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Antibacterial and Antioxidant Properties of Anthraquinones Fractions from *Morinda Citrifolia* Fruit

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Morinda citrifolia fruit have been used treatment numerous diseases such as dysentery, heart diseases, AIDS, cancers and others. The aim of this study was to determine the antibacterial and antioxidant activities of anthraquinones fractions from the fruits of *Morinda citrifolia*. Well diffusion assay, minimum inhibitory concentration and the minimum bactericidal concentration were used to test antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa* MRSA and *Helicobacter pylori*. DPPH radical scavenging activity and Superoxide dismutase activity assays were used to evaluate their antioxidant activity. LC-MS analysis was used to identify anthraquinones and SEM studies that revealed mode of action. Anthraquinones fraction from the fruit of *M. citrifolia* exhibited significant at *P* value < 0.05 inhibition against the test of bacteria including MRSA and *H. pylori*. Anthraquinones fractions from the fruit have high level 91.40% with significant at *P* value < 0.05 of antioxidant activities compared compare with control. LC-MS analysis of anthraquinones of the *M. citrifolia* identified specific compounds in this fraction. SEM results of these substances showed significant morphological changes of cell wall, membrane and destruction of bacterial cell. It could be concluded that the anthraquinones of the part of this plant had a good antibacterial and antioxidant effects. The results suggest the anthraquinones from *M. citrifolia* fruit can be a new source of antimicrobials against pathogenic bacteria and antioxidant source.

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Introduction

M. citrifolia belongs to the Rubiaceae family with 80 species. This plant is found in South East Asia, Caribbean countries, Australia and Central-South America [1, 2]. *M. citrifolia* has been used as a medicine for many ailments such as dysentery, heartburn, liver diseases, diabetes, high blood pressure, muscle aches, headaches, heart diseases, cancer, gastric ulcers and arthritis [3, 4]. *M. citrifolia* has approximately 200 phytochemical compounds which are distributed throughout the plant [5, 6]. Anthraquinones, a major bioactive compound, is present in different parts of the plant [3, 5]. Among the compounds found in the fruit of this plant are 2-methoxy-1,3,6-trihydroxyanthraquinone, 5,15-dimethylmorindol, 1,6-dihydroxy-5- methoxy-2-methoxymethyl anthraquinones, ,5,7-trihydroxy-6-methoxy-2-methoxymethyl anthraquinones, 1,3-dimethoxyanthraquinone and 1, 2-dihydroxyanthraquinone [7-10]. These compounds have antibacterial, antifungal and other biological activities [11]. The antibacterial and antioxidant properties of medicinal plants are being investigated because of the toxicological concerns associated with the synthetic antioxidants and preservatives.

The aim of this study is to determine the antibacterial and antioxidant activities anthraquinones fractions of from the fruit of *M. citrifolia*. LC-MS analysis and mode of action of anthraquinones fraction were also investigated. It was hypothesized that these compounds may display good inhibitory effects against pathogenic bacteria and therefore may aid in the development of antimicrobials and antioxidant supplements.

Materials and methods

Plant collection

The fresh ripe fruit of *M. citrifolia* were collected from Sendayan Valley, Seremban, Malaysia in November, 2010. This plant was identified at the herbarium under the registration numbers KLU 22480. All samples were washed under tap water and dried in an oven at 40ºC for 3 days. The plant materials were then put through a grinder with a mesh size of 2 mm.

Anthraquinones fraction from M. citrifolia fruit

This method is based on Smita, Sushma [12]. The dried powder of the fruit of this plant were added to 100 ml of methanol and 150 ml of distilled water and refluxed for 3 hours. Then, the extract was added to 4ml of concentrated HCl with 5% of methanolic solution and refluxed for 6 hr. Extraction was conducted with chloroform and filtered. Chloroform was then evaporated at 40ºC using a rotary evaporator until the solvent was removed (Heidolph WB2000, Germany). The product yield was 0.47% of the original material.

