The Antibacterial and Antioxidant Activity of *Moringa Oleifera* Seed Oil Extract Against Some Foodborne Pathogens

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Abstract

Moringa oleifera is one of the most important and useful plants because it is both a medical and nutritional plant. The aim of this study is to determine the antioxidant and antibacterial activity of *Moringa oleifera* seed oil extract. Petroleum ether solvent in Soxhlet apparatus was used to prepare the seed oil. Many tests were conducted included, Gas Chromatography-Mass Spectrum (GC-MS), evaluation of antioxidant activity utilizing 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay, total phenolic content. The oil extract of *Moringa oleifera* was investigated against some foodborne pathogens include *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Klebsiella pneumonia*. The results of GC-MS revealed to the identification of twenty two components, the prevalent compounds were Oleic acid (14.61 %), Butyric acid (11.55 %), 8-Octadecenoic acid, methyl ester (11.16 %) and 1-Pentanol (10.75 %). The radical scavenging activity of the seed oil was 75.81% in concentration 100 µl/ml, and the (EC_{50}) was 25 µl \ml. Furthermore, the total phenolic content of the seed oil extract increased gradually with the increases of concentration. The results of the antibacterial activity of *Moringa oleifera* oil extract showed that the oil extract have the antibacterial effect against all tested bacteria in all concentrations except *E. coli* and *K. pneumonia* which were resistant to the seed's oil extract in concentration 125 µl/ml. The highest antibacterial activity was observed on *Staph. aureus, B. cereus, E. coli* and *K. pneumonia* with inhibition zone 18.50, 14.16, 11.83 and 11.33 mm in concentration 500 µl/ml respectively. Furthermore, the MIC of the oil extract was 64µl/ml for *Staph. aureus*, 128 µl/ml for *Bacillus cereus* and 256 µl/ml for each *E. coli* and *K. pneumonia.*

Keywords: *Antibacterial activity, DPPH, MIC, Moringa oleifera, Seed oil extract, Total phenol.*

Introduction

Natural antioxidants have an important role in the inhibition of many diseases and improving health. It was also reported that phenolic compounds rich food may have a protective effect against certain diseases such as heart disease, inflammatory diseases, cancer and diabetes[1, 2, 3]. *Moringa oleifera* is a wonderful tree in the plant kingdom. It does not only possess rich nutritive values (e.g vitamin, mineral, protein, energy, carbohydrate and electrolyte) but contains a lot of medicinal value with important chemical constituents also [4]. A number of medicinal properties have been ascribed to various parts of this tree. Most parts of this plant: root, bark, gum, leaf, fruit (pods) flowers, seed and seed oil have been used in folk medicine in Africa and South Asia [5]. The effectiveness of *M. oleifera* as an antioxidant became evident after the identification of some natural antioxidants which include vitamin C, flavonoids, tocopherols and other phenolic compounds. It was reported that the Moringa plant provides a rich and rare combination of zeatin, quercetin, kaempferol and many other phytochemicals [6].

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Moringa oleifera seed oil is pleasant tasting, highly edible, and resembles olive oil in its fatty acid composition [7]. The characteristics of *M. oleifera* seed oil can be highly desirable especially with the current trend of replacing polyunsaturated vegetable oils with those containing high amounts of monounsaturated acids [8]. Furthermore, *M. oleifera* has been found to be a potential new source of oil especially with the advent of the need for oleo-chemicals and oils/fats derived fuels (Biodiesel) all over the world [9].Thus, the objectives of the present study include detection of the active ingredients in Moringa oleifera seed oil cultivated in Iraq and assessment of the antibacterial and antioxidant activity of oil on some foodborne pathogens.

Materials and Methods

Chemical reagents

The chemical reagents DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin Ciocalteu reagent were purchased from Sigma Aldrich chemicals (Sigma-Aldrich, Germany), Resazurin dye, Nutrient agar (Himedia, India), Muller-Hinton agar and broth (Oxoid, England).

Collection of *Moringa oleifera*

Moringa oleifera seed was obtained from the plantation of Al-Diwaniyah city, Iraq. The seed was identified by the specialist, Department of Biology, College of Science, University of Baghdad. The seed was washed with water and dried at room temperature, and shelled by using mortar and pestle. The husk and kernel were ground to a fine powder and stored at 4°C for further analysis.

