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Research Article
Major Chemical Composition of Fruit Extracts of *Morinda citrifolia* L. and their Antibacterial, Antioxidant and Cytotoxicity Properties

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Abstract

Background and Objective: *Morinda citrifolia* L. has been reported as a medicinal plant for treatment of abscesses and microbial infections. Thus, this study was conduced to isolate and identify the major constituents of the ethanolic extract of *M. citrifolia* fruit and also to evaluate their antibacterial, antioxidant and cytotoxicity properties. **Materials and Methods:** Isolation procedures included silica gel 60 column chromatography and thin layer chromatography. Identification of purified compounds was archived by spectroscopic methods. The antibacterial, antioxidant and cytotoxicity properties of purified compounds were carried out. **Results:** On the basis of the spectral data, the major compounds were isolated and identified as rutin (1) and asperulosidic acid (2) with yield of 9.28 and 7.71% (w/v), respectively. Antibacterial activity of crude extract and isolated compounds was evaluated by disc diffusion, MIC and MBC against *Staphylococcus aureu*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium* and methicillin-resistance *Staphylococcus aureus* SP6-106. The crude extract exhibited an antimicrobial activity at a dose level of 15 mg disc^{-1}, while the isolated compounds exhibited an excellent antimicrobial activity at a dose level of 3.75 μg disc^{-1} in all test micro-organisms for disc diffusion method. Compound 2 showed the lowest MIC (8 μg mL^{-1}) against *S. aureus*, while compound 1 showed the lowest MIC (16 μg mL^{-1}) against *E. coli*. However, compounds 1 and 2 had high MIC values (512 μg mL^{-1}) against *B. subtilis*. Compound 1 showed the lowest MBC (16 μg mL^{-1}) against *E. coli*, while the compound 2 showed the lowest MBC (8 μg mL^{-1}) against *S. aureus* whereas these compounds had high MBC values (512 μg mL^{-1}) against *B. subtilis*. The compound 1 had the highest antioxidant activity with SC_{50} values of 68.45 μg mL^{-1}, while compound 2 and the crude extract had antioxidant activity with SC_{50} values of 103.32 and 150.22 μg mL^{-1}, respectively. The cytotoxicity activity of the crude extract and isolated compounds was observed and showed weak cytotoxicity activity with IC_{50} values of 1352.67-2780.80 and 1524.19-2854.06 μg mL^{-1} toward L929 and HEK293 cell lines, respectively. **Conclusion:** It can be concluded that the major compounds isolated from the crude extract of *M. citrifolia* fruits in Nakorn Pathom, Thailand were rutin and asperulosidic acid, which have antibacterial, antioxidant and cytotoxicity properties. They may provide promising improvements in the therapeutic approach to infectious diseases and oxidative stress treatments.

Key words: Antibacterial activity, antioxidant activity, chemical composition, *Morinda citrifolia* L.

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

INTRODUCTION

Morinda citrifolia L. belongs to the family Rubiaceae, commonly known as Noni. The genus *Morinda* comprises some 80 species which all occur exclusively in tropical climate zones. It has a long tradition as a medicinal plant in Asia pacific countries. Typical uses have been reported as a treatment of boils and curs, abscesses, fungal infections, constipation as well as diarrhoea[1]. It has antimicrobial, anticancer, antioxidant, anti-inflammatory, analgesic and cardiovascular properties[2]. Many studies of *Morinda citrifolia* L. juice and isolated compounds from the fruit has been published including phenolic, volatile compounds and alkaloids[3]. Of the phenolic compounds, the most important reported are anthraquinones (damnacanthal, morindone and morindin, etc.) and also aucubin, asperuloside and scopoletin[3,4]. The main volatile compounds have been identified in the ripe fruit including organic acids (mainly caproic, caprylic, octanoic and hexanoic acids), alcohols (3 methyl 3-buten-1-ol), esters (methyl octanoate, methyl decanoate), ketones (2-heptanone) and lactones (E-6-dodeceno-g-lactone)[5,6], while the principal reported alkaloid is xeronine[7]. Due to the quality and quantity of the chemical components contained in this plant are directly related to the soil, climate, ripening and geographical location[8,9].

The purposes of this study were to evaluate antibacterial, antioxidant and cytotoxicity properties of the major compounds isolated from ethanolic extract of *Morinda citrifolia* L. fruit from Nakorn Pathom, where was a major location of Noni cultivation in Thailand.