Determination of antimicrobial Activities

Well diffusion assay

For this study, four species of bacteria were used. *S. aureus* (RF 122), *E. coli* (UT181), *B. cereus* (ATCC 14579) and *P. aeruginosa* (PA7) were procured from cultures maintained at the Fermentation Technology Laboratory in the Microbiology Division, Institute of Biological Sciences, University of Malaya, Malaysia. Other strains used in this study included methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BA-43) and *Helicobacter pylori* ATCC 43504. These strains of bacteria, except for *H. pylori*, were inoculated into Mueller-Hinton agar (Difco, Detroit, MI, USA) using cotton swabs. *H. pylori* was inoculated into Tryptic Soy agar (Difco, Detroit, MI, USA) with 5% defibrinated sheep blood for 3 days at 37°C under microaerophilic conditions. Wells of 6 mm in diameter were made on the media surface in petri plates. All fractions were dispensed into the wells (50 µl) and incubated overnight at 37ºC. Inhibition zones were observed in triplicate plates. The positive control used was 10 mg/ml of tetracycline and the negative control 5% DMSO. The protocol used in this study was modified from Saravanan *et al.* [13].

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

All fraction were determined for their MIC values using a standard protocol [14]. Nutrient broth (Difco, Detroit, MI, USA) was used as the medium to culture bacteria. One ml of this broth was added to the numbered tubes 1-9. One ml of the stock culture was added to tube 1 and successively diluted until tube number 7. The last 1 ml of tube 7 was discarded. Tube number 8 was used as a negative control and the tube 9 as a positive control. The bacterial inoculum was cultured in nutrient broth and incubated overnight, but *H. pylori* was inoculated into Tryptic Soy broth with 5% fetal calf serum (Sigma, Aldrich GmbdH, Germany) and incubated at 37°C under microaerophilic conditions. All the tubes were inoculated with 1 ml of the test bacteria media, except tube number 8, and incubated for 24 hrs at 37ºC. MIC values were determined based on the tube which showed no growth. MBC values were determined by sub-culturing from the MIC assay tubes onto Muller- Hinton agar (Difco, Detroit, MI, USA) and then determining the dilution at which growth was detected. McFarland standard (0.5) was used to determine the amount of colony forming units (CFU) of the bacteria in nutrient broth (1×10^8 CFU/ ml) based on optical density measurement at 620 nm.

Determination of antioxidant activities of plants

DPPH radical scavenging assay

Free radical scavenging activities were determined by using the method of Bozin et al. [15] with some modifications which included the number of samples and conditions of incubation (dark, 25°C for 2 hrs). The reagent of the assay is 2, 2- diphenyl-1- picrylhydrazyl solution (Sigma Aldrich GmbdH, Germany) (950 µl) that was added to 50 µl of the extract (10 mg/ml) and the volumes of the solutions made up to 4 ml by adding 95% ethanol. This mixture was shaken vigorously and incubated at room temperature for two hours in the dark. All samples were measured at 515 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The percentage of DPPH radical scavenging activity of the resulting solutions was calculated using the following equation:

DPPH radical scavenging activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$

Ascorbic acid (10 mg/ ml) was used as a positive control of the assay.

IC_{50} was calculated using linear regression plots. The IC_{50} values represent the concentrations of the sample that is required to scavenge 50% of DPPH free radicals.

Superoxide dismutase activity assay

The protocol used in this study was modified from Sakudo et al. [16]. The modifications included varying the amount of samples used and the incubation period. Superoxide dismutase (SOD) activity was determined using a SOD Assay Kit-WST (Dojindo Molecular Technologies, Gaithersburg). The samples (20 µl) were mixed with the reaction mixture in the kit. Then, the mixtures were gently shaken and incubated at 37ºC for 20 min. Antioxidant activity was measured at 450 nm using a Genesys 20 Thermo Scientific (USA) spectrophotometer. The positive control was ascorbic acid (10 mg/ml). The negative control to measure inhibition rates of SOD activity used all treatments without sample.

