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Research Article

Antibacterial Effect of the Ethanol Leaves Extract of *Moringa oleifera* and *Camellia sinensis* against Multi Drug Resistant Bacteria

Amal Sabry Othman and Nashwa Abbas Ahmed

Department of Microbiology, Faculty of Applied Medical Sciences, October 6th University, Egypt

Abstract

Background: *Moringa oleifera* and *Camellia sinensis* have antibacterial properties and may affect the multidrug-resistant bacteria. **Objectives:** The present study was undertaken to evaluate the potentiality of *M. oleifera* and *C. sinensis* extracts on MDR bacteria and reassessment of the antibiotic susceptibility after herbal treatment. It was also aimed at detecting the active compound present in the extract and its mode of action on bacterial cell. **Materials and Methods:** Thirty clinical isolates of *E. coli* and *S. aureus* were identified biochemically. Multi drug resistance isolates were determined by antibiotic susceptibility test. Several concentrations were prepared from the extracts of both *M. oleifera* and *C. sinensis* and applied to the selected isolates. Reassessment of the antibiotic susceptibility test was carried out again after growing bacteria on the MIC of the herbal extract. The active chemical compounds were detected by GC-MS and the effect of the plant extract on the cell morphology and whole bacterial proteins were determined. **Results:** Five isolates were MDR. One Gram-positive and one Gram-negative were selected for further study. The ethanol extract of *M. oleifera* and *C. sinensis* showed inhibitory effect with MIC values ranging from 10-20 mg mL^{-1} and MBC ranging from 30- 40 mg mL^{-1}. Growing both isolates on the MIC of *M. Oleifera* extract rendered their sensitivity to the tested antibiotics with more significant effect on MDR *E. coli*. Phytochemical screening revealed an array of bioactive compounds which may have a direct effect on the cell morphology and the protein content as evidenced by TEM examination and protein profile analysis.

Key words: Multidrug resistant bacteria, *Moringa oleifera*, *Camellia sinensis*, *Staphylococcus aureus*, *Escherichia coli*, GC-MS analysis

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Corresponding Author: Amal Sabry Othman, Department of Microbiology, Faculty of Applied Medical Sciences, October 6th University, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Development of multidrug resistant bacterial strains is a growing public health concern[1]. There is increased evidence to proof that medicinal plants may represent an alternative treatment for non-severe cases of infectious diseases. They could also serve as possible source of new and cheap antibiotics to which pathogenic strains are not resistant and several studies provide scientific bases for the popular use of plants against infectious diseases[2].

Moringa oleifera and *Camellia sinensis* have been used extensively in traditional medicine for the treatment of several ailments, promotes digestion, skin diseases, diarrhea and as a stimulant in paralytic afflictions[3-6].

Several reports deduced that *Moringa oleifera* and *Camellia sinensis* have been shown to have antimicrobial effects against a variety of Gram positive and Gram negative bacteria (e.g., *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Enterococcus* spp.) and some fungi (e.g., *Candida albicans*)[7-9].

Recent studies demonstrated that the ethanolic extract of the leaves of both plants contains a group of chemical compounds which are known for its antibacterial activity against new multidrug resistant strains[10-12].

This study aimed firstly at exploring the antibacterial effect of the ethanol extract of the leaves of both *M. oleifera* and *C. sinensis* against two multidrug resistant clinical isolates (*E. coli* and *S. aureus*) and clarify its effect on changing the resistivity of the of the selected isolates to antibiotics. Secondly, to screen the chemical components of the most potent extract and determine its effect on the morphology and the whole cell proteins.

MATERIALS AND METHODS

Bacterial isolates: A total of thirty identified bacterial isolates (*Escherichia coli* and *Staphylococcus aureus*) were kindly obtained from hospitals in Giza governorate. They were isolated from wounds and abscesses in infected patients (25-45 years old) and identified biochemically. The identified clinical isolates were sub-cultured on nutrient agar slant and stored at 4°C in a refrigerator until they were used.

Standardization of the bacterial cultures: A loopful of the test organism was inoculated on a nutrient agar plate and incubated at 36±1°C for 24 h. Discrete colonies on the 24 h culture plate were collected using a sterile wire loop and inoculated into a 5 mL sterile nutrient broth and incubated for 30 min. The test tubes were shaken thoroughly and the turbidity of the bacterial suspension were adjusted by comparing it with the 0.5 McFarland standard tube equivalent to10^8 CFU mL^{-1}.