Preparation of seeds oil extract

Soxhlet apparatus was used to extract oil from seeds, 100 g of *Moringa oleifera* whole crushed seeds (husk and kernel) were put in a thimble and 700 ml of petroleum ether was added within 40-60 ^{o}C for 6 hours. The solution was filtered through a filter paper Whitman No.1 and evaporated under vacuum at 40° C by a rotary evaporator to get rid of petroleum ether; the oil was stored in glass vials at 4°C until analyzed [10].

Gas chromatography mass spectrophotometer analysis (GC-MS)

Analysis of the moringa oleifera seed oil extract was carried out on GC-MS equipment. The experimental conditions of the equipment are: HP-5MS ultra inert capillary non-polar column, dimensions: 30 mm × 0.25 mm; ID: 0.25 mm, film thickness: 0.25 µm. The flow rate of mobile gas: 1.0 ml/min. The oven temperature for the gas chromatographic part was 50°C raised to 300°C at 7oC/min for 10 min. The nature and structure of compounds were identified by the mass spectrometer. The spectrum of unidentified components was compared with the spectrum of identified components stored in the national institute standard and technology (NIST) library [11].

Evaluation of the Antioxidant activity (DPPH assay)

The radical scavenging activity of samples was determined according to Kedare and Singh [12]. 5ml of a freshly prepared 0.004% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was mixed with 100 µl of different concentrations (20, 40, 60, 80 and 100 µl \ml) of the seeds oil extract, then the mixture was left to stand for 30 min. The absorbance was measured at 517 nm. All tests were performed in triplicate. The percentage of DPPH reduction was calculated as:

% Reduction = (Abs DPPH – Abs Dil.) / Abs DPPH × 100

Where:

Abs DPPH = average absorption of the DPPH solution

Abs Dil. = average absorption of the three absorption values of each dilution.

With the obtained values, a graphic was made using Microsoft Excel. The EC_{50} of each extract (concentration of extract or compound at which reduced 50% of DPPH) was taken from the graphic.

Determination of total phenolic contents

Total phenolic content of *Moringa oleifera* seed oil extract was determined spectrophotometrically using the Folin-Ciocalteu method described by Jayaprakasha [13]. Two ml of Folin-Ciocalteu reagent (diluted 10 times) was mixed with 1.6 ml of 7.5% sodium carbonate solution and 0.4 ml of *Moringa oleifera* extracts. The volume was completed to 5 ml by adding distilled water. The tubes were covered with parafilm for 30 min. at room temperature, and then the absorbance was read at 760 nm spectrophotometrically.

Bacterial isolates

Bacillus cereus, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella peneumonia*e isolated from food, obtained from the Department of Food Sciences, College of Agricultural Engineering Sciences, University of Baghdad, and emphasize diagnoses by using VITEK-2 System.

Antibiotic susceptibility test

Kirby-Bauer method was followed as described by WHO [14], to carry out the antibiotics susceptibility test for 10 different antibiotics. The bacterial suspension was prepared by picking 1-2 isolated colonies of bacteria from the original culture and introduced into a test tube containing 4 ml of normal saline to produce a bacterial suspension of moderate turbidity compared with the standard turbidity solution. This approximately equals to 1.5×10^8 CFU/ml. By a sterile cotton swab, a portion of bacterial suspension was transferred and carefully and evenly spread on Mueller- Hinton agar medium, and then it was left for 10 min. Thereafter the antimicrobial discs were placed on the agar with a sterile forceps pressed firmly to ensure contact with the agar. Later the plates were inverted and incubated at 37ºC for 18-24 hours. Inhibition zones developed around the discs were measured by a millimeter (mm) using a metric ruler according to Clinical Laboratories Standards Institute [15].