LC-MS analysis

Anthraquinones fraction from the fruit of *M. citrifolia* were identified through the Agilent 6530 quadrupole time-of-flight liquid chromatography mass spectrometer (Agilent Technologies, USA) with binary pump and automatic sampler by the method Kose et al. [17]. All fractions were filtered by 0.22 µM filters before injection. The solvents were A: 2% acetonitrile in water with 0.1% formic acid and B: 2% water in acetonitrile with 0.1% formic acid. A step gradient of solvent B was used to run the column as follows: 2-30% for 0-30 min, 30-98% for 30-40 min and 98-8% for 55-60 min and the volume of injection 5µl. The flow rate 100

µl/ min. The mass range between 50 to 3100 *m/z* and electrospray ionisation with positive ion polarity, the capillary voltage 3.5 KV, gas temperature 300°C, nebulizer pressure to 40 psi, sheath gas temperature 350°C and gas flow 8 L/ min. The data were gathered by Agilent MassHunter Workstation Software B.01.03.

Effect of anthraquinones fraction from M. citrifolia fruit by scanning electron microscope

Bacterial culture (*B. cereus*) was incubated into nutrient broth overnight at 37^{O}C. This culture (1 ml) was added to one milliliter of bioactive fraction of anthraquinones from *M. citrifolia* fruit. All treated samples and untreated samples were kept for 4 hr. at 37^{O}C by the method Shami *et al.* [18]. This mixture was then centrifuged at 6500 g at 4^{O}C for 10 min. The pellet was washed twice with 50 mM sodium phosphate buffer (pH 7). The bacterial cells were re-suspended with buffer and 1 µl of suspension deposited on a membrane filter. Bacterial cells were fixed with 8% glutaraldehyde for 1 hr. The fixed cells were washed with buffer in distilled water in a ratio of 1:3 for 15 min. The bacterial cells were dehydrated in ascending concentrations of ethanol (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100%) with a 15 minute exposure period for each concentration. The bacterial cells were further dehydrated in different ratios of ethanol: acetone (3:1, 1:1 and 1:3) for 20 min for each mixture and then washed with pure acetone four times each for 20 min. These bacterial cells were subjected to critical point drying using liquid CO_2 and the cells mounted on a stub. The cells were then coated with gold and examined through a scanning electron microscope (Model: JEOL JBM 7001F, UK). The control used in this experiment is normal, untreated bacterial cells which were compared with *B. cereus* cells treated with anthraquinones fraction from this plant.

Statistical analysis

Data is expressed as mean ± SD Statistical analyses were carried out using SPSS version 17. One-way ANOVA followed by Duncan's multiple comparison were used to compare the values of samples with the control. A P value < 0.05 was deemed as indicating significant differences. Each treatment was duplicated thrice and each experiment was repeated at least twice.

Results

Antibacterial activity

Fig.1 shows the zones of inhibition for the anthraquinone extract from the fruit of *M. citrifolia* as 21.00 mm for *S. aureus,* 19.66 mm for *E. coli,* 21.33 mm for *B. cereus,* 21.66 mm for MRSA and 13.66 mm for *P. aeruginosa* .
MIC and MBC values for anthraquinone fraction of the fruit from this plant were 25 mg/ml for *S. aureus*, *E. coli* and *H. pylori* while *B. cereus* had MIC/MBC of 12.5 mg/ml. *(*Table 1).

Bacteria	Plant extracts (mg/ml)	
	MIC	MBC
S. aureus	<25	>25
E. coli	>25	25
B. cereus	12.5	<12.5
P. aeruginosa	50	50
MRSA	25	25
H. pylori	25	25

Table 1. MIC and MBC of anthraquinones extracts of the fruit (ANMF) from *M. citrifolia* on selected microorganisms.

