Open Access **Baghdad Science Journal** **P-ISSN: 2078-8665**
2020, 17(2):452-462 **E-ISSN: 2411-7986**

DOI: http://dx.doi.org/10.21123/bsj.2020.17.2.0452

Investigation of the Biochemical and Ultrastructural Mechanisms Underlying the Antimicrobial Activity of *Mimusops* spp. Extracts

Gehad Abdelmohsen [1] ***Gouda T. M. Dawoud*** [1] ***Mahmoud S.M. Mohamed*** [2*]

Received 2/7/2019, Accepted 30/12/2019, Published 1/6/2020

Abstract:

Antibiotic resistance is the major growing threat facing the pharmacological treatment of bacterial infections. Therefore, bioprospecting the medicinal plants could provide potential sources for antimicrobial agents. *Mimusops*, the biggest and widely distributed plant genus of family Sapotaceae, is used in traditional medicines due to its promising pharmacological activities. This study was conducted to elucidate the antimicrobial effect of three unexplored *Mimusops spp.* (*M. kummel, M. laurifolia and M. zeyheri*). Furthermore, the mechanisms underlying such antibacterial activity were studied. The *Mimusops* leaf extracts revealed significant antibacterial activities against the five tested bacterial strains with a maximum inhibition zone diameter of 22.0 mm against *B. subtilis* compared with standard antibiotic ciprofloxacin. The minimal inhibitory and bactericidal concentration values against tested Gram-positive and Gram-negative bacterial strains ranged from 3.15-12.5 µg/ml. However, weak antifungal effect was recorded against *Candida albicans* with MIC value >25 µg/ml. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay showed that *M. caffra* was the best antioxidant (IC_{50}=14.75±0.028 µg/ml), while *M. laurifolia* was the least one (IC_{50}=34.22±0.014 µg/ml). The phenolics in plant leaves extracts were identified and quantified by high performance liquid chromatography (HPLC) which revealed the presence of seven phenolic acids and four flavonoids. The abundant phenolic compounds were rutin (5.216±0.067 mg/g dried wt.) and gallic acid (0.296±0.068 mg/g) followed by myricetin (0.317±0.091 mg/g) then kaempferol (0.113±0.049 mg/g) as flavonoids. The antibacterial mechanism of *M. laurifolia* extract, as a representative species, induces ultrastructural changes in the model bacterium *Staphylococcus aureus* with cell wall and plasma membrane lysis as revealed by transmission electron microscopy. Overall, *Mimusops* species (*M. laurifolia*, *M. kummel* and *M. zeyheri*) are promising natural alternative sources for antimicrobial agents.

Key words: Antimicrobial, *Mimusops*, Phenolic, *Staphylococcus aureus*, Ultrastructure

Introduction:

The progressive increase in antibiotic resistance is the major threat facing the pharmacological treatment of bacterial infections (1). For instance, many stains of *Staphylococcus aureus* have evolved resistance mechanisms against most of the known antibiotics (2, 3). Therefore, there is a great demand to search for new cost effective drugs, especially those having natural origins (4). In this regard, utilization of plant-derived nutraceuticals as antimicrobial agents has attracted much attention (5). Plants contain vast array of phytochemicals that have been proved to exert several pharmacological applications including toxicity against fungal and bacterial pathogens (6, 7).

Among these, phenolic compounds, the most diverse group of plant secondary metabolites, have been reported to possess antioxidant, antifungal and antibacterial properties (8, 9). Moreover, the total polyphenols content of plants is regarded as a good indicator for their biological activities (6,10). In fact, the phenolic ingredient of plants varies, both quantitatively and qualitatively, depending on several factors such as plant species, growth stage and environmental conditions. In this context, members of Sapotaceae family have been regarded as potential sources of bioactive phytochemicals, particularly phenolics (11).

The family Sapotaceae is flowering plants categorized into five tribes, taxonomically it is belonging to order Ericales, the family contain 53 genera and about 1250 species. It is characterized by their wide range of chemical constituents like

[1] Phytochemistry Lab. Applied Research Center for Medicinal Plant (NODCAR), Giza 12561, Egypt
[2] Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza 12613, Egypt
[*]Correspondence author: msaleh@sci.cu.edu.eg
[*]ORCID ID: https://orcid.org/0000-0003-2218-3947

polyphenols, flavonoids, and saponins, which have antibacterial, antifungal, antioxidant and anti-inflammatory activities (11). *Mimusops*, the most important and biggest genus of family Sapotaceae, is native to tropical and subtropical regions of Asia, Africa, Australia, and various oceanic islands (12). Several species of *Mimusops* species are utilized in traditional medicine as anthelmintic, tonic and astringent agents (13). Therefore, bioprospecting in the genus *Mimusops* could provide potential sources for antimicrobial agents as an approach to face the growing antibiotic resistance.

The antibacterial activity of phenolic rich extracts from several *Mimusops* spp. have been investigated, however *M. elengi* and *M. caffra* are the most studied species of the genus. They have been recognized as potent agents against several human pathogens (10, 14, 15). Unfortunately, the mechanisms underlying such antimicrobial activity are not elucidated. Therefore, regarding its importance for drug industry, exploring the mode of action of *Mimusops* phytochemicals on the physiology and ultrastructure of pathogenic bacteria represent a promising aim. Therefore, the current study was conducted to assess the antimicrobial activities of three unexplored species of the genus *Mimusops* (*M. laurifolia*, *M. kummel* and *M. zeyheri*), as compared to the well-studied one, *Mimusops caffra*. Moreover, the mechanisms underlying such antimicrobial potential were explored at both biochemical and ultrastructural levels.

Materials and Methods:

Chemicals

Methanol, ethyl acetate, dimethyl sulfoxide (DMSO), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and all standards required for HPLC were purchased from Sigma-Aldrich (St. Louis, USA). Aluminum choloride and Folin-Ciocalteu's reagent were obtained from Merck (Darmstadt, Germany). Ciprofloxacin, amphotericin-B, tryptone soy and Müller-Hinton media were purchased from Oxoid (Basingstoke, UK).

Plant collection and extraction

Fresh plant material of some *Mimusops* spp. (*Mimusops caffra* (E. Mey.ex A. D.C. in DC.), *Mimusops kummel* (Bruce ex A. D.C. inDC.), *Mimusops laurifolia* (Forssk.) Friis and *Mimusops zeyheri* (Sond.) was collected during summer 2014 from Giza Zoo (30°1'25.44"N; 31°12'50.35"E), Orman garden (30°1'44.66"N; 31°12'46.67"E), El-Zohria (30°2'47.42"N; 31° 13'32.91'E), Giza, Egypt and Aswan botanical garden (24°5'37.72"N; 32°53'12.89"E), Aswan, Egypt, identified by Cairo University Herbarium (CAI); authenticated specimen kept in (CAI).

