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Evaluation of Antioxidant and Antibacterial Properties of *Peaganum Harmala* Seed Extracts

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ABSTRACT

This study consist to evaluate the antioxidant and antibacterial activities of the phenolic fractions extracted from *Peaganum harmala* seeds by many organic solvents. The antioxidant activities of these extracts were studied by two complementary test; FRAP and NBT assay. The antibacterial effects were carried out using different bacterial strains; (*E. coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 13311, *Acinetobacter baumanii* ATCC 19606, *klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC10876, *Enterococcus faecalis* ATCC 49452 *Lysteria monocytogenes* ATCC 15313, *Citrobacter freundii* ATCC8090). The results indicate that the highest total polyphenols and flavonoids contents were recorded in chloroform extract (CHE) (66.29 mg gallic acid equivalent/mg of extract and 13.88 mg quercetin equivalent/mg of extract, respectively). In NBT assay CHE exhibited the best effect with an IC_{50} of 0,395mg/ml. The extracts exhibited a good reducing power, especially for CHE which gave the greatest reducing power (TAP = 10.422 mM Fe^{+2}/g of extract). The antimicrobial activity was tested on 11 bacteria using the agar diffusion method. The ethyl acetate extract (EAE) have shown a significant antimicrobial activity against Gram (+) and Gram (-) bacteria.

Keywords: *Peaganum harmala,* antioxidant, antibacterial, seed extract

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INTRODUCTION

Natural antioxidants can protect the human body against free radicals that may cause some chronic diseases [1, 2]. Studies conducted on the antioxidant activities of some plants as natural antioxidants generally focused on the herbs and aromatic plants [3, 4].

Plants being a rich source of novel biologically active molecules may serve as a natural remedy against these pathogens. Also the high cost of conventional antimicrobial treatments particularly in Asian and African countries necessitates the use of plant based natural medicine for therapeutic purpose. Many studies devoted to substances extracted from plants have shown an antimicrobial activity. It was established that essential oils, extracted from plants such as laurel, sage and rosemary, inhibited the growth of bacteria (*Staphylococcus aureus, Klebsiella pneumoniae*, *Enterococcus feacalis, Escherichia coli, Staphylococcus epidermidis* ....) [5-7].

Peganum harmala L. (Syrian rue) is a wild growing flowering plant belonging to the Zygophylaceae family and is considered as an important medicinal plant. The seeds are known to possess hypothermic and hallucinogenic properties [8,9].

P. haramala was also shown to possess antihelmitic, lactogogue, antispasmodic, antipyretic, abortifient, emetic and emmenagogue properties [10].

This plant has also a wide spectrum of pharmacological actions for example monoamine oxidase inhibition [11], binding to benzodiazepin receptors [12] and antioxidative action [13]. Moreover *Peganum harmala* was shown to have a cardiovascular actions [14], and DNA topoisomerase inhibition in cancerous cell-lines [15], and the extract is useful in wound healing [16].

Our study was divided into two main steps: initially, a chemical extraction and quantification of phenolic fractions from *P. harmala* seeds grown in Algeria were performed, then the antioxidant and the antimicrobial properties of obtained extracts were examined.

MATERIALS AND METHODS

Materials

Peganum harmala L. was collected from N'gaouss, Batna, Algeria in July 2010 and identified by Pr. Odjhih (Department of Agriculture, Faculty of Agriculture, University of Batna). All other reagents were purchased from Sigma Chemicals (Germany), Fluka and Prolabo.

Extraction Procedure

The extraction was carried out using various polar and non-polar solvents (17). The powdered plant material (100g) was extracted with methanol (MeOH) (85% and 50%), at room

temperature overnight. The MeOH fractions were combined and concentrated under reduced pressure on a rotary evaporator. MeOH extract (CE) successively extracted with hexane, chloroform and ethyl acetate. Each fraction was evaporated to dryness under reduced pressure to give hexane (HE), chloroform (CHE), ethyl acetate (EAE), and the remaining aqueous (R-H_2O) extracts.