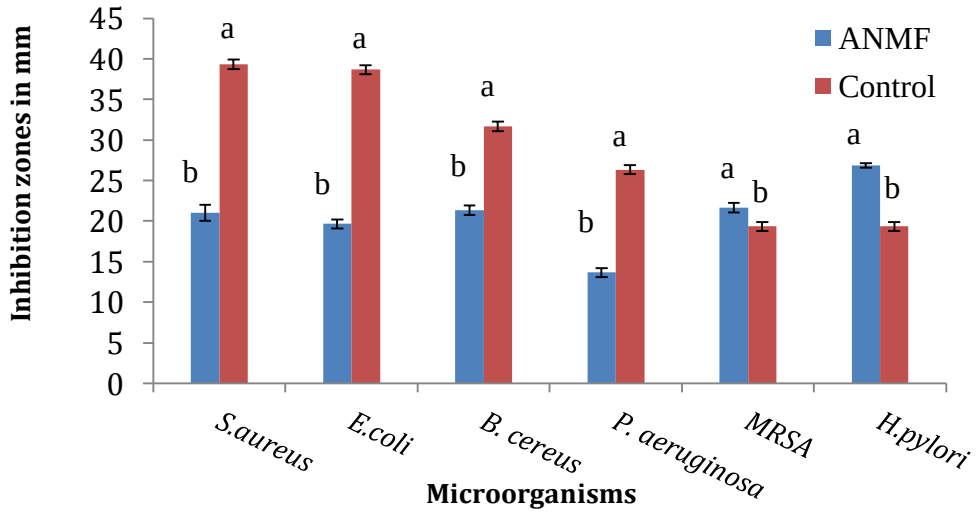

Fig. 1. Inhibition zones of anthraquinones fraction of *M. citrifolia* fruit (ANMF) on the test microorganisms in mm. The positive control (10 mg/ml of Tetracycline).

Antioxidant activity

Fig. 2 shows the percentage of DPPH radical scavenging activity of the anthraquinone fraction from *M. citrifolia* fruit as 91.40% (IC_{50} 5.48 mg/ml). The SOD activity inhibition rate of the anthraquinone fraction from the fruit of *M. citrifolia* was 91.75% (Fig. 3).

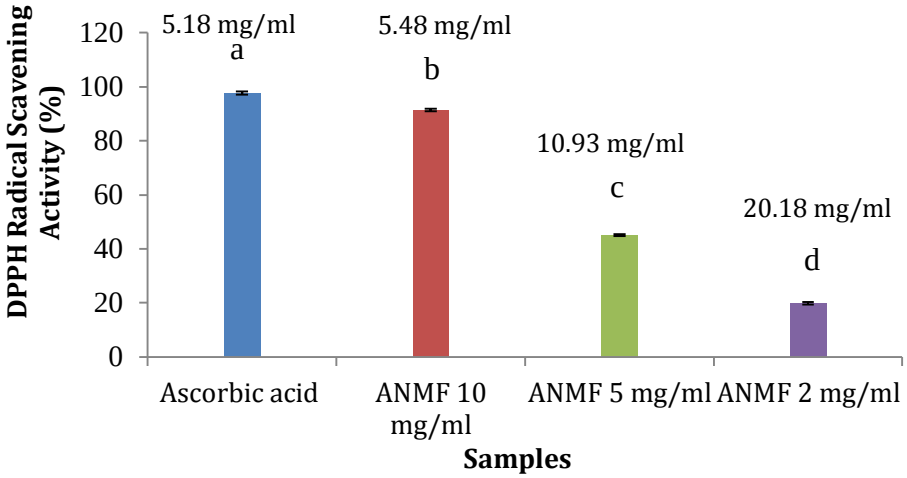

Fig. 2. DPPH scavenging activity with IC_{50} of anthraquinones fractions from *M. citrifolia* fruit. The IC_{50} values represent the concentrations of the sample that are required to scavenge 50% of DPPH free radicals. The positive control (10 mg/ml) of ascorbic acid.