MATERIALS AND METHODS

Plant material and extraction procedure: The ripen fruits of *Morinda citrifolia* L. were collected from the local areas around Silpakorn University in Nakorn Pathom, Thailand, between September, 2017 and January, 2018. The samples were washed with running tap water and separated before being chopped into small pieces. They were oven dried at 45°C for 3 days and ground to powder. The dried fruit powder (500 g) was extracted with 1 L of 95% ethanol for 3 days at room temperature. The aqueous extracts were filtered using Whatman filter paper (No. 1) and then concentrated *in vacuo* at 40°C using a Rotary evaporator. The residues obtained (350 mg) were stored in a freezer at -20°C until further studies.

Isolation of the compounds: The crude extract was dissolved in methanol to perform the bio-autography assays[10]. The major compounds were isolated by silica gel 60 (230-400 mesh, Merck) column chromatography and eluted with dichloromethane:ethyl acetate (1:2). Fractions were monitored by thin layer chromatography (TLC) (Kieselgel 60 F254, Merck) and spots were visualized under ultraviolet light and by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. Fractions with spots of the same retention factor (Rf) values were combined and rechromatographed in appropriate solvent systems until pure isolates were obtained. The results showed two major spots with Rf = 0.86 (compound 1; 65 mg) and Rf = 0.71 (compound 2; 54 mg). Preparative TLC on the material was carried out and the two fractions with Rf = 0.86 and 0.71 separated and characterized. IR, UV-Visible, ^{1}H-NMR, ^{13}C-NMR and MS spectra of each fraction was taken.

Antibacterial assay: An *in vitro* plate assay technique was used to test the inhibitory effects of crude extract and purified compounds on the tested bacteria using the paper disk method according to Clinical Laboratory Standard Institute[11]. Sterile paper discs (6 mm, Whatman 2017-006) were loaded with 30 mL of two-fold dilution of 1000 mg mL^{-1} of crude extract or 1 mg mL^{-1} of purified compounds. Four bacterial species were used in this study: *S. aureus* ATCC 25932, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 10536, *Salmonella typhimurium* ATCC 23564 and methicillin-resistance *Staphylococcus aureus* SP6-106 (the clinical isolate), these bacteria were cultured in nutrient broth at 37°C for 24 h. Dilutions of bacterial suspensions were prepared using McFarland standard tubes (1×10^8 CFU mL^{-1}). The air-dry discs with various concentration of the crude extract and purified compounds were placed on a lawn of bacterial spread on Muller Hinton agar. The plates were incubated at 37°C for 24 h. The diameter of the formed inhibition zones around each disc was recorded. The experiment was carried out in triplicates using penicillin (10 units disc^{-1}) (Oxoid, UK) as a reference for antimicrobial activity control.

Minimum inhibitory concentration (MIC): The MICs of the crude extract and purified compounds were determined by NCCLS micro-broth dilution methods[12]. The agents were dissolved in dimethyl sulfoxide (DMSO). Then, 10 µL of the bacterial suspension (10^5 cells mL^{-1}) was inoculated into each well of a 96-well microplate, each containing a different concentration of the test agents. It performed doubling dilutions of the test agents. Penicillin was used as a reference for antibacterial activity. The range of sample dilutions was 512-0.5 µg mL^{-1} in nutrient broth supplemented with 10% glucose (NBG) and a final concentration of the test agent that

inhibited bacterial growth was determined as indicated by the absence of turbidity. Test agent-free broth containing 5% DMSO was incubated as growth control. Minimum microbicidal concentration was determined by inoculating onto nutrient agar plates 10 µL of medium from each of the wells from the MIC test which showed no turbidity. The plates were incubated at 37°C for 24 h. Minimum bactericidal concentration (MBC) was defined as the lowest concentration of the test agent at which no microbial growth was observed on the plates.

Radical-scavenging activity-DPPH assay: The antioxidant activity of the crude extract and purified compounds was evaluated by monitoring their ability in quenching the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to a slightly modified method[13]. Spectrophotometric analysis was used to measure the free radical-scavenging capacity and to determine the scavenging concentration (SC_{50}) of the crude extract and purified compounds. The DPPH quenching ability was expressed as SC_{50} (the concentration required to inhibit radical formation by 50%). Six different ethanol dilutions of the crude extract or each compound (2.5 mL) at 250, 125, 62.5, 31.25, 15.62 and 7.81 µg mL^{-1} were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. Ethanol (1.0 mL) plus the crude extract or each compound (2.5 mL) was used as a blank. The absorbance was measured at 518 nm by using a UV-VIS spectrophotometer after 30 min of reaction at room temperature. The radical was prepared daily and protected from light. Relative properties were calculated from the calibration curve of L-ascorbic acid standard solution working in the same experimental conditions. Scavenging capacity (SC%) was calculated according to the following equation:

$$SC\ (\%) = 100 - \left[\frac{\left(Abs_{sample} - Abs_{blank}\right)}{Abs_{control}}\right] \times 100$$

where, Abs_{sample} is the absorbance of the test compound and $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test agent). The SC (%) was plotted against sample concentration and a linear regression curve was established in order to calculate the SC_{50}. Tests were carried out in triplicate. Correlation coefficients were optimized.

Cytotoxicity activity assay: In order to evaluate the cytotoxicity activity of the crude extract and purified compounds, a cytotoxicity test was performed and the effect of the median inhibitory dose (IC_{50}) on the murine fibroblast cell (L929) and embryonic kidney cell (HEK293) lines were assessed. Different concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg mL^{-1}) of the crude extract and purified compounds were prepared and used in the cytotoxicity test. To measure the cytotoxicity, 5×10^4 cells were seeded in 96-well plates and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum containing different concentrations of the test agents at 37°C for 24 h in 5% CO_2 incubator. The wells were washed with a serum-free medium. Vehicle control groups were added with double distilled water.

In the tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay, yellow MTT is reduced to purple formazan in the mitochondria of viable cells. One hundred microliters of the MTT working solution (0.5 mg mL^{-1}) were added to each well and incubated at 37°C for 5 h. Next, the media were removed, wells were washed with phosphate buffer saline and 100 µL of DMSO was added to solubilize the formazan crystalline product. The absorbance was measured with a plate reader (Packard AS10000 Spectrocount, USA) at 590 nm. The production of formazan dye was proportional to the number of viable cells.

The inhibition of the cell lines cytotoxicity rates for each test agents with different concentrations was calculated according to the following equation:

$$Inhibition\ (\%) = 100 - \left[\frac{\left(Abs_{sample} - Abs_{blank}\right)}{\left(Abs_{control} - Abs_{blank}\right)}\right] \times 100$$

where, Abs_{sample} is the absorbance of the test agent and $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test agent). The inhibition (%) was plotted against sample concentration and a linear regression curve was established in order to calculate the IC_{50}. Tests were carried out in triplicate. Correlation coefficients were optimized.

RESULTS

The TLC and column chromatography on silica gel with dichloromethane: ethyl acetate (1:2) as the mobile phase resulted in the separation of two major compounds with Rf = 0.86 and Rf = 0.71. The ^{1}H-NMR, ^{13}C-NMR, UV-visible, IR and MS spectra of these compounds were taken.

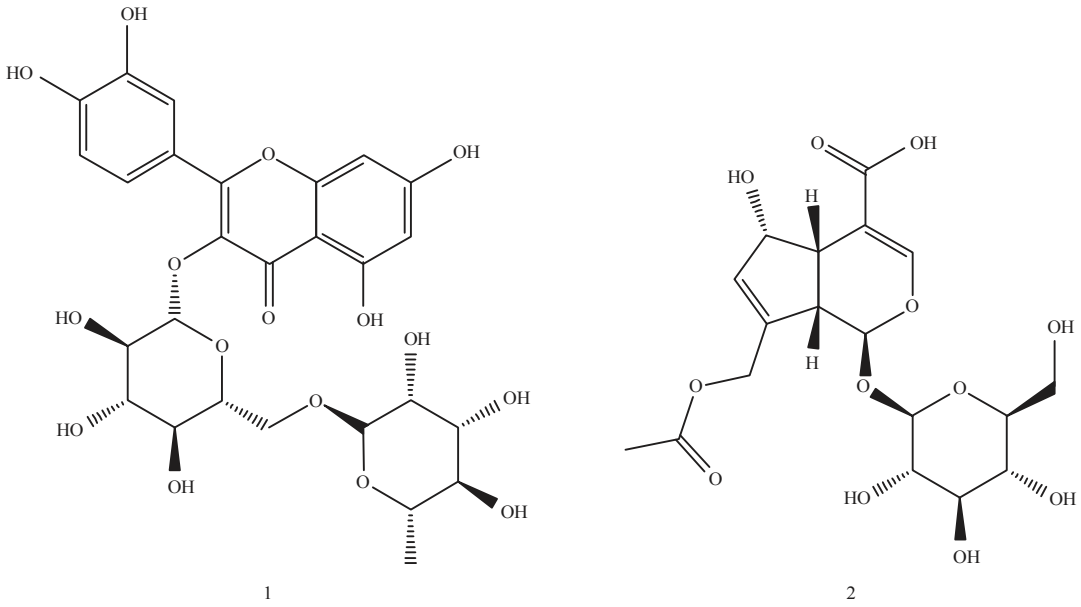

Fig. 1: Chemical structures of major components isolated from the crude extract of *Morinda citrifolia* L. fruits. (1) Rutin and (2) Asperulosidic acid

