Besides, phenolic compounds and flavonoids are also widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory or anti-carcinogenic [12]. While, flavonoids are a group of polyphenolic compounds with known properties, which include free compounds with known properties, which include free enzymes and anti-inflammatory action [13]. Hence, the aim of this part of the study was to extract and analyze the antioxidant property of different extracts from *Peganum harmala* seeds.

EXPERIMENTAL SECTION

Plant material

Seeds of *Peganum harmala* were collected in July 2012 from mature fruits growing in the region of Kasserine (Sbeïtla: 35°13′ N 9°03′E). The authenticity of this plant was confirmed by Dr. Lamia Hamrouni research at the National Institute for Research in Rural Engineering Water and Forests, (INRGREF) Tunisia. A voucher specimen (PH. 013) was deposited at the herbarium division of the INRGREF. Samples were dried in an oven at 60°C and were weighed each day until the difference between successive weights was less than 5%. The dried seed were pulverized by an electrical blender to get powder form.

Extraction procedure

Two techniques were used for the extraction of polyphenol compounds from the seeds of *Peganum harmala* powder. The extraction was carried out using various polar and non-polar solvents.

Maceration extraction:

Pulverized seeds (200 g) were subjected to maceration with methanol/water under room temperature. The methanol/water extract obtained from each sample was evaporated in order to remove the methanol, and the aqueous phase was extracted successively with hexane, ethyl acetate (EtOAc) and methanol (MeOH).

Soxhlet extraction procedure:

Dried coarse powder of the seeds of *Peganum harmala* was placed into the extractor of a Soxhlet. The extraction was carried out by using solvents of increasing polarity starting from hexane, and follow by ethyl acetate and methanol. At the end of the extraction the respective solvents were concentrated by evaporation.

Qualitative phytochemical screening

The different extracts from *Peganum harmala* seeds were screened for phytochemical constituents (alkaloids, flavonoids and tannins) using simple qualitative methods described by Paris and Nothis.

Total phenolic content (TP):

The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). According to the method described by Slinkard and Singleton; Singleton and Rossi, a 20 µl of the sample was added to 100 µl of Folin-Ciocalteu reagent. After 8 min, 300 µl of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 765 nm. The calibration curve was prepared with gallic acid solutions ranging from 0 to 500 mg/l, and the results are given as gallic acid equivalents (GAE) per 100 milligram of extract (mg GAE/100 mg of extract).

Total flavonoids content (TF):

The flavonoids content was determined spectrophotometrically, using a method based on the formation of a complex flavonoid–aluminium, having the absorptivity maximum at 430 nm. Quercetin (Q) was used to make the calibration curve. 1 ml of sample was separately mixed with 1 ml of 2% aluminium chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430nm and the

flavonoids content was expressed in µg of quercetin equivalents (QE) per 100 milligram of extract (mg QE/100 mg of extract).

Total tannin content (TT): The amount of total tannins was determined using the Folin -Denis method (Oyaizu, 1986). Briefly, 0.2 ml aliquot of extract was separately mixed with 0.5 ml of Folin-Denis reagent and 2.5 ml of Na_2CO_3 solution. After incubation for 30 min at room temperature, the absorbance versus a blank was determined at 760 nm. The total tannins concentrations of extracts were expressed as milligrams of tannic acid equivalents (TAE) per 100 milligram of extract (mg AT/100 mg of extract).

Antioxidant activity and free radical scavenging potential

Determination of free radical scavenging activity by DPPH method

Free radical scavenging activity of the sample extracts was determined spectrophotometrically using the method of Blois. This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Different concentrations of plant extract in methanol were added to methanol solution of DPPH (1.0110^4 M). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 515 nm by using a double-beam UV-Visible Camspec M550 spectrophotometer. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(AB - AS)/AB] \times 100$$

Where, AB is the absorbance of the control reaction (containing all reagents except the test compound), and AS is the absorbance of the test compound. Butylatedhydroxytoluene (BHT) was used as positive control. The tests were carried out in triplicate.

