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Characterization and Antimicrobial Effect of *Moringa Oleifera* and *Moringa Peregrina* Essential oils Against Some Pathogenic bacteria

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THE CHEMICAL composition and antimicrobial activity of two essential oils extracted from the seeds of *Moringa oleifera* (MO) and *Moringa peregrina* (MP) against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Pseudomonas aeruginosa, klebsiella pneumoniae, Salmonella typhi, Escherichia coli* and *Shigella flexneri* strains were investigated. Antibacterial diffusion method was used, GC-MS analysis was done to the most effective oil and TEM was done to detect the internal effect on the highly affected bacteria. MO essential oil was the most effective and *Ps. aeruginosa* was the highly affected bacterial strain. GC-MS analysis showed that MO essential oil contains several fatty acids especially oleic acid, *6-Octadecenoic acid* (11.27%). This acid could rupture the cell membrane of the bacteria and release all internal content as detected by TEM. In conclusion, MO essential oil may be further developed as a natural promising antibacterial agent. Future studies of synergism and compatibility may potentiate the antimicrobial activity.

Keywords: *Essential oil, Bactericidal, Moringa oleifera, Gas Chromatograph, Transmission Electron Microscope*

Modern pharmacological studies have been directed towards the plants and herbs as a source of antimicrobial and therapeutic agents (Mousavi *et al.*, 2016), (Newman and Cargg, 2016) and (Raskin and Waterman, 2015). The antimicrobial properties of plants lead the scientists to use them as safe and natural products against bacteria (Bugno *et al.*, 2007). Plants produce these antimicrobial compounds as secondary metabolites like alkaloids, phenolic compounds, etc. Using alternative medicine has been increased nowadays in developing countries as a direction of World Health Organization due to its role in treating infections (Dilhuydy, 2003).

New researches focused and carried out on species of Moringaceae family (Raskin and Waterman, 2015), (Elsayed *et al.*, 2016) and (El-Awady *et al.*, 2015). The Moringaceae family consists of up to twelve species (Majali *et al.*, 2015). MO has many benefits and best known as a medicinal plant and consumed

by humans (Iqbal *et al.*, 2006). It is very rich of vitamins A, B, C, D, E and K (Anwar *et al.,* 2005). MO extract possess antimicrobial activity and had an inhibitory effect against many harmful bacteria, including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Pseudomonas aeruginosa* (Saadabi and Zaid, 2011). Its mechanism of action is either to kill microorganism (bactericidal) or to prevent its growth (bacteriostatic) (Lockett and Louis, 2000) and (Anwar and Rashid, 2007). This study will investigate the active ingredients and compare the antibacterial activity of MO and MP essential oils against some Gram positive and Gram negative pathogenic bacteria.

Materials and Methods

Bacterial strains

Bacterial strains used for examination in this study were include, *Staphylococcus aureus* (ATCC: 25923), *Staphylococcus epidermidis* (ATCC: 12228), *Streptococcus pyogenes* (ATCC: 19615) *Pseudomonas aeruginosa* (ATCC: 27853), *klebsiella pneumoniae* (ATCC: 13883), *Salmonella typhi* (ATCC: 19430), *Escherichia coli* (ATCC: 25922) and *Shigella flexneri* (ATCC: 12002). Bacteria were grown on Brain Heart Infusion (BHI) broth medium and maintained on nutrient agar slants at 4°C (Cappuccino and Sherman, 1995).

Extraction of MO and MP essential oil

Seeds of MO and *Moringa peregrina* were obtained from Ankit Agrowal, India. It was identified taxonomically by conventional methods. The seeds were cleaned and dried directly by sunlight and grinded by a mechanical grinder (500 gms) and then in blender (Marrufo *et al.,* 2013).

Preparation of the essential Oil

MO and MP seed powder were hydro distillated for 3 hours. as described by the *European Pharmacopoeia* (Medicin EBPtQo, 2008). The oil was dried by anhydrous sodium sulphate and stored at 4 °C till analysis (Marrufo *et al.,* 2013).

Antimicrobial susceptibility tests

Antimicrobial susceptibility test was done by disk diffusion method. Results were determined according to National Committee for Clinical Laboratory Standards (CLSI) break point criteria (Breakpoints, 2011). Antimicrobial drugs included penicillin group (amoxicillin); glycopeptide group (vancocin), aminoglycosides (amikin) and cephalosporin (cephradine and imipenem). Multidrug resistant strains (MDR) were detected and defined as the non-susceptible strains to one agent in three or more antimicrobial categories (Magiorakos *et al.,* 2012).

