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Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MAC) of seed methanolic extract of *Peganumharmala L.* against Gram negative bacteria

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Summary

Peganum harmala L. (Zygophyllaceae) used in traditional medicine of Iraq and itis one of the most famous medicinal plants. The most bacteria pathogen is resistance for synthesis antibiotic drugs resulting from abuse uses of the patient or produce resistance strains for microorganism therefor, the aim of this search is the effect antibacterial of seed alcoholic extract of *peganum harmala* on some Gram negative bacteria pathogen. ***Methods*:** The broth serial dilution assay method determined the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of seed methanolic extract of *Peganum harmala* were tested for antibacterial effect against bacterial isolates (*salmonlla enteric , klebsiela pneumonia multidrugresitant E. Coli* and *highly resist E.coli*), used phytochemical analysis of seed mathanolic extrac of *peganumharmala*. ***Result:*** showed good antibacterial activity against strain *Salmonella enteric* and *Klebsella pneumonia*, were MBC at concentration (25 mg/ml) and no effect of low concentration of MIC. The MBC value against strain *multidrugresistant Escherichia coli*, was at concentration (3.125 mg/ml) and MIC at (25 and 12.5 mg/ml), while MBC of highly resist *Escherichia coli* was in (12.5mg/ml) and MIC value at 50 mg/ml compared with standard antibiotics synthesis. Phytochemical analysis of component seed methanolic extract of *peganumharmala* showed presence of flavonoids, alkaloids, saponins, tannins, glycosides, terpenoids and steroids.

Keywords: Peganum harmala, seed methanolic extract, antibacterial activity.

تحديد الحد الادنى لتركيز تثبيطي والحد الادنى لتاثيرقاتل لمستخلص الميثانول لبذور الحرمل *لفصيلة الغردقية ضد* جراثيم السالبة لصبغة الغرام

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فرع الفسلجة والادوية / كلية الطب البيطري / جامعة الكوفة

الخلاصة:

بذور الحرمل او غلقة الذئب الفصيلة الغرقديّة هي واحدة من النباتات الطبية الأكثر شهرة المستخدمة في الطب التقليدي في العراق. معظم البكتريا المرضية تكون مقاومة للمضادات البكتيرية نتيجة اساءة استخدامها من قبل لمرضى او انتاج سلالات مقاومة للكائنات الحية الدقيقة لذا الهدف من هذا البحث تأثير المضاد البكتيري لمستخلص الميثانولي لبذور الحرمل ضد بعض البكتيريا السالبة المرضية الطريقة. بطريقة تخفيف الميديا المغذية تم فحص المضاد الجراثيم ضد العزلات البكترية لمستخلص

الميثانول لبذور الحرمل لتحديد MIC و MBC .واستعمل التحليل الكيميائي لمستخلص الكحولي لبذور الحرمل. النتائج: أظهرت فعالية جيدة المضادة للجراثيم ضد عزلات *salmonlla enteric* و *Klebsiella pneumonia* لفحص تركيز الحد الأدنى قاتل للجراثيم (MBC) بتركيز 25 ملغم /مل ولا يوجد اقل تركيز الحد الأدنى المثبطة (MIC) . اما قيمة (MBC) MDR *E. coli* ، بتركيز 3,125 ملغم / مل و(MIC) بتركيز 25و12.5 ملغم /مل اما قيمة (MB) *E. coli* Hilghly resistant بتركيز 12,5 ملغم /مل . وقيمة (MIC) بتركيز 50 ملغم/مل وقد اظهرت اختبارات الكيمياوية لمستخلص الكحولي لبذور الحرمل وجود الفلافونويد والصابونين والقلويدات والتربنويد.