Agar well diffusion method

Agar well diffusion method was employed for the determination of this study. Muller- Hinton agar plates were swabbed (sterile cotton swabs) with broth culture of respective bacteria. Wells 6 mm diameter was made in each of these plates using a sterile cork borer. 100 µl from each concentration (125 , 250 and 500 µl/ ml) of the seed oil extract was put in each hole by using micropipette and allowed to diffuse at room temperature for 30 min. The plates were incubated at 37 ºC for 18-24 hours. All tests were performed in triplicate. The diameter of any resulting zone of inhibition was measured in millimeters[16].

Determination of the minimum inhibitory concentration (MIC) of the *Moringa oleifera* oil extract

Broth microdilution method was used to determine the MIC of *Moringa oleifera* oil extract using the 96-well microtiter plate. The extracts were prepared in a double concentration, the desired final concentration as it will be diluted with an equal amount of bacteria in broth. 200 µl of the prepared oil extract was introduced into the first wells in columns 1-4 (in row A). Rows B-H in columns 1-4 had 100µl of broth alone. Twofold serial dilutions using micropipette was done systematically down the columns 1-4 (from rows B-H). 100 µl was removed from the starting concentrations (columns 1-4 in row A) and transferred to the next row with the 100µl broth, properly mixed, and the procedure was repeated up to the last row (H) where the last 100µl was discarded. This brings the final volume in all the test wells with the extracts to 100 µl except the 5th, 7th, 9th and 11th columns which had 200 µl of the broth that served as sterility negative control (Broth only). The columns 6th, 8th, 10th and 12th served as positive control (Bacteria + Broth). 100µl of the 1×10^6 CFU/ ml bacterial inoculum was transferred into all the wells except sterility negative control columns to give us the desired final inoculum load of 5×10^5 CFU/ml. Microtiter plates were incubated at 37ºC for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for some minutes to observe any color changes. The minimum inhibitory concentrations were determined visually in broth microdilution as the lowest concentrations of the extracts at which color

changed from blue to pink in the resazurin broth assay [17].

Statistical Analysis

The Statistical Analysis System [18] was used to detect the effect of difference factors in study parameters. Least significant difference-LSD test was used to significant compare between means in this study.

Results and Discussion

Gas chromatography mass spectrophotometer analysis

The GC-MS analyses of the *Moringa oleifera* seed oil extract lead to the identification of twenty two components. The prevalent compounds were Oleic acid (14.61 %), Butyric acid (11.55 %), 8-Octadecenoic acid, methyl ester (11.16 %) and 1-Pentanol (10.75 %); all compounds were identified and listed in Table 1. Efeovbokhan [19] in his investigation to study the effect of different solvents on composition of *Moringa oleifera* seed's oil referred to the presence of different percentage of palmitolic acid in the seed oil extracted by different solvents. Furthermore, it was reported that oleic acid present in crude extract of *Moringa oleifera* seed oil [20, 21].

Table 1: Identified compounds by GC-MS in *moringa oleifera* seed oil extract

No.	Possible compound name	Ret. time (min)	Peak area (%)	Molecular formula	Molecular weight (g/mol)
1	Hexanoic acid	5.497	6.5	$C_6H_{12}O_2$	116.160
2	pentanoic acid	6.844	2.54	$C_5H_{10}O_2$	102.133
3	Butyric acid	7.235	11.55	C_3H_7COOH	88.106
4	1-Pentanol	7.728	10.75	$C_5H_{12}O$	88.150
5	Decane	8.565	8.57	$C_{10}H_{22}$	142.286
6	Ether, 6-methylheptyl vinyl	9.473	2.59	$C_{10}H_{20}O$	156.26
7	Malonic acid	10.257	1.15	$C_3H_4O_4$	104.061
8	Hexyl octyl ether	10.506	0.27	$C_{14}H_{30}O$	214.39
9	Heptanoic acid	10.720	0.81	$C_7H_{14}O_2$	130.187
10	3-Acetoxytridecane	11.390	0.30	$C_{15}H_{30}O_2$	242.4
11	6,8-Doixatetradecane	11.657	0.27	$C_{12}H_{26}O_2$	202.33
12	Cyclohexanecarboxylic acid	12.702	1.84	$C_7H_{12}O_2$	128.171
13	Isoamyl levulinate	14.577	1.75	$C_{10}H_{18}O_3$	186.25
14	Oxalic acid	15.195	0.69	$C_2H_2O_4$	90.034
15	Isophytol	15.747	1.80	$C_{20}H_{40}O$	296.539
16	Pyrrolidine	16.744	2.23	C_4H_9N	71.123
17	Heptadecyl heptafluorobutyrate	18.091	0.44	$C_{21}H_{35}F_7O_2$	452.49
18	Palmitoleic acid	20.163	0.42	$C_{16}H_{30}O_2$	254.414
19	Isopropyl myristate	20.513	2.26	$C_{17}H_{34}O_2$	270
20	8-Octadecenoic acid, methyl ester	23.065	11.16	$C_{19}H_{36}O_2$	296.48
21	Heptadecanoic acid,16-methyl- methyl ester	23.344	1.36	$C_{19}H_{38}O_2$	298.50
22	Oleic acid	24.602	14.61	$C_{18}H_{34}O_2$	282.47

