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Evaluation of Moringa Peregrina (Forsk) Fiori, Leaf and Seed Extract Against Multidrug Resistant Strains of Bacteria and Fungus of Clinical Origin

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Abstract

The emergence of antibiotic resistant microorganism strains has become a critical concern in the treatment of infectious diseases and makes the search of an alternative therapy a must. The study was designed to evaluate the in vitro antimicrobial activities of the *Moringa peregrina* (MP) leave (MPL) and seed (MPS) extracts. Antimicrobial assays were performed using a microplate growth inhibition assay against 11 multidrug-resistant (MDR) strains. Following qualitative analysis, dose-response assays were performed using the MTT colorimetric assay. The results showed a strong correlation between the MPL and MPS extract concentration and growth inhibition ($P<0.001$). MP extract revealed a remarkable antimicrobial effect and inhibited the growth and survival of MDR pathogens which include *Escherichia coli; Pseudomonas aeruginosa; Klebsiella pneumonia; Acinetobacter baumannii; Staphylococcus aureus* between (88.6-94.7 %) and between (62.3- 88.7%) against *Candida Kefyer; Candida parapsilosis; Candida albicans; Candida glabrata; Aspergillus flavus and Fusarium oxysporum*. MIC_{50} ranging from ≤6.25 to 25 mg/mL. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were the most susceptible to MP extracts (MIC50 < 6.25 mg/mL). These results support the use of MP in Arab traditional medicine as natural antimicrobial agents. Additionally, the use of such naturally occurring adjuvant derived from medicinal plants can be used as an adjuvant with synthetic antibiotics to combat bacterial resistance and to enhance the antibacterial potential. Further studies are recommended on isolation and purification of novel antimicrobial molecules to treat the infections caused by microbes.

1. Introduction

Worldwide, infectious diseases are a significant cause of morbidity and mortality with the World Health Organization (WHO) estimating it to account for 50% of all deaths in tropical countries. The current increase in health casualties associated with bacterial or fungal infections is because of treatment failures related to the growing bacterial resistance to most anti-infective agents that greatly lessen their efficacy **(Tchana *et al.*, 2014)**. Thus, widespread multidrug-resistant (MDR) strains of bacteria necessitate a regular substitute of new drug sources for the effective treatment of infectious diseases **(Khan *et al.*, 2009)** which includes newer classes of antibacterial from either synthetic or natural sources and inhibit these resistance mechanisms **(Soares *et al.* 2019)**. One of the most effective, safe and reliable sources of antimicrobial agents are medicinal plants and their metabolites or derivatives **(Lalas *et al.*, 2012)**. For instance, an appropriate combination of antibiotics and natural antimicrobial substances are potential approaches for combating MDR microorganisms **(Breijyeh *et al.*, 2020)**. Hence, there is a great need for new sources of antimicrobial agents to fight MDR strain infection and the WHO has recommended the member states to develop effective drugs to fight against this issue **(Silver and Bostian, 1993)**. The genus Moringa, called miracle tree is a member of the family Moringaceae. The species *Moringa peregrina* (MP) drumstick or alyusr tree is widely grown and cultivated in Saudi Arabia. MP has been used since ancient times and in many culture and traditions as a food as well as a medicinal plant owing to its medicinal value. All parts of *M. peregrina* are known to possess antibacterial activity **(Saleh *et al.*, 2017)**. The leaves and roots decoction is used for the treatment of malarial fever, stomach ailments, to regulate and control high blood pressures and hyperglycemia **(Elbatran *et al.*, 2005)**. The tender leaves are used to accelerate wound healing process **(Mekonnen *et al.*, 1999)**. In Saudi Arabian folk medicine, *M. peregrina* is used for the treatment of various disease conditions such as skin diseases, respiratory troubles, oral and ear infections, diabetes, anaemia and certain **cancers (Nawash & Al-Horani, 2011; Patel *et al.*, 2010; Emmanuel *et al.*, 2014; Kalkunte *et al.*, 2006; Jung *et al.*, 2014; Tiloke *et al.*,2013)**. In some earlier studies, various parts of the MP have been shown to possess antibacterial activity **(Al_husnan and Alkahtani, 2016)**. In recent years, *M. peregrina* is gaining added recognition due to its traditional, nutritional, industrial and medicinal values. Since various parts of this plant have a wide range of therapeutic uses, thus, it has been biologically screened