Antimicrobial susceptibility tests: Multi drug resistant strains (MDR) were determined by antibiotic susceptibility test, using the disc diffusion method, as recommended by the CLSI[13]. Commercial antimicrobial discs (Oxoid), used in this experiment include: Amoxicillin/clavulanic acid, amoxicillin and oxacillin from penicillin group, rifampicin from rifamycins, cefuroxime and cephalexin from cephalosporin group and sulfamethoxazole/trimethoprim from sulfa group. Hundred microliters of 24 h old culture of standardized inoculums (of each tested bacteria) was spread onto nutrient agar (Difco Laboratories) plates and left for 30 min. Commercial antibiotic discs with standard concentration were carefully placed on the seeded plates and incubated at 37°C for 24 h. Antibiotic susceptibility was evaluated by the diameter of inhibition zone (mm).

The MDR were detected and defined as the non-susceptible strains to one or more classes of antimicrobial agents[14].

Preparation of the plant extracts: Leaves of *Moringa oleifera* and *Camellia sinensis* (green tea) were kindly obtained from Faculty of Pharmacy, Cairo University.

The leaves were washed with water, air-dried for 2 weeks and then pulverised using an electric mill. About 500 g of the powdered plant materials was macerated with 1000 mL (1 L) of 70% v/v ethanol for 48 h with intermittent shaking. The percolates were then filtered with Whatman's No. 1 filter paper and the filtrate was concentrated *in vacuo* at 40°C under reduced pressure using a rotary evaporator to calculated:

$$\text{Percentage yield of the plant extract} = \frac{\text{Dry weight of extract (g)}}{\text{Initial weight of plant sample(g)}} \times 100\%$$

The concentrated extract was stored at 4°C until further use[15].

Antimicrobial assay of the ethanolic leaf extracts: The Minimum Inhibitory Concentrations (MICs) of *M. oleifera* and *C. sinensis* ethanolic leaf extracts were identified in triplicates such that 0.5 mL of different concentrations of the extracts of either plants (from 10-100 mg mL^{-1}) and 2 mL of nutrient broth were added, 100 µL of the standardized inoculum of either organism was added to each tube, all broth samples were incubated at 37°C for 24 h. The antimicrobial

results were measured by observing turbidity at 600 nm wavelength[16]. An inoculum was taken from the tubes showing no visible sign of growth or turbidity and was inoculated onto sterile nutrient agar plates by streak plate method. The plates were then incubated at 37°C for 24 h. The concentration of the extract that causes the complete absence of the growth of tested organisms was taken as the Minimum Bactericidal Concentration (MBC).

Reassessment of antibiotic sensitivity after growing the MDR bacterial isolates on the MIC of the most effective ethanol extract: After determination of the MIC of the most effective extract against MRSA and MDR *E. coli*, they were grown on nutrient broth media (2 mL) containing 100 µL bacterial suspension previously adjusted to McFarland standard and 0.5 mL of MIC detected for both strains. Each strain was then inoculated on Muller Hinton agar medium and the antimicrobial susceptibility test was done again by disk diffusion method.

Phytochemical screening of *M. oleifera* ethanol leaves extract via GC-MS analysis: The phytochemical investigation was carried out using Perkin-Elmer GC Clarus 500 system. For analysis TG-SQC column {15 m (length)×0.25 mm (internal diameter)×0.25 µm (Film thickness)} was used, the oven temperature was programmed from 50°C with an increase of 7°C min^{-1} to 150°C, then 5°C min^{-1} to 250°C with 5 min time hold, then 10°C min^{-1} to 290°C with 2 min hold time. Mass spectra were taken at 70 eV; a scan interval of 0.5 sec and fragments from 45-450 Da. The solvent delay time was set at 0.2 min. Methane was used as the carrier gas at a flow rate 1 mL min^{-1} and the total GC-MS running time was 45 min.

The spectrum of the unknown components was compared with the spectrum of known components stored in the National Institute Standard and Technology (NIST) library. The name, molecular weight and structure of the components of were detected[17].

Transmission Electron Microscope (TEM) examination: Conventional TEM microscopy is selected to visualize the ultrastructural damage on both cell wall and cytoplasmic membrane of entire microbes using a fixative material[12].

At ultrastructural 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[11].

Protein profile analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): About 5x sample buffer (10% w/v SDS, 10 mM dithiothreitol, or β-mercapto-ethanol, 20% v/v glycerol, 0.2 M tris-HCl, pH 6.8 and 0.05% w/v bromophenol blue) should add up to 8 M urea for really hydrophobic proteins 1×running buffer (25 mM tris-HCl, 200 mM glycine and 0.1% (w/v) SDS).