DPPH assay

The antioxidant activity of oil extract was 45.21% in concentration 20 µl/ml and the activity was 75.81% in concentration 100 µl/ml as shown in Table 2. The effective concentration 50 (EC_{50}) is an important parameter to evaluate the antioxidant activity of materials and it could be used to compare the antioxidant capacity of various materials. The radical scavenging capacity (EC_{50}) of the seed oil extract was 25 µl \ml.

The antioxidant activity of seed's oil may be attributed to the presence of 8-octadecenoic acid, methyl ester, oleic acid [22] and Isopropil myrstate [23].

Table 2: Radical scavenging activity of *moringa oleifera* seed oil extract

Concentration (µl \ml)	Oil Seed extracts (µl \ml)
20	45.21 ± 0.21
40	57.94 ± 0.18
60	66.98 ± 0.07
80	69.23 ± 0.03
100	75.81 ± 0.13
LSD value	0.450 *

Total phenolic content of seed oil extract

The results of total phenolic content of seed oil extract increased gradually with increases of concentration, with significant differences (P≤0.05) as shown in Table 3. The result in the current study is disagreement with Ogbunugafor [24] which referred in his investigation to study the antioxidant properties of *Moringa oleifera* seed oil, the total phenolic content was 40.17 ± 0.01 mg/g.

Table 3: Total phenolic content of *moringa oleifera* seed oil extract

Concentration (µl \ml)	Seed oil (mg\g)
62.5	14.38 ± 1.05
125	18.96 ± 0.22
250	23.93 ± 0.06
LSD value	2.154 *

* (P≤0.05)

Antibiotic susceptibility test

The antibiotic susceptibility of the bacterial isolates was performed on ten antibiotics represented in Table 4. The antibiogram of the studied isolates revealed that *B. cereus* was resistance to Cefotaxime, Chloramphenicol, Doxycycline, Erythromycin, Penicillin and Trimethoprim, *Staph aurous* was resistance to Cefotaxime and penicillin, *E. coli* was resistance to Erythromycin, Penicillin and Tetracycline and *K. pneumonia* was resistance to Erythromycin and Penicillin, while the isolates were sensitive and intermediate to another antibiotics used in this study.

Over the past decades, overreliance and use of antibiotics have led to the emergence and dissemination of multidrug resistant strains of several groups of microorganisms [25]. Due to the increase in resistant clinical isolates, there is a paramount need to develop new and innovative antimicrobial agents. Therefore,