Leafy branches were transferred to lab and dried in shade at room temperature (RT), then dried in oven (40 °C) until constant weight. The dried material was minced in a coffee grinder and 5 grams of powdered plant material were defatted with n-hexane (500 ml) using Soxhlet extractor (Gerhardt, Germany) and dried over night at 24 °C, then extracted by methanol: water (70%, v/v) in a Soxhlet extractor for one hour. The extract was concentrated using rotary evaporator (Heidolph, Germany) at 40 °C. After complete evaporation of the solvent, the obtained extracts were further purified using ethyl acetate, concentrated, evaporated and then weighed. The extracts were kept in sterile dark bottles in refrigerator at 4 °C until next use. Aliquot of each extract were dissolved in DMSO and used in antimicrobial activity assay, another was dissolved in HPLC grade methanol then filtered through a 0.45 µm Millipore membrane filter before injection into HPLC (16).

Phytochemical screening

In order to detect the presence or absence of different classes of secondary metabolites, qualitative phytochemicals analysis was performed. Preliminary phytochemically evaluation of different extracts was performed to determine the presence of terpenoids, alkaloids, saponins, tannins, steroids, anthraquinones, glycosides and carbohydrates according to standard procedure (17). The colours change or the formation of precipitate was considered as indicative of positive results to these tests as described by Harborne (17).

Evaluation of antimicrobial activity of the plant extracts

Microbial strains

The reference bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC); *Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 19659, *Pseudomonas aeruginosa* ATCC 27853 and the yeast *Candida albicans* ATCC 10231. All bacterial strains were stored at –80 °C in glycerol (15%, v/v). Bacteria were recovered in tryptone soya broth (TSB) overnight at 37 °C and then sub-cultured on tryptone soya agar (TSA) plates to obtain single colonies.

Antimicrobial assay

The *Mimusops* spp. extracts were first screened using well-diffusion assay for antimicrobial activity according to (15). The extracts were prepared to obtain 100 µg/ml concentration using DMSO as a solvent, *C. albicans*

inoculum was prepared as described by (18). Plates containing 15 ml Müller-Hinton (MH) agar medium or Yeast Peptone Dextrose agar medium (Laboratories Conda SA, Madrid, Spain) were prepared and allowed to solidify at 24 °C for bacteria and yeast respectively. The agar surface was inoculated with 100 µl of bacterial suspension (0.5 MacFarland), after that; 6 mm wells were punched in the agar using sterile corn borer. The wells were inoculated with 50 µl of the tested plant extract (50 µg/ml). Ciprofloxacin (5 µg/ml) was used as positive control for bacterial strains, whereas Amphotericin-B (20 µg/ml) was used as positive control for *C. albicans*. A negative control well was filled with only DMSO (50 µl). Plates were incubated at 37 °C for 24 h. All experiments were done in triplicate and the results were recorded as a mean inhibition zone.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

In order to determine the MIC and MBC values of the *Mimusops* spp. extracts, the broth micro-dilution method was performed using sterile 96-well micro-titre plates as described previously (19). In brief, stock solutions of studied plant extracts were prepared in 5.0% (v/v) DMSO. A total of 100 µl of bacterial suspension containing 10^6 cfu/ml were added to each well, then serial dilution of plant extract ranging from 3.15 to 50 µg/ml was added, then the volume was completed up to 200 µl using MH broth. A well containing bacterial suspension was used as positive control and a well containing MH broth only was used as negative control. The plates were incubated at 37 °C for overnight. After incubation, the result was observed visually, and the MIC is defined as the lowest concentration of the extract that completely inhibits visible bacterial growth. Experiments were done in triplicate, and the results were recorded. The MBC was determined by plating 10 µl of each well on TSA medium and further incubation at 37 °C for 24 h. The lowest concentration of extract with no visible bacterial growth on subculture agar medium, indicating 99.9% killing of the bacterial inoculum, was regarded as MBC (19). All experiments were repeated three times.

DPPH radical scavenging assay (Antioxidant activity)

The extract free radical scavenging activity were evaluated using DPPH system as described previously with minor modification (20). The extract stock solutions of different species were prepared as 1.0 mg/ml concentration in methanol, each solution was diluted to final concentrations of 50, 30, 25, 20, 10 and 4 µg/ml in methanol. Ascorbic acid was prepared (1.0 mg/ml) and used as standard antioxidant, then diluted in methanol to obtain final concentrations as the following 250, 125, 50, 25, 10 and 5 µg/ml.

One ml of DPPH methanolic solution (0.3 mM) was added to 2.5 ml of the solutions of the tested extracts and standards. Each sample was compared to a blank, where the DPPH is replaced with 1 ml methanol. Negative control was prepared by mixing 1 ml the DPPH solution with methanol (2.5 ml). All samples were incubated in the dark for 30 min at RT. The resulting mixture absorbance was recorded at 515 nm. The experiment was performed in triplicate, and the average absorbance (Abs) for each concentration was recorded.

The absorbance was converted into antioxidant activity percentage (AA%) by the following formula: AA% = 100 – (Abs sample – Abs blank) × 100 /Abs control.

The antioxidant activities of tested extracts were expressed by IC_{50} values which indicate (the concentration of tested extracts that causes 50% quenching of the UV absorption of DPPH was calculated.

Determination of the plant extracts total phenolics and flavonoids

The content of total phenolic compounds in tested extracts was measured with a colorimetry method based on oxidation/reduction reaction by Folin-Ciocalteau phenol according to British Pharmacopoeia (21). The blue colour absorbance was measured at 650 nm against a water-reagent blank after incubation for 30 min. Quantification was acquired by reporting the absorbance in the calibration curve prepared with 20, 40, 60, 80 and 100 mg gallic acid (GAE)/l, and then expressed as mg GAE equivalent per ml extract. All tests were carried out in triplicate

The total flavonoids contents were determined as previously described (22). From each extract 50 µl were mixed with 1.45 ml of distilled water, then 75 µl of 5% sodium nitrate solution were added. After 6 min, 150 µl of 10% aluminum chloride solution were added and the mixture was incubated before addition of 0.5 ml of 1 M sodium hydroxide for 5 min. Afterwards, the mixture was completed with distilled water up to 2.5 ml and mixed well. The absorbance was measured immediately at 510 nm. The concentration of total flavonoids was calculated using a standard curve of catechin (CE) and expressed as mg CE/ml extract.