Preparing the sample: Mix the protein 4:1 with the sample buffer. Heat the sample by boiling for 5-10 min.

Running the sample on gel: Clamp in your gel and fill both buffer chambers with gel running buffer according to the instructions for your specific apparatus. Pipette the sample into the gel adjusting the volume according to the amount of protein in the sample. Be sure to include a lane with molecular weight standards. Now attach your power leads and run the gel until the blue dye front reaches the bottom with 250 V constant which in a 4-20% mini gel needs about 30 min total run time, but adjust to the thickness of your gel, the power supply used and the resolution desired. Remove the gel for the power supply and process further-visualize the proteins using coomassie brilliant blue, silver stain or any of the other protein stains[11].

RESULTS

Antimicrobial susceptibility test: Table 1 showed the effects of different groups of antibiotics on the 30 clinical bacterial isolates collected from patients admitted to a hospital in Giza governorate. A total of 5 isolates were found to be Multi Drug Resistant (MDR) (three isolates of *E. coli*; E_5, E_6 and E_8 and two isolates of *S. aureus*; S_2 and S_7). They were resistant to more than one group of antibiotics.

The two isolates E_6 (Gram-negative bacteria) and S_2 (Gram-positive) were selected for further experiments as they were resistant to most of the antibiotics used.

Extraction yield percentage: From Table 2 it was found that the extraction of 300 g of the pulverized *M. oleifera* leaves using 1 L of 70%v/v ethanol gave a yield of 11.56% while, that of *C. sinensis* gave a yield of 10.87%.

Table 1: Susceptibility pattern of the bacterial isolates to different groups of antibiotics

		Mean diameter of inhibition zone (mm)						
		Penicillin group			Cephalosporin group		Rifamycins	Sulfa group
Bacterial isolates		Amoxicillin/clavulanic acid	Amoxicillin	Oxacillin	Cefuroxime	Cephalexin	Rifampicin	Sulfamethoxazole/trimethoprim
E. coli	E_1	20	23	22	15	16	22	20
	E_2	21	19	16	22	23	19	20
	E_3	25	26	23	20	22	21	22
	E_4	20	23	22	15	20	22	20
	E_5	10	22	00	09	08	10	00
	E_6	00	00	00	10	00	00	00
	E_7	18	22	23	26	22	12	20
	E_8	00	10	10	13	00	09	21
	E_6	21	19	20	20	23	24	21
	E_{10}	20	21	21	20	14	21	22
	E_{11}	23	25	22	20	15	18	20
	E_{12}	16	18	19	20	20	23	21
	E_{13}	20	23	22	15	16	22	20
	E_{14}	21	19	16	22	23	19	20
	E_{15}	25	26	23	20	22	21	22
S. aureus	S_1	18	22	23	26	22	12	20
	S_2	00	00	00	15	09	00	16
	S_3	20	19	20	20	18	22	22
	S_4	18	19	18	16	17	25	24
	S_5	16	18	19	20	20	23	21
	S_6	20	23	22	15	16	22	20
	S_7	00	00	00	22	10	00	20
	S_8	25	26	22	20	22	21	22
	S_9	22	23	18	20	25	23	20
	S_{10}	20	21	23	23	18	16	18
	S_{11}	19	18	16	18	18	19	16
	S_{12}	22	22	19	13	18	19	21
	S_{13}	21	19	20	20	23	24	21
	S_{14}	20	21	21	20	14	21	20
	S_{15}	23	25	22	20	15	18	20

Table 2: Yield percentage of *M. oleifra* and *C. sinensis* ethanolic leaf extracts

	Weight (g)		
Plant	Dry leaves	Ethanolic extract	Yield percentage
M. oleifera	300	34.7	11.56
C. sinensis	300	32.6	10.87

Antimicrobial assay of the ethanol leaves extracts: From Fig. 1 it was found that the different prepared concentrations from the ethanol extract of *M. oleifera* leaves were more effective than the corresponding ones of *C. sinensis* leaves. The MIC values were detected to be 10 and 20 mg L^{-1} for the two extracts, respectively. While from Fig. 2 it was found that the different prepared concentrations from the ethanoic extact of *M. oleifera* leaves were more effective than the corresponding ones of *C. sinensis.* The MBC of the tested bacterial isolates was found to be 30 and 40 mg mL^{-1} for the ethanolic extracts of *M. oleifera* and *C. sinensis,* respectively.

