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The Effect of Anthraquinones Fractions from *Morinda Citrifolia* Leaves against Pathogenic Bacteria with Antioxidant Activities

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A B S T R A C T

The objectives of this study were to evaluate the antibacterial and antioxidant activities from anthraquinones fractions from leaves of *Morinda citrifolia*. Anthraquinones fraction from the leaves of *M. citrifolia* exhibited significant inhibition against the test of bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Helicobacter pylori* (*H. pylori*). Anthraquinones fraction of the leaves have antioxidant activities. LC-MS analysis of anthraquinones of the *M. citrifolia* leaves identified specific compounds in these fractions. Scanning electron microscope 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 of the part of this plant can be a new source of antimicrobials against pathogenic bacteria and antioxidant source.

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Introduction

Morinda citrifolia belongs to the *Rubiaceae* family and comprises 80 species. This plant is found in South East Asia, Caribbean countries, Australia and Central-South America [1, 2]. *Noni*, Indian mulberry, *nuna*, and *mengkudu* are common names for this plant [1, 3]. A medium-sized tree, it is 3-10 metres tall with abundant wide elliptical leaves and small tubular white flowers, which are grouped together. The petioles leave ring-like marks on the stalks [2, 4]. The oval-shaped fruit of this plant has an embossed appearance. It is initially green to yellow in colour but the ripe fruit is white and covered with small reddish brown buds containing seeds [5]. The seeds are medium sized, ovoid in shape, reddish brown and with a distinct air chamber at the end probably for widespread seed dispersal by water [1, 3, 6]. *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 [5, 7]. *M. citrifolia* has approximately 200 phytochemical compounds which are distributed among the plant [8, 9]. Anthraquinones, a major bioactive compound, is present in different parts of the plant [5, 8]. *M. citrifolia* leaves contain to 2-methoxy-1, 3, 6-trihydroxyanthraquinone, 5,15-dimethylmorindol, 1,3-dihydroxy-2-methylol-9,10-anthraquinone, 1,2-dihydroxyanthraquinoneand 1,3-dihydroxy-2-methylanthraquinone [10, 11]. These compounds have antibacterial, antifungal and other biological activities [12]. Until now, there are no reports that demonstrate these anthraquinones fraction of this plant have been tested for antibacterial activity on pathogenic bacteria including MRSA and *H. pylori* along with antioxidant activity and mode of action. The aim at this study is to determine the antibacterial and antioxidant activities anthraquinones fraction of from the leaves of *M. citrifolia*. LC-MS analysis and mode of action of anthraquinones fractions 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 leaves of *Morinda citrifolia* were collected from Sendayan Valley, Seremban, Malaysia in November, 2010. This plant was identified with 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 fractions of M. citrifolia leaves

This method is based on Smita, Sushma [13]. The dried powder of the leaves of this plant (50 g) was added to 100 ml of methanol and 150 ml of distilled water and refluxed for 3 hours. Then, the fraction was added to 4ml of concentrated HCl with 5% of methanolic solution and refluxed for 6 h. Fractionation 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 *Staphylococcus aureus* (RF 122), *Escherichia coli* (UT181), *Bacillus cereus* (ATCC 14579) and *Pseudomonas 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 to 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.

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

All fractions 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 h 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 x 10^8 CFU/ ml) based on optical density measurement at 620 nm.

Determination of antioxidant activities

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 h). 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 fraction (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 2 h of 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 (%) = [($A_{control} - A_{sample}$) / $A_{control}$] x100

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 are 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 of 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 fractioned from *Morinda citrifolia* leaves were identified with the Agilent 6530 quadrupole time-of-flight liquid chromatography mass spectrometer (Agilent Technologies, USA)

with binary pump and automatic sampler. 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 fractions from the leaves of M. citrifolia 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 fractions of anthraquinones of *M. citrifolia* leaves. All treated samples and untreated samples were kept for 4 hr. at 37^{O}C. 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. 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 fractions of 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 the samples compare 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 fractions of the leaves of the plant inhibited all test bacteria with the mean of inhibition zones ranging from 17.33 mm to 11.66 mm compared to the positive control (Tetracycline 10 mg/ml) with zones of inhibition ranging from 39.44 mm to 19.33 mm.

MIC and MBC values for anthraquinone fraction of leaves of 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. However, MIC/MBC for *P. aeruginosa* was at 50 mg/ml *(*Table 1).