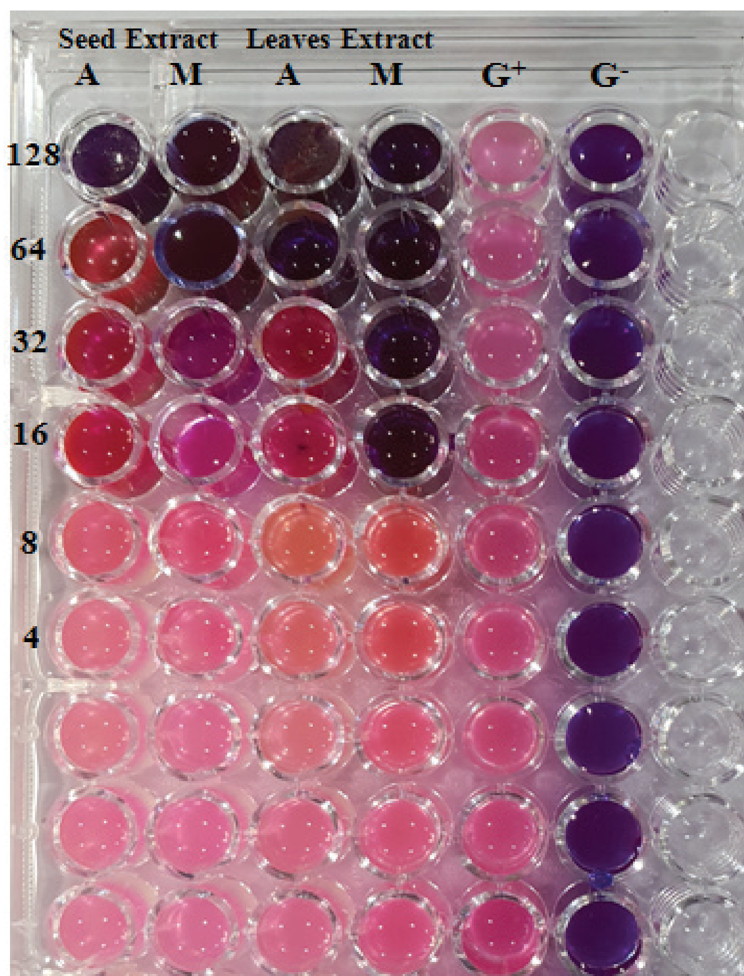
researchers are looking for new leads in the discovery of better alternatives against multidrug resistant microbial strains. Among the potential sources of new agents, plants have long been investigated owing to their popular use as remedies for diverse infectious diseases because they contain many bioactive compounds that could be interest in therapeutics [26].

Table 4: Antibiotic Sensitive Test

Antibiotics	Microorganisms			
	B. cereus	*Staph. aurous*	*E. coli*	*K. pneumonia*
Amikacin	S	S	S	S
Cefotaxime	R	R	S	S
Chloramphenicol	R	S	S	S
Ciprofloxacin	S	S	S	S
Doxycycline	R	S	I	S
Erythromycin	R	I	R	R
Gentamicin	S	S	S	S
Penicillin	R	R	R	R
Tetracycline	R	S	R	S
Trimethoprim	I	S	S	I

S=Sensetive, R=Resistant, I=Intermediate

Antibacterial activity of seed oil extract



The antibacterial activity of *Moringa oleifera* seed's oil extract was reported in Table 5. The oil extract had antibacterial effect against all tested bacteria in all concentrations except *E. coli* and *K. pneumonia* which were resistant to the seed's oil extract in concentration 125 µl/ml. The highest antibacterial activity was observed on *Staph. aureus* with inhibition zone 10.50 ± 0.28, 13.16 ± 0.16 and 18.50 ± 0.28 mm in concentrations (125, 250 and 500 µl/ml) respectively, and the lowest effect was observed on *E. coli* with inhibition zone of 7.33 ± 0.33 mm in 250µl/ml concentration with significant difference (P≤0.05). Abdulrasheed [27] revealed that *Moringa oleifera* seed oil was inactive against all the tested organisms even at 100% concentration. Results obtained by Othman [28] showed that *M.oleifera* seed oil had low effect on gram positive and gram negative test bacteria.

Table 5: Antibacterial activity of *moringa oleifera* seed oil extract

Isolate	Inhibition zone (mm)			LSD value
	125 (µl/ml)	250 (µl/ml)	500 (µl/ml)	
Staph. aureus	10.50 ± 0.28	13.16 ± 0.16	18.50 ± 0.28	2.173 *
B. cereus	6.83 ± 0.33	11.16 ± 0.16	14.16 ± 0.16	1.335 *
E. coli	0.00 ± 0.00	7.33 ± 0.33	11.83 ± 0.16	0.982 *
K. pneumonia	0.00 ± 0.00	7.66 ± 0.33	11.33 ± 0.33	0.967 *
LSD value	0.719 *	0.859 *	0.815 *	---