Reassessment of the antibiotic sensitivity test: Table 3 indicated that there was great differences in the antibiotic sensitivity test results before and after growing both isolates with *M. oleifera* ethanol leaves extract (10 mg mL^{-1}). The multi drug resistant *E. coli* (E_6) restore its antibiotic sensitivity for all the tested antibiotics. On the other hand for the multi drug resistant *S. aureus* (S_2) it became sensitive to rifampicin and become more sensitive to the other antibiotics.

Phytochemical screening of *M. oleifera* ethanolic leaf extract: As *M. oleifera* ethanolic extract was the most effective against the *E. coli* and *S. aureus* it was analysed phytochemically using GC-MS to detect its chemical composition (Fig. 3).

Table 4 showed that *M. oleifera* ethanolic contained 12 chemical compounds. The higher percentage was for the fatty acid $C_{16}H_{22}O_4$ (41.77%). The table also indicated that these chemical compounds were limited to fatty acids, Ketones, phenolic compounds and steroids.

The chemical structure of each of the 12 analysed compounds is illustrated in Fig. 4.

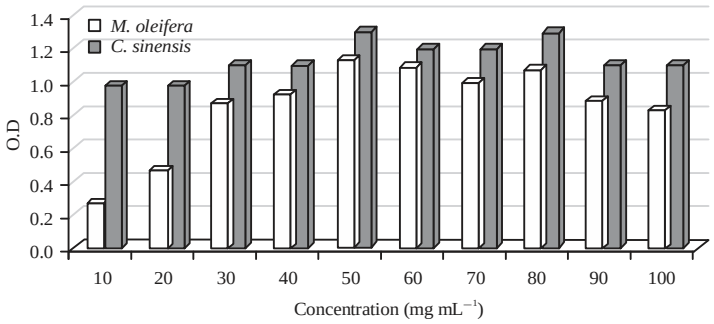

Fig. 1: Effect of different concentrations of *M. oleifera* and *C. sinensis* ethanolic leaf extracts on the selected bacterial isolate E_6

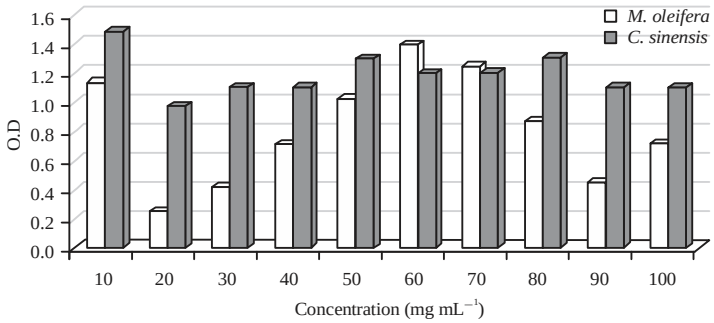

Fig. 2: Effect of different concentrations of *M. oleifera* and *C. sinensis* ethanol leaves extracts on the selected bacterial isolate S_2

Table 3: Re-assessment of the antibiotic sensitivity test of the selected multidrug resistant isolates

	Mean diameter of inhibition zone (mm)						
	Penicillin group			Cephalosporin group		Rifamycins	Sulfa group
Bacterial isolates	Amoxicillin/clavulanic acid	Amoxicillin	Oxacillin	Cefuroxime	Cephalexin	Rifampicin	Sulfamethoxazole/trimethoprim
E_6	00	00	00	10	00	00	00
*E_6	08	08	10	17	15	12	18
S_2	00	00	00	15	09	00	16
*S_2	00	00	00	20	12	09	20

*E_6 and *S_2: Selected multi drug resistant isolates after treatment with the MIC of *M. oleifera extract*, E_6 and S_2: Isolates before treatment

Table 4: List of the compounds detected by GC-MS analysis in the ethanol extract of *M. oleifera* leaves