Chromatographic analysis

Standard preparation and calibration curves

Standard stock solutions were prepared by dissolving 1 mg of each phenolic compound (gallic acid, chlorogenic acid, catechin, ellagic acid, tannic acid, caffeic acid, rutin, myricetin, ferulic acid,

quercetin, luteolin, apigenin and kaempferol) in 1 ml of methanol (HPLC high grade), then stored in dark glass bottles at 4 °C. These working standard should be freshly prepared before injection (23).

Analytical curves for each compound were prepared, plotted over different concentration rang. Calibration graph was used to determine linearity. The graph was further analysed by using an increasing of concentration and further evaluated by visual inspection of a calibration graph. The calibration curves was constructing by plotting absorbance against concentration to calculate regression equation (24).

HPLC condition

HPLC analysis was performed on an Agilent 1260 infinity (Agilent Technologies, USA) chromatograph using a diode array detector (DAD) detector (UV/Vis). The separation was performed at 25°C on an Econosil^{TM} C18 reversed-phase column (4.6×250 mm) with 5 µm particle size. The ChemStation software (Agilent technologies, USA) was used for the data analysis.

Identification and quantification of phenolic compounds

An elution system with acetic acid 1.5%, adjusted to PH 3 (solvent A), acetonitrile 100% (solvent B) at the follow gradient flow was used: at 0-10 min, A: B (80: 20, v/v), staring at 10.1 min, A: B (60: 40, v/v) (25). The flow rate was kept constant at 1 ml/min, using an injection volume of 20 µl, and the chromatograms were recorded at 280 λ up to 10 min, then changed to 330 λ started at 10.1 min. The peaks were characterized by comparison of their retention times with the reference standards.

Transmission electron microscopy (TEM) of *S. aureus* treated with *M. laurifolia* extract at different times points

For investigating the antibacterial mechanism of *M. laurifolia*, as a representative species, extract on the model bacteria *S. aureus*, plant extracts (6.25 µg/ml) were incubated with bacterial cells (10^6 cfu/ml) in sterile falcon tubes. Treated bacterial cells were collected after 8, 16 and 24 h, then centrifuged for 10 min at 5000 rpm and washed twice with 0.1 M phosphate buffer pH 7.4, and subsequently fixed for 2 h with 3% (v/v) glutaraldehyde at RT. Afterwards, the samples were rinsed using buffer and post fixed at 4 °C with 1% (w/v) osmium tetroxide (Carl Roth, Germany) for 3 h. Next, using a graded ethanol series (once at 20%, 50%, 60%, 70%, 80% and 90% and 100%), the samples were gradually dehydrated. Next, the samples were gradually impregnated, starting with pure propylene oxide and finishing with 100% Spurr (6). The ultra-thin sections (100 nm) were prepared on a Reichert Jung ultramicrotome by a diamond knife. Then the sections were placed on formvar-coated copper grids and stained with 2% aqueous solution of uranyl acetate and subsequently examined at an accelerating voltage of 80 kV under a JEM-1400 transmission electron microscope (JEOL Korea Ltd., Korea) at National Research Centre, Giza, Egypt.

Statistical analysis

The chemical analysis and antimicrobial activities of investigated *Mimusop* spp. extracts was performed in triplicate. The obtained data are presented as mean value ± standard deviation (SD). The mean values were recorded and compared using the Duncan test and the differences were considered statistically significant difference (P<0.05).

Results:

Antimicrobial assay

The antimicrobial assay revealed that all the tested plant extracts showed significant antibacterial activity against both Gram positive as well as Gram negative ATCC strains compared to positive control (Table 1). The plant extracts MIC values ranged from 3.15-12.5 µg/ml with slight variability in the inhibitory concentrations of each extract for given bacteria (Table 2). The most sensitive bacterial strain was *B. subtilis* while no significant antifungal activity was recorded against *C. albicans* and it was completely resistant.

Baghdad Science Journal
P-ISSN: 2078-8665
E-ISSN: 2411-7986

Table 1. Antimicrobial activity of the *Mimusops* species extract against authenticated microbial strains as measured by inhibition zone (mm) ± SE

		Plant extract			
Test organisms	**Positive control***	***M. caffra***	***M. kummel***	***M. laurifolia***	***M. zeyheri***
Gram negative					
E. coli	14.2±0.05^{b}	15.5±0.11^{d}	08.4±0.14^{a}	14.8±0.15^{c}	08.2±0.08^{a}
K. pneumoniae	10.0±0.12^{d}	09.0±0.14^{c}	08.2±0.14^{b}	09.4±0.14^{c}	07.0±0.12^{a}
P. aeruginosa	07.6±0.08^{d}	05.2±0.11^{c}	03.0±0.12^{a}	08.4±0.05^{e}	04.3±0.12^{b}
Gram positive					
B. subtilis	19.3±0.12^{b}	22.0±0.09^{d}	17.2±0.05^{a}	21.3±0.12^{c}	17.5±0.11^{a}
S. aureus	15.4±0.14^{b}	16.3±0.08^{c}	14.4±0.10^{a}	17.2±0.05^{d}	14.2±0.06^{a}
Yeast					
C. albicans	14.3±0.30	-**	-	-	-

* ciprofloxacin (5 µg/ml) was used as positive control for bacterial strains and Amphotericin-B (20µg/ml) was used as positive control for *C. albicans*. ** -; no inhibition zone detected

Each value is the mean±SE of 3 different experiments, different letters indicate significant difference (P<0.05) as analysed by Duncan test.

Table 2. MIC and MBC of the *Mimusops* species extracts against tested microbial strains

Test organisms	**Positive control***	**Plant extract (MIC/MBC (µg/ml))**							
		M. caffra		***M. kummel***		***M. laurifolia***		***M. zeyheri***	
Gram negative		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	0.15	6.25	12.5	12.5	25	6.25	12.5	6.25	12.5
K. pneumoniae	0.50	3.15	6.25	<3.15	3.15	<3.15	3.15	<3.15	3.15
P. aeruginosa	1.00	6.25	12.5	3.15	6.25	6.25	12.5	6.25	12.5
Gram positive									
S. aureus	0.50	6.25	12.5	6.25	12.5	6.25	12.5	6.25	12.5
B. subtilis	1.00	<3.15	3.15	<3.15	3.15	<3.15	3.15	<3.15	3.15
Yeast									
C. albicans	0.98	>25	>25	>25	>25	>25	>25	>25	>25

*Positive control for bacterial strains was ciprofloxacin (µg/ml) and Amphotericin-B (µg/ml) was positive control for *C. albicans*

Determination of total phenolics and flavonoids of the plant extracts

The quantitative estimation of total phenolic content ranged from 5.7 to 9.9 mg GAE/g dried extract, whereas the total flavonoids level varied from 0.33 to 0.79 mg/g with significant differences within the different *Mimusops* species as shown in Fig. (1). In all extracts, the level of total phenolics was higher than that of total flavonoids as expected. *M. kummel* had the highest level of total phenolics and the lowest level of total flavonoids; however the highest content of flavonoids was recorded in *M. caffra*.

