In Vitro antibacterial activity of Yemeni medicinal plants (Ocimum basilicum and Peganum harmala) against some human pathogenic bacteria

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Abstract. This study aims to evaluate the antimicrobial potential of ethanolic and water extracts of Ocimum basilicum and Peganum harmala cultivated in Yemen against some pathogenic bacteria (Escherichia coli, Kebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus cereus and Staphylococcus aureus). The antimicrobial activities and minimum inhibitory concentrations (MIC) of the plant extracts have been determined using Agar well diffusion method. The activity was expressed as inhibition zone diameter (IZD). A significant difference was noticed in the antibacterial activities and in the values of MIC at a significant level of α = 0.05. The aqueous extract of O. basilicum caused the highest IZD (2.2±0.28 cm) against E. coli at 20%, whereas for alcoholic extract, the highest IZD (2.90±1.27 cm) was achieved against K. pneumoniae at the same concentration. The increasing of aqueous and alcoholic extract concentration reduces the ability of the solvent to extract the active compounds from plants and weakens their inhibitory effect. The MIC values were varied depending on the source of extract, the type of bacteria and the type of solvent. In conclusion, the plant extracts used in this work could be of great value as natural antimicrobials. Further studies are needed to develop new alternative pharmacological possibilities for applications.

Keywords: Pharmacological, Staphylococcus aureus, Antibacterial activity, Extracts, Peganum harmala.

1. Introduction

Recently, due to the increased use of commercial antimicrobial drugs, multiple drug resistance has been emerged in human pathogenic microorganisms [1], [2], [3]. Also, due to the high cost of synthetic drugs, medicinal plants producing natural antioxidants and antimicrobial activity against antibiotic-resistant bacteria [4], [5] have attracted high interest as an alternative source of medicinal agents, in addition to their safe presumption compared to the synthetic drug agents [3], [4], [6].

Medicinal plants and their extracts have been used for thousands of years for several purposes such as food preservatives, pharmaceuticals, natural therapies and alternative medicine. These plants are important source of natural active biochemical molecules with potential therapeutic effects [1], [7]. The antioxidant and radical scavenging activity properties of the medicinal plants are often the attributes of their therapeutic benefits [8], [9]. Thus, natural antibacterial, antioxidants, antiviral, fungicidal, cytotoxic agents have gained popularity recently. Many species of medicinal plants are also used as flavoring agents, spices and cosmetics additives [7], [10].
Basil and harmal are widely distributed in Yemen, their cheapness, and other properties enable them to be used as natural alternative medicine due to their antimicrobial and antioxidant activities [4], [11]. *Ocimum basilicum* (Lamiaceae family) is one of the more than 60 *Ocimum* species included in *Ocimum* genus and commonly known as basil [12]. The extracts of leaves and flowers in addition to essential oils of *Ocimum* plant are used as natural food additives as well as effective drugs in folk medicine and employed as a natural source of antibacterial and antioxidant agents. The plant derived essential oils are used to treat different diseases like upper respiratory tract infections, headaches, diarrhea, eye problems, skin disease, coughs, fevers, pneumonia, and conjunctivitis [7], [13], [14]. The parts of *O. basilicum* are used as aromatic, antispasmodic, digestive, carminative, gastro-enteritic, migraine, insomnia, depression, gonorrhoea, dysentery, tonic and stomachic treatment agents [1], [15].

*Peganum harmala* plant (Zygophylacea family) is one of the medicinal plants, and traditionally its burning seeds have been used as disinfectant and antiseptic agents [16], [17], [18], [19]. The roots and seeds of *P. harmala* contain several active pharmacological alkaloids. Recently, the antiviral property of alkaloids contained in the seeds of this plant has attracted considerable attention [3], [20], [21], [22]. *P. harmala* possess anthelmintic, antispasmodic, antipyretic, lactogogue, abortifient and emetic properties [9], [23], insecticidal effect, caving malaria [16], anti-histaminic, vasorelaxant effect, wound healing [24], anti-leishmanial [19], anti-oxidant activity, immuno-modulator properties [25], hypoglycemic effect [26], leukemic healing [27], analgesic, anti-inflammatory properties [28], antiviral, antibacterial and antifungal effects [29].