*Rt (min)	Compound name	Area (%)	Molecular formula	Molecular weight	Compound nature
10.65	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one				
31.30	$C_6H_8O_4$	144	Ketone		
14.09	1H-benzotriazole, 5nitro phenol	2.62	$C_6H_4N_4O_2$	164	Phenolic compound
24.00	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester				
41.77	$C_{16}H_{22}O_4$	278	Fatty acid ester		
24.82	Hexadecadienoic acid, methyl ester				
0.33	$C_{17}H_{30}O_2$	266	Fatty acid ester		
24.87	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	0.43	$C_{24}H_{46}O_2$	366	Fatty acid ester
26.63	Oleic acid	4.82	$C_{18}H_{34}O_2$	282	Fatty acid
26.68	Hexadecanoic acid	8.31	$C_{16}H_{32}O_2$	256	Fatty acid
28.17	9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester				
0.99	$C_{21}H_{36}O_4$	352	Fatty acid ester		
28.55	Ethyl iso-allocholate	0.38	$C_{26}H_{44}O_5$	436	Steriod
28.63	Isochiapin B	0.30	$C_{19}H_{22}O_6$	346	Sesqiterpeniod
29.94	Linoleic acid ethyl ester	3.06	$C_{20}H_{36}O_2$	308	Fatty acid ester
30.02	9,12-octadecadienoic acid	4.76	$C_{18}H_{32}O_2$	280	Fatty acid

* Rt: Retention time in minutes

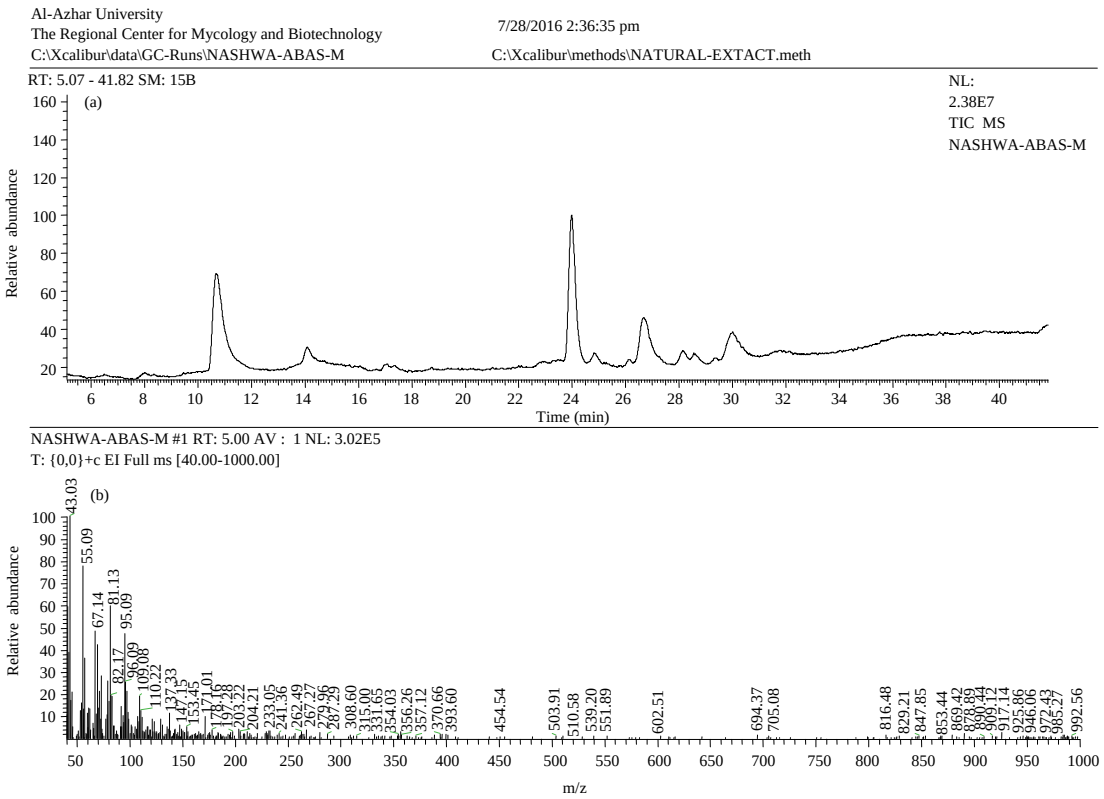

Fig. 3(a-b): GC-MS chromatogram of the ethanol extract of *Moringa oleifera* leaves