Antioxidant activity and IC_{50} of the plant extracts

The DPPH radicals reduction was determined by the decrease in its absorbance at 518 nm. Results indicated a decrease in the DPPH radical concentration owing to the scavenging capability of the soluble constituents in the *Mimusops* species extracts. The scavenging effect of *M. caffra*, *M. Zeyheri*, *M. kummel* and *M. laurifolia* hydro-methanolic extracts on the DPPH radical at a concentration of 20 µg/ml was 67, 56, 42 and 31%, respectively. The calculated IC_{50} value revealed that *M. caffra* was the best antioxidant while *M. laurifolia* was the least one (Fig. 1).

Baghdad Science Journal
P-ISSN: 2078-8665
E-ISSN: 2411-7986

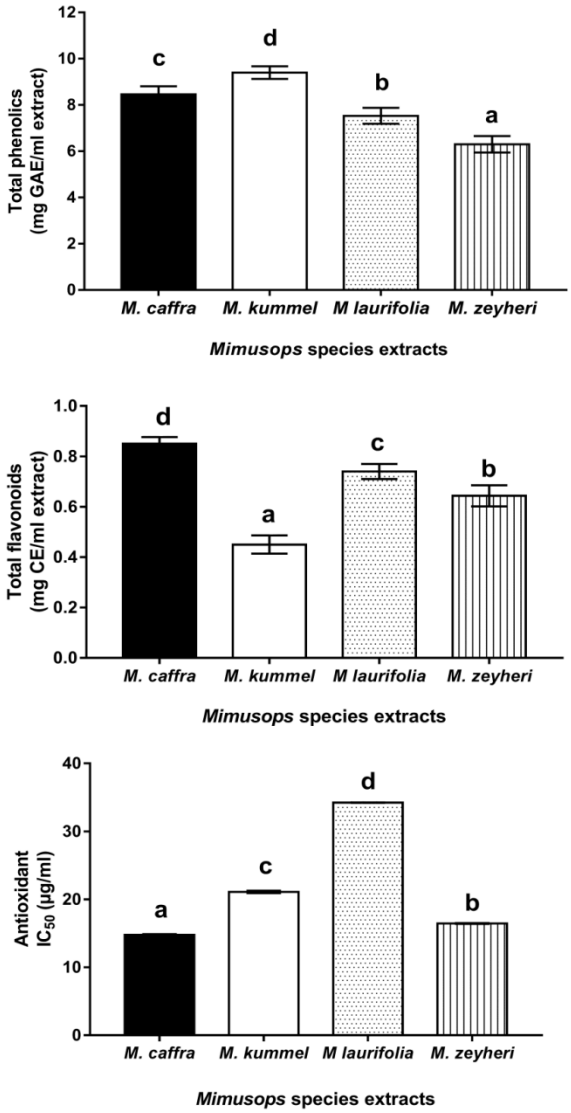

Figure 1. Total phenolics (mg GAE/ml extract) (A), total flavonoids (mg CE/ml extract) (B) and antioxidant IC50 (µg/ml) (C) of the *Mimusops* species extracts. Each value is the mean±S.D. of three different experiments. Different letters indicate significant difference (P<0.05) as analyzed by Duncan test.

Phenolome identification and quantification of the plant extracts by HPLC

The results of the HPLC analysis of the ethyl acetate fraction of hydro-methanolic extracts of the *Mimusops* species extracts are represented in Table 3. Eleven phenolic compounds were successfully isolated and identified as chlorogenic acid, gallic acid, catechin, tannic acid, caffeic acid, rutin, ferulic acid, myricetin, luteolin, apigenin and kaempferol. These compounds peaks were recorded at 280 nm and 330 nm and identified by comparison to standards.

The major phenolic compounds obtained were gallic acid and rutin marked at the peak at 2.59 and 6.49 min (Fig. 2) followed by a series of compounds between the retention times of 2.5-17 min at 280 λ. *M. Kummel* had the highest content of rutin (5.216±0.067 mg/g dried wt.) and gallic acid (0.296±0.068 mg/g dried wt.). The values of phenolic contents varied significantly, and ranged from 0.075–0.0296, 1.16–5.216, 0.002–0.113 and 0.03–0.39 mg per gram for gallic acid, rutin, kaempferol and myricetin respectively. Ellagic acid was not detected and catechin was detected only in *M. zeyheri*.

The major flavonoids were kaempferol and myricetin in all plant extracts at retention times 16.9 and 14.1 min at 330 λ, luteolin was also present with low concentration in all plant extracts. The highest content of myricetin was found in *M. zeyheri* (0.317±0.091 mg/g dried wt.)

