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Original Article

Antidiabetic, tyrosinase inhibitory, and free radical scavenging activities of the purified extracts from *Morinda citrifolia* unripe fruits

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Abstract

The crude extracts from medicinal plants are increasingly important for bioactive activities. Several parts of *Morinda citrifolia* have been utilized in traditional medicine, such as diabetes, anticancer, and hypertension. Therefore, the purpose of this research was to investigate the biological activities, e.g., antioxidant, antidiabetic, and tyrosinase inhibitory activity of the crude extracts of *Morinda citrifolia* unripe fruits. The highest percent extraction was found in methanol. The purified methanoic fraction displayed the highest total phenolic content and was a potent tyrosinase inhibitor. The fraction of ethyl acetate exhibited the highest flavonoid content and DPPH free radical scavenging capacity. Furthermore, the ethyl acetate and methanol fractions were investigated as potent α-glucosidase and α-amylase inhibitors. Additionally, the cytotoxicity test of the bioactive fractions was explored in human keratinocyte cell lines.

Keywords: *Morinda citrifolia* unripe fruit extract, tyrosinase inhibition, glucosidase inhibition, amylase inhibition, free radical scavenging

1. Introduction

Skin pigment production is related to melanin accumulation produced by melanogenesis in melanocytes. In the past decade, tyrosinase was reported as a key target of the rate-limiting step for melanin pigment synthesis (You *et al.*, 2015). Tyrosinase (EC 1.14.18.1), an enzyme in the type-3 copper protein family, catalyzed two steps, namely the hydroxylation of tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to *ortho*-quinone (dopaquinone) (Criton & Le Mellay-Hamon, 2008). Various natural compounds from plants, fungi, and bacteria have been investigated to provide tyrosinase inhibitory activity (Zolghadri *et al.*, 2019). Therefore, the natural inhibitors have been greatly attractive for developing skin-whitening cosmetic and pharmaceutical applications (Pillaiyar, Manickam, & Namasivayam, 2017).

Diabetes mellitus (DM) is a metabolic disorder involving chronic hyperglycemia. It was found that 80–90% of diabetes resulted from type-II diabetes mellitus (T2DM)

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associating with the post-prandial hyperglycemia process (Taha *et al.*, 2017). The abnormal hyperglycemia causes retinopathy and cardiovascular disorders, e.g., myocardial infarction, coronary heart disease, and atherosclerosis (Kazmi *et al.*, 2018). Consequently, inhibition of the carbohydrate digestion process catalyzed by α-amylase (EC. 3.2.1.1) and α-glucosidase (EC. 3.2.1.20, α-D-glucoside glucohydrolase) can control the blood glucose level of type-II diabetes. α-Amylase enzyme catalyzes the hydrolysis of the α-linked polysaccharides to provide oligosaccharides or disaccharides. The α-glucosidase enzyme, found in the small intestine, then hydrolyzes the oligosaccharide or disaccharide to obtain monosaccharide (Teng & Chen, 2017). Therefore, α-glucosidase inhibitors have been developed for antidiabetic drugs. Although various oral diabetic drugs, including voglibose, acarbose, and miglitol, have been widely employed in clinical therapeutics, these drugs have several side effects such as abdominal discomfort, diarrhea, and flatulence (Kashtoh *et al.*, 2016). Diverse medicinal plants have been isolated to determine the antidiabetic inhibitory activity to improve the inhibitory efficiency and diminish side effects (Dirir, Daou, Yousef, & Yousef, 2021; Yin, Zhang, Feng, Zhang, & Kang, 2014).

Morinda citrifolia L. (Rubiaceae), also called noni, is a small tropical tree that grows in the Pacific Islands and Southeast Asia, including Thailand. The leaves, roots, barks, flowers, and fruits of *M. citrifolia* have been utilized in the traditional medicine for many diseases such as hypertension and diabetes (Nerurkar, Hwang, & Saksa, 2015). Therefore, all parts of *M. citrifolia* have been investigated for biological activities such as antimicrobial, antiseptic, antifungal, antioxidant, anti-inflammatory, anti-arthritic, anticancer, antidiabetic, and anti-viral activities (Reem *et al.*, 2017). The *M. citrifolia* ripe fruits exhibited several volatile compounds, including carboxylic acids, alcohols, esters, terpenes, aldehydes, ketones, and sulfur compounds (Farine, Legal, Moreteau, & Le Quere, 1996; Visagaperumal, 2019). The volatile compounds such as aldehydes, ketones, acids, alcohols, esters, terpenes, and sulfur compounds were also found in noni juice (Ali, Kenganora, & Manjula, 2016). The fresh noni juice showed the phenolic content and free radical scavenging activity (Yang, Paulino, Janke-Stedronsky, & Abawi, 2007). The extraction of the fermented fruit juice of *M. citrifolia* presented antioxidant activity and α-glucosidase inhibition (Liu *et al.*, 2007; Simamora A, 2019). The *n*-butanol partition of the fruit juice was also investigated to have various compounds (Samoylenko *et al.*, 2006). The *M. citrifolia* fruits were isolated to provide several lignans, which exhibited copper-induced low-density lipoprotein oxidation (Kamiya, Tanaka, Endang, Umar, & Satake, 2004) and cyclooxygenase-2 (COX-2) inhibition (Deng *et al.*, 2007). Besides, the ethanoic extract of the *M. citrifolia* seeds showed elastase and tyrosinase inhibition (Masuda *et al.*, 2009) and considerable inhibition of the melanogenesis process without cell proliferation (Masuda *et al.*, 2012). *Morinda lucida Benth* leaf's aqueous extract also inhibited α-amylase and α-glucosidase (Kazeem, Adamson, & Ogunwande, 2013). In recent years, the methanolic extract of *M. citrifolia* unripe fruit was demonstrated for treating alcohol dependence using the mouse conditioned place preference test (Khan & Pandy, 2016). Although *M. citrifolia* unripe fruit has been utilized in traditional medicine, such as for the antiemetic activity (Chuthaputti, Permpipat, & Techadamrongsin, 1996). The *M. citrifolia* unripe fruit extracts have not been reported yet for the α-glucosidase, α-amylase, and tyrosinase inhibition.