Table 1. MIC and MBC of anthraquinones fractions of the leaves (ANML) from *M. citrifolia* on selected microorganisms.

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

The positive control was bacteria without plant extracts

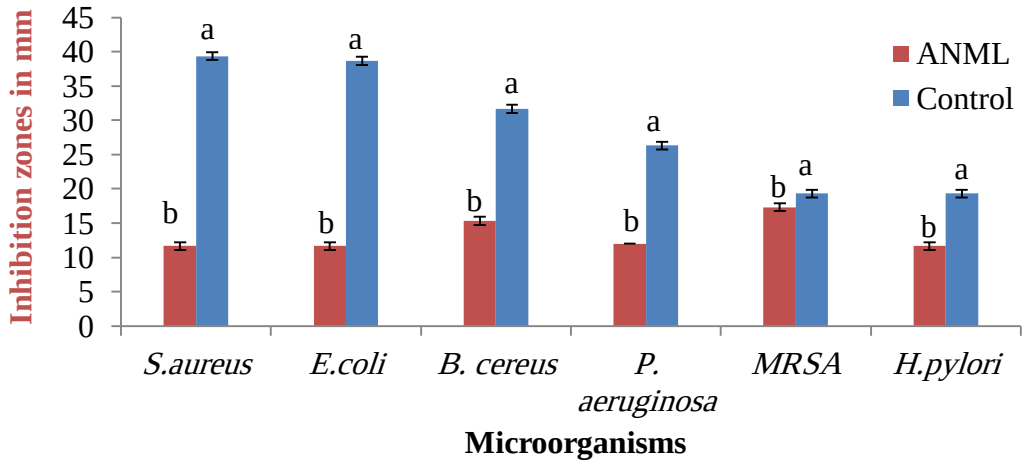

Fig. 1. Inhibition zones of anthraquinones fractions of *M. citrifolia* leaves (ANML) on the test microorganisms in mm. The positive control (10 mg/ml of Tetracycline)

Antioxidant activity

Fig. 2 shows the percentage of DPPH radical scavenging activity of the anthraquinone fraction of *M. citrifolia* leaves as 64.48% (IC_{50} 7.76 mg/ml) compared to ascorbic acid as a positive control at 96.59% (IC_{50} 5.18 mg/ml). The SOD activity inhibition rate of the anthraquinone fraction of the leaves of this plant displayed the lowest antioxidant activity at 23.28% (Fig. 3).

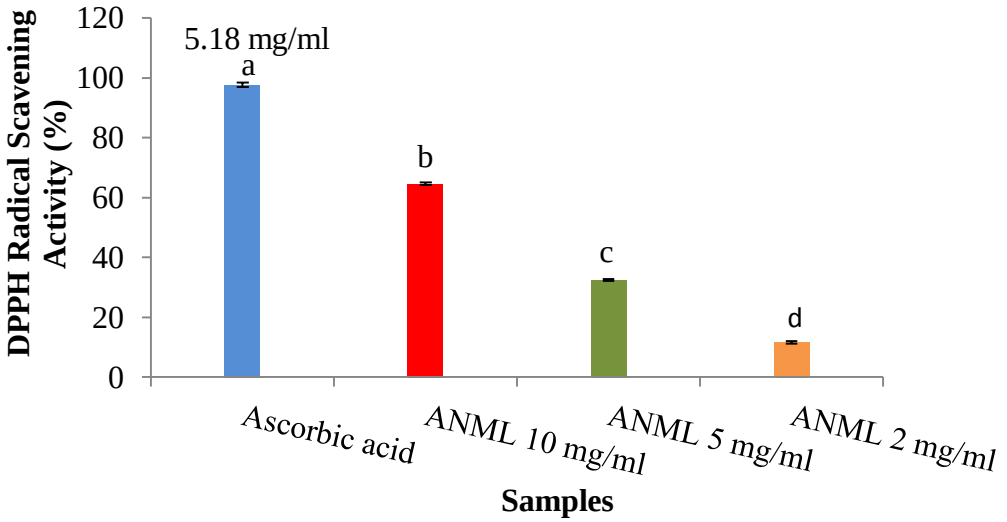

Fig. 2. DPPH scavenging activity with IC_{50} in mg/ml of anthraquinones fractions from *M. citrifolia* leaves (ANML). . The IC_{50} values represent the concentrations of the sample that are required to scavenge 50% of DPPH free radicals. The positive control (10 mg/ml) of Ascorbic acid.

