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Antioxidant, Antibacterial, and Phytochemical Screening of Ethanolic Crude Extracts of Libyan *Peganum harmala* Seeds

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NJS and AAA managed the analyses of the study. Author IAA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Peganum harmala is one of the most famous medicinal plants and natural products commonly used in traditional medicine and extensively spread in Middle and East Asia and North Africa. This study aimed to evaluate the radical scavenging activity of ethanolic crude extracts of *P. harmala* seeds from Libya using DPPH assay; to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of the *P. harmala* seeds crude extracts against Gram-negative *Salmonella typhi* ATCC14028 and *Escherichia coli* ATCC 25922 and also Gram-positive *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 33591; and to screen the phytochemicals in the ethanolic crude extracts of the Libyan *P. harmala* seeds using gas chromatography-mass spectrometry (GC-MS). The IC_{50} value of *P. harmala* seeds extract was 179.62±7.32 µg/mL. The MIC ranged from 1.95 to 31.25 mg/mL while the MBC ranged from 7.80 to 62.50 mg/mL. The eight compounds identified were harmine, harmaline, leptalorine, hexahydro fluorene, 3-methoxy-6-methyl, 1H-pyrido [3,4-b]indole,2,34,9- tetrahydro-6methoxy-1-methyl, 9,12-

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octadienoic acid ethyl ester, linoleic acid ethyl ester, 9,12-octadienoic acid (z,z). This study has, therefore, revealed the antixodant and antibacterial efficacy of *P. harmala* ethanolic extract. Thus, it could be further developed as a substitute for chemical antioxidants and as antibacterials agent.

Keywords: Peganum harmala; minimum inhibitory concentration; minimum bactericidal concentrations; harmine; harmaline.

1. INTRODUCTION

Phytochemicals provide significant health benefits and basic nutrition [1-4]. The bioactive substances in medicinal plants such as essential oils, polyphenols, and flavonoids have antioxidant and antimicrobial properties, in addition to their chemotherapeutic potential [5-8]. Efforts to find alternative antioxidants and antibacterial agents from natural sources have been intensified in recent times because of their effectiveness, safety, and efficacy. Natural antioxidants have proved to be as effective antimicrobials as synthetic antibiotics [9-11].

The development of new drugs is unprecedently on a decline thus energizing the advocacy for the use of herbal therapeutics both as botanical preparations and dietary supplements. The vast biosynthetic capacity of medicinal plants is majorly responsible for their complex phytochemicals [12]. Furthermore, potential phytochemicals have been successfully studied in targeting lung-chronic disease biomarkers thus creating a novel paradigm for the discovery of more potent drugs against myriads of chronic diseases [13].

Peganum harmala (Syrian rue or wild rue in English) is one of the most famous medicinal plants and natural products commonly used in traditional medicine. It is a perennial herbaceous plant that grows in arid conditions as well as in plains and sandy soils [14]. *P. harmala* is commonly referred to as “Espand” in Iran, “Harmel” in North Africa as well as “Mexican rue,” “African rue,” or “Turkish rue” in the United States [15]. *P. harmala* belongs to the Zygophyllaceae family. It is a perennial herbaceous, glabrous plant that grows in semiarid conditions, steppe areas, and sandy soils *P. harmala* is native to the eastern Mediterranean area and is extensively spread in Middle and East Asia and North Africa. *P. harmala* total alkaloids extract has been reported to be an effective antibacterial on phytopathogenic bacteria [11]. Several known and undescribed triterpenoids have been isolated from *P. harmala* L. with potent anti-proliferative activities through the induction of apoptosis in HeLa cells [16]. The anti-corrosive potential of the aqueous *P. harmala* seed extract has been reported as a cost-effective and green source of amine-rich molecules such as harmalol, harmaline, harmane, harmol, vasicinone, and vasicine [17].