In Yemen, the herbal medicine is employed widely for treatment of various diseases [30]. Some investigations were published during the last decade [11], [31], [32], [33], [34], [35] about the pharmacological and chemical properties of a minority of traditionally used medicinal herbal plants. Considering to the importance of these untapped research area, the aim of this study was to evaluate the in vitro potential of two traditionally used medicinal plants (*O. basilicum* and *P. harmala*) grown in Yemen, and their hydrolytic and ethanolic medicinal extracts as antibacterial agents. This research was growing on these plants as cheap, safe, and more acceptable for people than synthetic antibiotics.

2. Materials and methods

2.1. Plant materials

*O. basilicum* and *P. harmala* plants were collected from the farms of Hadhramout, Yemen in March 2020. The two plants were free from treatment by any chemicals, and free from any defects. The leaves of *O. basilicum* and the seeds of *P. harmala* were selected for this investigation, washed, dried in room temperature, powdered with an electric mill and stored at 4°C. The extraction was mediated using 80% ethanol (BDH chemical) and water de-ionized by Milli-Q Plus system (Millipore, Bedford, USA).

2.2. Bacterial strains

In vitro antibacterial studies were carried out against five human pathogenic Gram negative and Gram positive bacteria, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*. The strains were supplied by the National Center for Public Health Laboratories, Mukalla branch, and maintained on nutrient agar (HiMedia – Mumbai – India). Bacterial identification was confirmed by the researchers in the Microbiology Laboratory of the College of Environmental Sciences and Marine Biology, Hadhramout University.

2.3. Media used

MacConkey-Agar, *Pseudomonas* Isolation Agar, Eosin methylene blue Agar, Nutrient Agar and broth, Brain–Heart Infusion Agar and broth, peptone water and Muller Hinton Agar were purchase from HiMedia – Mumbai – India, and used for inoculum preparation, preservation and confirmatory identification of bacteria and evaluation of antibacterial activity.
2.4. Preparation of inoculum
Liquid Luria broth medium (LB) was used for preparation of the bacterial inoculum which was growing in a shaking incubator at 37°C for 24 h. The actively growing inocula were adjusted to a final density of 10⁶ cfu/ml [36], [37] as initial cell counts using peptone water.

2.5. Preparation of aqueous extracts
Ninety grams of fresh leaves powder of *O. basilicum* and 90 grams of fresh seeds powder of *P. harmala* were mixed separately with deionized water (200 ml), blended for 15 min, then filtered using sterile gauze to remove the solid parts from both plants, centrifugation followed the filtration at 4500 rpm for 30 min at 20 °C. Using vacuum pump (Heidolph Instruments GmbH, Germany), the supernatant was filtrated through Whatman filter paper no. 1. The extract of each plant was collected separately and stored finally in a refrigerator at 4°C [38].

2.6. Preparation of ethanolic extracts
Fifty grams of each powdered plant material were extracted separately with 200 ml of 80% ethanol in conical flasks and left for 24 h closing with rubber corks. The resulting liquid was filtered using Whatman filter paper no. 1. The filtrates resulting from four time repeated process were concentrated in a rotary evaporator (IKA-WERKE, Germany) at 45°C to eliminate ethanol. Samples were dried in an oven 45°C (Daihan Lab-Tech Co.) for about 48 h to remove the water from the aqueous residue [39].

2.7. Determination of antibacterial activity
The antibacterial activities of the prepared alcoholic and aqueous crude extracts of both plants were determined basing on the measurement of inhibition zone diameter (IZD) by agar well diffusion method. The inoculum density of the five tested bacteria was standardized using a BaSO₄ turbidity (0.5) McFarland standard. Each standardized suspension of test bacteria was uniformly spread using a sterile cotton swab on a sterile petri dish of Muller Hinton agar (MHA) (HiMedia, Mumbai, India), separately. Using a 6 mm diameter sterile borer, six wells were made in the inoculated plates. Using 1% dimethyl sulfoxide (DMSO) (BDH chemical), five serial dilutions were prepared for the final product of each plant extract yielding concentrations of 20, 40, 60, 80 %, in addition to the final concentration 100% was used without dissolvement [40]. 90μl from each extract dilution were introduced into each well in the inoculated media using a micropipette. Sterile aqueous DMSO (1%) was used as a negative control. Under aerobic conditions, the inoculated dishes were incubated for 24 h at 36 ± 1°C. Tests were performed in duplicate. After incubation, the growth of bacteria was observed. The bacterial growth inhibition was measured in centimeters using a ruler [41], [42]. The lowest concentration of each extract which completely inhibited microbial growth is known as MIC value.