Characterization of the compound 1 with Rf = 0.86: It was isolated as a yellow powder, the negative APCI MS exhibited a pseudomolecular ion peak at m/z 609 $[M-1]^-$ and the positive APCI MS showed a significant pseudomolecular ion peak at 611 $[M+1]^+$, which together with the ^{1}H- and ^{13}C-NMR indicated a molecular formula of $C_{27}H_{30}O_{16}$. The ^{1}H-NMR showed signals at δ 12.58 (1H, s, 5-OH), 7.49 (2H, m, H-2, 6), 6.78 (1H, d, J) 9.0 Hz, H-5), 6.34 (1H, br s, H-8), 6.18 (1H, br s, H-6), 5.26 (1H, d, J) 7.4 Hz, H-glu-1), 4.34 (1H, H-rha-1) and 3.00-3.82 (sugar protons), 1.00 (3H, d, J) 6.0 Hz, H-rha-6). The ^{13}C-NMR (50 MHz, in DMSO-d_6) showed data at δ 177.2 (s, C-4), 164.1 (s, C-7), 161.3 (s, C-5), 156.6 (s, C-9), 156.4 (s, C-2), 148.3 (s, C-4), 144.8 (s, C-3), 133.2 (s, C-3), 121.5 (s, C-1), 121.1 (d, C-6), 116.2 (d, C-5), 115.1 (d, C-2), 103.8 (s, C-10), 101.1 (d, C-glc1), 100.5 (d, C-rha-1), 98.4 (d, C-6), 93.7 (d, C-8), 76.4 (d, C-glc-3), 75.8 (d, C-glc-5), 74.1 (d, C-glc-2), 71.8 (d, C-glc-4), 70.5 (d, C-rha-3), 70.3 (d, C-rha-2), 69.8 (d, C-glc-4), 68.2 (d, C-rha-5), 67.1 (t, C-glc-6) and 18.0 (q, C-rha-6). On the basis of these spectral data, this component was identical with those of rutin[14,15].

Characterization of the compound 2 with Rf = 0.71: It was isolated as a colorless oil. Its molecular formula, $C_{18}H_{24}O_{12}$, was deduced from negative APCI MS, which showed a pseudomolecular ion peak at m/z 431 $[M-1]^-$, the positive APCI MS, which exhibited a significant pseudomolecular ion at m/z 450 $[M+NH_4]^+$ and the ^{1}H- and ^{13}C-NMR. The ^{1}H-NMR spectrum showed signals at δ 7.63 (1H, br s, H-3), 6.01 (1H, br s, H-7), 5.05 (1H, d, J) 9.0 Hz, H-1), 4.70 (1H, d, J) 7.6 Hz, H-1), 3.85 (1H, d, J) 11.4 Hz, H-6), 3.18-3.68 (m, H-2, 3, 4, 5, 6), 3.01 (1H, m, H-5) and 2.60 (1H, t, J) 8.3 Hz, H-9). The ^{13}C-NMR showed signals at δ 172.8 (s, C=O), 170.7 (s, C=O), 155.4 (d, C-3), 145.8 (s, C-8), 131.7 (d, C-7), 100.8 (d, C-1), 99.8 (d, C-1), 78.6 (d, C-5), 77.8 (d, C-3), 75.4 (d, C-6), 74.7 (d, C-2), 71.5 (d, C-4), 63.7 (t, C-10), 62.8 (t, C-6), 46.2 (d, C-9), 42.4 (d, C-5) and 20.8 (q, $COCH_3$). On the basis of these spectral data, this compound was identical with those of asperulosidic acid[15-17]. The structures of these compounds are shown in Fig. 1.