Baghdad Science Journal
P-ISSN: 2078-8665
E-ISSN: 2411-7986

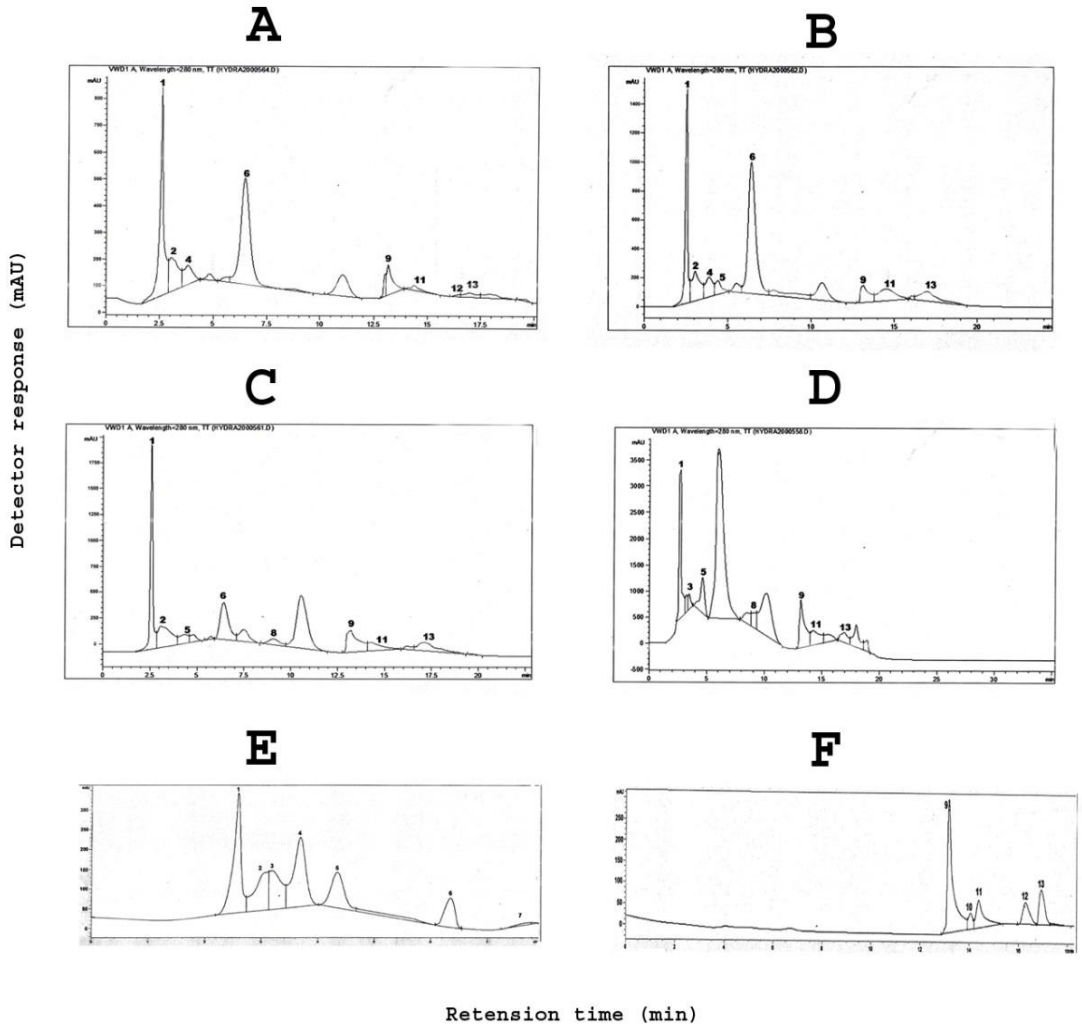

Figure 2. High Performance Liquid Chromatography (HPLC) profile of phenolic and flavonoid compounds detected in *Mimusops spp.* plant extract (A-D). A, *M. caffra*; B, *M. Kummel*; C, *M. laurifolia* and D, *M. Zeyheri*. E and F were standard mixture of phenolic and flavonoid compounds respectively. The peaks numbers representing each compound were (1) gallic acid, (2) Chlorogenic acid, (3) Catechin, (4)Tannic acid, (5) Caffeic acid, (6)Rutin, (7) Ellagic acid, (8) Ferulic, 9) Myricetin, (10) Quercetin, (11) luteolin, (12) Apigenin, and (13) Kaempferol.

Table 3. Endogenous phenolic compounds (mg/g extract) in the four *Mimusops* species extracts as identified by HPLC

No.	R.T.	Phenolic compounds	Plant peak area			
			M. kummel	*M. caffra*	*M. zeyheri*	*M.laurifolia*
1	2.59	Gallic acid	0.296 ± 0.068 [c*]	0.075 ±0.007 [ab]	0.013 ± 0.002 [a]	0.159 ± 0.037 [b]
2	3.00	Chlorogenic acid	0.045 ± 0.014 [b]	0.026±0.002 [ab]	ND	0.031 ± 0.012 [ab]
3	3.10	Catechin	ND	ND	0.003 ± 0.001	ND
4	3.85	Tannic acid	0.0313 ± 0.014 [a]	0.060 ± 0.006 [b]	ND	ND
5	4.52	Caffeic acid	0.007 ± 0.001 [a]	ND	0.070 ± 0.013 [b]	0.009 ± 0.002 [a]
6	6.49	Rutin	5.216 ± 0.067 [c]	1.56 ± 0.071 [b]	ND	1.160 ± 0.075 [a]
7	7.8	Ellagic acid	ND	ND	ND	ND
8	9	Ferulic acid	ND	ND	0.039 ± 0.024 [a]	0.031 ± 0.009 [a]
		Flavonoids				
9	13.1	Myricetin	0.039 ± 0.010 [a]	0.015 ± 0.003 [a]	0.317 ± 0.091 [b]	0.115 ± 0.066 [a]
10	14.1	Quercetin	ND	ND	ND	ND
11	14.3	Luteolin	0.021 ± 0.006 [a]	0.001 ± 0.001 [a]	0.061 ± 0.015 [b]	0.001 ± 0.001 [a]
12	16.5	Apigenin	ND	0.001 ± 0.001	ND	ND
13	16.9	Kaempferol	0.113 ± 0.049 [b]	0.002 ± 0.001 [a]	0.013 ± 0.002 [a]	0.014 ± 0.001 [a]

[*] Values are mean ± standard error of the three replicates, different letters indicate significant difference (P<0.05) as analyzed by Duncan test. **R.T**.: retention time (min); **ND**, not detected.

Baghdad Science Journal
P-ISSN: 2078-8665
E-ISSN: 2411-7986

Transmission electron microscopy (TEM) of *S. aureus* treated with *M. laurifolia* extract

Photomicrographs of control cells (*S. aureus*) show the presence of cell wall, plasma membrane, cytoplasm and electron dense materials (Fig. 3, A&B). On the other side, extensive cell damage was observed in bacteria treated with *M. laurifolia* extract. After 8 h of incubation, significant ultrastructure changes were observed such as partial rapture in cell wall (Fig. 3, C) that increased over 16 h, to include cell wall and plasma membrane (Fig. 3, D&E). Moreover, cells were completely vacuolated, cell wall and plasma membrane disintegrated and the cytoplasm leached out from the cell after 24 h incubation (Fig. 3, F).