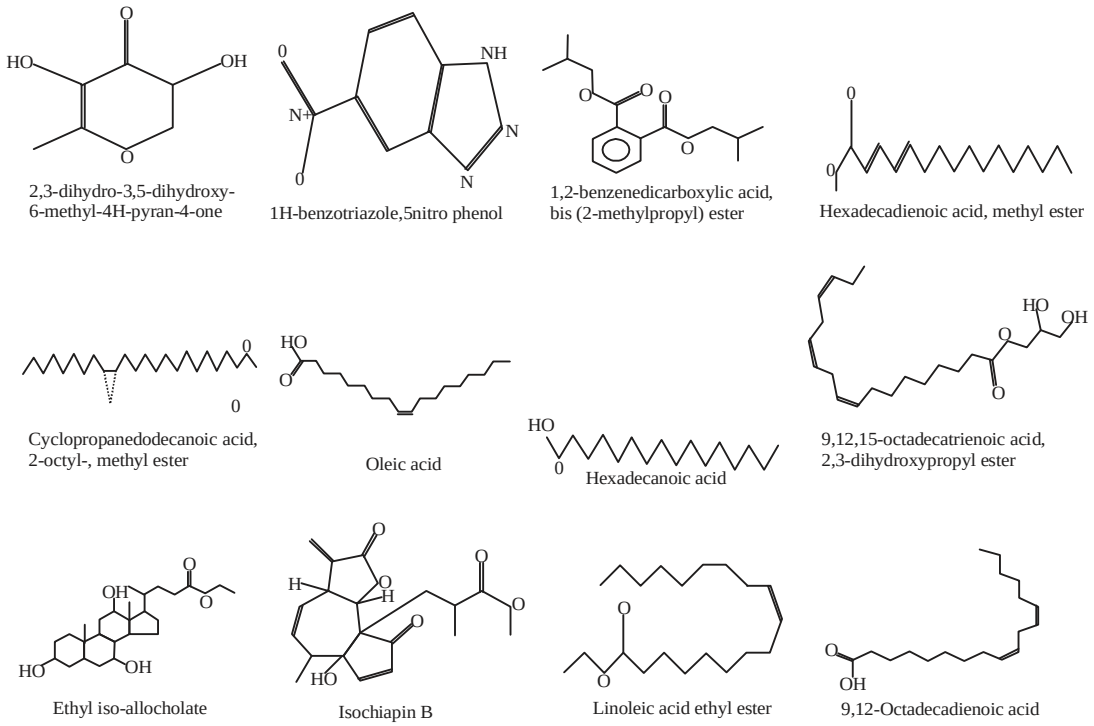

Fig. 4: Chemical structure of the major phytochemical compounds detected in the ethanol extract of *M. oleifera* leaves