Evaluation of antibacterial activity of the essential oils against bacterial strains

Essential oils of MO and MP were examined for their antimicrobial activity against the tested bacteria by agar diffusion method. Dilutions of essential and fixed oils were carried out in DMSO (dimethyl sulfoxyde) with concentrations ranging from 10% to 100% (v/v). Plates were incubated at 35±2ºCfor 16-20 h.

The mean inhibition zone diameter was measured. Each assay was carried out in triplicate.

Preparation of fatty acid methyl ester (FAME)

Forty grams of dried seeds were ground into powder using mortar and pastle, then dichloromethane (0.6 ml), sodium methoxide (4.0 ml of 0.5N) and boron triflouride-methanol complex (14%w/v) were added. (Firestone, 1990) and (Rezanka and Rezankova, 1999). Tubes were incubated at 50°C for 30 min. in a mechanical shaker. The reaction was stopped by adding 5.0 ml of water containing 0.2 ml of glacial acetic acid. The extraction of the esterified fatty acids was done by 3.0 ml petroleum ether (40- 600C). The clear fraction was kept at -20°C until further analysis.

Separation condition of fatty acids on GC/MS

HP 6890 Series Gas Chromatograph System instrument with an HP 5973 Mass Selective Detector was used. The FAME in hexane (1 µl) was injected into the column with a split ratio of 100:1. The injector and detector temperature were adjusted at 200 and 250°C, respectively. Helium at a flow rate of 1.5 ml/min was used as the carrier gas. Separation was carried out on a TR-FAME (30 mm × 0.25 mm ID) (Thermo 260 M142 P) with a film thickness of 0.25 υm film in capillary column (70% Cyanopropyl Polysilphphenylene siloxane). The column temperature was programmed from 100 to 160°C at 2°C/min and then to 250°C at 4°C/min and finally held at 250° C for 20 min. The individual FAME weight was calculated on the basis of their relative peak area compared to that of internal standard, and then they were corrected using the corresponding GC response factors for each fatty acid.

Transmission electron microscope (TEM) examination

Conventional TEM microscopy is selected to visualize the ultra structural damage on both cell wall and cytoplasmic membrane of entire microbes using a fixative material (Hammer *et al*., 2010). At ultra structural level, the negative staining for TEM (JEM-1400 TEM, JEOL- Japan) can reveal changes on the mechanism of membrane disruption by antimicrobial proteins and peptides (AMPPs). Fixation is done using aldehydes, then osmium tetraoxide is used for post-fixation. Dehydratation was done for this thin film and it was embedded in Epoxy resin to allow the observation of membrane and cytoplasmatic alterations (Torrent *et al.,* 2209).

Results

The sensitivity of the tested strains were investigated as summarized in Table 1. Results showed that all the tested bacteria were not multidrug resistant, however 4 (50%) strains of them were resistant to vancomycin, 1 (12.5%) was

resistant to cephradines and 1 (9%) was resistant to amoxicillin while all of them (100%) were susceptible to imipenem and amikacin.

TABLE 1. Sensitivity of the tested strains to different antibiotics and detection of the multi-drug resistant strains (mm).

Antibiotics / Bacterial strains	Mean diameter of inhibition zone (mm)				
	Penicillin group	Cephalosporin group		Aminoglycosides group	Glycopeptide group
	Amoxicillin	Imipenem	Cephradine	Amikacin	Vancomycin
S. aureus	32	40	15	28	18
S. epidermidis	22	45	32	40	00
St. pyogenes	30	45	32	30	20
Ps.aeruginosa	30	44	33	28	28
k. pneumonia	22	18	35	25	18
Salmonella typhi	14	38	28	22	00
E.coli	23	32	30	18	00
Shigella flexneri	28	28	00	32	00

S. aureus, S. epidermidis and St. pyogenes representing Gram positive strains were affected by MO essential oil in different manor as shown in Fig. 1. *St. pyogenes* is the highly affected one and the inhibition effect was increased with the higher concentrations of MO oil. Fig. 2 illustrated that only two Gram negative strains were affected namely, Ps. aeruginosa and *S. typhi*. From the eight bacterial strains tested, Ps. aeruginosa was the highly affected bacterial strain showing largest inhibition zone. The highly effective concentrations were 60 and 100%. On behalf of MP oil extract, Fig. 3 showed very low effect on tested Gram positive bacteria. For Gram negative strains, Ps. aeruginosa was the highly affected bacterial strain with largest inhibition zone but less than that of MO's as shown in Fig. 4.