The objectives of this study were to evaluate the radical scavenging activity of ethanolic crude extracts of *P. harmala* seeds using DPPH assay as well as to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of *P. harmala* seeds crude extracts against two Gram-negative (*Salmonella typhi* ATCC14028 and *Escherichia coli* ATCC 25922) and two Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 33591); and to screen the phytochemicals in the ethanolic crude extracts of Libyan *P. harmala* seeds using gas chromatography-mass spectrometry (GC-MS).

2. MATERIALS AND METHODS

2.1 Sample Collection

P. harmala seeds were purchased from a local grocery store in Gharyan Libya on 2nd June 2019. The authentication of the sample’s identity was carried out by a botanist, Mr. Abdulsslam Elmogasapi, Director, Assetans Database of Herbarium of Cyrenaica, University of Benghazi, Libya.

2.2 Chemicals

Ethanolic (Merck, Germany), Brain Heart Infusion (BHI) agar, BHI broth (Merck, Germany), and Muller Hinton (MH) agar, and MH broth (Merck, Germany), 1,1-diphenyl-2-picrylhydrazyl (cat. no.

D9132, Sigma-Aldrich Co., St. Louis, MO, USA), ascorbic acid (cat. no. 536954, Sigma–Aldrich Co., St. Louis, MO, USA), and gentamicin sulphate (Bio world, USA) were used. The media was prepared according to the manufacturer's instructions. All the media were autoclaved (TOMY Autoclave SS-325 (Japan) at 121ºC and 1 atm for about 15 min.

2.3 Seeds Extraction

The seeds extraction was conducted according to the method by Rebey et al. [18] The collected *P. harmala* seeds were first washed under distilled water and air-dried in the shade at room temperature. Using a blender, the seeds were then grounded to a fine powder. The extraction of the dry powdered seeds was carried out using 96% ethanol (1:10 sample: solvent) in a shaker at room temperature initially for four hours. The residue was further re-extracted again with 96% ethanol for additional two hours. The collected crude extracts were filtered through double-layered muslin followed by centrifugation at 5000 rpm for 5 mins to get a clear supernatant. Then, the supernatant was concentrated under reduced pressure at 50ºC using a rotary evaporator to obtain the final ethanolic crude extract.

2.4 DPPH of Radical Scavenging Assay

The antioxidant activity of *P. harmala* seeds' extract was evaluated using the 2,2- dipheny1-1 picrylhydrazyl (DPPH) method using a microplate reader, according to the literature [5] (Sridhar & Charles, 2019). About 24 mg DPPH was dissolved in 100 mL methanol. Then, 20 µL (1 mg/mL) plant extract/standard solution was added into the wells, followed by 180 µL methanolic DPPH. Ethanol was used as a blank. The reaction mixture was kept in the dark for 45 mins before measuring the absorbance at 517 nm [19]. The percentage of DPPH scavenging activity was calculated using the following formula:

% scavenging = [Abs control – Abs sample]/[Abs control] * 100%

2.5 Bacteria Culture and Maintenance

The bacteria cultures used were Gram-negative (*Salmonella typhi* ATCC14028 and *Escherichia coli* ATCC 25922) and Gram-positive bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 33591). All the bacterial strains were obtained from the Microbiology Laboratory collection, Department of Biomolecular Science, Universiti Teknologi MARA. For long-term storage, all test microorganisms were stored in 30% glycerol stocks at -20°C. Subculturing was done routinely and maintained at 4°C on BHI Agar Nutrient Agar (BD, USA).

2.6 Antimicrobial Activity

The antimicrobial activity of the ethanolic extract of *P. harmala* seeds was evaluated using the method proposed by Mohsenipour & Hassanshahian [20] and Cheruiyot et al. [21]. A stock solution of 250 mg/mL was first dissolved in a little amount of DMSO and made up with Muller Hinton broth (MHB). Two-fold serial dilutions of the stock were prepared to10-levels concentration of (125 mg/mL – 0.24 mg/mL). Gentamicin sulphate (10 µg/mL) was also prepared as a positive control.