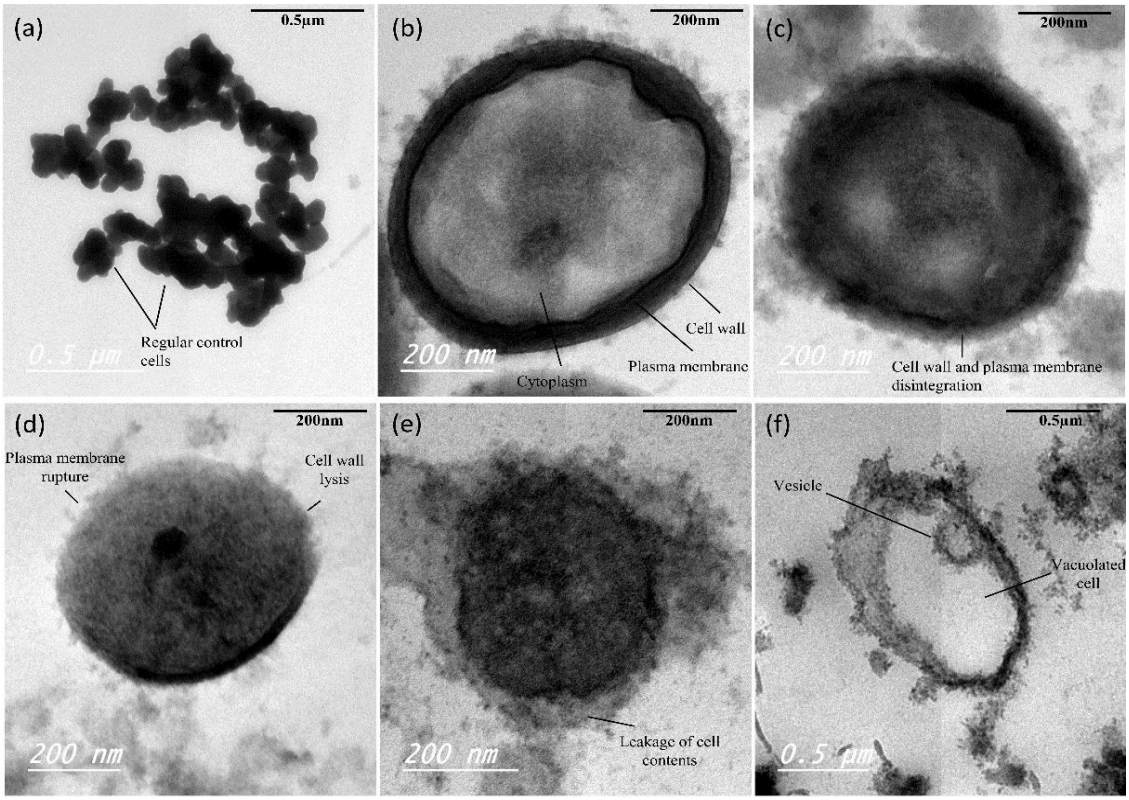

Figure 3. Transmission electron micrographs of ultra-sections in the *S. aureus* cell in MH culture medium without (A & B) or treated with *M. laurifolia* extract at concentration of 6.25 µg/ml (c–f). A, 6,000×; B, 12,000×; C, 8,000×; D, 8,000×; E, 9,000×; F, 12,000×. A: overview of the untreated bacterial cell, B: normal bacterial cell with regular shape including (capsule, cell wall, plasma membrane, cytoplasm and electron dense), C: bacterial cell treated with the extract after 8 h incubation showing raptured of capsule, D & E: bacterial cell treated after 16 h incubation, D: show partially disraptured of cell wall and, E: show plasma membrane disintegration, F: bacterial cell treated after 24 h incubation showing cell vacuolated and cytoplasmic content leakage.

Discussion:

Plants contain a vast array of bioactive phytochemicals that are believed to play a role in defense reactions against infections by pathogenic microorganisms (26). Among these, phenolic acids and flavonoids are known to be biologically active against tumors, viruses and other microbes and are also considered as antioxidant agents (27). Recently, there are a growing interest in developing novel antimicrobial agents from different sources like plant extracts, essential oils and pure secondary metabolites to cope with microbial resistance (6, 7, 28). Herein, the four studied species have antibacterial activity against both Gram positive and negative strains, this is in line with previous studies which reported that the *M. elengi* extracts had antibacterial activity against *P. aeruginosa, Salmonella typhi, Vibrio cholera, Streptococcus pneumonia, E. coli, B. subtilis* , *S. enterica*, and *S. mutans* (14, 15). However, no antifungal activity was reported against *C. albicans* (29). Similar results were obtained in our study with no antifungal activities recorded against *C. albicans*. The *Mimusops* species extracts in our study had significantly inhibited the bacterial growth with quite different strengths because all the tested plants belong to the same genus. In this context, ethyl acetate extract of bark of *M. elengi* inhibited 84.5% of *B. subtilis* growth (MIC = 0.6 mg/ml). In this

study, lower MIC and MBC values, indicated enhanced antibacterial activities, were recorded compared to the previously characterized family specie *M. elengi* which may be attributed to many factors such as plant genetic factors, cultivation conditions and plant geographical locations**.**

Antioxidants are derived from different sources such as herbs, essential oils and plant extracts. The antioxidant activities of extracts of the four *Mimusops* species were evaluated using DPPH scavenging assay. Similarly, previous studies revealed that *M. elengi* is a good source of natural antioxidants (30). It is well known that morphological, environmental and genetic factors have influences on both qualitative and quantitative variations in the secondary metabolites production, including phenolics. (31, 32). The quantitative estimation of total flavonoids and phenolics showed significant variations in their levels amongst the extracts of the *Mimusops* species. In line with that, difference in the phenolics and flavonoids levels among the different plant species and yet between the different parts of plant was reported previously (6).

HPLC, coupled with uv/vis detection, was used for the identification as well as quantification of phenolic compounds of the tested *Mimusops* species extract by comparing the retention times of the detected peaks with those of the pure standard compounds. In our study, we focused on analysis of phenolic compounds, which consist of phenolic acids and flavonoids as the hydrophilic extracts were purified by ethyl acetate fractionation followed by hydrolysis. In consistence with the present results, gallic acid and myricetin are considered the most two important bioactive components in *Mimusops* genus (14, 33).

The observed antimicrobial and antioxidant activities in our study were probably due to combination of bioactive constituents. In this context, *M. caffra* is the source of a variety of biologically active phenolic compounds which are responsible for antimicrobial and antioxidant activities (29). In contrast, the chemical constituents of *M. kummel*, *M. zeyheri* and *M. laurifolia* are rarely investigated (34). To understand the mechanism of antibacterial activity of *Mimusops* spp. we studied the ultrastructural changes induced by *M. laurifolia* extract in the affected bacteria at different time points. TEM provide useful insights on the mode of action of antibacterial agents by explaining the changes in the morphological structure induced in the bacterial cells. *M. laurifolia* extract induce complete cell vacuolation after 24 h of incubation due to the observed cell wall disnaturing and plasma membrane disintegration. The phenolic and flavonoid compounds recorded in our studied plants were previously reported to induce cell abnormalities and damage to the cell wall and cytoplasmic membrane of bacteria, triggering cell lysis and leakage of cytoplasmic contents (35, 36). It is known that phenolic compounds have the ability to alter cell permeability and disrupt the membrane lipid (6). Furthermore, the regulation and function of cell wall synthesis enzymes may also be affected leading to alternation in microbial cell growth and morphogenesis (37, 38).