In this research, the powder of *M. citrifolia* unripe fruits was extracted by hexane, ethyl acetate, and methanol, followed by purification by silica column chromatography. The total phenolic and flavonoid contents of the purified extract fractions were investigated. For the biological activities, the purified fractions were also elucidated, including free radical scavenging activity and the inhibitory activities of α-glucosidase, α-amylase, and tyrosinase.

2. Materials and Methods

2.1 Preparation and isolation of plant material

The *Morinda citrifolia* unripe fruits were collected from Pathumthani, Thailand. The pericarp (909 g) was cut to a thin slice and separated from the seed, followed by drying in the hot air oven at 60°C for 48 h, and then ground by a mechanical blender. The green powder (130 g) was extracted with stirring for 3 days with the following solvents (650 mL); hexane, ethyl acetate, and methanol, respectively. The supernatant of each solvent was separated by the filter paper and then evaporated under reduced pressure to provide the crude extracts: hexane (1.3127 g), ethyl acetate (0.8364 g), and methanol (10.3413 g). Each crude extract, i.e. hexane, ethyl acetate, and methanol, was further purified by column chromatography by eluting 3–5% EtOAc/hexane, 20% EtOAc/hexane–50% MeOH/EtOAc, and 10–40% MeOH/EtOAc, respectively.

2.2 Phytochemical screening

The crude extracts of *Morinda citrifolia* unripe fruits were tested for their phytochemical profile (Hossain, Al-Raqmi, Al-Mijizy, Weli, & Al-Riyami, 2013). For the alkaloid analysis, Mayer's reagent was prepared from the solution of $HgCl_2$ (0.2716 g) and KI (1.0 g) in deionized water (20 mL). Each crude extract (0.01 g) was dissolved in 28% NH_3 (1.5 mL) and mixed for 5 min. To the reaction solution, $CHCl_3$ (5.0 mL) was added and mixed for 10 min. The mixture was then filtered using filter paper and evaporated in a water bath, followed by the addition of Mayer's reagent (1.5 mL). The presence of alkaloids was indicated by the formation of a white or cream-colored precipitate.

The fourth method was used to determine the presence of saponin. The crude extracts (10.0 mg) were dissolved in deionized water (5.0 mL) and boiled for 5 minutes in a water bath. Three drops of olive oil were added to the filtrate (2.0 mL) in deionized water (5.0 mL) followed immediately by shaken. The solution was treated at ambient temperature for 30 min. Saponins were detected by the production of emulsion and persistent bubbles.

Qualitative analysis of tannin was investigated by ferrous (III) chloride. $CHCl_3$ (1.0 mL) was added to the stock solution (3.0 mg/mL) of the crude extracts, followed by the addition of Ac_2O (1.0 mL). The reaction mixture was then added *conc.* H_2SO_4 (1.0 mL) by the side of the test tube. The formation of green color suggested the presence of tannins.

The steroids were determined by the Salkowaski reaction. $CHCl_3$ (2.0 mL) was added to the crude extracts (10.0 mg) and shaken immediately. On the side of the test tube, *conc.* H_2SO_4 (2.0 mL) was added. The presence of steroids was indicated by the production of red color in the upper layer and yellow color with green fluorescence in the sulphuric acid layer.

A few drops of 10% NaOH were added to the stock solution (2.0 mg/mL) of crude extracts to give a yellow solution. A few drops of 10% HCl were added to the reaction solution to obtain a colorless solution, suggesting the presence of flavonoids.

2.3 Determination of total phenolic contents

The total phenolic determination of the sample fractions was investigated by the Folin-Ciocalteu method (Hossain, Al-Raqmi, Al-Mijizy, Weli, & Al-Riyami, 2013). The standard calibration curve of gallic acid and the stock solution (1.0 mg/mL) of the fractions were prepared in methanol. 10% Folin-Ciocalteu reagent in deionized water (500 μL) was added to the sample solution (100 μL) and then incubated at ambient temperature under the dark condition for 5 min. After that, 5% Na_2CO_3 (500 μL) was added to the reaction solution and then treated under the dark condition for 2 h. A PerkinElemer Lambda 35 UV/Visible spectrometer was used to measure the absorbance at 750 nm. The phenolic content of the purified fraction was determined as mg of gallic acid equivalent (GAE) per g of the purified fraction.