Each of the bacterial cultures was first grown overnight on MHA plates and incubated at 37°C. A loopful of each culture was then inoculated into fresh MHB at 1:100 dilution and continue to grow at 37°C for approximately three to four hours until it reached the exponential phase whereby the bacterial cells were most active. The bacterial cultures were then adjusted to 0.5 McFarland standard which is equivalent to 1×10^8 CFU/mL and then diluted to 1×10^6 CFU/mL.

2.7 Preparation of Resazurin Stock Solution

Resazurin dye ($C_{12}H_7NO_4$) stock solution of 0.01% (wt/vol) was prepared by dissolving 0.01 g of resazurin sodium salt (Sigma USA) in 100 mL of sterile distilled water and sterilized by filtration through a 0.2 µm syringe filter. The prepared stock solution was kept at 4°C for 1-2 weeks and thawed before use.

2.8 Resazurin Microtiter Based Assay

About 100 µL of bacteria culture was prepared and incubated in each of the wells of the microtiter plate except for column 11 which was left empty. Following that, 100 µL of serially diluted extract (0.24 – 250 mg/mL) and positive control (0.0024 – 2.5 mg/mL) was added to the first ten wells. The wells were gently mixed. The

negative control contained 100 µL of the bacterial culture only without any extracts or antibiotics. The microtiter plates were then incubated at 37ºC in a static condition for 18-24 hours. Following that, 20 µL of 0.01% (wt/vol) resazurin stock solution was added to each well and the plate was further incubated at 37ºC for five hours. The changes in the color of resazurin were then recorded. Non-viable cells were colored blue while for viable cells, resazurin was reduced to resorufin, as shown by the bright pink color of the cells. Each experiment was done in at least three replicates.

2.9 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria), used to evaluate the antimicrobial efficacy of various compounds. The MIC was determined by observing the changes in the color of resazurin. The value of MIC was taken as the lowest concentration which showed blue resazurin thus indicating no sign of bacteria growth.

2.10 Determination of Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a bacterium over a fixed, somewhat extended period, such as 18 hours or 24 hours, under a specific set of conditions. The MBC is complementary to the MIC (Ohikhena et al., 2017). All the wells with no bacteria growth observed on the microtiter plate were plated on BHI agar and incubated at 37ºC for 24 hours. Following that, bacteria growth on the agar plate was observed. The lowest concentration of the extract that inhibited the bacteria growth on the agar plate was the MBC.

2.11 Gas Chromatography- Mass Spectrometry Analysis

The phytochemical compounds in the crude ethanolic extract of *P. harmala* seeds samples were analyzed using GCMS (Agilent 7890A) equipped with an auto-injector (Agilent 7683 Series injector) and connected to a mass detector (Agilent Technologies 5973 Inert mass selective detector). The phytochemical compounds were separated on a capillary column (HP-5 MS; Agilent 0.25 mm × 30 m 0.25 µm) equipped with an auto-sampler. The initial temperature of 50°C was held for 3 mins, increased to 180°C at 10°C/min, increased to 220°C at 5°C/min, and held for 10 mins. The column temperature was elevated to 230°C at 5°C/min and held at the final temperature for 3 mins. Helium was used as the carrier gas at a flow rate of 3.0 mL/min. The temperature of the detector and injector was set at 240°C. Results were expressed as a relative proportion of the total area of peaks [22].

2.12 Statistical Analysis

The IC_{50} values were generated with GraphPad Prism v8.01 (GraphPad Software, La Jolla, CA, USA) presented as Mean±SD. Other calculations and graphs were performed with Microsoft Excel (2016, Microsoft Corp., Redmond, WA, USA). Each test was repeated three times and the results were represented as mean±standard deviation [23].