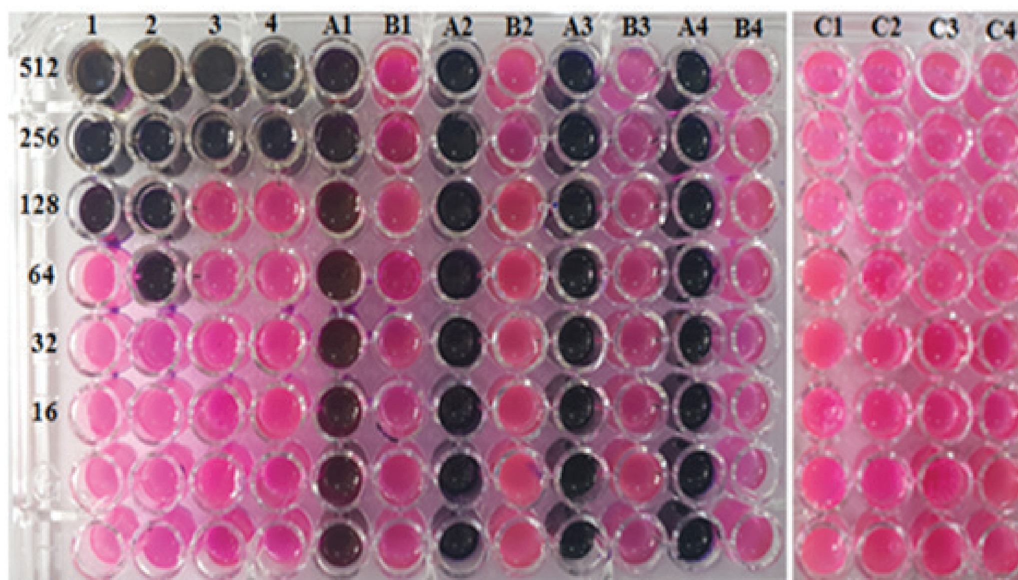
*** (P≤0.05)**

The antibacterial activity of *Moringa oleifera* seed oil extract attributed to the presence of oleic acid (fatty acid) , 8-Octadecenoic acid, methyl ester (fatty acid ester) [22], Butyric acid and Hexanoic acid, Pyrrolidine [29], Heptadecyl heptafluorobutyrate, Isophytol [30], and other compounds that exhibit antimicrobial activity.

Minimum Inhibitory Concentration (MIC) of seed oil extract

Different standard methods have been used to evaluate the antimicrobial activities of plant's extracts. However, dilution methods have been favored over others for the determination of MIC and MBC. A method using the oxidation-reduction colorimetric indicator resazurin has been proposed for the determination of drug resistance and MIC of antimicrobial agents against pathogenic organisms [31]. The results of seed oil extract showed that the MIC was 64µl/ml for *Staph. aureus*, 128 µl/ml for *Bacillus cereus* and 256 µl/ml for each *E. coli* and *K. pneumonia* as shown in Figure 1.

Phenolic compounds of plants are of noticeable interest due to their antioxidant and antibacterial properties [32, 33]. The means by which microorganisms are inhibited by phenolic compounds involves a sensitization of the phospholipids bilayer of the cell membrane, causing an increase in permeability and leakage of vital intracellular constituents, or impairment of bacterial enzyme systems. Phenolic compounds act by inhibiting the amino acid decarboxylase in target bacteria [34].



(1): *Bacillus cereus*, (2): *staph. aureus*, (3): *K. pneumoniae*, (4): *E. coli*, (A): Media+ oil, (B): Bacteria + Media, (C): Bacteria + DMSO

Figure 1: MIC of *Moringa oleifera* seed oil extract

Conclusion

Moringa oleifera seed oil extracts have a wide variety of fatty acid like Oleic acid, Butyric acid, 8-Octadecenoic acid, methyl ester and 1-Pentanol that show antioxidant activity and antimbacterial agent against both gram positive bacteria (*Staph. aureus* and *B. cereus*) and gram negative bacteria (*E. coli* and *K. pneumonia*).

Conflict of Interest: Nil

Source of Funding: Self

Ethical Clearance: Not required

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