Determination of total flavonoid contents

Total flavonoid content in each extract was determined by a colorimetric method as described by Bahorun et al [18]. Each sample (1 ml) was mixed with 1 ml of aluminium chloride ($AlCl_3$) solution (2%) and allowed to stand for 15 min. Absorbance of the mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as equivalent quercetin and rutin (mg quercetin or rutin/g dried extract).

Determination of total polyphenols

Total polyphenols were measured using Prussian blue assay described by Price and Butler [19] modified by Graham [20]. Phenolics were expressed as gallic acid equivalents. Briefly 0.1 mL of each sample, were dissolved in methanol, 3 mL of distilled water was added and mixed, then 1 mL of $K_3Fe(CN)_6$ (0.016 M) was added to each sample followed by the addition of 1 mL of $FeCl_3$ (0.02 M dissolved in 0.1 M HCl), and 5 mL stabilizer (30 mL gum Arabic, 1%; 30 mL H_3PO_4, 85% and 90 mL of distilled water) were added to the sample and mixed. The absorbance was measured at 700 nm. Phenols were expressed as gallic acid equivalents (mg gallic acid/g dried extract) ranging from 0.00 to 200 µg/mL.

Antioxidant activity

Measurement of superoxide anion scavenging activity (NBT assay)

The superoxide scavenging ability of the *Peaganum harmala* seed's extracts (PHSE) was assessed [21]. superoxide anions were generated in samples that contained 100µl of 1mM NBT, 3mM NADH and 0.3mM PMS and the final volume was adjusted to 1ml with 0.1M phosphate buffer (pH7.8) at room temperature. The reaction mixture (NBT and NADH) was incubated with or without extracts at ambiant temperature for 2 min and the reaction was started by adding PMS. The absorbance at 560 nm was measured; against blank samples for 3min. Decrease in absorbance in the presence of various plant extracts indicated superoxide anion scavenging activity. The percent scavenging activity was calculated using the following formula.
Superoxide scavenging activity (%) = Control OD-Sample OD / Control OD

Ferric reducing antioxidant power (FRAP) assay

The reducing power of PHSE was determined by using FRAP assay described by Benzie and Strains [22], with some modifications [23]. Briefly, the FRAP reagent contained 2.5 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3$ and 25 ml of

0.3M acetate buffer, pH 3.6, was freshly prepared. The absorption of the reaction mixture was measured at 593 nm. In the FRAP assay the antioxidant potential of sample was determined from a standard curve plotted using $FeSO_4$ $7H_2O$ at a concentration range between 100 and 2000 µM.

The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM FeSO4.

Antimicrobial activity

The antimicrobial activity were performed on 11 reference bacteria : *E. coli* ATCC 25922, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 13311, *Acinetobacter baumanii* ATCC 19606, *klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC10876, *Enterococcus faecalis* ATCC 49452 *Lysteria monocytogenes* ATCC 15313, *Citrobacter freundii* ATCC8090.

In the test of antimicrobial activity, the agar disc diffusion method was adopted. The bacterial strains were incubated on Mueller-Hinton medium. Overnight cultures of bacteria were used to prepare dilutions in saline solution (0.9%, w/v) to a turbidity of 0.5 Mac Farland standards (108 CFU/ml). Once the agar on Petri dishes (90 mm) seeded, disks of Whatman No. 6 paper (6 mm in diameter), were soaked, each, of a fixed volume of 10 µl of plant extract. Discs were deposited at equal distances on the surface of the agar. A negative control disc impregnated with solvent without extract [24]. After incubation for 24 h at 37°C, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) of two /three replicates. Where applicable, the data were subjected to one-way analysis of variance (ANOVA), where the differences between extracts to each other were determined by Tukey's multiple comparison test for comparison between extracts and standards, using GraphPad program. p value ≤ 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Determination of total phenolic and flavonoid contents of different PHSE were carried out using Prussian bleu assay and $AlCl_3$ methods. CHE contains high amounts of phenolics and flavonoids.

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The total phenolic content was reported as mg gallic acid equivalent per gram dried extract. The results showed that the amount of phenolic compounds in PHSE were in the following order: CHE > EAE > CE. The total flavonoid contents of different fractions were reported as mg quercetin and rutin equivalent per g dried extract (Table 1).