2.8. Statistical analysis
The resulted values of growth inhibition zone diameters evaluated with the crude extracts were expressed as mean value ± standard deviation. Statistical differences between the antibacterial activities of aqueous and alcoholic extracts of each plant were detected using SPSS program, the analysis of variance (Two way-ANOVA) followed by LSD test were conducted to know the differences between the types of bacteria and the dimensional comparisons between the ratios of the aqueous and alcoholic extract concentrations. P value lower than 0.05 (p < 0.05) was considered significant.

3. Results
The antibacterial activity of *O. basilicum* and *P. harmala* extracts (aqueous and alcoholic) was tested in the current study, and their potency was evaluated by measurement of IZD. The antagonistic activity of tested materials varied according to the type of plant, the extraction solvent, employed concentration and bacterial species.
3.1. Antibacterial effects of aqueous and alcoholic extracts of *O. basilicum*

In general, *O. basilicum* extracts were more effective against Gram negative bacteria comparing to Gram positive. The highest activity (IZD 2.30 cm) was recorded against *P. aeruginosa* using the aqueous extract at 40% concentration, but not with other concentrations. The same plant extract showed activity against *K. pneumoniae* at all tested concentrations with variable degrees; the highest IZD (2.30±0.14 cm) was recorded at 80% concentration. This aqueous extract showed inhibitory effect against *E. coli* (IZD 2.2±0.28 cm) at 20% concentrations, but no detectable activity was occurred at concentrations of 60% to 100%. The activity was less pronounced against Gram positive bacteria, only at 20% and 40% of aqueous extract of *O. basilicum*, the mean IZD (1.0±0.0 and 0.85±0.07 cm) were recorded against *B. cereus*. Surprisingly, the highest antibacterial effect of aqueous extract (IZD 1.15±0.21) against *S. aureus* was noticed at the lowest concentration used (20%) but a negative effect was observed at the highest concentration (100%) (Table 1).

Table 1. Average values of inhibition zone diameter (IZD) resulting from the impact of different concentrations of basil aqueous and alcoholic extracts.

| Concentration (%) | Type of extract | *E. coli* | *P. aeruginosa* | *K. pneumoniae* | *B. cereus* | *S. aureus* |
|-------------------|-----------------|----------|----------------|----------------|-------------|--------------|
| 20                | Aqueous         | 2.2±0.28 | 0.0            | 1.1±0.14       | 1.0±0.0     | 1.15±0.21    |
|                   | Alcoholic       | 0.0      | 0.0            | 2.9±1.27       | 0.9±0.0     | 1.35±0.64    |
| 40                | Aqueous         | 0.75±0.21| 2.30±0.14      | 1.35±0.07      | 0.85±0.07   | 0.90±0.14    |
|                   | Alcoholic       | 1.0±0.0  | 0.0            | 1.8±0.14       | 0.0         | 0.80±0.0     |
| 60                | Aqueous         | 0.0      | 0.0            | 1.1±0.14       | 0.0         | 0.90±0.0     |
|                   | Alcoholic       | 0.90±0.14| 0.0            | 1.55±0.07      | 0.0         | 0.80±0.0     |
| 80                | Aqueous         | 0.75±0.21| 0.8±0.0        | 0.85±0.07      | 0.0         | 0.95±0.07    |
|                   | Alcoholic       | 0.0      | 0.0            | 1.05±0.07      | 0.0         | 0.0          |
| 100               | Alcoholic       | 0.7±0.14 | 0.8±0.0        | 1.7±0.28       | 0.0         | 0.80±0.0     |

The mean ± standard deviation of the mean values of the diameters of the bacterial inhibition zones.