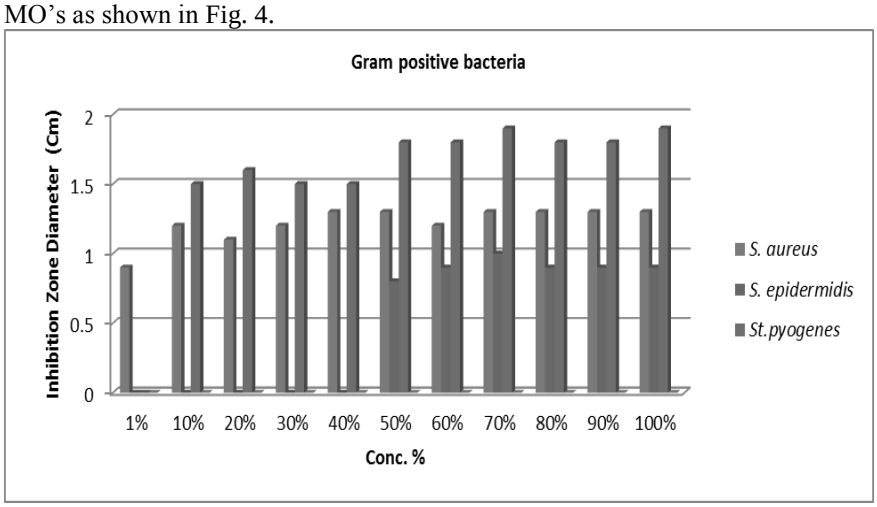


Fig. 1. Effect of Moringa oleifera essential oil on gram positive bacteria tested.

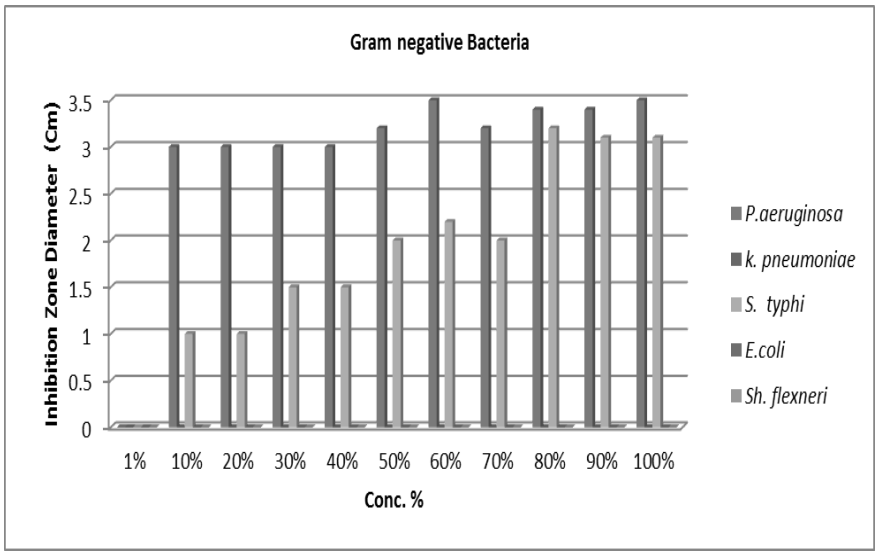


Fig. 2. Effect of Moringa oleifera essential oil on gram negative bacteria tested.