Introduction

(*Peganum harmala*L. family *Zygophyllaceae*) is a persistent, glabrous plant which grows naturally in sandy soils. Iraq and other countries have been used as a traditional medicine to therapy the diseases of respiratory and digestive systems and use as antibiotics for treatment of human and animals disease resulting many of microorganisms is multidrug resistant. **(1) and (2)**

The olden time were used herbal plants for healing and treatment diseases, have been noteworthy, because these plants have mechanism of protection against microorganism and produce secondary metabolites, the antioxidant components. (**3)**

Antibiotics used to treatments common infectious diseases, were associated with many problems such as side effects unwanted and resistance. Plants can be considered as a substitute for chemical drugs. Therefore, today used herbal medicine in extensive researches have been carried out in traditional medicine, in different fields of medical sciences . **(4**)

Peganumharmala has great variety of pharmacological and biological activities such as antibacterial, antifungal, analgesic and anti-inflammatory, that due to contain harmaline, harmine, harmalol and harmol are the main beta carboline alkaloids **5** also has been spontaneous effect , insecticidal effect , caving malaria , anti-leishmanial, anti-spasmodic, anti-histaminic, vasorelaxant effect, wound healing , anti-oxidant activity, leukemic healing , hypoglycemic effect, immuno-modulator properties. **(6)** This plant used for treatment of a variety of human ailments, such as lumbago, asthma, colic, jaundice and as a stimulant emmenagogue . **(7**) therefore, in this study assist to determination of antibacterial activity of the seed methanolic extract of *peganumharmala* against some negative pathogenic bacteria.

Materials and methods

Collection of material

Peganumharmala seeds were brought from the local market of Iraq, the extraction of seed *peganumharmala* was performed at physiology and pharmacology department the microbiology work including bacterial strains isolation, identification and antibiotic sensitive testing was performed at the Microbiology department

Methanolic extraction

The seed crude extract of the *Peganumharmala* was gained according to (Harbone, and Mabray, 1975) (**8**) The dry seeds of *Peganumharmala* were purified washed and dried in fresh air in room, Seeds sample was taken and grinded in ground in electrical grinder to become powder of the seeds. 50 grams of seed powder were extracted by soaking in 250 ml of 70% methanol with magnetic stirrer at 45℃ for about twenty four hours, then filtered by whatman number 1 then evaporated by evaporator (Buchi made Germany). The yield percentage was calculated according to the following equation:

$$\text{The yield \%} = \frac{\text{weight of Dry extract}}{\text{weight of plant powder}} \times 100$$

Phytochemicals detection of the active components

Phytochemical tests were carried out on seed methanolic extracts by using procedures to essist the constituents of seed extract according to follows:

- Detection of Phenolic accorging to Harbone,1973 . (9)
- Detection of Tannins, Saponin, Resins Components, and Glycosides Components according to Harborne,1984 . (10)
- Detection of Coumarins Components according to Geissman, 1962 . (11**)**
- Detection of Flavonoids Components according to Jaffer et al., 1983 **(**12**)**
- Detection of Steroids and Terpenoids Components according to Al-Bid, 1985. (13)
- Test for alkaloids according to Sofowora, 1993. (14)

Isolation of pathogenic strains

Pathogenic bacterial strain isolate using Sallmonlla Shegella (SS), Eosin Methylene Blue (EMB) , MacConky, and Nutrient agar plate. The isolated strains were identified using , Indole, motility, urase agar (IMU) , Himedia, Simmons citrate test and kliglears iron agar test (IKA). Strain were chosen (*Sallmonlla enterica*, *Klebsiella pneumonia*, sensitive *Escherichia coli*, and resist *Escherichia coli)* isolate from the clinical cases of Al-Sadder Hospital/ Najef.

Bacterial counting:

Bacterial counting was done using spectrophotometer (spectrumlab 22PC, USA) the amount of light of a specific wavelength 600-800 that is absorbed standards at 0.1 – 0.08 by a culture is related to the number of bacteria cells to obtain bacterial concentration of (10^7 cfu/ml)) (**15)** and inoculated with seed extract of *peganumharmala* , then culturing were spread-plate by swap on the account agar plate (Fluka, spain) and kepted in incubator (BINDER, USA) for period 24 hrs, after that counting of the colonies of bacteria by colony counter (BioCote, Belgieca) to assess the effect of seed extract of *peganumharmala* on gram negative bacteria whether it is sensitive , intermitted or resistance.