Evaluation of total antioxidant capacity by phosphomolybdenum method

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. [14]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract (25 µg/ml, 50 µg/ml, and 100 µg/ml) was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.3 ml of methanol was used in place of extracts. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 95°C for 90 min. After cooling at room temperature, the absorbance of the solution was measured at 695 nm using a spectrophotometer. The antioxidant capacity of each sample was expressed as ascorbic acid (A.A) equivalent using the following linear equation established using ascorbic acid as standard:

$$[A = 0.0037C + 0.0343; R^2 = 0.991]$$

Where A is the absorbance at 695 nm and C the concentration as ascorbic acid equivalent (µg/ml). The calibration curve was prepared with gallic acid. The values are presented as the means of triplicate analysis.

Reducing power assay (iron reducing activity)

The reducing power of *Peganum harmala* seeds extracts was determined according to the method previously described by Oyaizu. Different concentrations of seeds of *Peganum harmala* extracts (0-1 mg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid, tannic acid and gallic acid were used as standards. Phosphate buffer (pH 6.6) was used as blank solution. All analyses were run in triplicate and results averaged.

Antimicrobial activity

The different extracts were tested for their antibacterial activity against four reference bacterial strains: *Bacillus subtilis* ILB 142B and *Staphylococcus aureus* ATCC 25922 (Gram-positive), and *Pseudomonas aeroginosa* ATCC 27853 (Gram-negative). The strains were cultured on nutrient agar and incubated at 37°C for 24 h and were then maintained in their appropriate agar medium at 4°C throughout the study and used as stock cultures.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC):
The MICs were determined using the broth microdilution assay as previously described by Bouhdid et al. with slight modifications, agar at 0.15% (w⁄v) was used as emulsifier and resazurin was used as bacterial growth indicator. Firstly, 50 µl of Mueller Hinton Broth (Oxoid, UK) were distributed from the second to the 12thwell of a 96-well polystyrene microtiter plate. The different extracts dilution were prepared in methanol (0.5% w/v), 100 µl of these suspensions were added to the first test well of each microtiter line, and then 50 µl of scalar dilution were transferred from the second to the 11th well. The initial concentration of the different extracts was 100 mg/ml and 40% (v⁄v) for the seeds oil. The 12thwell was considered as growth control. Then, 50 µl of a bacterial suspension were added to each well at a final concentration of approximately 10^6 CFU/ml. Plates were incubated at 37°C for 20 h. After incubation, 5 µl of resazurin were added to each well to assess bacterial growth as indicated by. After further incubation at 37°C for 2 h, the MIC was determined as the lowest seed extracts concentration that prevented a change in resazurin colour. Bacterial growth is detected by reduction of blue dye resazurin to pink resorufin. Experiments were conducted in triplicate. The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the seed extracts yielding negative subcultures after incubation at 37°C for 24 h. It is determined by spotting 2 µl from negative wells on LB plates. Experiments were also conducted in triplicate.

RESULTS AND DISCUSSION

In this study the extracts obtained we have were two aspects: the oily brown colour extract was found for hexane solvent and the sticky pastered–brown colour extract was found for other solvents of extraction.

Qualitative phytochemical screening
The results of phytochemical screening of *Peganum harmala* seeds extracts are shown in Table 1. The phytochemical analysis was carried out to detect the presence of phytoconstituents (alkaloids, tannins and flavonoids) in all extracts of *Peganum harmala*. Phytochemical screening of *Peganum harmala* indicated the presence of tannins, alkaloids and flavanoids in aqueous, methanolic and ethyl acetate extracts mainly obtained by the two methods of extraction. However, the hexanic extracts were deficient in alkaloid and tannins (Table 1).

Table 1: Phytochemicals detected in *Peganum harmala* seed extracts

Type of extract	Alkaloid	Flavonoid	Tannin
Successive Soxhlet extraction			
Methanolic extract	+	+	+
Hexanic extract	_	+	_
Ethyle acetate extract	+	+	+
Maceration extraction			
Aqueous extract	+	+	+
Hexanic extract	_	+	_
Ethyle acetate extract	+	+	+
Methanolic extract	+	+	+

(Where, – absent and + present)

Furthermore, Benbott et al. [15] showed that *Peganum harmala* extracts contains: flavonoids, saponins, tannins, compounds reducers, volatile oils, anthraquinones, triterpenes, sterols and alkaloids. Indeed, several authors have shown that different types of chemical compounds highlighted in the extracts of this plant have therapeutic effects such as: narcotic, anthelmintic, malaria and in some cases against rheumatism [16-18]. Plants have an almost limitless ability to synthesize aromatic substances and derivatives which form the phytochemical constituents [19,20]. Phytochemical constituents and derivatives are commonly used for medicinal purposes against number of disease such as analgesic, antimalarial, bactericidal and antiseptic [21].