2.4 Determination of total flavonoid contents

Aluminum chloride colorimetric test was used to measure the total flavonoid content (Sembiring, 2018). The standard calibration curve of quercetin was prepared in methanol. 10% $AlCl_3$ (50 μL) was added to the solution of the fractions (1.0 mg) in methanol (900 μL). After that, CH_3COONa (1.0 M, 50 μL) was added to the reaction solution and incubated at ambient temperature for 40 min in the dark. A UV-vis spectrophotometer was used to measure absorbance at 415 nm. The total flavonoid content was determined in milligrams of quercetin equivalents (QE) per gram of the purified fraction.

2.5 DPPH radical scavenging activity

The antioxidant activity was investigated using DPPH assays (Sembiring, 2018). The sample solution (10 μL) was added to the DPPH solution (1.0 mM, 90 μL). After incubation at room temperature for 30 min under the dark condition, the absorbance at 517 nm was measured by a UV-vis spectrophotometer.

2.6 α-Glucosidase inhibition assays

The inhibitory activity of α-glucosidase was investigated according to the modified procedure (Kazeem, Adamson, & Ogunwande, 2013). The stock solutions (1.0 mg/mL) of each fraction were prepared in 5% DMSO/deionized water. In the total volume (100 μL) of the reaction, the sample solution (10 μL) and 4-*p*NPG (5.0 mM, 5 μL) were mixed in sodium phosphate buffer (50 mM, pH 6.8, 80 μL). The α-glucosidase solution (0.05 mg/mL, 5 μL) was added into the reaction solution and then incubated at 37°C for 20 min. To stop the enzyme activity, Na_2CO_3 (0.5 M, 100 μL) was added to the reaction mixture. A UV-Vis spectrophotometer was used to quantify the α-glucosidase activity at 405 nm. The percent of α-glucosidase inhibition was calculated as the following equation;

$$\%\text{Inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where $A_{control}$ was the absorbance of the enzyme activity and A_{sample} was the absorbance of the enzyme activity in the addition of the sample solution. The IC_{50} values were determined by using Grafit 5.0 computer software (Erithacus Sofware, Horley, UK).

2.7 α-Amylase inhibition assays

α-Amylase inhibition assays were elucidated by the previously modified procedure (Kazeem, Adamson, & Ogunwande, 2013). The stock solutions (1.0 mg/mL) were prepared in deionized water. The sample solution (10 μL), α-amylase (0.5 mg/mL, 10 μL), and amylose (0.5 mg/mL, 10 μL) were added in sodium phosphate buffer (20 mM, pH 6.8, 70 μL) and then incubated at 37°C for 30 min. To stop the enzyme activity, the solution was soaked in boiling water for 5 min. The released glucose molecules were determined using peroxidase-glucose oxidase assay. The coupled enzyme (100 μL) and ABTS (100 μL) were added to the solution and then incubated for 30 min at 37°C. The percent inhibition and IC_{50} values were measured by the absorbance at 475 nm and calculated in the same method of α-glucosidase inhibition.

2.8 Mushroom tyrosinase inhibition assays

The tyrosinase inhibition assays were elucidated by modifying the previous literature (Sari, Barut, Özel, Kuruüzüm-Uz, & Şöhretoğlu, 2019). The stock solutions (1.0 mg/mL) of each fraction were prepared in deionized water. The sample solution (10 μL) and mushroom tyrosinase (0.025 mg/mL, 5 μL) were mixed in sodium phosphate buffer (20 mM, pH 6.8, 80 μL), followed by pre-incubation for 15 min at 37°C. L-DOPA solution (0.25 mM, 5 μL) in sodium phosphate buffer (20 mM, pH 6.8) was added to the reaction solution. After incubation for 20 min at 37°C, the dopachrome formation was determined by the absorption at 492 nm. The percentages and IC_{50} values of tyrosinase inhibition were calculated in the same manner as for α-glucosidase inhibition.

2.9 Cytotoxicity test

Human skin keratinocyte (HaCaT) cell line was cultured in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin/streptomycin (100 UI/mL), and 5% L-glutamine in a 95% humidified atmosphere of 5% CO_2 at 37°C. The cells were dissociated with 0.25% trypsin, 0.04% EDTA, 0.1% glucose in PBS.

The cytotoxicity test of the H10, E11, and M5 fractions was performed using a modified colorimetric MTT assay (Joompang *et al.*, 2020). Briefly, HaCaT cells were prepared by seeding 1.0×10^5 cells/well. They were incubated at 37°C to allow attachment and cell growth for 24 h. After incubation at 37°C for 12 h, the solutions of the fractions were discarded, and the cells were further treated in MTT (1.0 mg/mL, 100 µL) in free serum culture medium at 37°C for 4 h. The supernatant was removed and replaced with DMSO (100 µL) to solubilize the formazan crystal. The absorbance of the solution was carried out at 570 nm to calculate the cell viability according to the following equation:

$$\text{Cell viability (\%)} = \frac{A570_{treatment} \times 100}{A570_{control}}$$

Where $A570_{treatment}$ is the absorbance of cells treated with the fractions and $A570_{control}$ is the absorbance of cells without treatment.