3. RESULTS AND DISCUSSION

3.1 Antioxidant Activity

DPPH assay is a rapid and sensitive way to identify the antioxidant of extracts [5,24] The maximum radical scavenging activity of the ethanolic extract of the seeds of *P. harmala* was 59.16±0.14% at 250 µg/mL while the minimum radical scavenging activity was 19.90±1.33% at 15.6 µg/mL. The IC_{50} values of ascorbic acid and the extract were also estimated. The result of the DPPH radical scavenging activity of the ethanolic extracts of seeds of *P. harmala* is shown in Table 1. The IC_{50} values of ascorbic acid and the extracts of Libyan *P. harmala* seeds were 72.76±2.17 µg/mL and 179.62±7.32 µg/mL, respectively.

The results are comparable with *P. harmala* ethanolic extract reported in Iraq by Kaskoos [25] with an IC_{50} of 198.64±6.17 µg/mL. However, the IC_{50} of *P. harmala* ethanolic extract reported in India by Singh et al. [26], in Morocco by Khadhr et al. [27], in Jordan by Mazandarani et al. [28] were 71.97±3.79 µg/mL, 66.55±4.29 µg/mL, 15.45±0.01 µg/mL, and 53.64±0.5 µg/mL,

respectively. Other studies have also reported DPPH radical scavenging activities of *P. harmala* methanolic extracts with varying values IC_{50}. For instance, in Iran, Abolhasani et al. [29] reported that *P. harmala* methanolic extract had an IC_{50} value of 348.82±2.79 mg/mL. In Pakistan, Abderrahim et al. [30] reported an IC_{50} value of 240.32±50.56 mg/mL for *P. harmala* leaves methanolic extract while in Tunisia, an IC_{50} value of 142.5±1.65 µg/mL was reported for *P. harmala* seed methanolic extract [31] as well as an IC_{50} value of 70.16± 3.30 mg/mL for *P. harmala* seed methanolic extract [32]. The differences in the IC_{50} based on the DPPH radical scavenging might be due to differences in the extraction solvent in addition to other factors such as geographical location, extraction techniques, and seed varieties.

3.2 MIC and MBC of Libyan *P. harmala*

The results of the antimicrobial activity of the ethanolic extract of Libyan *P. harmala* against *S. typhi* ATCC 14028 are shown in Table 2. In the wells of columns 1-8 representing the concentration of Libyan *P. harmala* between 125-1.95 mg/mL, the resazurin remained blue which indicates growth inhibition of the bacteria whereas in the wells of columns 9-10 whereby the concentration of *P. harmala* was between 0.97 and 0.24 mg/mL, respectively, resazurin was oxidized, turning to pink color, thus indicating an actively growing bacteria. For the growth control, the resazurin turned to pink color, thus indicating that the *S. typhi* ATCC 14028 was actively growing. Similar results were obtained for the ethanolic extract of *P. harmala* against *E. coli* ATCC 25923. Hence, the MIC for *P. harmala* was 7.8 mg/mL. The MIC values for the ethanolic extract of Libyan *P. harmala* seeds against *B. subtilis* ATCC 6633 and *S. aureus* ATCC 29213 were 1.95 mg/mL and 15.62 mg/mL, respectively. The positive control (gentamicin) also showed a great lethal activity on all the test organisms.

3.3 Phytochemical Screening of Libyan *P. harmala*

Eight phytochemical compounds were identified from the ethanolic extract of Libyan *P. harmala* seeds by using gas chromatography-mass spectrometry through matching with the National Institute of Standards and Technology (NIST) library. The 8 compounds are shown in Table 3. The compounds were harmine, harmaline, leptalorine, hexahrydrofluorene, 3-methoxy-6-methyl, 1H-pyrido[3,4-b]indole,2,3,4,9-tetrahydro-6methoxy-1-methyl, 9,12-octadienoic acid ethyl ester, linoleic acid ethyl ester, 9,12-octadienoic acid (z,z). The harmine, harmaline, and leptalorine that were found to be in the highest abundance in the ethanol extract have antioxidant and anticancer properties [33]. Several biological and pharmacological activities such as antispasmodics, antioxidants, antibiotics, antimicrobials, fungi, antineoplastic agents, cytotoxic, and antidotes have also been reported [34]. They have also been suggested to have both antidepressant and anti-inflammatory properties. Therefore, it may be a potential candidate for the treatment of depression [35]. They exhibited a dose-dependent inhibitory effect against a well-known botanical insect growth control (azadirachtin) and exhibit resistance to many insecticides [36]. It can be used analgesic and vasorelaxant [37].