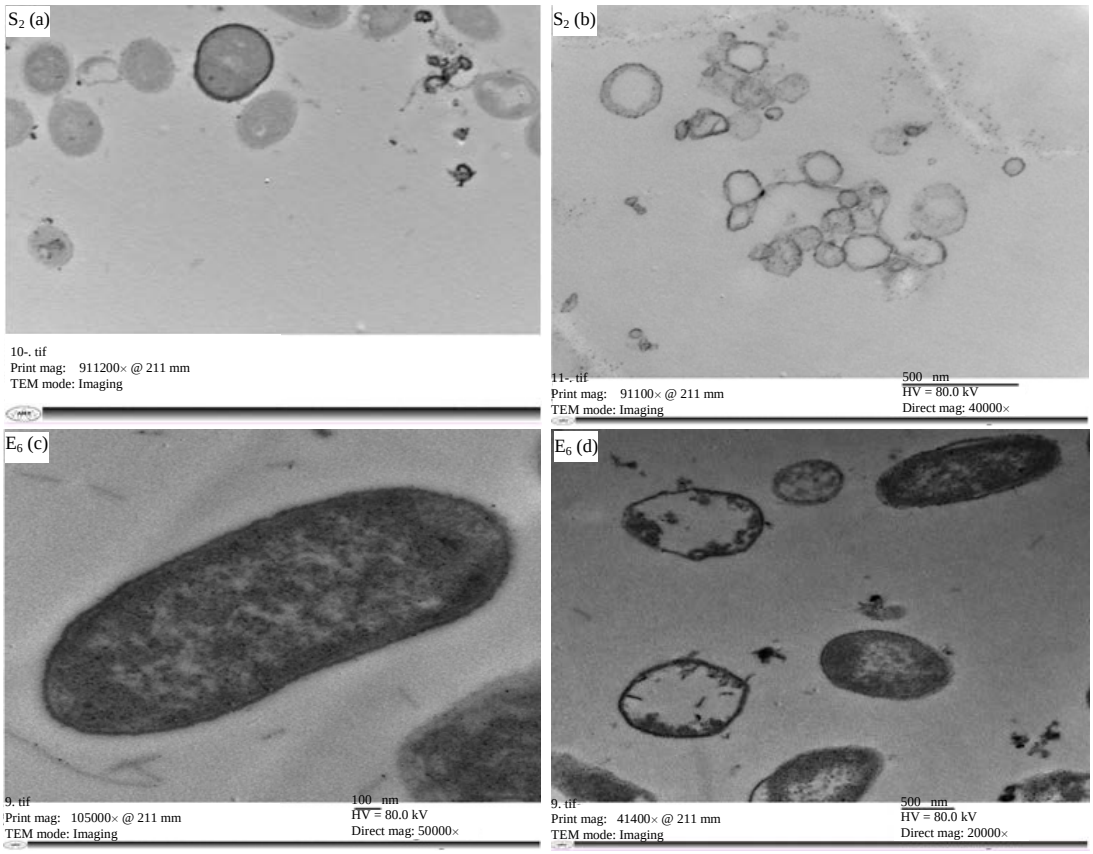

Fig. 5(a-d): TEM for E_6 and S_2 (a, c) Before and (b, d) After growing on the MIC of *M. oleifera* ethanol leaf extract

Figure 5 showed that there were many chemical compounds present in *M. oleifera* ethanolic extract. These compounds are illustrated with its percentage in Table 4.

Transmission Electron Microscope (TEM) examination: The TEM was done to detect the internal effect of the extract on both MDR bacteria (Fig. 5). The figure indicated that after growing MRSA on *M. oleifera* extract the cells lost its internal components and appeared as washed out cells (Fig. 5b) while in case of MDR E. coli the bacteria changed in shape and lost its internal components also (Fig. 5d).

Protein profile analysis: The protein profile of the two clinical isolates reveals the disappearance of the protein bands in case of growing with 10 mg mL^{-1} of the plant extract, which indicates the alteration of the protein synthesis in both. Three clear bands were detected in case of E_6, while one clear band only is detected in case of S_2 after treatment (Fig. 6).

DISCUSSION

The emergence of antibiotic resistant bacterial strains become an important issue that create problems in the treatment of infectious diseases and makes the search of an alternative therapy is a must[18-20].

During this study, 30 clinical isolates previously identified as *E. coli* and *S. aureus* were obtained from clinical samples collected from out patients admitted to a hospital at Giza. The results of the antimicrobial susceptibility test revealed that 16.6% of the isolates were found to be resistant to two or more of the used antibiotics and this reflects the emergence of new strains of *E. coli* and *S. aureus* which are resistant to classes of antibiotics that it was sensitive to before (Table 1). Several studies assessed the role of *M. oleifera* and *C. sinensis* leaves in folk medicine[21-23]. The current study revealed that the ethanolic leaf extract of *M. oleifera* and *C. sinensis* at a concentrations ranging from 10-100 mg mL^{-1} have shown promising bacterial inhibiting properties against the two selected multidrug resistant isolates (Fig. 1, 2), *M. oleifera* ethanol leaves extract was found to be more effective showing MIC values10 and 20 mg mL^{-1} for E_6 and S_2, respectively. Similar researches deduced that the ethanol leaves extract of both plants showed antibacterial activities against broad spectrum cultures[24]. In the present study, tried to overcome the bacterial resistance by growing the MDR isolates on the MIC of *M. oleifera* and re-assess the antibiotic