2.10 Statistical analysis

The percent inhibitions of α-glucosidase, α-amylase, tyrosinase, and radical scavenging activity were expressed as the means and standard deviation of means (means ± SD) for triplicate determination. The statistical analysis was calculated by one-way analysis of variance (ANOVA), followed by the Duncan method. Values of $p < 0.05$ were considered to be statically significant.

3. Results and Discussion

3.1 Purification and phytochemical analysis

The percent extracts of *Morinda citrifolia* unripe fruit powder using solid-liquid extraction yielded 1.0%, 0.6%, and 8.0% from hexane, ethyl acetate, and methanol, respectively. Phytochemical screening of the crude extracts was elucidated, including alkaloids, saponins, tannins, and steroids (Table 1). Flavonoids were present in the crude extract of ethyl acetate and methanol. Tannins were detected in the crude extract of hexane and ethyl acetate. In addition, alkaloids and steroids were only found in the crude extract of hexane. Each crude extract was further isolated by silica column chromatography method to obtain several fractions, such as hexane (H1–H10), ethyl acetate (E1–E11), and methanol (M1–M5), as shown in Figure 1.

Table 1. Phytochemical analysis of the crude extracts of *Morinda citrifolia* unripe fruit

Crude extract	Hexane	Ethyl acetate	Methanol
Alkaloids	+	–	–
Saponins	–	+	–
Tannins	+	+	–
Steroids	+	–	–
Flavonoids	–	+	+

Note: + : Present, – : Absent

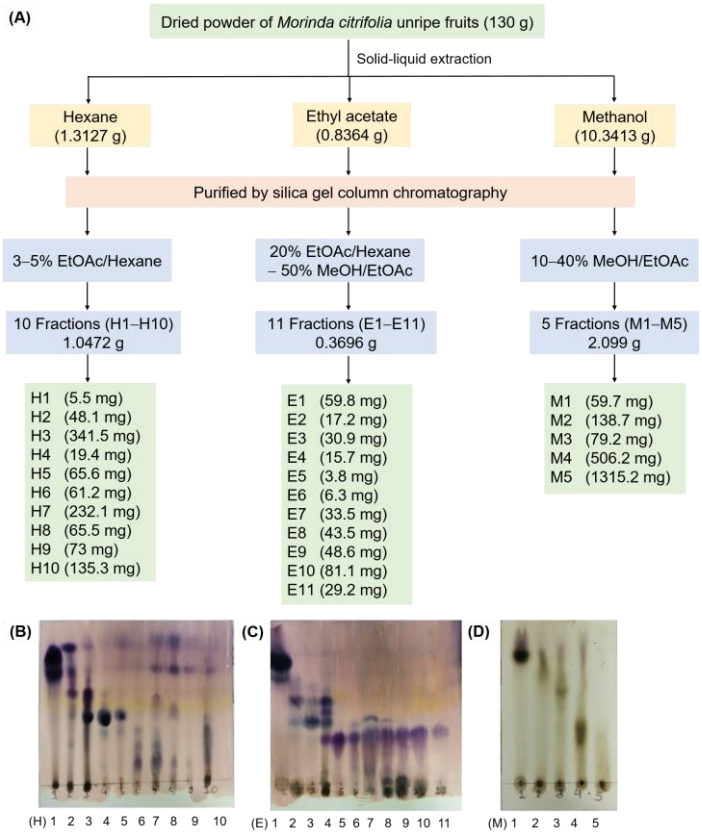

Figure 1. (A) Flow-chart diagram of the extraction and isolation procedure of *Morinda citrifolia* unripe fruit powder and TLC results of the purified fractions such as (B) hexane (H1-H10), (C) ethyl acetate (E1-E11), and (D) methanol (M1-M5) in 30% EtOAc/hexane

3.2 Total phenolic and flavonoid contents

The total phenolic contents of hexane (H1−H10), ethyl acetate (E1−E11) and methanolic (M1−M5) fractions were determined by the Folin-Ciocalteu method. The standard calibration curve of gallic acid was utilized to determine total phenolic contents based on the equation of y = 11.35x + 0.0426, R^2 = 0.9968. The total phenolic contents of the methanolic fractions (38.7−83.2 mg GAE/g extract) were found to be higher than in ethyl acetate (12.9−38.6 mg GAE/g extract) and hexane (4.8−65.4 mg GAE/g extract) fractions, as described in Table 2.

For the total flavonoid determination, the ortho-hydroxyl group of B rings for flavonoid derivatives could form the complex with aluminum chloride (Sembiring, 2018). Among the ethyl acetate fractions, the highest total flavonoid content of the E11 fraction using the 50% MeOH/EtOAc eluent was found to be 94.0 mg QE/g extract based on the standard calibration curve of quercetin equivalents from the equation of y = 14.223x - 0.0078, R^2 = 0.9998. The total flavonoid contents of the methanolic fractions were found in the range of 13.1−22.5 mg QE/g (Table 2). For the *Morinda citrifolia* leaves, the flavonoid content of the ethanolic extract was determined about 2.8% from the calibration curve of rutin (Setyani & Setyowati, 2018).