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(Anwar and Rashid, 2007). Earlier, an anti-cancer potential of Moringa leaves and bark extract has been reported **(Al Asmari *et al*., 2015)**. Moreover, a broad range of biological activities are also attributed to Moringa Spp. **(Senthilkumar *et al*., 2018)**; such as antidiabetic **(Rao *et al*., 2001)**, antispasmodic **(Sadraei *et al*., 2015)**, antioxidant **(Taniyama & Griendling, 2003), antimicrobial (Saleh *et al*., 2017)**, anti-hepatotoxic **(Elabd *et al*., 2017)**, anticholesterolemic **(Rouhi-Broujeni *et al*., 2013)**, anti-gastric ulcer (**Senthilkumar *et al*., 2018)**, anti-phlogistic **(Koheil *et al*., 2011)**, and memory-enhancing activities **(Elsaey *et al*., 2016)**. Although, various biological activities of *M. peregrina* have been exhaustively studied; the present investigation was undertaken of locally grown *Moringa peregrina* ethanol extract of leaves and seed kernel for their antimicrobial activity on multidrug-resistant bacteria and fungi isolates.

2. Material and methods

2.1 Plant material collection and extraction

The fresh leaves (L) and seeds (S) of *Moringa peregrina* were collected from Riyadh area. They were identified and authenticated by an expert taxonomist and a voucher specimen has been deposited at the CAM division of the center for future reference.

2.1.1 Preparation of leaves extract

Shade dried leaves of MP were coarsely powdered in an electric blender; extracted with 96% ethanol using soxholet apparatus. The solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) to get semi solid viscous mass. Extract thus obtained was preserved at 4°C until further use.

2.1.2 Preparation seed extract

The dried MP seeds coat were removed manually and grinded to powder, soaked in 96% of ethanol and continuously shaken for 24 h. The filtrate was collected and filtered through Whatman filter paper. The solvent was eliminated under reduced pressure using rotary evaporator (Buchi, Switzerland).

2.2 Evaluation of antimicrobial activity

In this study, we focused upon the effect of *Moringa peregrina* extracts from leaves (MPL) and seeds (MPS) to observe its efficacy as an anti-microbial agent on a panel of 5 bacterial strains belonging to Gram-negative and positive bacteria including multidrug-resistant strains *Escherichia coli* (*E. coli*) ATCC 25922, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC27853, extended spectrum beta lactamase producing *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 700603 and *Acinetobacter baumannii* (*A. baumanii*) clinical isolate and multidrug resistant *Staphylococcus aureus* (*S. aureus*) ATCC 25923; and (4) Pathogen yeast including *Candida Kefyer* (*C. kefyer*), *Candida parapsilosis* (*C. parapsilosis*) *Candida albicans* (*C. albicans*) and *Candida glabrata* (*C. glabrata*); and 2 molds including *Aspergillus flavus* (*A. flavus*) and *Fusarium oxysporum* (*F. oxysporum*). The ATCC strains were obtained from the American Type Culture Collection. *Acinetobacter baumannii*, and candida species were clinical isolates, a generous gift from the Medical Microbiology Division, Prince Sultan Military Medical City, Riyadh, Saudi Arabia.