Antibiotic Sensitivity testing

Turbidity of bacterial strains used in the study was measured according to 0.5 MaCcferland standards for 10^7 cfu/ml. **15** . The bacterial strains were cultured on Mueller-Hinton agar (HIMEDIA, India), different commercial antibiotic disks were spread on the agar and they included amikacin (AK), meropenem (MRP), levofloxacin (LE), ciprofloxacin (CIP), piperacillin (PI), gentamicine (GNT), cefixime (CFM) and cephalexine (CL), disks, at stander concentration and pressed lightly onto the agar surface then overnight incubation at 37^oC , the zone of inhibition (ZI) was measured for each bacterial growth around each disc and results were set according to Clinical and Laboratory Standards Institute CLSI (2017) **(16)** .The measured ZI of cefixime (CFM) and cephalexin (CL) according to CLSI (2013). (**17)**

Determination of MIC and MBC assay methods

A serial dilution of stock of seed methanolic extract of *peganum harmala* at concentrations 50 mg/ml and done was 4

tubes of folds at different concentrations (3.125, 6.25, 12.5, 25 mg/ml) **2** were performed 1 ml of actively growing culture of pathogenic bacteria at a concentration of (10^7 colony-forming unit/mL) were mixed with extract dilution in tubes at the laminar cabinet II (Gusto, USA). All the tubes were incubated at 37 °C for 24 hrs. MIC and MBC were determination by culturing strains on counting plate and colonies were counted after 24 hrs of culturing. The result of MIC and MBC were calculated according to bacteria colonies counting on plates 1 ≥ colony number considered as sensitive, 2≤ colony number as intermitted and 10 ≤ colony number consider as resistant .

Results and Discussion

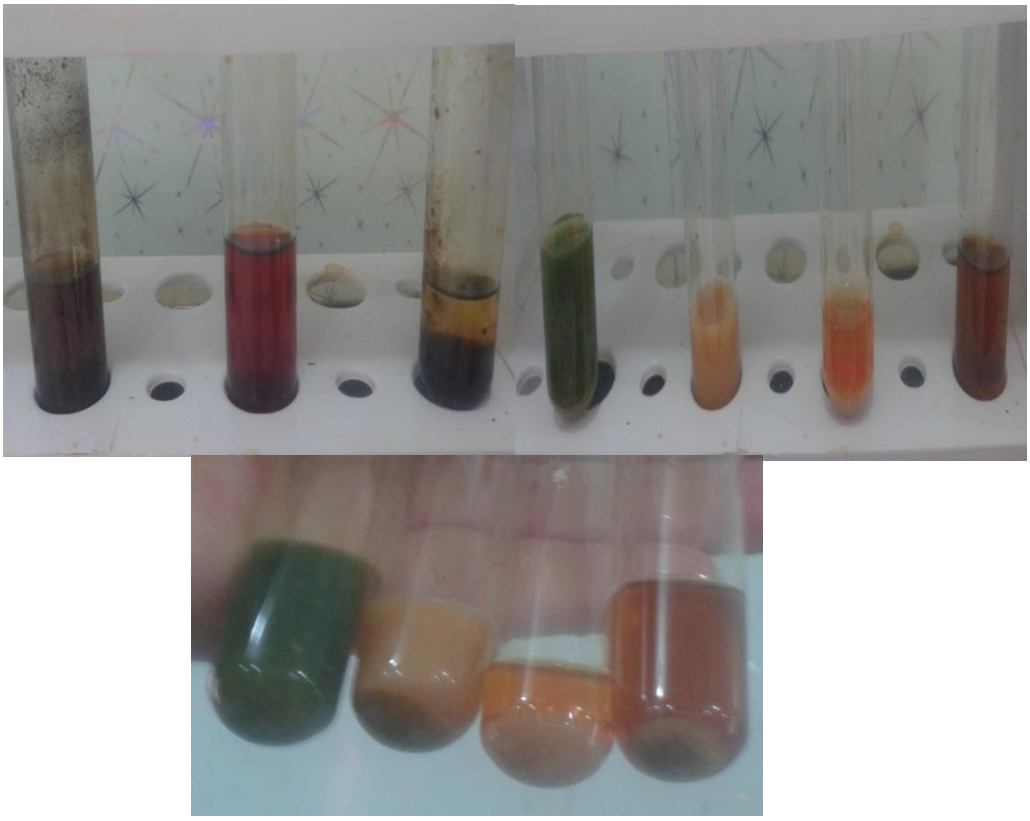
Phytochemicals of seeds extract of *P. harmala*