The alcoholic extract of *O. basilicum* exhibited antibacterial activity against *E. coli* at all concentrations except at 20%, while its activity against *P. aeruginosa* was only observed at high concentrations (80% and 100%) recording average IZD of 0.8±0.0 cm for both concentrations. On the other hand, growth of *K. pneumoniae* was inhibited by all tested concentrations with variable degrees, the highest activity (IZD 2.90±1.27 cm) was recorded when the lowest concentration (20%) was used. In respect to Gram +ve bacteria, *B. cereus* was only inhibited at the lowest concentration of *O. basilicum* (20%) resulting an IZD of 0.90±0.0 cm. In case of *S. aureus*, this alcoholic extract affected the bacterial growth with different degrees, the largest IZD (1.35±0.64 cm) was achieved at 20% concentration, while the lowest activity was achieved at 40% and 60% concentrations (Table 1).

3.2. Antibacterial effects of aqueous and alcoholic extracts of *P. harmala*

The antibacterial activity of *P. harmala* aqueous and alcoholic extracts was expressed against all tested pathogens (Table 2). Growth of *E. coli* was only affected by concentrations above 20%. The measured IZDs were ranged between 1.0 cm and 0.7 cm. On the contrary, growth of *P. aeruginosa* was only inhibited by the lowest alcoholic used extract (20%) and by the aqueous extract (80%) causing IZDs of 1.15 and 0.85 cm, respectively, and not affected by other concentrations. The growth of *K. pneumoniae* was inhibited by all tested concentrations of both extracts with considerable IZDs ranging from 1.0 to 2.45 cm. Considering to the Gram+ bacteria, it was noticed that *B. cereus* was only inhibited by the aqueous extract of 20%, whereas alcoholic extract of the same plant caused inhibition at concentrations of 20, 40 and 60%. The aqueous and alcoholic prepared extracts of *P. harmala* showed considerable effect against the growth of *Staphylococcus aureus* at all tested concentrations with IZDs ranging from 0.9 to 1.95 cm for aqueous extracts and from 2.0 to 3.5 cm for alcoholic extracts (Table 2).
Table 2. Average values of inhibition zone diameter (IZD) resulting from the impact of different concentrations of aqueous and ethanolic extracts of harmal plant.

| Extract concentration (%) | Type of extract | Diameter of inhibition zone(cm) |
|--------------------------|----------------|---------------------------------|
|                          |                | E. coli | P. aeruginosa | K. pneumonia | B. cereus | S. aureus |
| 20                       | Aqueous        | 0.0     | 0.0           | 1.05±0.07    | 0.80±0.0  | 0.90±0.14 |
|                          | Alcoholic      | 0.0     | 1.15±0.07    | 2.45±0.07    | 0.90±0.0  | 2.0±0.28  |
| 40                       | Aqueous        | 0.90±0.0| 0.0           | 1.05±0.07    | 0.0       | 1.35±0.21 |
|                          | Alcoholic      | 0.7±0.14| 0.0           | 2.45±0.07    | 0.95±0.07 | 2.5±0.14  |
| 60                       | Alcoholic      | 1.0±0.14| 0.0           | 1.0±0.28     | 0.0       | 1.9±0.0   |
|                          | Aqueous        | 0.90±0.14| 0.0          | 1.80±0.28    | 0.95±0.07 | 2.55±0.07 |
| 80                       | Alcoholic      | 0.90±0.14| 0.0          | 1.65±0.21    | 0.0       | 3.40±0.85 |
|                          | Aqueous        | 0.75±0.07| 0.0          | 1.2±0.0      | 0.0       | 1.95±0.07 |
| 100                      | Alcoholic      | 0.95±0.07| 0.0          | 1.55±0.50    | 0.0       | 3.50±0.07 |

The mean ± standard deviation of the mean values of the diameters of the bacterial inhibition zones.