2.2.1 Bacterial strains and antimicrobial activity

Bacterial species were Sub-cultured and maintained in Tryptic Soy Broth (TSB; Oxoid Ltd, Basingstoke, UK). at 4°C while, yeast was sub-cultured and maintained in Sabouraud Broth (SB; Oxoid Ltd, Basingstoke, UK) at 4°C. The microbial inoculums (bacterial, fungal) were prepared from an overnight culture, diluted in 0.85% NaCl to achieve 0.5 McFarl and (108 cells/mL). The suspension inoculum was carried out in MicroScan Inoculum water (Siemens Healthcare Diagnostics Inc. USA) from a colony alone. This suspension, after shaking in vortex by 15s was adjusted to 0.5 of McFarland scale, resulting in a concentration of $1x10^6$ CFU/mL, diluted, 1:10 in TSB for bacterial strains and in RPMI 1640 medium with GlutaMAX™ supplement (Gibco, Life Technologies, NY, USA) for yeast and fungi strains. The *Moringa pregrina* extracts dissolved in 2.5% dimethyl sulfoxide (DMSO), which is maximum volume of DMSO that could be used to dissolve solid extracts, were first dilution to the final concentration (200 mg/ml) for each extract and then serial two-fold dilution was made in concentration range 10~200 mg/ml in 10 ml sterile test tube containing 2.5% DMSO. The solvent DMSO (2.5%) that would not inhibit growth of the microorganisms was used as the negative control for all the experiments.

2.2.2 *Agar dilution method*

Two-fold serial dilutions of *Moringa peregrina* ethanol extract (leaves, barks and seeds) were made in molten TSA or SDA medium cooled down to 45°C to obtain the desired final concentrations. Bacterial suspensions (0.1 mLwith10^6 CFU/mL) were then inoculated on solid TSA or SDA. Agar plates were incubated aerobically at 37°C for 48 h for all tested organisms. Negative controls included ethanol in amounts corresponding to the highest quantity present in the agar dilution assay. Inoculated agar plates without added plant extract served as positive controls and the negative control contained DMSO in the concentration used to dilute the extracts. As antimicrobial control, we used three agents of different classes: Amphotericin B, Ampicillin and Gentamicin.

2.2.3 Growth inhibitory assay

In vitro susceptibility of bacterial and yeast isolates was performed using broth micro-dilutions according to the methodology recommended by the Clinical and Laboratory Standards Institute – CLSI in M27-A3 protocol (2008) and NCCLS/CLSI in M2–A9 and M7–A7 (2007). The cells were seeded in 96-well plates at a density of 3×10^8 cells/well and treated with *M. peregrina* leave and seed extracts concentrations ranging 1.5-100 mg/ml, and incubated at 35 ± 2°C, for 24 h. Microbial growth was detected former by optical density at the end of the exposure period and after by addition of 20 µL of a solution at 5 mg/mL of 3-(4, 5-imethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to each single well and incubation for another 3 h at 35°C. The plates were read at absorbance of solubilized MTT in (HIDEX Oy, Turku, FINLAND) at 570 nm. Positive growth controls were inoculated as described above, without addition of plant extract. Viable bacteria are quantified by measuring cleavage of the yellow tetrazolium salt MTT into purple formazan in the presence of metabolically active bacterial/fungal cells. Inhibition (100%) was calculated as follows: [(Initial control absorbance - final absorbance) / (Initial control absorbance)] x 100. Determination of the concentration of MP extract causing 50% inhibition (MIC50) in reduction of the dye was calculated from the regression curve generated using GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA). The results were recorded as means ± SE of the triplicate experiment.

2.3 Data analysis

The results were expressed as mean ± standard error of mean (SEM), and statistical comparisons were made using analysis of variance (ANOVA) by Tukey test to compare means. A value of $P \leq 0.05$ indicated significance.

3. Results and Discussion

The results of this study validates the effects of ethanol extract of the leaves and seeds of *Moringa peregrina* (MP) as antimicrobial agents to fight various pathogens in Saudi folk medicine. In the present investigation, ethanol extracts of *Moringa peregrina* leaves and seeds clearly showed favorable antibacterial and antifungal activities on the tested bacteria including Gram-negative and Gram-positive and multidrug-resistant bacterial strains using various techniques. The antibacterial activity of leaf and seed extracts have exhibited significant inhibitory effects on the growth of a wide range of microorganisms. Surprisingly, the moringa extracts used in this study was found to be more effective than synthetic antibiotics. The obtained data on the growth of 11 multidrug-resistant (MDR) pathogens and yeast showed significantly potent antibacterial activity, this inhibitory effect recorded as dose-dependent in the culture media. Results are shown in the figure 1 & figure 2.