Table 1- phytochemical of the seed methanolic extract constituents

Component	seeds methanolic extract
Flavonoids	+++ +
Alkaloids	+++ +
Tannins	++++
Saponins	++
Glycosides	+
Polyphenol	+
Steroids and terpenoids	++ +

++++ : high quantity, +++: good containe + : less contained ; - : Absent

The results of phytochemical tests of the seed extract *P. harmala* has shown higher component of flavonoids, alkaloids, tannins and good contained of saponins , terpenoids with less contained of polyphenol and glycoside in table -1- figure (1) the antimicrobial effects of these agents were proven in the previous literature. result similar to our results were reported by Benbott, *et al*., 2013 **(18**). That shown that different types of chemical compounds highlighted in the seed extract especially alkaloids showed that the highest percentage also agreement with the reported by Hemadri ., *et al* , 2017 (**19)**



1- Polyphenol, 2- tannins , 3 flavonoid , 4- glycosides,
5- terpenoids and steroids, 6 –alkaloids, 7 – saponins

Figure- 1-phytochemical analysis of component of seed methanolic extract of *peganumharmala*

The MIC and MBC results showed that the seed methanolic extract of *P.harmala* was effect of inhibiting the bacterial activity of *salmonlla enteric , klebsiela pneumoniae* and two strain of *E Coli.* where the 1≥ colony number , therefore the strain of *Salmonlla enteric and klebsiela pneumonia* showed sensitivity to seed extract *of peganumharmala* in concentration 25 mg/ml and no effect of low concentration MIC where the strains resistance for seed extract that showed 10 ≤ colony number. The reported by Talaro, 2009. **(20)** showed active seed extract as antibacterial effect against *K. pneumonia* especially was remarkable compared with synthetic antibiotic.

MDR *E. coli* showed the MBC value at concentration 3.125 mg/ml while highly resistant *E. coli* in concentration 12.5 mg/ml. The least MIC value showed growth inhibition of colonies more than two colony consider intermitted activity antibacterial that appeared in MDR *E.coli* at concentrations 25 mg/ml and 12.5 mg/ml while highly resistant *E. coli* at concentration 50 mg/ml were showing in table (2) and figure (2). our results were nearly similar to results of the MIC and MBC values for the results reported by Khademalhosseini1, *et al*., 2015. **(15)** seed methanolic extract of *P. harmala* against the two Gram-negative strains *E. coli* and *K. pneumoniae* were the same (1.3 mg/mL) disagree with results of the reported by Darabpour, *et al*., 2011. **(6)** who showed was concentration of seed methanolic extract *P. hamala* against *E. coli* was equal (0.625 mg/ml).

The results agreement with reported by Akbary1, *et al*., 2015 (**21)** that among the evaluated parts of *P. harmala*, the root and seed extracts presented antibacterial activity at the lowest concentration against all of tested bacteria *Escherichia coli* and *Klebsiella pneumonia*. it can be related to the nature and number of active phytochemicals in the seed methanolic extract of *P. harmala*. due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, tannins, trepenoids and sterols and also it is related to the virulence factor of bacteria.

Table -2- different concentration of seed methanolic extract of *Peganum harmal* on study group bacteria

Microorganism	Concentration of seed extract mg/ml				
	3.125		**12.5**	**25**	**50**
Salmonella enterica	R	R	R	S	R
Klebsialla pneumonia	R	R	R	S	R
Escherichia coli Multidrug resist (MDR)	S	R	I	I	R
Escherichia coli Highly Resistant	R	R	S	R	I