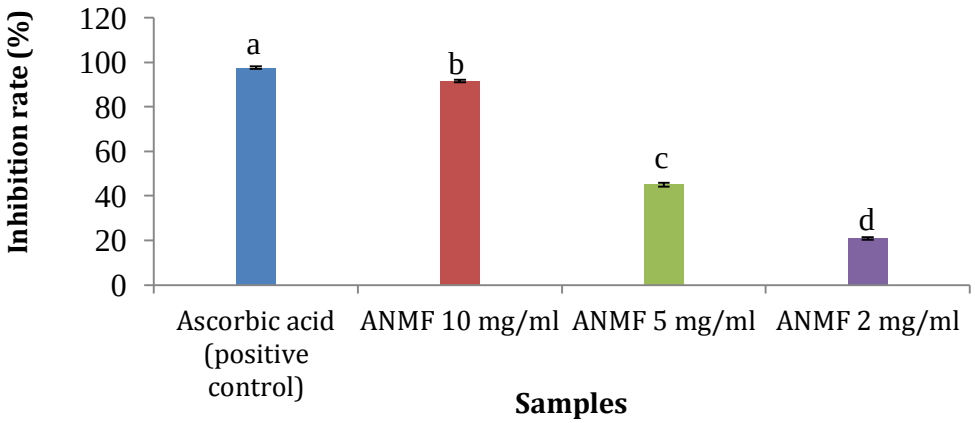

Fig. 3. Inhibition rate of SOD activity of anthraquinones fraction from M. citrifolia fruit. The positive control (10 mg/ml) of ascorbic acid.

LC-MS analysis

Fig. 4 shows the LC chromatograms and MS data of the major anthraquinone fraction from the fruit of *M. citrifolia.* The peak at retention time 42.417 min exhibiting an $[M + H]^+$ at *m/z* 239.2117 as 1-hydroxy-2-methylanthraquinone was identified. The peak at retention time 42.798 min exhibiting an $[M + H]^+$ at *m/z* 329.3721 as 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinones was identified. The peak at retention time 44.349 min exhibiting an $[M + H]^+$ at *m/z* 345.2424 as morindolin was identified. The peak at retention time 45.256 min exhibiting an $[M + H]^+$ at *m/z* 315.1353 as 1,1-*O*i-O-methyl morindolin was identified. The peak at retention time 46.353 min exhibiting an $[M + H]^+$ at *m/z* 241.221 as alizarin or 1, 2-dihydroxyanthraquinone was identified. The peak at retention time 50.732 min exhibiting an $[M + H]^+$ at *m/z* 287.2385 as

1,3-6 Trihydroxy-2-methoxyanthraquinone was identified.