Table1. Total polyphenols and flavonoids of *Peaganum harmala* seed extracts

Extracts	% yield (W/W)	Total polyphenol and Flavonoids	
		mg Equivalent gallic acid/g	mg Equivalent quercetin/g
Crud	1.65 ± 0.27	27.91 ± 0.98	7.39 ± 0.68
Chloroform	2.19 ± 0.16	66.29 ± 1.58	13.88 ± 0.13
Ethyl acetate	2.41 ± 0.31	58.1 ± 2.87	12.18 ± 0.08

Value are expressed as mean ± SEM, n =3.

Antioxidant capacity by (PMS-NADH-NBT) assay

The inhibition of cytochrome c reduction is due to dual effect of extracts as demonstrated previously by Baghiani et al [25]. Firstly, these compounds inhibit the XO activity and secondly, some of them scavenge $O_2^{\cdot-}$ approximately at the same concentrations. To give a clear cut that PHSE have $O_2^{\cdot-}$ scavenging activity or not the PMS-NADH-NBT system was used as a nonenzymatic method to measure $O_2^{\cdot-}$ scavenging activity. Which was assessed spectrophotometrically by following the decrease in the reduction of NBT (Nitroblue tetrazolium) to formazan (λmax = 560 nm) produced chemically by superoxide in the PMS-NADH. The different extracts showed an antioxidant activities in a dose-dependent manner (Figure 1).

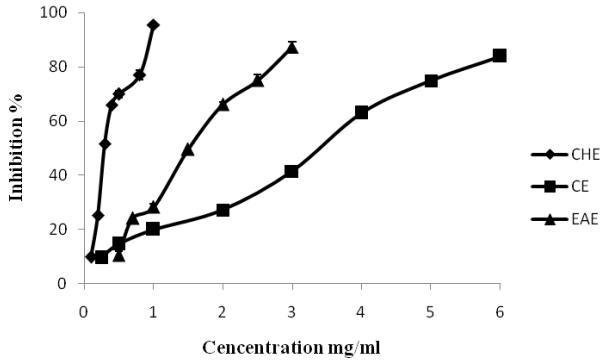

Fig. 1. Superoxide- scavenging effect of *P. harmala* extracts using NBT assay, where $O_2^{\cdot-}$ was generated by NADH/PMS system. Values were expressed as mean ± SD (n=3).

The most potent scavenger for ($O_2^{\bullet-}$) radical was observed with CHE IC_{50} of 0,395± 4,912 mg /ml followed by EAE with IC_{50} of 1,644± 0,023 mg /ml. However, CE gave a scavenging effect lower than those of previous extracts (IC_{50} = 3,335 ± 0,047 mg /ml) ($p \leq 0.001$). Our results were compared with the positive control quercetin which gave an IC_{50} of 33.69 ± 0.35 µg/ml, (figure 2).

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All the tested extracts in this study showed a significant linear correlation between their scavenger effects on superoxide anion and their phenolic compounds content's. Hanasaki and their collaborators [26] showed that flavonoids such as epicatechin, myricetin and catechin are able to scavenge superoxide radical. Accordingly, our result showed that the superoxide-scavenging effect due to the non-glucosilic phenolic compounds (CHE).

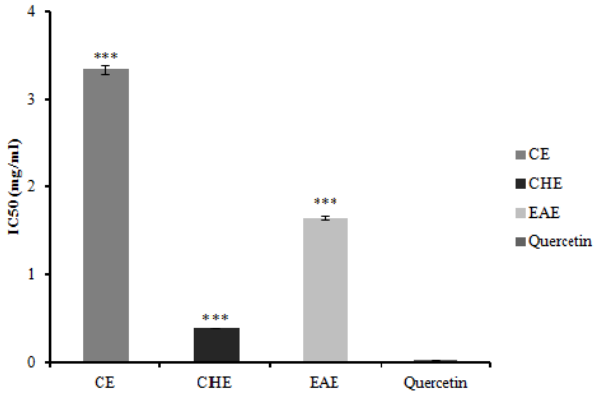

Fig. 2. IC_{50},inhibitory concentration of *P. harmala* extracts for 50% of superoxide generation using NBT assay. Comparison was realized against Quercetin as standard,***: $p \leq 0.001$.