S-sensitive , R- resistant, I- intermitted

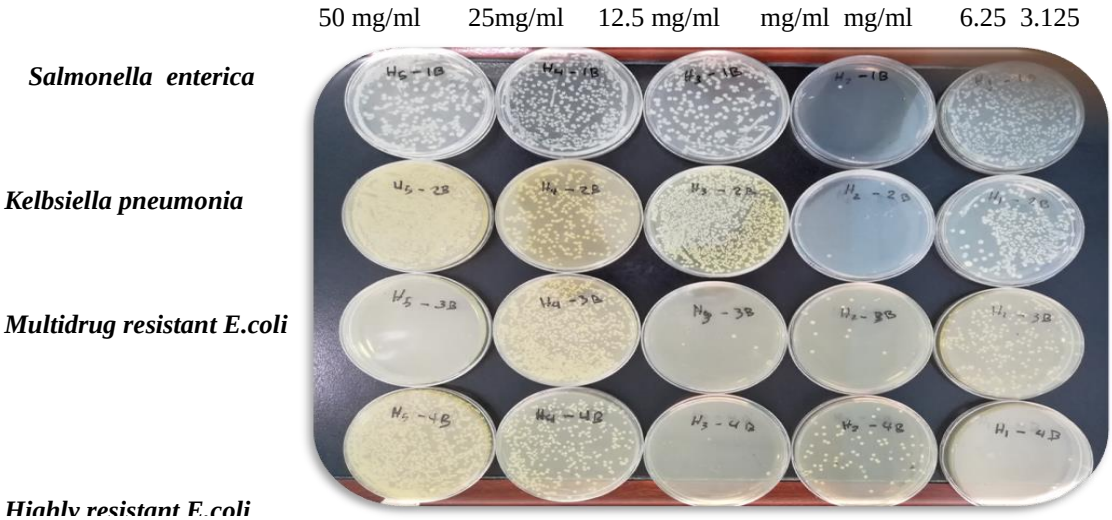


Figure -2- appearance the MIC and MBC of seed methanolic of *Peganumharmal* extract on four strains

Antibiotic profile :

The results of sensitive antibiotic tests were done against four gram negative bacteria were *Salmonella enterica* and *Kelbsiella pneumonia* showed resistant for all selectively antibiotic that present in table (3) compering to sensitivity to seed methanolic extract of *peganumhamala* at concentration 25 mg/ml in table (4). During the last decade, increasing the antibiotic resistance and multidrugresistance of *Salmonella* spp that used for treatment diseases of humane and animals with abuse uses in development countries, therefore, various *Salmonella* become serovars resistant to conventional antibiotics **(22)**

The reported by Dhanashree , 2007 (**23)** show multidrugresistance for among the nontyphoidal *Salmonella enterica.* Bacteria, making them resistant to multiple families of antibiotic drugs, since can acquire multiple different genes for resistance to most an antibiotic is used. therefore, strains present the greatest clinical challenge become resistant to it such multiple drug resistant.**(24**)

MDR *E.coli* was sensitive to antibiotics MRP, LE and CIP and it was sensitive for seed methanolic extract at concentration 3.125mg /ml where, the highly resistant *E.coli* was resistance to (CL, PI, MRP, GNT), intermitted to CIP and sensitive for another selectively antibiotic, table 3 and it showed sensitive for seed methanolic extract at concentration 12.5 mg/ml. table (4)

Table - 3 –Antibiotic profile for the study group bacteria

	Antibiotic disc w ith zone inhibition (ZI) (mm)							
	AK 30µg	**MRP 10 µg**	**LE 5 µg**	**CIP 5 µg**	**PI 100 µg**	**CL 30 µg**	**GNT 10 µg**	**CFM 5 µg**
Salmonella enterica	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Kelbsiella pneumonia	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
E. coli Multidrug resist	**R**	**S**	**S**	**S**	**R**	**R**	**R**	**R**
E. coli Highly resistant	**S**	**R**	**S**	**I**	**R**	**R**	**R**	**S**

R-resistant, S- sensitive, I- intermitted

Table -4- comparison of bacterial sensitivity to certain antibiotics and seed extract of *peganumharmala* with different concentration

Microorganism	**Antibiotic disks**					**extract concentrati mg/ml**
	AK 30 **µg**	**CL 30 µg**	**GNT 10 µg**	**CIP 5 µg**	**CFM 5 µg**	
Salmonella enterica	**-**					**25**
Kelbsiella pneumonia	**-**					**25**
E. coli Multidrug resist	**-**	**S**	**S**	**S**	**-**	**3.125**
E. coli Highly resistant	**S**	**-**	**S**	**I**	**S**	**12.5**

R-resistant, S- sensitive, I- intermitted

In conclusion: Seed methanolic extract of *P. harmala* act as antibacterial compounds for treatment of infections by resistant bacterial strains.

Recommendation

- further investigation on and more studies about the action of *peganumharmal* as an antibacterial against positive (+ ve) and negative (- ve) bacterial strains, different extraction methods should be tested
- further investigation of the effect antioxidant, antiinflamatory, analegesic and anticancer

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