The crude extract from the fruits of *M. citrifolia* showed a dark brown color. The crude extract yield was 700 mg kg^{-1} while the percentage yields of the purified compounds 1 and 2 were about 9.28 and 7.71% (w/w), respectively. The antibacterial activity of the crude extract and purified compounds is summarized in Table 1. Various concentrations of crude extract and purified compounds were tested using agar disc diffusion assay. A zone of inhibition >8 mm in diameter was interpreted as sensitive. All of the susceptible strains were sensitive to the crude extract at 15 mg $disc^{-1}$. The crude extract showed the highest activity against *E. coli* and *S. typhimurium* at 30 mg $disc^{-1}$ with the average zones of inhibition being 33.30±10.56 and 46.60±15.81 mm, respectively. However, this crude extract showed low activity against MRSA at 30 mg $disc^{-1}$ with the average zones of inhibition 13.30±4.17 mm and also showed moderate activity against *S. aureus* and *B. subtilis* at 30 mg $disc^{-1}$ with the

Table 1: Diameters of inhibition zones of the crude extract and purified compounds on the tested micro-organisms

	Diameters of inhibition zones on tested micro-organisms (mm)				
Test agents/concentrations	S.a.	B.s.	E.c.	S.t.	MRSA
Crude extract (mg disc^{-1})					
3.75	NZ	NZ	NZ	NZ	NZ
7.5	10.00±5.77	NZ	16.60±9.58	17.60±10.16	NZ
15	16.60±2.55	15.00±2.55	25.00±9.64	21.60±6.69	11.60±3.44
30	21.60±2.55	20.50±1.96	33.30±10.56	46.60±15.81	13.30±4.17
Compound 1 (mg disc^{-1})					
3.75	8.27±2.84	7.41±2.63	11.74±2.57	13.22±2.58	7.85±2.43
7.5	12.74±3.66	10.28±3.87	16.42±3.81	18.49±3.43	9.78±2.17
15	17.22±4.84	12.72±3.02	20.53±3.88	29.22±3.57	11.73±3.24
30	23.35±4.13	14.66±3.93	24.25±3.44	37.33±3.21	13.66±3.93
Compound 2 (mg disc^{-1})					
3.75	7.33±2.66	7.65±2.41	12.26±2.25	14.77±3.14	7.72±2.12
7.5	11.18±3.62	9.54±2.77	17.14±3.36	17.73±4.06	9.22±2.75
15	15.50±4.25	11.60±3.66	21.27±3.64	27.52±4.05	10.53±3. 72
30	22.05±3.77	13.62±3.88	26.32±3.63	38.31±4.81	12.56±3.33
Penicillin					
10 Units disc^{-1}	25.00±17.50	33.30±13.33	36.60±16.60	26.60±14.40	25.00±14.60

S.a.: *Staphylococcus aureus* ATCC25932, B.s.: *Bacillus subtilis* ATCC6633, E.c.: *Escherichia coli* ATCC10536, S.t.: *Salmonella typhimurium* ATCC23564, MRSA.: methicillin-resistance *Staphylococcus aureus* SP6-106. Results represent the Mean±SD. NZ: No inhibition zone

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of crude extract, purified compounds on tested micro-organisms

	Antibacterial activity of the test agents							
	Crude extract (mg mL^{-1})		Compound 1 (mg mL^{-1})		Compound 2 (mg mL^{-1})		Chloramphenicol (mg mL^{-1})	
Test micro-organisms	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S.a.	64	64	64	64	8	8	4	512
B.s.	512	512	512	512	512	512	8	>512
E.c.	64	64	16	16	64	64	4	>512
S.t.	64	64	64	64	64	64	8	>512
MRSA	256	256	256	256	16	16	4	512

S.a.: *Staphylococcus aureus* ATCC25932, B.c.: *Bacillus subtilis* ATCC6633, E.c.: *Escherichia coli* ATCC10536, S.t.: *Salmonella typhimurium* ATCC23564 and MRSA: Methicillin-resistance *Staphylococcus aureus* SP6-106

average zones of inhibition 21.60±2.55 and 20.50±1.96 mm, respectively. Compounds 1 and 2 showed the highest activity at 30 µg disc^{-1} against *S. typhimurium* with the zones of inhibition ranging from 37.33±3.21-38.31±4.81 mm and *E. coli* with the zones of inhibition ranging from 24.25±3.44-26.32±3.63 mm, respectively. They showed moderate activity against *S. aureus* at 30 µg disc^{-1} with the zones of inhibition ranging from 22.05±3.77-23.35±4.13 mm and also showed low activity against *B. subtilis* and MRSA at 30 µg disc^{-1} with the zones of inhibition ranging from 12.56±3.33-14.66±3.93 mm. Sensitive results were not obtained with discs containing 3.75 mg disc^{-1} of the crude extract in all tested micro-organisms.