Quantitative phytochemical screening
Total phenolics, flavonoids and tannins determination:
Phenolic compounds are secondary plant metabolites, which contribute to the overall antioxidant activities of plants mainly due to their redox properties. The total phenolic content of *Peganum harmala* seed extracts were determined by Folin-Ciocalteu assay using gallic acid as a standard phenolic compound. This method can be useful in characterizing and standardizing plant samples. The total phenolic contents of *Peganum harmala* seed extracts were calculated with a regression equation based on a standard curve using gallic acid and shown in Table 2.

Table 2: Total phenolic and flavonoid compound of *Peganum harmala* seed extracts

Extraction Type	Extract	Total Phenolic	Total Flavonoid	Total Tannin
		(mg GAE*/100 mg extract)	(µg QE/100 mg extract)	(mg TAE/100 mg extract)
Soxhlet extraction	Hexanic extract	5.34 ± 1.23	49.3 ± 4.23	-----
	Ethyl acetate extract	4.83 ± 0.89	148.46 ± 5.23	6.53 ± 2.76
	Methanolic extract	5.11 ± 2.34	76.02 ± 1.34	13 ± 3.12
Maceration extraction	Aqueous extract	19.68 ± 1.56	113.9 ± 4.21	13.60 ± 2.65
	Hexanic extract	6.02 ± 2.13	43.47 ± 1.45	-----
	Ethyl acetate extract	5.52 ± 1.43	71.5 ± 3.12	8.21 ± 0.23
	Methanolic extract	9.11 ± 1.65	134.34 ± 3.56	13.64 ± 1.32

*GAE, gallic acid equivalents; QE, quercetin equivalents; and TAE, tannic acid equivalents

The total phenolic content was reported as mg gallic acid equivalent per 100 mg dried extract. In the present work, the aqueous extract showed the highest amount of the phenolics. However, these results showed that the amount of phenolic compounds in extracts for two methods of extraction were in the following order: Methanolic extract>Ethyle acetate extract>Hexanic extract. The content of total flavonoids was also measured spectrophotometrically by using the aluminium chloride colorimetric assay. The flavonoid content of *Peganum harmala* seeds was expressed as quercetin equivalents in µg /100 mg extract and a calibration curve of quercetin was used. The results are showed a large dominance for methanol extract of the maceration extraction (134.34 µg EQ/100 mg extract) and ethyl acetate extractof the soxhlet extraction (148.46 µg EQ/100 mg extract) in total flavonoids contents (Table 2). For the content of total tannins the results obtained show that the extracts of the maceration extraction showed a higher content of total tannins than that in extracts of the soxhlet extraction (Table 2). Mazandarani et al. [22] showed that the total phenolic content of the ethanolic extract from *Peganum harmala* seeds was 61.55 mg GAE/g dry weight and TF content 42.21 mg EQ/g. Moreover, flavonoids are phenolic compounds found in *Peganum harmala*; these substances in the diet are powerful antioxydant by scavenging the superoxide anion [23], singlet oxygen [24], lipid peroxy radicals [25] and by stabilizing free radicals involved in oxidative processes through either hydrogenation or complexing with oxidizing species [26].

***In-vitro* antioxidant activity and free radical scavenging potential**

Free radical scavenging potential:

DPPH assay is rapid and sensitive way to survey the antioxidant activity of a specific compounds or plant extracts. The DPPH radical scavenging capacity in the study was reported after 30 min reaction time for all samples investigated. The results obtained are shown in Figure 1 (panels A, B and C). Results show that extract concentration was directly proportional to the antioxidant activity and the extracts of the maceration extraction showed, in general, a higher antioxidant activity (Figure 1 panel B) than that in extracts from the soxhlet extraction at all concentrations (Figure 1 panel A). Compared with BHT the hexane extracts have a higher antioxidant activity than those of all extracts of *Peganum harmala* seeds (Figure 1 panel A). Furthermore, Raad showed that the maximum inhibition produced by ethanolic, hydroalcoholic and aqueous extracts of *Peganum harmala* seeds were 66.55 ± 4.29, 78.98 ± 5.19 and 86.37 ± 3.46 %, respectively at 1 mg/ml concentration level.