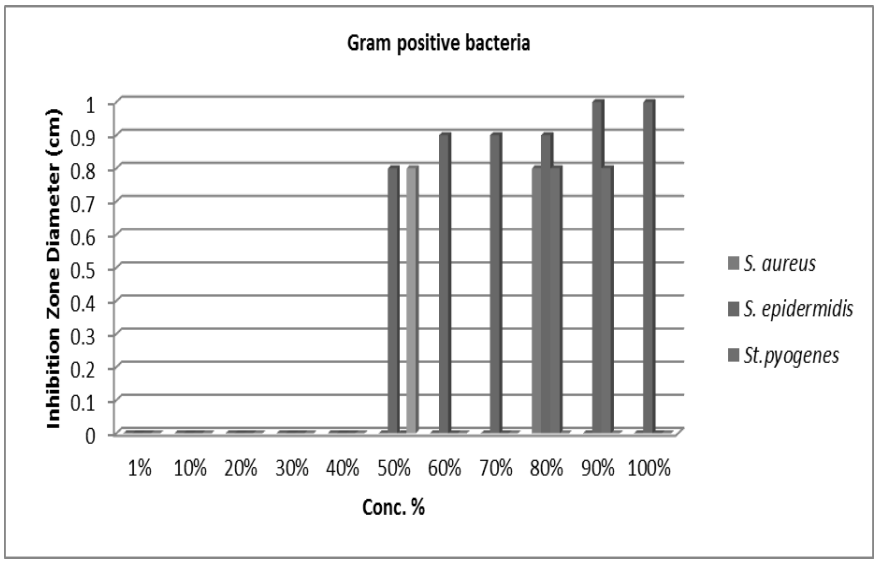


Fig. 3. Effect of Moringa peregrina essential oil on gram positive bacteria tested.

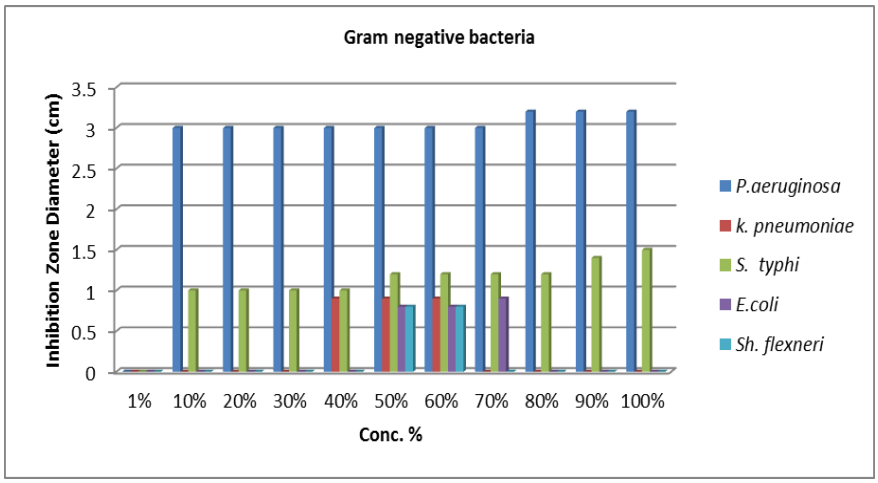


Fig . 4. Effect of MP essential oil on Gram negative bacteria tested.

GC-MS analysis of MO essential oil constituents was determined and summarized in Table 2. It is showed that MO essential oil contains many fatty acids. It contains Oleic Acid, 6-Octadecenoic acid (11.27%). Total chromatogram for GC-MS analysis of MO essential oil constituents was determined as shown in Fig. 5.