Adopting a classification based on MIC values proposed by Kuete[18] and Kuete and Efferth[19], the antibacterial activity of a plant extract is considered significant when the MICs are below 100 µg mL^{-1}, moderate when 100≤MIC≤512 µg mL^{-1} and weak if MIC>512 µg mL^{-1}. Consequently, where the activity of the crude extract showed MIC values equal to 256 and 512 µg mL^{-1} for MRSA and *B. subtilis*, respectively (Table 2). It was therefore, considered a moderate inhibitor against these micro-organisms. Compounds 2 showed the lowest MIC (8 µg mL^{-1}) against *S. aureus*. It was followed by the MIC values (16 µg mL^{-1}) against MRSA, while compound 1 showed the lowest MIC (16 µg mL^{-1}) against *E. coli*. It was followed by the MIC values (64 µg mL^{-1}) against *S. aureus* and *S. typhimurium*. Compounds 1 and 2 had high MIC values (512 µg mL^{-1}) against *B. subtilis*. They were therefore considered an activity inhibitor against these micro-organisms excepted *B. subtilis*. Compounds 1 showed the lowest MBC (16 µg mL^{-1}) against *E. coli*, while the compound 2 showed the lowest MBC (8 µg mL^{-1}) against *S. aureus* whereas, these compounds had high MBC values (512 µg mL^{-1}) against *B. subtilis*.

Table 3: Antioxidant activity of the crude extract and tested compounds

Test agents	SC_{50} (μg mL^{-1})[a]	Standard deviation
Crude extract	150.22[b]	34.68
Compound 1	68.45[b]	21.35
Compound 2	103.32[b]	29.82
L-ascorbic acid	45.66	12.67

[a]SC_{50} values represent the concentration required to inhibit radical formation by 50%, [b]Represents a significant ($p<0.05$) difference from the positive control

Table 4: IC_{50} of the crude extract, purified compounds against normal cell lines after 24 h using the MTT assay

	IC_{50a} values of crude extract, purified compounds on tested cell lines (mg mL^{-1})	
Test agents	L929[b] cells	HEK293[c] cells
Crude extract	2780.80	2854.06
Compound 1	1735.28	1875.77
Compound 2	1352.67	1524.19

[a]IC_{50} values represent the concentration causing 50% growth inhibition. They were determined by linear regression analysis. [b]L929, murine fibroblast cell line. [c]HEK293, human embryonic kidney cell line

The free radical-scavenging capacity of the crude extract and the compounds was assessed by the decoloration of the ethanolic solution of DPPH. In the presence of an active radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric at a selected range with respect to the degree of reduction. Ethanolic solutions of DPPH served as a control and the calibration curve made with L-ascorbic acid was used to compare the activity as a positive control, since its standard antioxidant activity was well established. Table 3 shows the antioxidant activity of the crude extract and purified compounds. Compounds 1 and 2 had antioxidant activity with SC_{50} values of 68.45 and 103.32 μg mL^{-1}, respectively, which were comparable to that of a positive control, L-ascorbic acid with SC_{50} value of 45.66 μg mL^{-1}.

To evaluate the cytotoxicity activity of the crude extract and purified compounds against L929 and HEK293, the cell lines were incubated with different doses of two-fold dilution (1-512 μg mL^{-1}) of the crude extract and purified compounds. After 24 h of incubation, cell viability was determined by MTT assay. The crude extract and purified compounds induced cell cytotoxicity in a concentration-dependent manner. The corresponding IC_{50} was calculated and the results are presented in Table 4. The cytotoxicity activity of the crude extract and purified compounds was observed and showed weak cytotoxicity activity with IC_{50} values of 1352.67-2780.80 and 1524.19-2854.06 mg mL^{-1} toward L929 and HEK293 cell lines, respectively.