In a view of the current study, plants of the *Mimusops* genus are natural sources for potential antimicrobial agents as well as antioxidants. The polyphenolic constituents of the tested plants are largely responsible for these activities. To best of our knowledge, this is the first study to describe the underlying antibacterial activity mechanisms of *M. laurifolia*. Therefore, extraction, purification and production of phytochemicals from *Mimusops* plants represent a promising approach to develop different therapeutic applications, the subject of future work.

Acknowledgements

The authors thank Prof. Azza El Hadidy and Prof. Rim Hamdy (Cairo University Herbarium, Giza, Egypt) for their help in the taxonomic identification of all plant materials.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Cairo University.

References:

1. Hosseinzadeh S, Jafarikukhdan A, Hosseini A, Armand R. The Application of Medicinal Plants in Traditional and Modern Medicine A Review of *Thymus vulgaris*. Int J Clin Med. 2015;6:635–642.
2. Davies J , Davies D. Origins and Evolution of Antibiotic Resistance Julian. Microbiol Mol Biol Rev. 2010;74(3):417–33.
3. Metwally M, Ali S, Khatab I, El-Sayed M. Antibacterial potential of some seaweeds species to combat biofilm-producing multi-drug resistant *Staphylococcus aureus* of Nile Tilapia. Egyptian Journal of Botany, 2020; 60(1): 9-24.
4. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod. 2016;79(3):629–61.

5. Marcus AC, Edori OS, Maduagu MC. Phytochemical and Anti-microbial Screening of *Phyllantus fratenus* and *Taraxacuim officinale* Leaves. Biochem Anal Biochem. 2019;8(376):1009–2161.
6. Mohamed MSM, Saleh AM, Abdel-Farid IB, El-Naggar SA. Growth, hydrolases and ultrastructure of *Fusarium oxysporum* as affected by phenolic rich extracts from several xerophytic plants. Pestic Biochem Physiol. 2017;141:57–64.
7. Mohamed MSM, Abdallah AA, Mahran MH, Shalaby AM. Potential alternative treatment of ocular bacterial infections by oil derived from *Syzygium aromaticum* flower (Clove). Curr Eye Res. 2018;43:873–881.
8. Ghasemzadeh A, Ghasemzadeh N. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. J Med plants Res. 2011;5(31):6697–703.
9. Fu L, Lu W, Zhou X. Phenolic compounds and in vitro antibacterial and antioxidant activities of three tropic fruits: persimmon, guava, and sweetsop. Biomed Res Int. 2016;2016: 1-9.
10. Vaquero MJR, Serravalle LRT, De Nadra MCM, De Saad AMS. Antioxidant capacity and antibacterial activity of phenolic compounds from argentinean herbs infusions. Food Control. 2010;21(5):779–85.
11. Baky MH, Kamal AM, Elgindi MR, Haggag EG. A Review on Phenolic Compounds from Family *Sapotaceae*. J Pharmacogn Phytochem. 2016;5(2):280–7.
12. Govaerts R, Frodin DG, Pennington TD. World checklist and bibliography of *Sapotaceae*. Royal Botanic Gardens; 2001. 372 p.
13. Kadam P V, Yadav KN, Deoda RS, Shivatare RS, Patil MJ. *Mimusops elengi*: a review on ethnobotany, phytochemical and pharmacological profile. J Pharmacogn Phytochem. 2012;1(3):64-74.
14. Kumar HAK, Mandal BK, Kumar KM, babu Maddinedi S, Kumar TS, Madhiyazhagan P, et al. Antimicrobial and antioxidant activities of *Mimusops elengi* seed extract mediated isotropic silver nanoparticles. Spectrochim Acta Part A Mol Biomol Spectrosc. 2014;130:13–8.
15. Lalitha V, Kiran B, Raveesha KA. *In vitro* evaluation of *Mimusops elengi* plant extract for antibacterial activity and phytochemical analysis. Pharmacophore. 2011;2(1):78–85.
16. Chernonosov AA, Karpova EA, Lyakh EM. Identification of phenolic compounds in *Myricaria bracteata* leaves by high-performance liquid chromatography with a diode array detector (HPLC-DAD) and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Rev Bras Farmacogn. 2017;27:576–579
17. Harborne AJ. Phytochemical methods a guide to modern techniques of plant analysis. springer science & business media; 1998.
18. Rossoni RD, Fuchs BB, De Barros PP, dos Santos Velloso M, Jorge AOC, Junqueira JC, et al. *Lactobacillus paracasei* modulates the immune system of *Galleria mellonella* and protects against *Candida albicans* infection. PLoS One. 2017;12(3):0173332.
19. Mohamed MSM, Mohamed SH, Khalil MS, Mohamed WS, Mabrouk MI. Antibiofilm activity of papain enzyme against pathogenic *Klebsiella pneumoniae*. J Appl Pharm Sci. 2018;8(06):163–8.
20. Mensor LL, Menezes FS, Leitão GG, Reis AS, Santos TC dos, Coube CS, et al. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phyther Res. 2001;15(2):127–30.
21. Pharmacopoeia B. British pharmacopoeia, 2016; Appendix XIN, V-A336.
22. Sakanaka S, Tachibana Y, Okada Y. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food Chem. 2005;89(4):569–75.
23. Tallini LR, Pedrazza GPR, de Bordignon SAL, Costa ACO, Steppe M, Fuentefria A, et al. Analysis of flavonoids in *Rubus erythrocladus* and *Morus nigra* leaves extracts by liquid chromatography and capillary electrophoresis. Brazilian J Pharmacogn. 2015;25(3):219–27.
24. Saran S, Menon S, Shailajan S, Pokharna P. Validated RP-HPLC method to estimate eugenol from commercial formulations like *Caturjata Churna*, *Lavangadi Vati*, *Jatiphaladi Churna*, *Sitopaladi Churna* and clove oil. J Pharm Res. 2013;6(1):53–60.
25. Cabral B, Siqueira EMS, Bitencourt MAO, Lima MCJS, Lima AK, Ortmann CF, et al. Phytochemical study and anti-inflammatory and antioxidant potential of Spondias mombin leaves. Brazilian J Pharmacogn. 2016;26(3):304–11.
26. Vijayaraghavan K, Rajkumar J, Seyed MA. Phytochemical screening, free radical scavenging and antimicrobial potential of *Chromolaena odorata* leaf extracts against pathogenic bacterium in wound infections–a multispectrum perspective. Biocatal Agric Biotechnol. 2018;15:103–12.
27. Korkina LG, Afanas' Ev IB. Antioxidant and chelating properties of flavonoids. Adv Pharmacol. 1996;38:151–63.
28. Helal N, Ibrahim N, Khattab, H. Phytochemical analysis and antifungal bioactivity of *Pulicaria undulata* (L.) methanolic extract and essential oil. Egyptian Journal of Botany, 2019; 59(3): 827-844.
29. Gupta PC. *Mimusops elengi* Linn.(Bakul)-A potential medicinal plant: A review. Int J Pharm Phytopharm Res. 2017;2(5):332–9.
30. Mannan MA, Alam MS, Mustari F, Kudrat-E-Zahan M, Ali R, Haque ABMH, et al. In vitro Antioxidant, Antimicrobial, Insecticidal and Cytotoxic Activities of the Medicinal Plants: *Allamanda cathartica* and *Mimusops elengi*. European J Med Plants. 2017;1–12.
31. Cirak C, Radusiene J, Ivanauskas L, Jakstas V, Çamaş N. Changes in the content of bioactive substances among *Hypericum montbretii* populations from Turkey. Brazilian J Pharmacogn. 2014;24(1):20–4.
32. Sivaci A, Duman S. Evaluation of seasonal antioxidant activity and total phenolic compounds in stems and leaves of some almond (*Prunus amygdalus*