Table 2. Total phenolic content, total flavonoid content of the selected extract fractions of *Morinda citrifolia* unripe fruits including hexane (H1−H10), ethyl acetate (E1−E11) and methanol (M1−M5)

Fractions	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
H1	49.6 ± 0.4^{hi}	1.6 ± 0.1^{abcd}
H2	62.0 ± 1.2^{j}	2.1 ± 0.1^{bcd}
H3	33.2 ± 0.5^{e}	10.4 ± 0.3^{g}
H4	65.4 ± 0.4^{j}	9.0 ± 0.3^{f}
H5	29.1 ± 0.1^{de}	10.8 ± 0.6^{g}
H6	33.2 ± 0.3^{e}	16.9 ± 0.4^{i}
H7	13.3 ± 0.1^{b}	2.1 ± 0.2^{bcd}
H8	45.6 ± 0.1^{gh}	1.1 ± 0.0^{ab}
H9	12.3 ± 0.4^{b}	0.3 ± 0.1^{a}
H10	4.8 ± 0.1^{a}	ND
E1	42.4 ± 0.3^{fg}	6.4 ± 0.1^{e}
E2	126.3 ± 0.7^{l}	5.4 ± 0.0^{e}
E3	19.2 ± 2.6^{c}	0.8 ± 0.1^{ab}
E6	19.3 ± 2.2^{c}	2.2 ± 0.1^{bcd}
E7	12.9 ± 0.2^{b}	2.6 ± 0.2^{cd}
E8	38.6 ± 0.7^{f}	9.9 ± 0.6^{fg}
E9	30.5 ± 5.6^{e}	1.2 ± 0.3^{abc}
E10	30.6 ± 7.7^{e}	2.8 ± 0.5^{d}
E11	25.4 ± 2.1^{d}	94.0 ± 3.4^{l}
M1	38.7 ± 2.9^{f}	20.8 ± 0.2^{j}
M2	62.2 ± 1.8^{j}	15.6 ± 0.7^{h}
M3	51.2 ± 4.7^{i}	22.5 ± 1.2^{k}
M4	83.2 ± 0.8^{k}	17.0 ± 2.6^{j}
M5	64.4 ± 7.0^{j}	13.1 ± 1.9^{cd}

Note: GAE: gallic acid equivalents; QE: quercetin equivalents; ND: Not detected

3.3 HR-MS characterization of the biological fractions

The highest biological activities of the purified fractions from *M. citrifolia* unripe fruits were analyzed by a high-resolution mass method, as shown in Figure 2. The peaks in HRMS spectra of H10, E11, and M5 fractions were identified with the reported chemical constituents of *M. citrifolia* plant. The HRMS spectrum of H10 fraction displayed two peaks at *m/z* 391.2863 $[C_{16}H_{22}O_{11}+H]^+$ and 479.3463 $[C_{30}H_{48}O_3+Na]^+$, which were identified as the major iridoids including deacetylasperulosidic acid (calcd. for *m/z* 391.1235, $[M+H]^+$) (West, Deng, & Jensen, 2011) and ursolic acid (calcd. for *m/z* 479.3495, $[M+Na]^+$), respectively (Lv, Chen, Ho, & Sang, 2011; Xiao-Long, Meng-Yuan, Kun-Lung, & Ji-Kai, 2009). The HRMS spectrum of E11 showed several peaks at *m/z* 391.2680 $[C_{16}H_{22}O_{11}+ H]^+$, 347.2011 $[C_{15}H_{22}O_9 + H]^+$, and 291.0900 $[C_{15}H_{14}O_6 + H]^+$, corresponding to deacetylasperulosidic acid (calcd. for *m/z* 391.1235, $[M+H]^+$), aucubin (calcd. for *m/z* 347.1337, $[M+H]^+$), and catechin (calcd. for *m/z* 291.0863 $[M+H]^+$, respectively (West, Deng, & Jensen, 2011). Furthermore, HRMS spectra of M5 fraction showed the peak at *m/z* 433.1762 $[C_{18}H_{24}O_{12} + H]^+$, which was verified as asperulosidic acid (calcd. for *m/z* 433.1341, $[M+H]^+$) (Xiao-Long, Meng-Yuan, Kun-Lung, & Ji-Kai, 2009). Other interesting peaks also exhibited at *m/z* 217.0699 $[C_6H_{10}O_7 + Na]^+$ and 203.0515 $[C_6H_{12}O_6 + Na]^+$ corresponding to glucuronic acid (calcd. for *m/z* 217.0319, $[M+Na]^+$) and galactose (calcd. for *m/z* 203.0526, $[M+Na]^+$), respectively (Reem *et al.*, 2017).