DISCUSSION

The antimicrobial activity of *M. citrifolia* may have been its first observed property, indeed, the fruit contains relatively large amounts of sugars that are not fermented even when fruits are stored in closed containers at ambient temperature. This property is used to transport the fruit by boat from the scattered pacific islands to processing plants without specific treatment[2]. It has been reported a significant antimicrobial effect on various strains of *Salmonella* sp., *Shigella* sp., *E. coli*, *S. aureus*, *B. subtilis*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Citrobacter* sp., *Klebsiella* sp., *Proteus morganii*, *Providencia* sp., *Vibrio* sp., *Mycoplasma* spp.[5,20-27]. Another study showed that an acetonitrile extract of the dried fruit inhibits the growth of *P. aeruginosa*, *B. subtilis*, *E. coli* and *Streptococcus pyrogenes*[22]. It has also been found that ethanol and hexane extracts of *M. citrifolia* L. fruits have an antitubercular effect as they inhibit the growth of *Mycobacterium tuberculosis*[28] by 89-95%. Furthermore, it was showed that the antimicrobial effect is highly dependent on the stage of ripeness and on processing, being greater when the fruit is ripe and undried[5,20].

Its antibacterial activity was attributed to the presence of phenolic compounds such as aucubin, L-asperuloside, alizarin and scopoletin[29]. The major components identified in the hexane extract are E-phytol, cycloartenol, stigmasterol, b-sitosterol, campesta-5,7,22-trien-3-b-ol and the ketosteroids stigmasta-4-en-3-one and stigmasta-4-22-dien-3-one. Another antimicrobial *in vitro* assay was conducted on methanol, ethyl acetate and hexane Indian *M. citrifolia* fruit extracts against a wide range of organisms including the following: *B. subtilis*, *S. aureus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *P. aeruginosa*, *Salmonella typhi*, *E. coli*, *Vibrio harveyi*, *Klebsiella pneumonia*, *Shigella flexneri*, *Salmonella paratyphi* A, *Aeromonas hydrophila*, *Vibrio cholera*, *Chromobacterium violaceum* and *Enterococcus faecalis*. Among the three tested extracts, methanol extract was the most effective, ethyl acetate was effective against some of the tested microorganisms and hexane extract was ineffective against all tested micro-organisms[30]. These results showed that the highest extraction yield of active compounds from *M. citrifolia* fruit was extracted with polar solvents. So in the present study, ethanol had a polarity which was used as solvent extraction. The antibacterial activity of *M. citrifolia* fruit extract was also assessed in an *in vitro* assay on *S. aureus*, *B. subtilis*, *E. coli*, *S. typhimurium* and MRSA SP6-106. *E. coli* and *S. typhimurium* were the most sensitive to *M. citrifolia* antimicrobial activity, while *S. aureus*, *B. subtilis* and MRSA sensitivity was lower. This activity was linked to the purified compounds of the fruit, particularly, rutin and asperulosidic acid. A similar result was obtained in antimicrobial activity of *M. citrifolia* fruit extract on *E. coli*, *Candida albicans* and *S. aureus*. *Candida albican* was the most sensitive to *M. citrifolia* antimicrobial activity, while *S. aureus* sensitivity

was the lowest[31]. Another report had demonstrated the antibacterial activity of leaf, stem and fruit of *M. citrifolia* against wide spectrum of gram positive and gram negative bacterial strains[32]. It was differ from the present study, *M. citrifolia* fruit was screened for antibacterial effect and the results obtained proved that moderate activity was seen in gram positive bacteria. Gram-negative bacteria were more susceptible to *M. citrifolia* fruit extract than gram positive bacteria. Membrane accumulator mechanism might play important role behind this perception[33-35]. These results were in accordance with the previous studies which suggest that rutin and asperulosidic acid might enable the extract to overcome the barrier in bacterial cell wall and membrane[36].

In the present study, antioxidant activity of ethanol extracts of *M. citrifolia* fruit was assessed by the decoloration of the ethanolic solution of DPPH. Due to the presence of phenolic hydroxyl groups of the compounds 1 was expected to exert radical-scavenging activity against DPPH radicals, while the antioxidant activity of the compound 2 was indicated to be as moderate active. It was similar report by Deng *et al*.[37] that asperulosidic acid isolated from *M. citrifolia* blossoms had moderate scavenging activity against DPPH radicals. This finding was differed from the report of Su *et al*.[38] that asperulosidic acid did not exhibited evident scavenging activity against DPPH radicals because of the IC_{50} values over 30 μM. In this study, L-ascorbic acid was utilized as a positive control, which showed significantly higher antioxidant activity than purified compounds; these results were due to the number and the position of the hydroxyl groups in their molecular structure. Similar findings were obtained in previous studies[39,40], which reported that the antioxidant activity depends on the numbers and positions of the hydroxyl groups. In addition, substitution of the hydroxyl groups with methoxyl groups reduces this activity. In the present study, the crude extract of *M. citrifolia* fruit showed an antioxidant activity 0.30 times lower than vitamin C. However, The anti-oxidant properties of ethanol extracts of *M. citrifolia* fruit have been assessed using the ferric thiocyanate method (FTC) and thiobarbituric acid test (TBA). It was found that the crude extract exhibited strong inhibition of lipid oxidation comparable to the same weight of pure a-tocopherol and butylated hydroxy toluene[41]. Radical scavenging activity of *M. citrifolia* juice had been also reported by the tetrazolium nitroblue (TNB) assay and lipids oxidation by superoxide anion radicals (SARs). The SAR scavenging activity of Australian *M. citrifolia* L. juice was shown to be 2.8 times higher than that of vitamin C, 1.4 times and almost the same magnitude as that of grape seed powder[3]. In other study, the optimum magnitudes of radical scavenging activity (RSA) and total phenolic content of Malaysian seedless *M. citrifolia* fruit methanol extract were 55.60% and 43.18 mg GAE 10 g^{-1}, respectively[3].