3.3. Values of Minimum Inhibitory Concentrations, MIC
The values of minimum inhibitory concentrations varied depending on the different types of bacteria and plants. In general, the lowest MIC value (20%) was depicted with the Gram positive bacteria compared to Gram negative using both extracts (Figure 1 and 2), the same MIC value was observed for aqueous extract of O. basilicum against E. coli and for alcoholic extract of P. harmala against K. pneumoniae. The highest MIC value (80%) was recorded in this study against P. aeruginosa using aqueous extract of P. harmala and alcoholic extract of O. basilicum. Average MIC values (40%) were obtained against E. coli using the alcoholic extract of both plants and aqueous extract of P. harmala (Figure 1 and 2).

Figure 1. Minimum Inhibitory Concentrations (MICs) of O. basilicum extracts against tested pathogens.
4. Discussion

Despite the great progress in the field of medicine, many bacterial diseases still pose a serious threat to human health, and this may be due to the emergence of many strains of bacteria that are resistant to antibiotics as a result of indiscriminate and excessive use of antibiotics [43]. Therefore, it was necessary to search new antimicrobial natural and ecofriendly compounds extracted from plants [44].

It was aimed, in this work, to assess the potency of the aqueous and ethanolic extracts of two medicinal herbs (basil and harmal) available in Yemen as antimicrobial agents. The extracts were tested against some Gram +ve and Gram-ve pathogenic bacteria. The antagonistic activity of prepared extracts was dependent on the solvent used for extraction and on the concentration of extracted material. Similar datum was found by Al-Maeeny et al. [45] and Al-Bayati and Sulaiman [36].

In the current work, the ethanolic extract of basil plant showed antagonistic effect against K. pneumoniae higher than that of aqueous extract at all tested concentrations except 80%. These results agree with the study of Al-Maeeny et al. [45] who found that no antimicrobial effects for the aqueous extract of O. basilicum leaves against the bacteria under the study, they attributed this result to the nature and properties of the main components present in the aqueous extract of O. basilicum and to the degree of polarity. The same results were obtained by Zakaria et al. [46] who reported that the alcoholic extract of leaves and stems of basil displayed the highest antibacterial activity. Other researchers like Sawar [47] attributed the appearance of alkaloids in the alcoholic plant extracts and their disappearance in aqueous extracts due to the fact that the alkaloids require heating during extraction, or because they do not dissolve in water and only dissolve in alcohol. In contrast to our data, Adiguzel et al. [48] studied the antibacterial activity of O. basilicum L. extracted using different solvents, they found that all extracts inhibited the growth of bacteria except the ethanolic extract.

The antimicrobial activity of alcoholic extract of P. harmala against S. aureus and K. pneumoniae was higher than all concentrations of aqueous extract because the active substances (chemical compounds) included within P. harmala plant can be dissolved in ethanol but not in water. Furthermore, Baradaran and Jalali [6] recorded that the ethanolic extract of harmal had inhibitory effect against MRSA. Our results agree with the results of Darabpour et al. [17] who recorded that the alcoholic extract of roots and seeds of P. harmala exhibited the highest antimicrobial activity against B. cereus, S. aureus, P. aeruginosa, E. coli and K. pneumoniae even at the lowest concentration. Inhibition zone diameter of 1.01 cm was estimated by Ghasemi and Atakishiyeva [49] as an inhibition effect of P. harmala extract (500 μg/mL) against K. pneumoniae.

The inhibition zone diameters conducted by alcoholic P. harmala extract against S. aureus were estimated between 3.40±0.85 cm and 3.50±0.07 cm at concentrations of 80% and 100%, respectively, this result agree with the study of Edziri et al. [9]. Our study showed that the growth of tested bacteria was inhibited by alcoholic P. harmala extract even at its lowest concentration (20%), but the
The antibacterial effect of aqueous extract was reduced or absent at the same concentration, due to the difference of secondary metabolites and chemical compounds in the extract such as terpenoids, alkaloids, flavonoids, sterols, derivatives of fatty acids, fluorides, tannins, resin and others having the ability to inhibit the growth of a number of Gram negative and Gram positive bacteria as a result of deprivation of substrate, disruption of membrane, binding to adhesins and proteins complex with cell wall, inactivation of enzymes and proteins [50], [51], [52], [53]. There are other factors affecting on the antimicrobial activity of plant extracts such as the type of active ingredients of the extract, the age of the plant and its storage and drying conditions as clarified by Mitscher et al. [54]. The difference of antimicrobial effect may be also attributed to the type of studied bacteria and to the differences in bacteria species in terms of their cellular nature, composition and the genetics of the target on which the extract is working, as reported by Frazier and Westhoff [55].