L.) varieties. Biol Res. 2014;47:1-5.
33. Pennington TD. The genera of Sapotaceae. R Bot Gard Bronx, New York Kew New York Bot Gard. 1991;1–13.
34. Eskander J, Lavaud C, Abdel-Khalik SM, Soliman HSM, Mahmoud II, Long C. Saponins from the leaves of *Mimusops laurifolia*. J Nat Prod. 2005;68(6):832–41.
35. Lee Y-S, Lee D-Y, Kim YB, Lee S-W, Cha S-W, Park H-W, et al. The Mechanism Underlying the Antibacterial Activity of Shikonin against Methicillin-Resistant *Staphylococcus aureus*. Evidence-Based Complement Altern Med. 2015;2015:1–9.
36. Adams DJ. Fungal cell wall chitinases and glucanases. Microbiology. 2004;150(7):2029–35.
37. Mohamed SH, Mohamed MS, Khalil MS, Azmy M, Mabrouk MI. Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant *Klebsiella pneumoniae*. J Appl Microbiol. 2018;125(1):84-95.
38. Abd El-Zaher E, El- Shouny W, Shabana S, Abd El-Salam O. Efficiency of Essential Oils as Antifungal Agents against *Aspergillus fumigatus* KY026061 Causing Allergic Bronchopulmonary Aspergillosis (ABPA). Egyptian Journal of Botany, 2019; 59(3): 595-604.

استكشاف آليات الكيمياء الحيوية والبنية التحتية الكامنة وراء نشاط جنس *Mimusops* المضاد للميكروبات

جهاد عبد المحسن[1] **جودة طلعت محمد داود[1]** **محمود صالح محمود محمد[2]**

[1] معمل كيمياء النبات، مركز الدراسات التطبيقية لبحوث النباتات الطبية (الهيئة القومية للرقابة والبحوث الدوائية).
[2] قسم النبات والميكروبيولوجي، كلية العلوم، جامعة القاهرة.

الخلاصة:

مقاومة المضادات الحيوية هي التهديد الرئيسي الذي يواجه العلاج الدوائي للالتهابات البكتيرية. *Mimusops* هو أكبر جنس في العائلة Sapotaceae ويستخدم على نطاق واسع في الأدوية التقليدية بسبب أنشطته الدوائية الواعدة. التنقيب البيولوجي لجنس *Mimusops* قد يوفر المصادر المحتملة للعوامل المضادة للميكروبات كنهج لمواجهة المقاومة المتزايدة للمضادات الحيوية. وقد أجريت هذه الدراسة لتوضيح تأثير مضادات الميكروبات من ثلاثة أنواع من جنس *Mimusops* الغير مستكشفة سابقا وهي (*M. kummel*, *M. laurifolia M. zeyheri*). علاوة على ذلك تمت دراسة الآليات الكامنة وراء مثل هذا النشاط المضاد للميكروبات. وأظهرت المستخلصات النباتية الغنية بالفينولات نشاطات مضادة للبكتيريا. وأوضحت النتائج أن قيم أقل تركيز مثبط وأقل تركيز قاتل للبكتيريا بواسطة المستخلصات النباتية قيد الدراسة تراوحت بين 3.15-12.5 ميكروغرام / مل ضد عزلات بكتيرية مختلفة. وأظهر كشف مضاد الأكسدة أن *M. caffra* هو أفضل نبات مضاد للأكسدة (تركيزه الذي يعطي نتيجة 50% اكسدة هو 14.75± 0.028)، بينما *M. laurifolia* هو أقل نبات له تأثير مضاد للأكسدة. وقد قمنا بتحديد الفينولات في المستخلصات النباتية وقياسها بواسطة جهاز التحليل الكروماتوجرافي السائل، والذي كشف عن وجود سبعة أحماض فينولية وأربعة فلافونويد. المركبات الفينولية الوفيرة كانت (Rutin : بتركيز 5.216± 0.067) يليه (gallic acid : بتركيز 0.296± 0.068)، بينما مركبات الفلافونويد الوفيرة كانت (myricetin : بتركيز 0.317 ± 0.091) يليه (kaempferol : بتركيز 0.113 ± 0.049).وبدراسة تأثير مستخلص نبات *M. laurifolia* علي البكتريا العنقودية تبين انها تتسبب في تغييرات في البنية التحتية كتحلل جدار الخلية وغشاء البلازما كما كشف المجهر الالكتروني. في النهاية وبشكل عام، تعد أنواع *Mimusops* مصادر بديلة طبيعية واعدة مضادة للميكروبات.

الكلمات المفتاحية: المكورات العنقودية ، *Mimusops* ، المركبات الفينولية ,*Staphylococcus aureus*التركيب الدقيق.