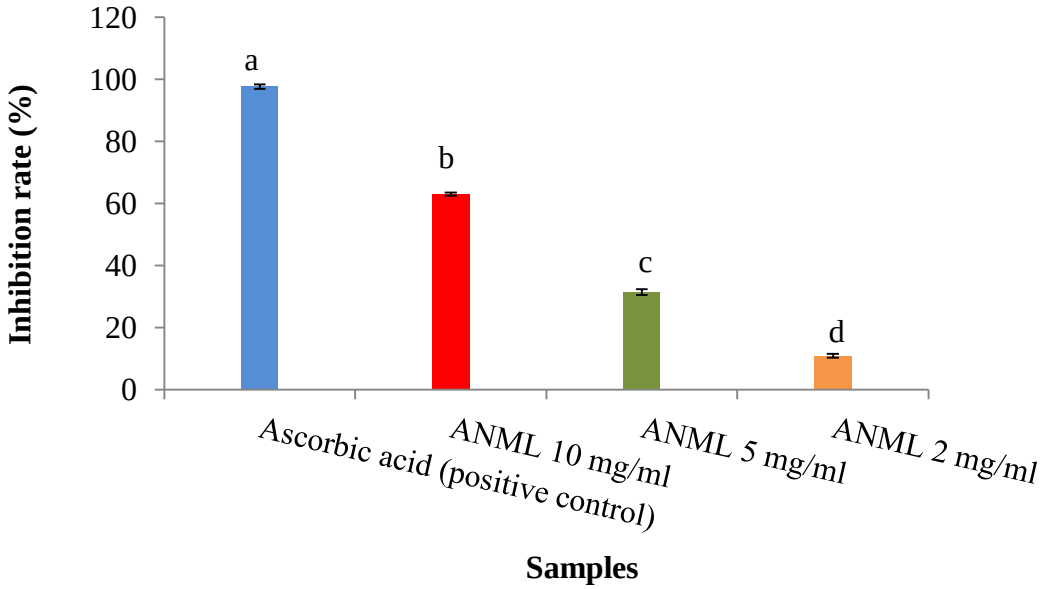

Fig. 3. Inhibition rate of SOD activity of anthraquinones fractions of *M. citrifolia* leaves (ANML). The positive control (10 mg/ml) of ascorbic acid.

LC-MS analysis

In the LC chromatograms five major anthraquinones were fractioned of *Morinda citrifolia* leaves (Fig. 4). The peak at retention time 38.871 min exhibiting a $[M + H]^+$ at *m/z* 241.1434 as 1,2-dihydroxyanthraquinone was identified. The peak at retention time 45.287 min exhibiting a $[M + H]^+$ at *m/z* 285.3341 as 1-hyroxy-2,3-methoxyanthraquinone was identified. The peak at retention time 46.668 min exhibiting a $[M + H]^+$ at *m/z* 301.1413 as 2,6-diroxy-1,3-methoxyanthraquinone was identified. The peak at retention time 47.001 min exhibiting a $[M + H]^+$

at *m/z* 255.2099 as 2-hydroxy-1-methoxyanthraquinone was identified. The peak at retention time 47.322 min exhibiting a $[M + H]^+$ at *m/z* 329.2678 as 2-hydroxy-1, 5-dimethoxy-6-(methoxymethyl)anthraquinone was identified.