Ferric reducing antioxidant power by (FRAP) assay

The FRAP values were calculated using the respective Fe (II) calibration curves, gallic acid was used for comparison of ferric reducing ability of studied extracts. The results were expressed as EC1 which is the concentration of antioxidants having a ferric reducing ability equivalent to that of 1mM $FeSO_4.7H_2O$, EC1 [23]. EC1 was calculated as the concentration of antioxidant giving absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1mM concentration of Fe^{+2} solution determined using the corresponding regression equation. Total antioxidant power (TAP) was calculated from EC1.

In contrast to Benzie and Strain [22] method, the absorbances of our extracts did not stabilize after 4 min in agreement with Pulido et al [23]; therefore the measurements were continued for 30 min (Fig. 3).

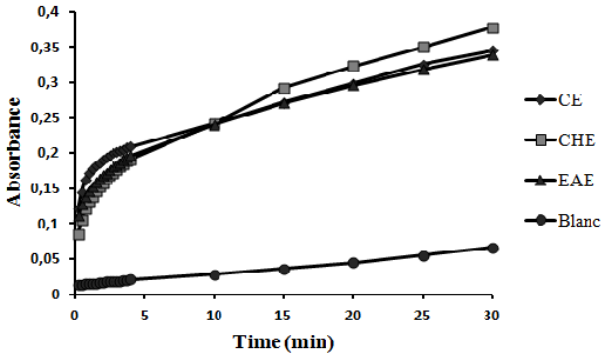

Fig. 3. FRAP reaction kinetics of reagent blank and *P. harmala* extracts (1,25 mg/ml). Values were expressed as mean (n= 3).

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The results of reducing power of extracts and gallic acid are presented in Table 2. At 4 min, CE showed the highest TAP, which was approximately, 3-folds lower than that of gallic acid, referring a good reducing power. This effect was followed by those of EAE and CE with TAP lower than that of gallic acid by 3.4-folds folds, and 3.6-folds, respectively (p <0.001). However, at 30 min, the extracts gave the same values of TAP, with 2.7-folds lower than that of gallic acid (Figure. 4). Our results revealed a proportional decreasing of the reducing power with the polyphenol content (Figure 4, Table 1). Numerous reports described antioxidant activity, seems that the reducing power method may be predominantly related to the presence of the phenolic compounds [27].

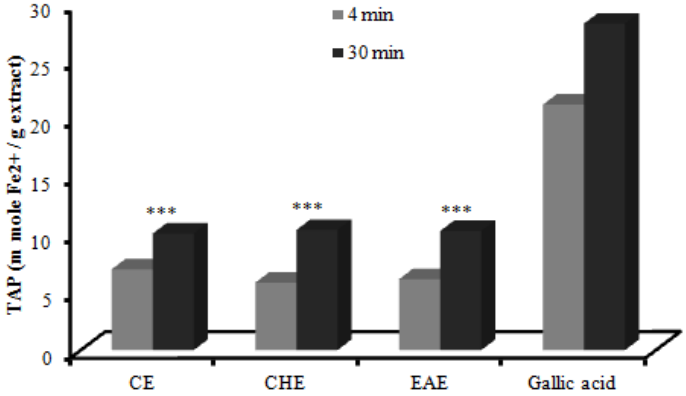

Fig. 4.Comparison between TAP of *P. harmala* extracts at 4 min and 30 min. TAP values were expressed as mean ± SD of triplicate. (*: $p \leq 0.001$).**

Table 2. EC1 and TAP values at 4 min and 30 min of reaction using FRAP assay.

	EC1 (4mn) (mg/ml)	EC1 (30mn) (mg/ml)	TAP (4mn) mM Fe^{+2} /g extract	TAP (30mn) mM Fe^{+2} /g extract
Gallic acid	0.048 ± 0.003	0.035 ± 0.002	21.287 ± 1.179	28.312± 1.458
CE	0.143 ± 0.001	0.099 ± 0.002	7.004 ± 0.059	10.102 ± 0.191
CHE	0.171 ± 0.004	0.096 ± 0.001	5.853 ± 0.146	10.422 ± 0.149
EAE	0.162 ± 0.005	0.096 ± 0.002	6.156 ± 0.218	10.330 ± 0.220

Results are expressed as means ± SD of 3 measurements

Antimicrobial activity

Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health, despite of the tremendous progress in human medicine. Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and resistance to old and newly produced drugs is on the rise [28]. The development of microbial resistance to available antibiotics due to random selection and possible side effects, have led some authors to investigate the antimicrobial activity of indigenous medicinal plants in many parts of the world [29, 30, 31].