3.4 Free radical scavenging activity

The antioxidant activities of the purified fractions (0.1 mg/mL) of *Morinda citrifolia* unripe fruits were investigated by DPPH radical scavenging, as shown in Table 3. The H10 fractions showed a high free radical scavenging capacity (53.9% inhibition) due to the presence of tannins and alkaloids confirmed by phytochemical analysis (Gan, Feng, He, Li, & Zhang, 2017). The E11 and M5 fractions exhibited a high antioxidant activity of 47.4% and 60.6%, respectively. The IC_{50} values of E11 were further found to be 1.19 ± 0.27, which was lower than M5 fractions (IC_{50}: 4.43 ± 0.12 mg/ml) because the ethyl acetate extract consisted of saponins, tannins, and flavonoids according to the preliminary phytochemical analysis. Interestingly, catechin, a secondary metabolite in the flavonoid family, was found in the E11 fraction, related to the highest total flavonoid content and DPPH free radical scavenging capacity. The antioxidant activity resulted from the redox properties of the free OH moiety of the phenolic and flavonoid derivatives (Baba & Malik, 2015; Hossain, Al-Raqmi, Al-Mijizy, Weli, & Al-Riyami, 2013). However, their DPPH scavenging activities were lower than that of ascorbic acid as a well-known antioxidant standard (6.89 ± 0.70 μg/mL). In the case of the fermented fruit juice, the free radical scavenging activity of the ethyl acetate extraction was revealed to be greater than that of ascorbic acid (Liu *et al.*, 2007; Reem *et al.*, 2017).

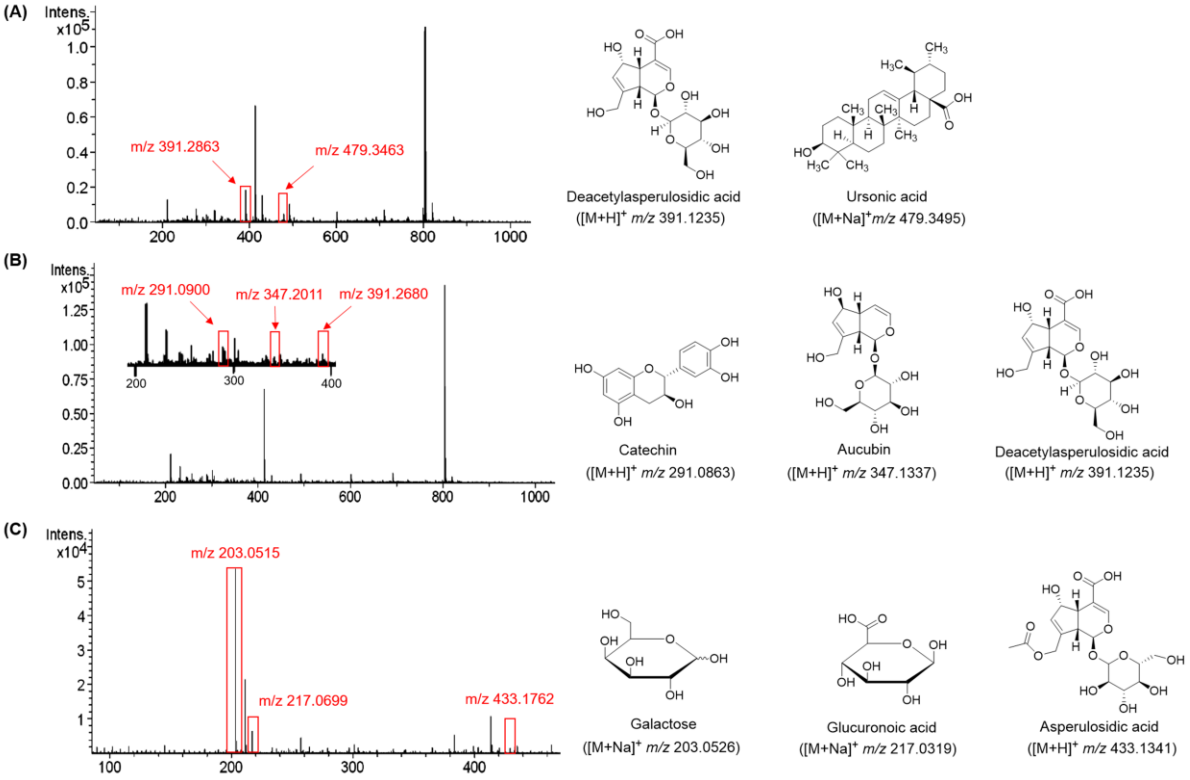

Figure 2. The HRMS spectra of (a) H10, (b) E11, and (c) M5 fractions

Table 3. The DPPH radical scavenging activity and α-glucosidase, α-amylase, and tyrosinase inhibition activities of the purified fractions (0.1 mg/mL) of *Morinda citrifolia* unripe fruits