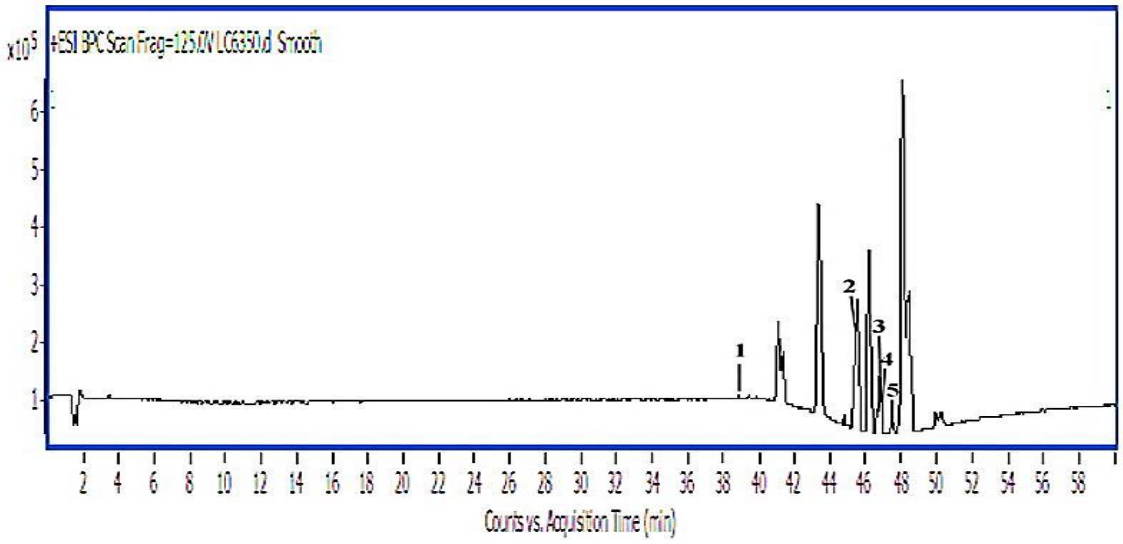

Fig. 4. LC chromatograms of the major anthraquinones fractioned from the leaves of *M. citrifolia* (1) 1,2-dihydroxyanthraquinone, (2) 1-hyroxy-2,3-methoxyanthraquinone, (3) 2,6-diroxy-1,3-methoxyanthraquinone, (4) 2-hydroxy-1-methoxyanthraquinone and (5) 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinones.

Effect of anthraquinone fraction of the leaves of M. citrifolia by scanning electron microscope

Fig. 5 shows the effect of anthraquinones fractioned of the leaves of *Morinda 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*.

Discussions

Anthraquinones fractions for leaves of *Morinda citrifolia* showed antibacterial effects against all test bacterial strains including MRSA and *H. pylori*. It is noteworthy that the anthraquinones fractioned from the leaves of the plant showed significant inhibition zones. According to many literature reviews documented, *M. citrifolia* has anthraquinones in the leaves [10, 11, 17]. These compounds have antibacterial, antifungal and other biological activities [12]. Comini et al. [18] reported that anthraquinones isolated from *Heterophyllaea pustulata* Hook f. (Rubiaceae) have antibacterial activity against *S. aureus* bacteriostatic and bactericidal effects. MIC and MBC values of anthraquinones fractions of the leaves of this plants showed that the fractions are very potent against the selected test bacteria with *B. cereus* being very sensitive. The anticipated finding of this current study is the first report for a test of antibacterial anthraquinones fractions for leaves 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* leaves have moderate percentage of DPPH radical scavenging activity with significant IC_{50} values. Therefore the anthraquinones fractions of *M. citrifolia* have good antioxidant activity. In addition, anthraquinones fractions of *M. citrifolia* leave have a high level of inhibition rate for SOD activity. Earlier studies show the leaves of this plant have rich

anthraquinones in the content so that this part of *M. citrifolia* has high antioxidant activity [8]. Kremer, Kosalec [19] reported anthraquinones have a strong natural antioxidant fractioned from plant. The results of LC-MS analysis of anthraquinones fractioned from *Morinda citrifolia* leaves identified five major compounds namely 1,2-dihydroxy anthraquinone, 1-hyroxy-2,3-methoxyanthraquinone, 2,6-diroxy-1,3-methoxyanthraquinone, 2-hydroxy-1-methoxyanthraquinone and 2-hydroxy-1,5-dimethoxy-6-(methoxymethyl) anthraquinones. These compounds isolated from the present study can be found in authentic standards and literature data but there are differences in the methods and instruments for isolation and identification of the anthraquinones [10, 11].

The SEM observations show that the anthraquinones fraction of the leaves of *Morinda citrifolia* causes changes and damage to the morphology of the treated bacteria (Fig.5 A, B). 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 enzyme [20, 21]. 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 [22]. As the result of the mode of action the cell dies because of loss of cell contents [21].

Conclusions

In conclusion, this is the first report that studied antibacterial activity, antioxidant capacity and mode of action in anthraquinones fractions of *M. citrifolia* leaves. This fraction of leaves of this plant have antibacterial effect on all pathogenic bacteria including important resistant bacteria such as MRSA and *H. pylori* in well diffusion, MIC and MBC assays. Also anthraquinones fraction of the leaves of this plant exhibited a high level of antioxidant activity with significant values of IC_{50}. SEM analysis of anthraquinones fraction of the leaves of *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* leaves 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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