The antibacterial effect of CE, CHE, EAE extracts of PHE was assessed by the disc diffusion method. The results showed that this plant has a very important antibacterial effect

on *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeroginosa* ATCC 27853 and *E. faecalis* ATCC 49452 strains.

The obtained results showed that the EAE has the highest antibacterial power in comparison with other extracts; it has the ability to inhibit all bacterial strains. The best inhibition zone was recorded *on S. aureus* strain with diameter of 35 mm higher than that obtained by the standard gentamycin (33 mm). However CE was less effective with an inhibition zone of 27mm (Table 3).

The most sensitive strains against CE are *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeroginosa* with inhibition zones arrange between 27 and 21 mm. CHE gave the lower effects against *P aeruginosa, S aureus, B cereus* and *E faecalis* with a diameter between 14 and 21mm. However, *Lysteria monocytogenes* is less affected by all extracts.
It was found that there is no correlation between phenolic content and antibacterial activity, similar results were obtained by [32] when evaluating the antibacterial activity of tea extracts, and [33]

Table3. Inhibition zones (mm) of *Peaganum harmala* extracts against different strains of bacteria

Bacteria	Extracts			Negative Control DMSO	Positive control Gentamycine
	CE	CHE	EAE		
***Peudomonas aeruginosa* ATCC 27853**	21	16	25	-	25
***Escherichia coli* ATCC 25922**	15	12	20	-	21
***Salmonella typhimurium* ATCC 13311**	18	10	16	-	15
***Acinetobacter baumanii* ATCC 19606**	14	09	20	-	11
***Citrobacter freundii* ATCC 8090**	17	12	22	-	13
***Klebsiella pneumoniae* ATCC 700603**	10	-	18	-	09
***Staphylococcus aureus* ATCC 25923**	27	21	35	-	33
***Bacillus cereus* ATCC 10876**	16	14	22	-	18
***Enterococcus faecalis* ATCC 49452**	25	14	24	-	30
***Lysteria monocytogenes* ATCC 15313**	08	08	14	-	11

Previous studies demonstrated that the polyphenols such as tannins and flavonoids as epigallocatechin, catechin, myricetin, quercetin, [34] and luteolin [35] 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 [36]. In addition, flavonoids possess ability to increase colonic water and electrolyte reabsorption, therefore plant containing flavonoids are used in the treatment of diarrhoea and dysentery [37, 38, 39]. El-Mahmood, [40] reported that glycosides and flavonoids are known to protect against

gastrointestinal infections. It has been shown that the mechanism of toxicity against the microorganisms is done either by deprivation of metal ions such as iron or by non-specific interactions such as the establishment of bridge hydrogens with proteins of the cell walls (adhesions) or enzymes in bacteria [41]

CONCLUSION

The antioxidant properties of different extracts of *P. harmal* seed were determined by two methods FRAP and PMS-NADH. Based on our results, the decreasing order of antioxidant activity among the extracts was found to be as follow, chloroform extract > ethyl acetate extract >methanol extract. This order is similar to the phenolic contents of the extracts. The antioxidant activity of the extract is in accordance with the amount of phenolics present in that extract. In the present study it is found that the chlorofotm extract of *P. harmala* seed contains substantial amount of phenolics and it is the extent of phenolics present in this extract is responsible for its marked antioxidant activity as assayed through various in vitro models. Since the chemical composition and structures of active extract components are important factors governing the efficacy of natural antioxidants, the antioxidant activity of an extract could not be explained on the basis of their phenolic content, which also needs their characterization. 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.

Ethyl acetate fraction from the methanol extract of the *Peaganum harmala* seed possess antimicrobial activity against Gram positive and negative bacteria species. which can be explored as remedy for human microbial infections and could justify the claimed ethno-medicinal uses of *Peaganum harmala* seed

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