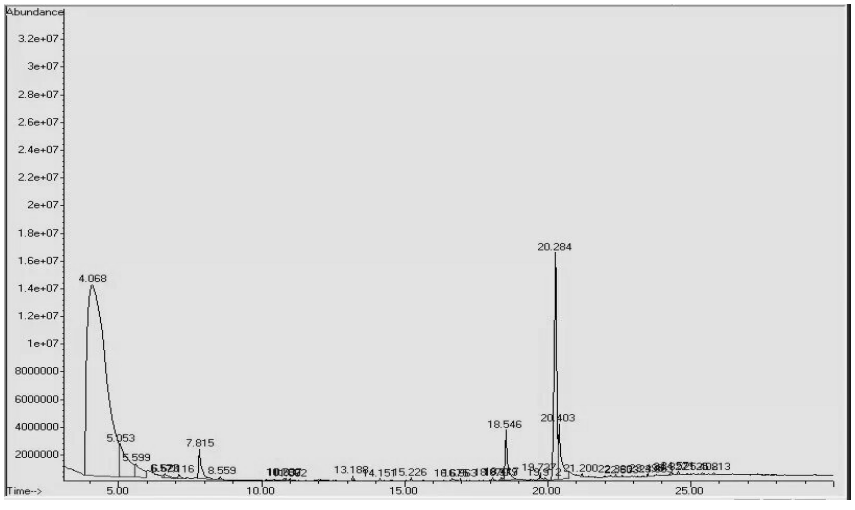


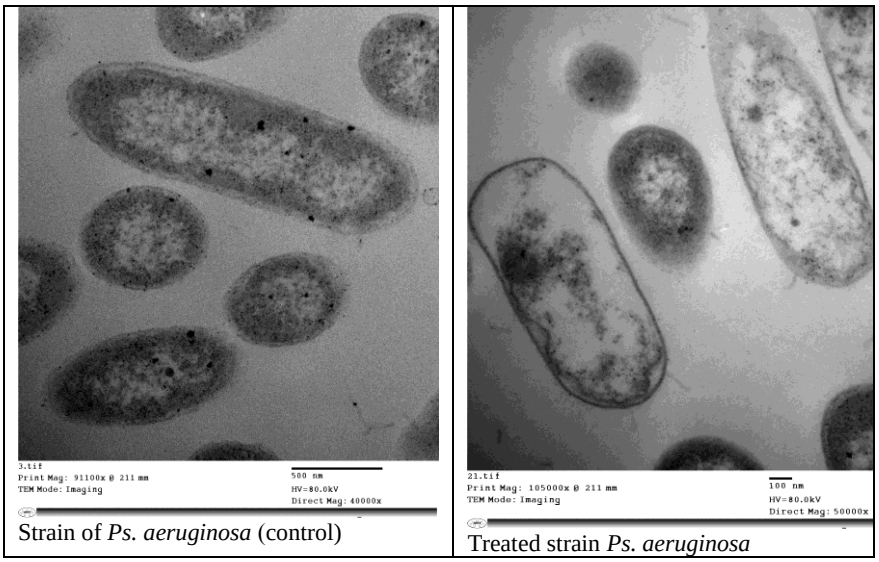
Fig. 5. Total chromatogram for GC-MS analysis of MO essential oil constituents.

TABLE 2. GC-MS analysis of MO essential oil constituents.

Peak No.	Compound	Relative Retention time (min)	Percentage (%)
1	Dimethyl-sulfoxide	4.068	72.01
2	Dimethyl-sulfoxide	5.053	5.31
3	Dimethylsulfoxonium formylmethylid	5.598	1.79
4	Dimethyl-sulfoxide	6.574	0.10
5	Dimethyl-sulfoxide	6.620	0.35
6	4-Methoxycyclohexanone 2-Aminomethyl-5-methylamino-1,3,4-oxadiazole	7.118	0.17
7	1,6-Octadien-3-ol, 3,7-dimethyl-3-Carene	7.814	0.11
8	Dodecamethylpentasiloxane	13.189	0.06
9	Ethyl8H-naphtho[2',1':5,6]pyrano4,3-b]quinoline-11-carboxylate Benzothiazole-2-carboxamide	14.149	0.05
10	N-(4'-Chlorophenyl)-8-fluoro-3-methyl-isoalloxazine	15.228	0.05
11	Ethylidene chloride	16.676	0.15
12	Propionic acid, 2-chloro-, ethyl ester	16.961	0.04
13	7- octadecatrienoic acid methyl ester, methyl ester	19.737	2.20
14	Hexadecanoic acid, methyl ester	18.072	0.09
15	Hexadecenoic acid, Z-11-cis-Vaccenic acid	18.378	0.06
16	Carbonic acid, heptadecyl propyl ester	18.414	0.07
17	n-Hexadecanoic acid, Octadecanoic acid	18.544	2.41
18	11-Octadecenoic acid, methyl ester	19.737	0.23
19	6-Aza-5,7,12,14-tetrathiapentacene	19.914	0.08
20	Oleic Acid, 6-Octadecenoic acid	20.284	11.27
21	9-Octadecenoic acid, (E)- cis-Vaccenic acid	20.401	3.25
22	2-ethoxy-2,3-dihydro-3,3-dimethyl-benzofuran-5-yl methanesulphonate	22.601	0.06
23	2,4-Cyclohexadien-1-one, 3,5-bis,1-dimethylethyl	23.494	0.05
24	1,4-Bis(trimethylsilyl)benzene	23.805	0.04

As Ps. aeruginosa was the highly affected bacterial strain it was scanned by transmission electron microscope (TEM) to investigate effect of MO essential oil on Ps. aeruginosa and evaluate the changes that occurred between the control and treated one as a clear shown in Fig. 6. The figure showed that MO essential oil affected the internal content of the bacterial cell as the cell appeared empty with some effect on bacterial membrane.