The current study indicated that there was a significant effect (P<0.05) in the relation between the average values of IZDs and the affecting concentrations of the basil extract against all tested bacteria except K. pneumonia (P<0.05), and there was a significant effect (P<0.05) in the relation between the average values of IZDs and the affecting concentrations of the harmal extract against all tested bacteria, there was a significant effect (P<0.05) in the relation between the average values of IZDs and the types of the dissolved extracts of basil and harmal (aqueous or alcoholic) against all tested bacteria except E. coli (P>0.05). A significant effect (P<0.05) occurred for the treatments of the type of the dissolved extracts of basil and harmal with their concentrations to the IZDs of all tested bacteria. The current study indicated that there were significant differences (P<0.05) between the average values of IZDs depending on the extract types of basil and harmal (aqueous or alcoholic) inhibiting all tested bacteria except E. coli, and there were significant differences (P<0.05) between the average values of IZDs depending the concentrations of the basil extract inhibiting all tested bacteria except K. pneumonia and between the average values of IZDs depending the concentrations of the harmal extract inhibiting all tested bacteria without exceptions.

In the current study, a significant difference was noticed in the antibacterial activities of the different plant extracts against the growth of different types of bacteria under study and in the values of the Minimum Inhibitory Concentration (MIC) at a significant level of α = 0.05, the same results were obtained by Kaya et al. [1] and Silva et al. [14]. This may be attributed to the variance of the secondary metabolites in the different sources of the plant extracts (O. basilicum and P. harmala), the different concentrations (20, 40, 60, 80, and 100 %), and the type of solvent used in the extraction (water and ethanol). Our results agree with the study of Amiri and Fozouni [3] who reported that the MIC of the harmal aqueous extract was significantly lower than that of the ethanolic extract (P<0.05), because the 8-hydroxy peganine and vasicine/peganine were mostly abundant bioactive compounds in the aqueous extract of P. harmala. The study of Al-Naqeb [4] showed that the methanolic extracts of Yemeni P. harmala inhibited the growth of B. subtilis and did not inhibit the growth of E. coli while Omoregba et al. [56] reported that the extract of O. basilicum had antibacterial activity against E. coli more than other bacteria.

In comparison to the study of Mostafa et al. [59] who employed 5 mg of Gentamycin contained in filter paper discs as a positive control against E. coli, P. aeruginosa, B. cereus and S. aureus and led to results of 1.56, 1.31, 1.68, 2.05cm as IZD values, the antibacterial activity of the plant extracts used in current study competed these results of Mostafa et al. [59] after use of Gentamycin as a positive control. Ghasemi and Atakishiyeva [49] reported an IZD value of 1.9 cm to indicate the sensitivity of K. pneumonia toward the antibiotic of tetracycline.

The above described reasons led to differences among the MIC values depending on the source of extract, the type of bacteria and the type of solvent. It is worth mentioned that the lowest MIC value (20%) appeared in the case of K. pneumoniae, B. cereus and S. aureus using the extracts of basil and harmal, and the higher MIC value (80%) appeared with P. aerogenosa using the alcoholic O. basilicum and aquatic P. harmala extracts. Our results resemble to some extent the results obtained by Gaio et al. [57] who reported that all tested bacteria were inhibited by the essential oil of O. basilicum except P. aeruginosa. Silva et al. [14] found that certain strains of P. aeruginosa were resistant to the essential oil of O. basilicum, but in the studies of Nascimento et al. [58] and Kaya et al. [1], the basil alcoholic extract had antibacterial activity only against P. aeruginosa.
The current results justified the use of some investigated plants; *O. basilicum* and *P. harmala* in the Yemeni ethnic medicine, and the present findings demonstrated that the investigated Yemeni plants "*O. basilicum* and *P. harmala*" could be used as natural alternative to synthetic antibiotics.

5. References

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