In the present study, rutin and asperulosidic acid were isolated as the major compounds from the crude extract. This was different from those in other reports, which rutin was not a major component in *M. citrifolia* fruits[38,42]. Rutin is a flavonoid known to have a variety of biological properties including antiallergic, anticarcinogenic, antiinflammatory, antioxidant, antiproliferative, cardioprotective, cytoprotective, neuroprotective and vasoprotective properties[43-50]. Rutin is extensively studied for antimicrobial activity against various strains of bacteria. It has demonstrated a profound degree of inhibition on growth of bacteria *E. coli*[51], *Enterococcus faecalis* and *Streptococcus mutans*[52]. Rutin, quantified in honey has also shown inhibitory effects over *Proteus vulgaris*, *Shigella sonnei* and *Klebsiella* sp.[53]. Antimicrobial activity against *P. auruginosssa* and *B. subtilis* has also been documented[54,55]. *In situ* antimicrobial activity of rutin and other polyphenols in the food system has been studied and the results demonstrate a promising involvement of flavonoids in the preservation of food[56]. It had been demonstrated that rutin inhibited DNA isomerase IV of *E. coli*[57]. It was also shown, rutin synergistically enhanced antibacterial activity of other flavonoids against *Bacillus cereus* and *Salmonella* enteritidis. Minimum inhibitory concentration value for kaempferol was remarkably decreased by the addition of rutin[58].

In the present study, the antioxidant activity of rutin was reported, these findings were in agreement with those report by Morishita *et al*.[59], who also showed a contribution of rutin on the antioxidant activity. Another reports also found that rutin exhibited strong DPPH radical scavenging activity. At the concentration of 0.05 mg mL^{-1}, ascorbic acid, butylated hydroxytoluene and rutin showed 92.8, 58.8 and 90.4% inhibition, respectively. In addition, rutin had effective inhibition of lipid peroxidation[60,61]. It also showed preventive effect on oxaliplatin-induced painful peripheral neuropathy based on its antioxidant properties[62]. The antioxidant activity of rutin had been described by the presence of a phenolic group, which was known to add hydrogen donation for scavenging the radiation-induced radicals and to inhibit radiation-induced oxidative stress[63]. This study regarding rutin as a major natural antioxidant from *M. citrifolia* fruits has no cytotoxic effects. Therefore, it could replace synthetic antioxidants in the food processing industry and have potential for use in preventive medicine.

CONCLUSION

The *M. citrifolia* fruits has been used for centuries in folk medicine. Different studies, some of them with controversial methodologies showed that this fruit contains several compounds. In the present study, the major compounds identified in ethanolic fruit extracts from Thailand are rutin and asperulosidic acid which have an antibacterial and antioxidant properties, but no cytotoxic effects. These compounds may provide promising improvements in the therapeutic approach to infectious diseases and oxidative stress treatments.

SIGNIFICANCE STATEMENT

This study reports the antibacterial, antioxidant and cytotoxicity properties of rutin and asperulosidic acid, which were isolated as the major compounds from the ethanolic extract of *Morinda citrifolia* fruits. This study will help the researchers to used rutin, asperulosidic acid from *Morinda citrifolia* fruit as a food and traditional medicine in optimum concentration. Our data indicate that these compounds are not toxic against normal cell lines *in vitro*. In addition, they have an antibacterial and antioxidant properties. Therefore, the optimum dose of rutin, asperulosidic acid and crude extract of Noni fruit used as a traditional medicine is required and needs to be studied further for the benefit of human health.

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