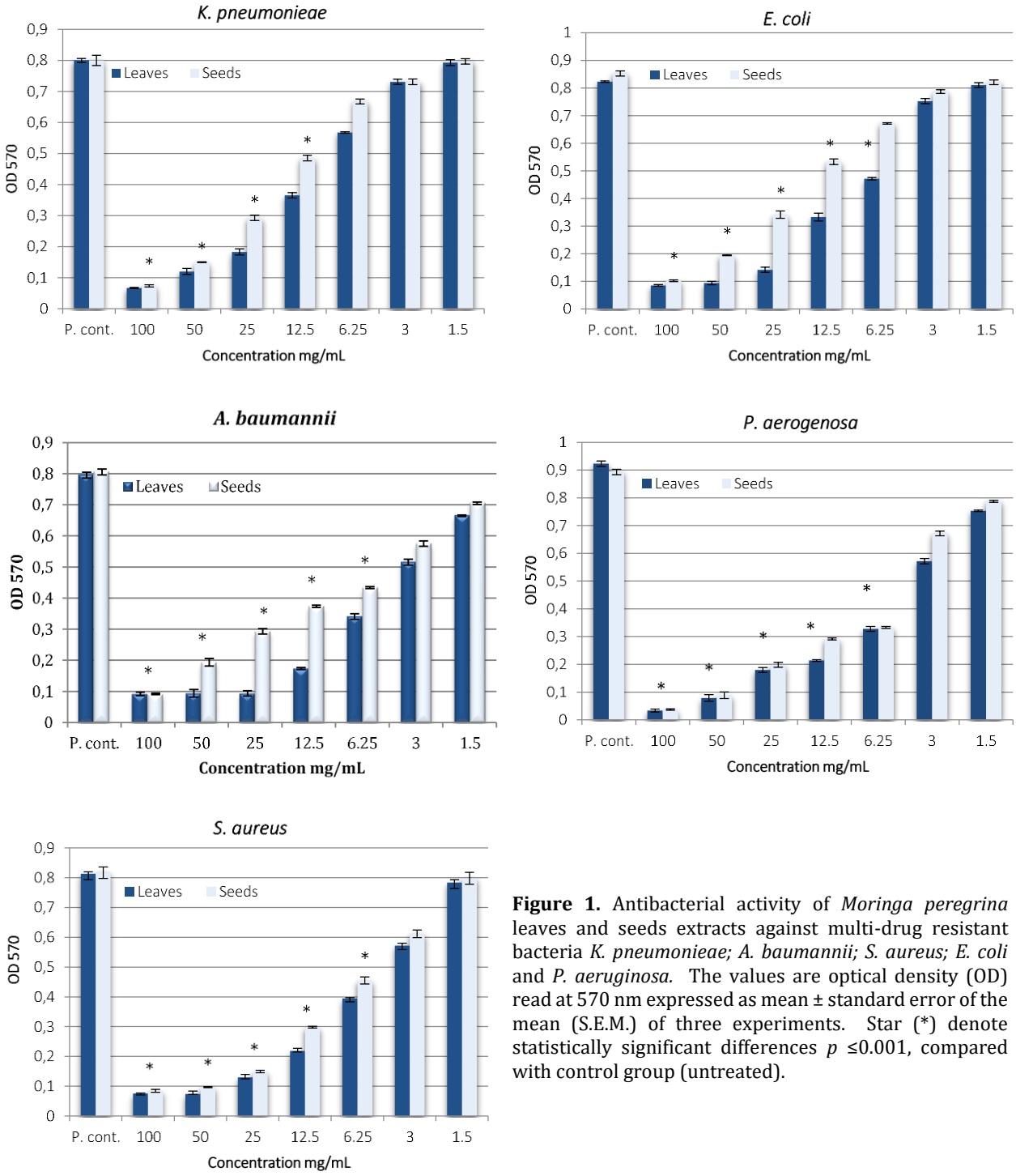

Figure 1. Antibacterial activity of *Moringa peregrina* leaves and seeds extracts against multi-drug resistant bacteria *K. pneumonieae; A. baumannii; S. aureus; E. coli* and *P. aeruginosa.* The values are optical density (OD) read at 570 nm expressed as mean ± standard error of the mean (S.E.M.) of three experiments. Star (*) denote statistically significant differences $p \leq 0.001$, compared with control group (untreated).