Table 1. DPPH radical scavenging activity of the ethanolic extracts of seeds of libyan *P. harmala*

Sample	IC_{50} (µg/mL)	R^2
P. harmala	179.62±7.32	0.9982
Ascorbic Acid	72.76±2.17	0.9955

Table 2. Resazurin broth microdilution with extracts of *P.* Libyan *harmala* seeds

	MIC	MBC
Bacteria	(mg/mL)	(mg/mL)
Salmonella typhi ATCC 14028	31.25	62.50
E. coli ATCC 25923	31.25	62.50
B. subtilis ATCC 6633	1.95	7.80
S. aureus ATCC 29213	15.62	31.25

Table 3. Phytochemical screening of Libyan *P. harmala*

S/No	RT	Name of compounds	Peak area (%)	Quality
1	21.351	Harmine	51.98	98
2	20.115	Harmaline	42.93	99
3	19.331	Leptalorine	1.69	97
4	19.331	Hexahrydrofluorene, 3-methoxy-6-methyl	1.69	96
5	19.331	1H-pyrido[3,4-b]indole,2,3,4,9-tetrahydro-6-methoxy-1-methyl	1.69	93
6	18..602	9,12-Octadienoic acid ethyl ester	1.29	99
7	18.602	Linoleic acid ethyl ester	1.29	99
8	17.943	9,12-Octadienoic acid (z,z)	0.12	96

RT = Retention time

P. harmala was used since ancient times to treat syphilis, malaria, hysteria, neuralgia, Parkinson's disease, and rheumatism [38]. It played a vital role in antitumor activities [39]. Harmine possesses several important biological activities, including antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic and anthelmintic, anticancer, anti-hyperglycemic, organ protective, and neuropharmacological activities. Additionally, some alkaloids have been identified to exert toxic effects on animal organs [40]. Also, leptaflorine reported applications of biocatalysts in the synthesis of pharmaceuticals [41]. The 9,12-octadecadienoic acid ethyl ester was found to be the highest abundance in the ethanolic extract of the seeds of *Brachystegia eurycomia* Harms. It has been reported for its antibacterial activity against *E. coli, Salmonella typhi,* and *S. aureus* [42] as well as against nasal congestion [43]. It may possess the potential to be a medicinal drug especially in breast cancer treatment [44]. Moreover, it has anti-cancer, antioxidant, anti-inflammatory, cancer preventive antiarthritic, antibacterial antitumor activities [45]. Linolenic acid has been found in the ethanol extract and can also be a source of natural antioxidant, antidiabetic, and antibacterial agents, in addition to its anti-inflammatory activity [46]. Some of these compounds have antimicrobial, antioxidant, hepatoprotective, hypocholesterolaemia as well as cancer preventive activities [44].

4. CONCLUSION

The IC_{50} value of *P. harmala* seeds extract was 179.62±7.32 µg/mL. The MIC ranged from 1.95 to 31.25 mg/mL while the MBC ranged from 7.80 to 62.50 mg/mL. The eight compounds identified were harmine, harmaline, leptalorine, hexahydro fluorene, 3-methoxy-6-methyl, 1H-pyrido[3,4-b]indole,2,3,4,9- tetrahydro-6methoxy-1-methyl, 9,12-octadienoic acid ethyl ester, linoleic acid ethyl ester, 9,12-octadienoic acid (z,z). This study has, therefore, revealed the antibacterial efficacy of *P. harmala* crude ethanolic extract on pathogenic bacteria. Thus, it has the potential to be further developed as a substitute for chemical antioxidants and antibacterials.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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