Total antioxidant capacity:

The total antioxidant activity for the different extracts of *Peganum harmala* seed extracts was evaluated by using the phosphomolybdate method. This assay is based on the reduction of Mo (VI) to Mo(V) in presence of the antioxidant compounds and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH, which is measured at 695 nm. Total antioxidant capacities of *Peganum harmala* seed extracts, expressed as equivalents of ascorbic acid (µg/mL of extract), is shown in Figure 2. All the extracts showed an increase in antioxidant capacity with an increase in dose. The extracts of the maceration extraction showed a higher antioxidant capacity than that in extracts of the soxhlet extraction this good antioxidant activity might be attributed to the presence of high amounts of polyphenols in these extracts.

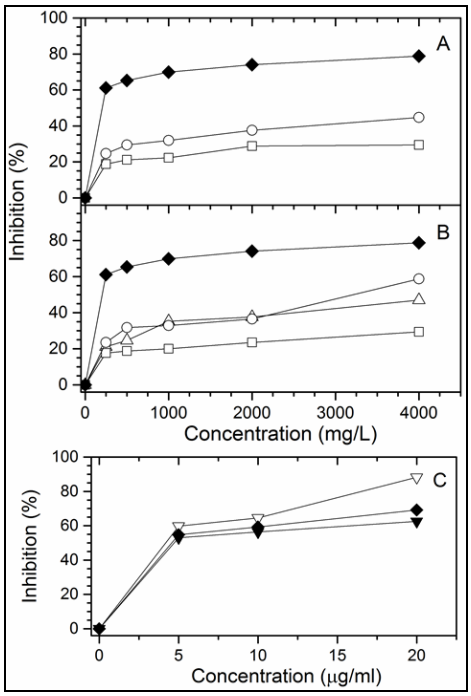

Figure 1: DPPH radical scavenging activity of *Peganum harmala* seed extracts obtained by soxhlet (A) or maceration (B) extraction, and activity of the hexanic extracts obtained by either of both methods(C). Symbols: Hexanic extracts (X by soxhlet and B by maceration), ethyl acetate extracts (∀), methanolic extracts (–), aqueous extract (8) and BHT (Λ).Experimental details in M&M. Each value represents the mean of three values

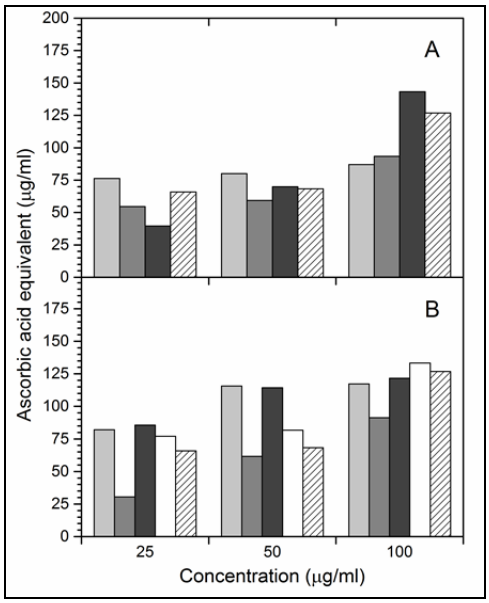

Figure 2: Total antioxidant activity of Peganum harmala seed extracts obtained by soxhlet (A) or maceration (B) extraction. Hexanic extract light grey boxes, ethyl acetate extract grey boxes, methanolic extract dark grey boxes, aqueous extract in white boxes and gallic acid as stripped boxes. Experimental details in M&M. Each value represents the mean of three values

Reducing power assay (Iron reducing activity):

In order to determine reducing potential of *Peganum harmala* seed extracts, we have employed ferric reducing assay. In this, the absorbance of the reaction mixture at 700 nm was found to increase with the increase in concentration of *Peganum harmala* seed extracts which indicates reducing potential of extracts. The result of power reducing activity observed in our extracts is showed in Figure 3.