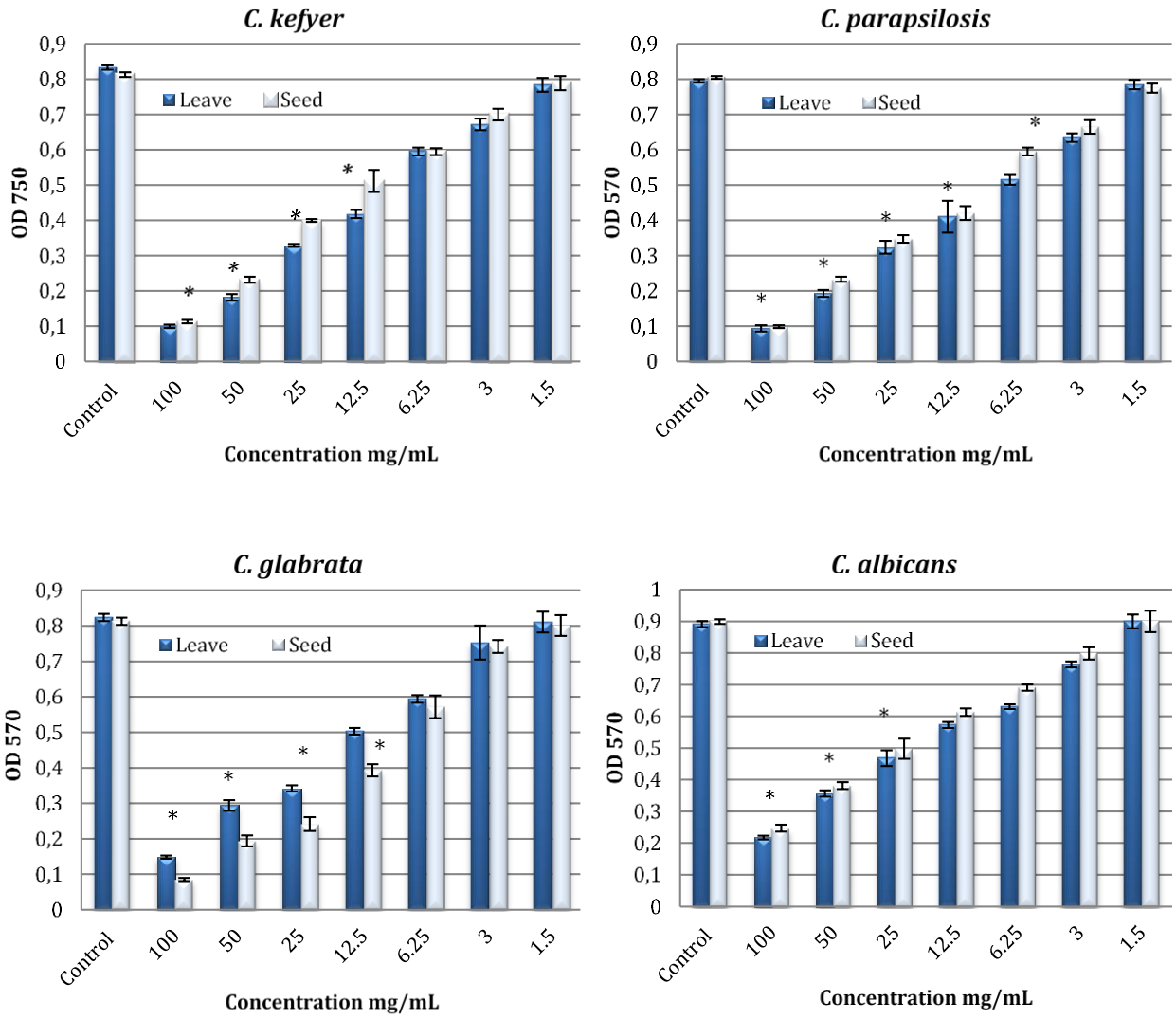

Figure 2. Antifungal activities of Moringa peregrina leave and seed extracts against multi-drug resistant yeast. The values are optical density (OD) read at 570 nm expressed as mean ± standard error of the mean (S.E.M.) of three experiments. Star (*) denote statistically significant differences p ≤0.001, compared with control group (untreated with extract of M. peregrina).

In various countries of Africa and elsewhere, Moringa species have been extensively used to purify water for antiseptic water treatment because of its potent antimicrobial activity **(Rani *et al.*, 2018)**. Innumerable naturally occurring bioactive compounds found in plants, herbs, fruits, vegetables and spices have been shown to possess antimicrobial properties and used as a source to destroy pathogens **(Kumar *et al.*, 2006)**. *Moringa peregrina* is reported to contain a wide range of chemical constituents including flavonoids **(Al-Owaisi *et al.*, 2014)** in the leaves, while arachidonic and linoleic acid, saturated and unsaturated fatty acids and isothiocyanates are present in the seed kernel **(Somali *et al.*, 1984)**.