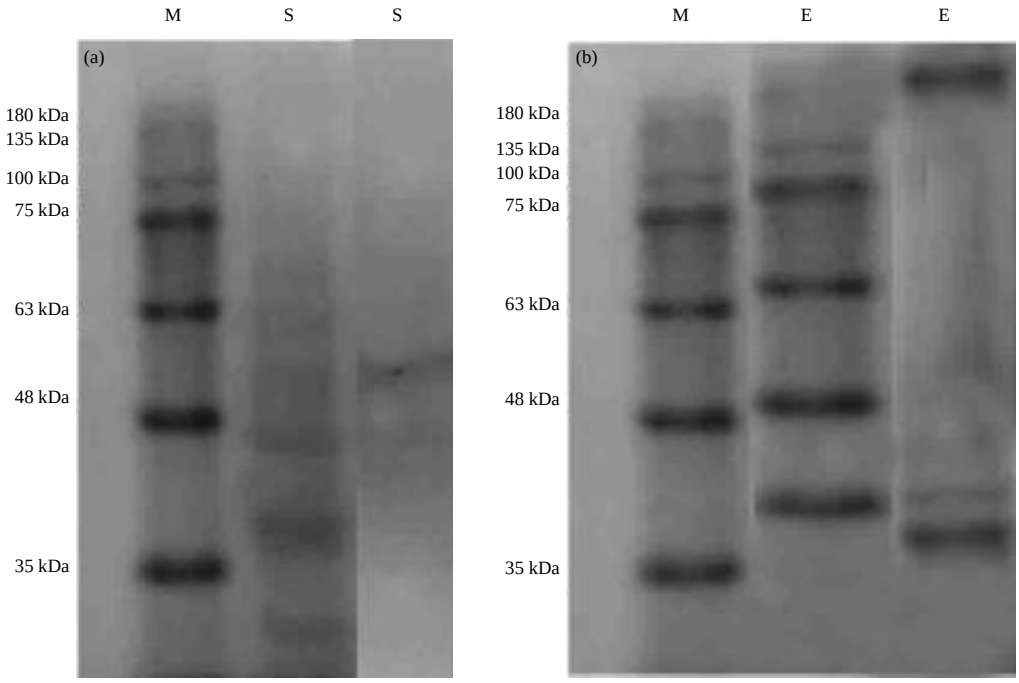

Fig. 6(a-b): Protein profile analysis of the isolates E_6 and S_2 before (E, S) and after (*E, *S) growing on the MIC ethanol extract of *M. oleifera* leaves, (a) *S. aureus* (S_2) and (b) *E. coli* (E_6)

sensitivity test after this treatment. Results of reassessment of the antibiotic sensitivity test of both isolates had led to modifying the behavior of E_6 towards the studied antibiotics as it became sensitive to the antibiotics that it was previously resistant to, while in MRSA the change was not great except for cephalixen and sulphmethoxazol/trimethoprime (Table 4). The phytochemical screening of the ethanol extract of *M. oleifera* leaves was performed via GC-MS analysis, the extract was a complex mixture of various constituents. Comparing the mass spectrum with the NIST data base library a total of 12 major compound were identified, their chemical structures and molecular weights were detected (Table 4, Fig 3, 4), these phytochemicals belongs to variety groups including fatty acids, terpenoids, esters and phenolics whose antibacterial and antifungal potentials were comparable to those of standard antibiotics[25-28]. The TEM examination revealed morphological changes induced by treatment with *M. oleifera* ethanolic leaf extract which include disruption and disintegration of the cell wall and extrusion of the cytoplasmic content.

Protein profile analysis is a method for detecting distantly related proteins by sequence comparison[29] in order to estimate the molecular weights of the proteins in the samples, the migration of each band was compared to the migration of the protein standards of known molecular weights in the molecular ladder. In case of the selected two multidrug resistant isolates *E. coli* (E_6) and *S. aureus* (S_2) there was a clear alteration of the protein profile after their growth on the MIC of the ethyl alcohol extract of *M. oleifera* leaves. A general disappearance of bacterial proteins was observed assigned to large scale protein degradation (Fig. 6). The deletion of the protein bands gives a good evidence of the ability of the bioactive compounds present in the plant extract to interrupt the biochemical pathways of protein synthesis and give an explanation of the antibacterial mode of action of these compounds besides its ability to modify the behavior of the multidrug resistant isolates under study. These findings were supported by those of Hajar and Gumgumjee[30] who reported that secondary plant metabolites including alkaloids, phenolic, steroids, terpeniods and fatty acids separated by ethanol may result in the change in the gene expression creating a new genotypes.

These results demonstrated that besides the clear effect of the ethanolic extract of *M. oleifera* leaves on the cell wall of the tested bacterial isolates there was also a significant effect on the whole cell protein of both isolates which is responsible for modifying the behavior of the bacterial isolates to the tested antibiotics.

CONCLUSION

From the previous results it was concluded that the comparable study of the antibacterial effect of the ethanol leaves extract of both *Moringa oleifera* and *C. sinensis*

revealed that the two plants have inhibitory effect on the tested multi drug resistant clinical isolates (*E. coli* and *S. aureus*). The *M. oleifera* extract have a significant modifying action on the antibiotic susceptibility behavior of the bacterial isolates. Phytochemical screening of the extract shows an array of bioactive compounds that have a significant effect on the cell wall and the cell proteins of both isolates.

This reveals that *M. oleifera* could be a promising naturally occurring antibacterial agent with potential applications in the pharmaceutical industry for controlling multidrug resistant microorganisms.

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