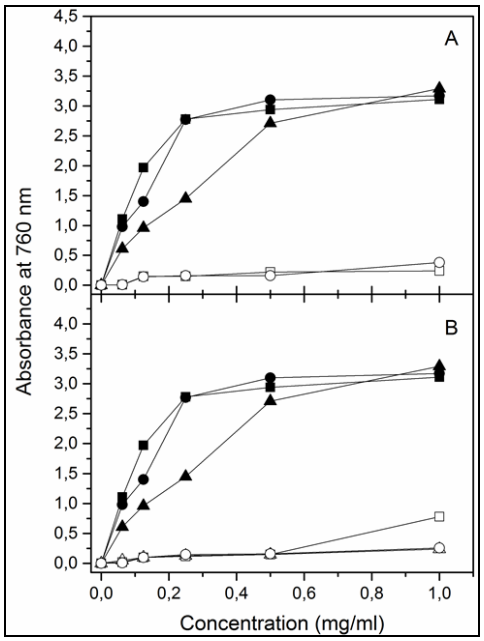

Figure 3: Iron reducing activity of *Peganum harmala* seed extracts obtained by soxhlet (A) or maceration (B) extraction. Symbols: Ethyl acetate extracts (∀), methanolic extracts (–), aqueous extract (8), gallic acid (¸), tannic acid (!) and ascorbic acid (7).Experimental details in M&M. Each value represents the meanof three values

Minimal inhibitory and bactericidal concentrations

The results of the different extracts which were evaluated for their antibacterial activity against *S. aureus* ATCC 25922, *P. aeroginosa* ATCC 27853, *B. subtilis* ILB142B and *E. coli* O128B12 are shown in Table 1. As can be noted in this finding, all the extracts tested were shown an important antibacterial effect expect for the hexanic extract obtained by soxhlet extraction. Indeed, the MIC values of the different extracts ranged from 0.097 to 0.78% (v/v) against strains studied. Regarding the MBC values of the different extracts tested (Table 3). We found that MBC values of the different extracts were ranged from 0.39% to 100% (v/v) against strains studied. It can therefore be interpreted that they act by a bactericidal action.

Table 3: MIC and MBC values for the crude extracts of *Peganum harmala* seeds

Extraction Type	Extract	MIC			MBC		
		Bs*	Sa	Pa	Bs	Sa	Pa
Soxhlet extraction	Hexanic extract	-	-	-	-	-	-
	Ethyl acetate extract	0.19	0.19	0.39	12.5	0.39	25
	Methanolic extract	0.78	0.78	0.39	25	50	50
Maceration extraction	Aqueous extract	0.39	0.39	0.78	100	0.39	50
	Hexanic extract	6.25	3.12	25	6.25	25	25
	Ethyl acetate extract	0.097	0.19	0.78	50	0.39	50
	Methanolic extract	0.19	0.39	0.19	1.56	12.5	12.5

* Bs, *Bacillus subtilis*; Sa, *Staphylococcus aureus*; and Pa, *Pseudomonas aeroginosa* .

Furthermore, by the disc diffusion method; Djarmouni et al. proved that the chloroform and ethyl acetate extracts from *Peganum harmala* seeds showed an important antibacterial activity. It was found that there is no correlation between phenolic content and antibacterial activity, similar results were obtained by Turkmen et al. [27]. Previous studies demonstrated that the polyphenols such as tannins and flavonoids as epigallocatechin, catechin, myricetin, quercetin [28] and luteolin [29] are important antibacterial substances. The antibacterial activity of extracts is mainly due to their chemical structure especially to their high phenolic groups, which are capable to binding to certain proteins and enzymes, thus altering the equilibrium enzyme [30]. In addition, flavonoids possess ability to increase colonic water and electrolyte reabsorption; therefore plant containing flavonoids are used in the treatment of dysentery [31]. El Mahmood [32] reported that glycosides and flavonoids are known to protect against

CONCLUSION

This work showed the antioxidant activity of the different extracts. Further studies are warranted for the isolation and identification of individual phenolic compounds and also in vivo studies are needed for better understanding their mechanism of action as antioxidant. Methanolic fraction of *Peganum harmala* seeds possesses antimicrobial activity against Gram positive and negative bacteria species which can be explored as remedy for human microbial infections and could justify the claimed ethnomedicinal uses of *Peganum harmala* seeds.

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