Our results showed a strong correlation between the MPL and MPS extract concentration and growth inhibition (P<0.001). *Moringa peregrina* extracts revealed a remarkable antimicrobial effect and inhibited the growth of almost all the tested strains in the concentrations ranging from 6 to 100 mg/mL (Tab 1). Among the Gram-negative and gram-positive tested bacteria, *A. baumannii* and *P. aeruginosa* were the most susceptible to *M. peregrina* leaves and seeds extracts (MIC_{50}<6.25) followed by *E. coli* (MIC_{50}≤6.25), S. *aureus* (MIC_{50}≥6.25) and *K. Pneumoniae* (MIC_{50}≤12.5). Results are shown in the Figure 1 and Table 1.

In recent years, the use of natural compounds that are derived from microbials, animals or plants have been shown to possess various antimicrobial activities **(Gyawali *et al.*, 2014; Moloney, 2016)**. The reported phytochemical components in this species include flavonoids, flavanol glycosides, rutin, quercetin, apegenin, glucosinolate and isothiocyanate, phenolic acid, β-sistosterols, alkaloids (lupeol), besides arachidonic and linoleic acids **(Rani *et al.*, 2018)**.

In general, the antibacterial activity of an agent, whether synthetic or natural, is largely attributed to a couple of mechanisms; by hampering chemically the synthesis and/or functions of essential ingredients of the pathogen. Secondly, by preventing the usual mechanisms of antibacterial resistance **(Khameneh *et al.*, 2016; Shakeri *et al.*, 2018)**. The main