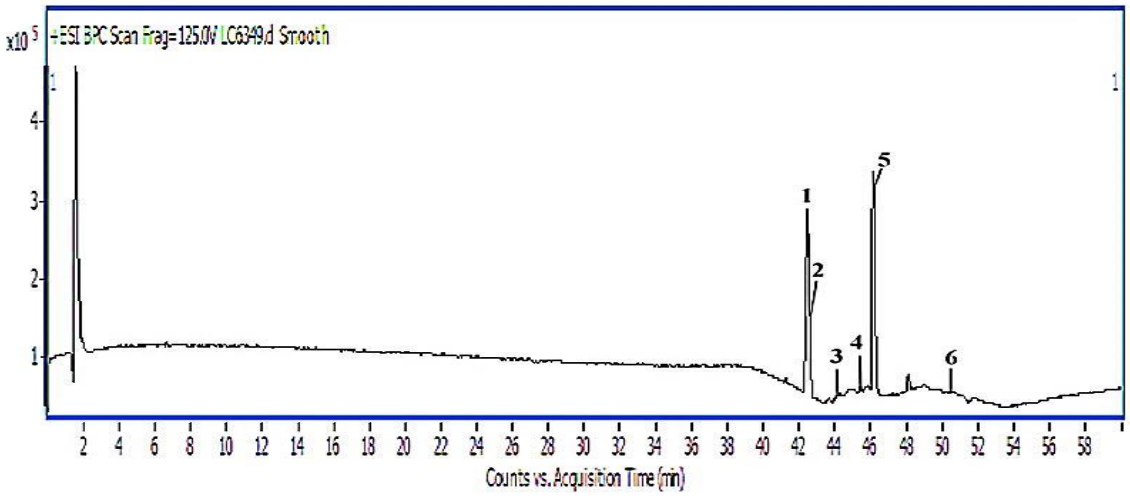

Fig. 4. LC chromatograms of the major anthraquinones fraction from the fruit of *M. citrifolia* 1-hydroxy-2-methylanthraquinones, (2) 2-hydroxy-1, 5-dimethoxy-6-(methoxymethyl) anthraquinones, (3) morindolin, (4) 1,1-*O*i-O-methyl morindol, (5) 1,2-dihydroxyanthraquinone and (6) 1, 3-6 Trihydroxy-2-methoxyanthraquinone.

Effect of anthraquinones extract from the fruit of M. citrifolia by scanning electron microscope

Fig. 5 shows the effect of anthraquinones fraction from the fruit of *M. citrifolia* against *B. cereus.* It was observed that the bacterial cells treated with antibacterial anthraquinone underwent morphological changes that included breakage in the cell wall membrane, roughening of the cell surface, leakage of cytoplasmic contents and a build-up of cell debris. Untreated cells displayed a normal surface and the typical rod shape of *B. cereus*.

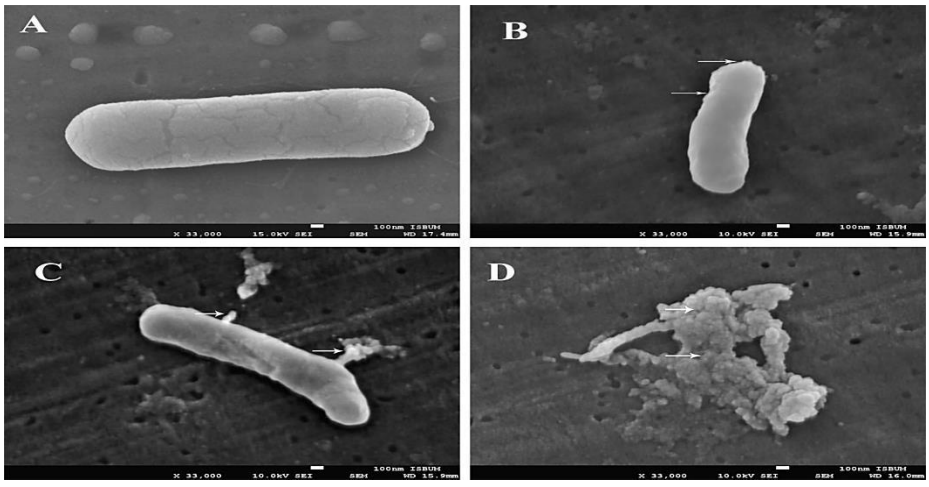

Fig. 5. Effect of anthraquinones fraction from the fruit of *M. citrifolia* by scanning electron microscope (A) Control: *B. cereus* without any changes (B) *B. cereus* treated anthraquinones after 1 hour with cell wall and cytoplasmic membrane

damaged, (C) *B. cereus* treated anthraquinones after 2 hour the membrane's ability to act as a permeability barrier and (D) *B. cereus* treated with anthraquinones after 4 hour with blasting and destroy.