Medicinal plants have great value and now increased in use (Heinrich, 2000) due to the harmful effect and undesirable side effects of chemical drugs (Uprety *et al.,* 2012). The activity of MO and MP ethanol extracts against bacteria was found to be due to the presence of putative compounds that can affect the antibacterial activity by disrupting protein or DNA structures (Nair *et al.,* 2005).



Strain of *Ps. aeruginosa* (control)

Treated strain *Ps. aeruginosa*

Fig. 6. Transmission electron microscope (TEM) for *effect of* MO essential oil on control and treated *Ps. aeruginosa*.

Discussion

The results of the current study showed that both MO and MP essential oils had varied antibacterial activities against the eight tested bacterial strains. The highly affected bacterial strain was *Ps. aeruginosa* showing greater inhibition zone by both essential oils giving the more effect to MO essential oil. This result was in agreement with (Habbal *et al.,* 2011) who found that Moringa leaf extracts (M. oleifera) can affect *S. aureus* and *Ps. aeruginosa* by inhibiting its growth. On contrast to this result other studies revealed that the antibacterial activity of the extract was greater against Gram positive species than Gram negative ones (Grosvenor *et al.,* 1995), (Kudi *et al.,*1999) and (Ali *et al.,* 2001).

In the present study MO essential oil was analyzed by GC-MS to detect the active antibacterial ingredient that could affect *Ps. aeruginosa*. It was found that the essential oil contained many fatty acids with different percentages. It contained *Oleic Acid, 6-Octadecenoic acid* (11.27%). Oleic acid, an 18-carbon mono-unsaturated fatty acid is found to be more abundant in this essential oil. Oleic acid has been reported to be the predominant bactericidal unsaturated fatty acid (Speert *et al*., 1979) and (Dye and Kaprel, 1981). The antibacterial mechanism of oleic acid is still not completely elucidated. The initial process probably involves adherence to the cell wall Salton (1968). This is probably necessary but not sufficient for the bactericidal effect, as oleic acid binds to resistant as well as to sensitive species of bacteria. The critical event may well be penetration to the cell membrane with consequent damage to its integrity, permitting release of essential intracellular materials (Hugo and Longworth, 1965).

Transmission Electron microscope was used to detect the internal effect that happened to *P. aeruginosa* before and after treatment with MO essential oil. It was clear that the internal bacterial cell content was affected and released and there was also some disruption in outer cell membrane. This result could confirm the effect of oleic acid after adherence to the cell membrane, disrupting it and releasing of internal components.

Conclusion

MO essential oil had greater effect on the tested eight bacterial strains. GC-MS analysis showed different concentrations of fatty acids. Oleic acid was the most abundant and had bactericidal activity especially against *Ps. aeruginosa*. Further investigations should be carried out for the detection of the antimicrobial agent present in MO seed oil.

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وصف وتحديد التأثير المضاد الميكروبي لزيوت المورينجا اوليفرا والمورينجا بريجرين الأساسية ضد بعض البكتيريا الممرضة

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تم تحديد التركيب الكيميائى والتأثير المضاد الميكروبى لاثنين من الزيوت الأساسية لبذور نبات المورينجا اوليفرا والمورينجا بريجنينا ضد الميكروب المكور العنقودى الذهبى والعنقودى ابيديرمس والسبحى بيوجينز والسيدموناس ايرجينوزا والكلبسيللا نيومونيا والسالمونيللا تايفى والايشيريشيا كولاى والشيجللا فليكسينيرى. كانت افضل النتائج للزيت المستخلص من المورينجا اوليفرا وكان التاثير الاكبر على ميكروب السيدوموناس ايروجينوزا. وباستخدام جهاز الجى-سى ماس اوضح احتواء زيت المورينجا اوليفرا على مجموعات مختلفه من الاحماض الدهنيه وخاصة حمض الاوليك- ٦- اوكتاديكونيك بنسبة (١١,٢٧%) وهذا الحمض له تأثير على الجدار الخلوى البكتيرى مما ادى الى تدميره وخروج مكونات الخليه البكتيريه وموتها كما هو واضح بتصوير الماسح الاكترونى لهذه البكتيريا. ويستخلص من ذلك انه يمكن مستقبلا استخدام هذا الزيت كمضاد بكتيرى طبيعى. وتوصى الدراسة بعمل دراسات اخرى لقياس التوافق بين هذا الزيت ومواد بكتيرية مضادة اخرى لاعطاء نتائج افضل.