Crude extract/Fractions	% Inhibition at 0.1 mg/mL			
	α-glucosidase	α-amylase	Tyrosinase	Radical scavenging activity
Hexane	33.5 ± 1.3^{b}	46.1 ± 2.2^{l}	41.1 ± 2.0^{e}	35.6 ± 1.7^{f}
H6	30.1 ± 0.5^{a}	17.5 ± 0.6^{a}	29.1 ± 0.8^{b}	20.9 ± 0.9^{a}
H7	37.6 ± 1.0^{c}	24.0 ± 0.9^{c}	36.2 ± 1.0^{cd}	25.2 ± 0.7^{b}
H8	40.1 ± 0.5^{d}	21.5 ± 1.0^{b}	49.6 ± 0.4^{g}	28.0 ± 0.4^{c}
H9	33.9 ± 0.3^{b}	28.6 ± 0.9^{ef}	40.5 ± 0.8^{e}	32.3 ± 0.3^{e}
H10	48.5 ± 0.8^{f}	36.3 ± 0.5^{i}	67.6 ± 0.4^{l}	53.9 ± 0.9^{l}
Ethyl acetate	64.4 ± 0.6^{i}	62.7 ± 2.8^{n}	63.5 ± 4.7^{k}	39.0 ± 1.3^{h}
E3	38.6 ± 0.6^{c}	25.2 ± 1.0^{cd}	24.7 ± 0.3^{a}	22.2 ± 0.5^{a}
E6	47.1 ± 0.5^{f}	26.9 ± 0.5^{de}	34.1 ± 0.7^{c}	27.4 ± 0.6^{c}
E7	51.0 ± 0.6^{g}	29.1 ± 1.0^{fg}	30.8 ± 0.4^{b}	25.4 ± 0.8^{b}
E8	43.3 ± 0.5^{e}	28.3 ± 0.7^{ef}	47.3 ± 0.9^{g}	41.1 ± 0.8^{i}
E9	55.8 ± 0.3^{i}	33.3 ± 0.9^{h}	35.3 ± 0.9^{cd}	30.5 ± 0.8^{d}
E10	59.4 ± 0.6^{h}	39.0 ± 0.8^{j}	37.7 ± 1.0^{d}	51.0 ± 0.5^{k}
E11	71.9 ± 0.9^{l}	42.4 ± 0.5^{k}	53.1 ± 0.6^{h}	60.6 ± 0.6^{m}
Methanol	66.5 ± 2.9^{j}	66.9 ± 0.3^{o}	65.0 ± 3.0^{k}	61.4 ± 2.6^{m}
M1	30.6 ± 0.6^{a}	33.1 ± 1.0^{h}	44.6 ± 0.7^{f}	31.5 ± 0.6^{de}
M2	33.0 ± 0.4^{b}	30.6 ± 0.6^{g}	42.1 ± 0.6^{e}	27.6 ± 0.9^{c}
M3	60.3 ± 0.9^{h}	42.9 ± 0.7^{k}	56.8 ± 0.8^{i}	30.5 ± 1.0^{e}
M4	68.2 ± 0.5^{k}	47.9 ± 1.0^{m}	60.5 ± 0.8^{j}	37.3 ± 1.0^{g}
M5	75.7 ± 0.9^{m}	63.5 ± 0.7^{n}	76.7 ± 0.6^{m}	47.7 ± 0.7^{j}
Acarbose	67.8 ± 3.2^{jk}	74.0 ± 0.3^{p}	NM	NM
Kojic acid	NM	NM	83.4 ± 0.8^{n}	NM
Ascorbic acid	NM	NM	NM	82.6 ± 0.9^{m}

Note: ± indicates the standard division of mean (SD) (n = 3). Values within each column followed by different superscript letter (a-p) are significantly different ($P < 0.05$). NM indicates not measurement.

3.5 α-Glucosidase inhibitory activity

For diabetes mellitus, the blood glucose level has been controlled by inhibiting the potent digestive enzymes such as α-glucosidase and α-amylase (López-Angulo *et al.*, 2019). Therefore, the inhibition of these enzymes from the medical plants has been greatly interested. The potential α-glucosidase inhibition by the purified fractions (0.1 mg/mL) was studied in Table 3. The H10, E11, and M5 fractions displayed greater percent inhibition than other fractions in hexane, ethyl acetate, and methanol, respectively. Furthermore, the α-glucosidase inhibitory activities of E11 (IC_{50} = 0.43 ± 0.02 mg/mL) was higher than those of M5 (IC_{50} = 0.85 ± 0.03 mg/mL) and H10 (IC_{50} = 1.51 ± 0.32 mg/mL), as shown in Table 4. The IC_{50} of the E11 fraction indicated that the potent α-glucosidase inhibitory activity was related to the high total flavonoid contents and saponins, corresponding to the reported literature (Dou, 2013; Kazeem, Adamson, & Ogunwande, 2013; Yin, Zhang, Feng, Zhang, & Kang, 2014). In addition, catechin and iridoids including deacetylasperulosidic acid and aucubin exhibited α-glucosidase inhibition, which was found in the E11 fraction confirmed by mass analysis (Figure 2) (Gan, Feng, He, Li, & Zhang, 2017; Milella *et al.*, 2016). Nevertheless, their α-glucosidase inhibition were less than that of acarbose as the standard inhibitor (IC_{50} = 0.13 ± 0.02 mg/mL).