Discussions

Anthraquinones fraction for the fruit of *M. citrifolia* showed antibacterial effects against all test bacterial strain including MRSA and *H. pylori*. It is noteworthy that the anthraquinones fraction from the fruit of the plant showed significant inhibition zones. According to many literature reviews documented, *M. citrifolia* has anthraquinones in the fruit [8, 19, 20]. These compounds have antibacterial, antifungal and other biological activities [11]. Comini, Núñez Montoya [21] reported that anthraquinones isolated from *Heterophyllaea pustulata* Hook f. (Rubiaceae) have antibacterial activity against *S. aureus* as bacteriostatic and bactericidal effects. MIC and MBC values of anthraquinones fraction of this plants showed that the fraction are very potent against the selected test bacteria with *B. cereus* being very sensitive. However, the anthraquinones fractions from the fruit have significant values of MIC and MBC. The anticipated finding of this current study is the first report for a test of antibacterial anthraquinones fraction for the fruit of *M. citrifolia* activity against *S. aureus*, *E. coli*, *B. cereus*, *P. aeruginosa* including important bacterial strains namely MRSA and *H. pylori*.

Results of antioxidant activity anthraquinones fraction of *M. citrifolia* fruit have a high percentage of DPPH radical scavenging activity with significant IC_{50} values. Therefore the anthraquinones fraction of *M. citrifolia* has good antioxidant activity. In addition, anthraquinones extract of *M. citrifolia* have a high level of inhibition rate for SOD activity. Earlier studies show the fruit of this plant have rich anthraquinones in the content so that this part of *M. citrifolia* has high antioxidant activity [5]. Kremer, Kosalec [22] reported anthraquinones have a strong natural antioxidant fraction from plants LC chromatograms and MS data identified the major compounds of anthraquinones fraction from the fruit of *M. citrifolia* are 1-hydroxy-2-methylanthraquinone, 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinones, morindolin, 1,1-*O*i-O-methylmorindol, 1,2-dihydroxyanthraquinone, 1, 3-6 trihydroxy-2-methoxyanthraquinone. Past studies isolated anthraquinones from the fruit of *M. citrifolia* such as 2-methoxy-1, 3, 6-trihydroxyanthraquinone, anthragallol 1, 3-di-*O*-methyl ether**,** 6-hydroxyanthragallol-1, 3-di-*O*-methyl ether, austrocortinin**,** morindone-5-*O*-methyl ether**,** anthragallol 2-*O*-methylether, 5,15-dimethylmorindol**,** 1,6-dihydroxy-5-methoxy-2-methoxymethylanthraquinones**,** 1,5,7-trihydroxy-6-methoxy-2-methoxymethylanthraquinones**,** 1,3-dimethoxyanthraquinone and 1, 2-dihydroxyanthraquinone**,** morindacin and lucidin [7-9, 23]. In the present study some compounds were not detected because of the differences in the methods and instruments for isolation and identification of the anthraquinones between our and previous studies.

The SEM observations show that the anthraquinones extract from the fruit of *M. citrifolia* causes changes and damage to the morphology of the treated bacteria (Fig.7 B, C and D). There were several mechanisms of action of anthraquinones. These bioactive compounds might bind to the cell surface and penetrate to the target sites and damage the bacterial cell wall by affecting the phospholipid of the cell membrane and prevent membrane-bond enzymes [24, 25]. The bacterial cell wall and cytoplasmic membrane damaged indicates a loss in structural integrity and the membrane's ability to act as a permeability barrier [26]. As the result of the mode of action the cell dies because of loss of cell contents [25].

Conclusions

In conclusion, this is the first report that studied antibacterial activity, antioxidant capacity and mode of action in anthraquinones fraction from *M. citrifolia* fruit. These fractions of the fruit of this plant have antibacterial effect against all pathogenic bacteria including important resistant bacteria such as MRSA and *H. pylori* in well diffusion, MIC and MBC assays. Also anthraquinones fraction from the fruit of this plant exhibited a high level of antioxidant activity

with significant values of IC_{50}. SEM analysis of anthraquinones fraction from *M. citrifolia* indicate a bactericidal effect of these compounds related to breakage in cell wall and membrane with roughening in cell surface and leakage of cytoplasmic contents. LC-MS analysis of anthraquinones fraction from *M. citrifolia* fruit identified important compounds which may be used to develop biopharmaceuticals against infectious diseases with antioxidants source in future.

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Conflict of interest

Author certifies that there is no actual or potential conflict of interest in relation to this article.

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