mechanism of antibacterial action of these substances is by bacterial protein biosynthesis (by inhibition of protein synthesis) **(Walsh, 2000)**. Thirdly, the biosynthesis of bacterial cell walls as the cell wall layer poses as a valid target for antibacterial agents that consequently weaken the cell wall **(Schneider *et al*., 2010)**. Furthermore, the inhibition of nucleic acid synthesis, as the enzyme of DNA gyrase is essential for synthesis, repair, replication and transcription process and considered as a suitable target for antibacterial agents. The gyrase enzyme is responsible for coiling and uncoiling of bacterial DNA and replication of DNA (**Maxwell, 1997)**. On the other hand, antifungal potential of the *M. peregrina* among tested fungi strains seem to be high on *C. albicans* (MIC_{50}≤12.5mg/mL) whilst, it has weak activity on *F. oxysporum* and *A. falvus* (MIC_{50}≥50mg/ml) (Tab 1). The results of antimicrobial activity obtained with the extracts of leaves and seeds of *Moringa peregrina* is in accordance of earlier studies **(Majali *et al*., 2015; Hajar and Gumgumjee, 2014; Saleh *et al*., 2017; El-Awady *et al*., 2015; Alrayes *et al*., 2019)**. The results are tabulated in Table 1. The obtained potent antibacterial and antifungal activity of the moringa leaf and seed extract is due to the presence of an array of bioactive molecules that play an important role in enhancing antibiotic activity against resistant pathogens through diverse mechanisms **(Farhadi *et al*., 2019; Mansour *et al*., 2019; Muhuha *et al*., 2018)**. The bioactive constituents of MP are regarded as potent antioxidants besides their multiple pharmacological activities **(Dehshahri *et al*., 2012)**.

Table 1. Inhibitory activity of *Moringa peregrina* ethanol extracts of leaves and seeds on the growth and survival of multi-drug resistant pathogens at different extract concentrations ranged from 1.5 -100 mg/mL

		% Inhibition [a]						
Pathogen	**Extracts**	100	50	25	12.5	6.25	3	1.5
Klebsiella pneumoniae	Leaves	94.29	89.63	77.13	58.33	42.04	28.63	1.88
	Seeds	88.67	78.13	63.38	49.33	16.54	8.63	1.37
Acinetobacter baumannii	Leaves	91.07	89.18	86.50	78.15	60.15	35.21	19.41
	Seeds	89.57	78.91	69.58	59.31	52.89	38.56	12.48
Pseudomonas aeruginosa	Leaves	94.95	89.29	79.80	69.67	57.96	40.06	18.45
	Seeds	93.25	88.10	75.91	61.73	53.31	34.78	21.84
Escherichia coli	Leaves	91.67	88.58	82.78	64.54	51.68	22.54	1.42
	Seeds	89.61	79.43	63.48	55.24	38.38	14.34	0.87
Staphylococcus aureus	Leaves	94.71	89.82	80.07	71.19	50.41	39.67	13.57
	Seeds	93.67	86.10	78.70	69.53	44.31	35.12	12.33
Candida kefyr	Leaves	88.61	79.43	66.48	55.24	38.38	4.34	0.27
	Seeds	86.67	74.58	59.78	48.54	39.68	21.54	9.09
Candida parapsilosis	Leaves	81.51	72.36	64.25	50.77	41.45	25.75	6.31
	Seeds	84.71	70.90	59.07	50.19	38.41	22.67	9.57
Candida albicans	Leaves	87.67	79.10	71.70	51.53	38.31	25.12	3.33
	Seeds	88.83	81.09	72.38	57.33	39.42	19.63	8.88
Candida glabrata	Leaves	80.46	70.64	61.40	50.28	37.20	15.54	1.19
	Seeds	85.91	71.15	62.78	54.17	42.53	24.61	2.47
Aspergillus flavus	Leaves	63.97	51.58	43.97	41.18	20.22	9.89	1.73
	Seeds	65.23	54.67	41.21	32.78	22.53	12.95	0.84
Fusarium oxysporum.	Leaves	63.87	51.58	42.07	31.48	23.25	11.71	1.31
	Seeds	62.25	60.91	41.04	34.17	21.96	13.01	3.25

[a] Inhibition (100%): [(Initial control absorbance - final absorbance) / (Initial control absorbance)] x 100.

5. Conclusion

These results support the use of MP in Arab traditional medicine as natural antimicrobial agents. Additionally, the use of such naturally occurring phytochemical components can be used adjacent with synthetic antibiotics to combat bacterial resistance and to enhance the antibacterial potential. Further studies are recommended on isolation and purification of novel antimicrobial molecules to treat the infections caused by microbes.

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Declaration of interest

The authors declare that they have no competing interest.

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