3.6 α-amylase inhibitory activity

The α-amylase inhibitory activities of the purified fractions of *Morinda citrifolia* unripe fruits (0.1 mg/mL) were determined (Table 3). All fractions exhibited the α-amylase inhibitory activity, such as hexane (17.5–36.3%), ethyl acetate (25.2–42.4%), and methanol (33.1–63.5%). The highest percent inhibitions of each purified fraction, including H10, E11, and M5 were investigated for IC_{50} values. The α-amylase inhibition activities of E11 (IC_{50} = 0.58 ± 0.09 mg/mL) and M5 (IC_{50} = 0.13 ± 0.02 mg/mL) indicated effective potential inhibition as same as acarbose as the standard inhibitor (IC_{50} = 0.08 ± 0.01 mg/mL), except the H10 fraction (IC_{50} = 2.95 ± 0.62 mg/mL). The potent inhibition against α-amylase was elucidated in the M5 fraction that may relate high total phenolic compounds and asperulosidic acid as an iridoid glucoside (Lin *et al.*, 2016; Milella *et al.*, 2016).

3.7 Mushroom tyrosinase inhibitory activity

The tyrosinase inhibitory activities of the purified fractions of *Morinda citrifolia* unripe fruits (0.1 mg/mL) were also elucidated, as shown in Table 3. The fractions in hexane (H1-H10), ethyl acetate (E3-E11), and methanol (M1-M5) presented tyrosinase activity inhibition in the range of 29.1–67.6%, 24.7–53.0%, and 44.6–76.7%, respectively. The inhibition percentage of H10, E11, and M5 fractions exhibited 67.6 ± 0.4%, 53.0 ± 0.6%, and 76.7 ± 0.6%, respectively. Consequently, the IC_{50} values of H10, E11, and M5 fractions were found to be 1.20 ± 0.25, 0.67 ± 0.02, and 0.37 ± 0.06 mg/mL, respectively (Table 4). For the tyrosinase inhibitor, the methanoic (M5) and ethyl acetate (E11) fractions displayed effective inhibition against mushroom tyrosinase as a result of the high amount of total phenolic and flavonoid contents, respectively (Chang, 2009; Panzella & Napolitano, 2019). However, all of these fractions inhibited tyrosinase less than kojic acid as the standard inhibitor (IC_{50} = 0.09 ± 0.01 mg/mL) in agreement with the previous report (Matsuda, Masuda, Murata, Abe, & Uwaya, 2013).

Table 4. The inhibition (IC_{50} value) of the H10, E11, and M5 fractions on α-amylase, α-glucosidase, and tyrosinase activities

Fractions	IC_{50} (mg/mL)		
	α-Glucosidase	α-Amylase	Tyrosinase
H10	1.51 ± 0.32^{d}	2.95 ± 0.62^{b}	1.20 ± 0.25^{d}
E11	0.43 ± 0.02^{b}	0.58 ± 0.09^{a}	0.67 ± 0.02^{c}
M5	0.85 ± 0.03^{c}	0.13 ± 0.02^{a}	0.37 ± 0.06^{b}
Acarbose	0.13 ± 0.02^{a}	0.08 ± 0.01^{a}	NM
Kojic acid	NM	NM	0.09 ± 0.01^{a}

Note: ± indicates the standard division of mean (SD) (n = 3). Values within each column followed by different superscript letter (a-d) are significantly different ($P < 0.05$). NM indicates not measurement.

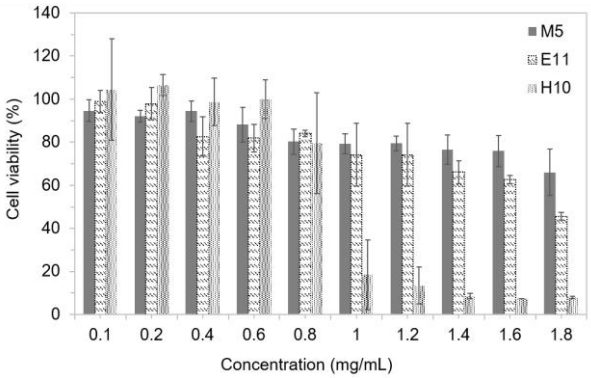

Figure 3. Cell viability of the purified fractions (H10, E11, and M5) from the *Morinda Citrifolia* unripe fruits

3.8 Cytotoxicity test

The cytotoxicity of the H10, E11, and M5 fractions from *Morinda citrifolia* unripe fruits was investigated in the Human skin keratinocyte (HaCaT) cell line, as shown in Figure 3. The results showed that these fractions exhibited no cytotoxicity in the concentration range of 0.1–0.8 mg/mL. However, the cytotoxicity of H10 showed a higher concentration of 1.0 mg/mL. From the results, the bioactive fractions demonstrated no cytotoxicity in the correlation of the IC_{50} values against tyrosinase inhibition, suggesting that both ethyl acetate and methanol fractions could be applied in living cells.

4. Conclusions

The crude extracts of *Morinda citrifolia* unripe fruits obtained an overall yield of 9.6% using solid-liquid extraction and then purified by silica column chromatography.

The total phenolic contents based on the Folin-Ciocalteu method were found in greater amount in the methanolic fraction. The total flavonoid content of the last ethyl acetate fraction was found in the highest, resulting in the high free radical scavenging capacity based on the DPPH assay. Furthermore, the ethyl acetate and methanol fractions inhibited both α-glucosidase and α-amylase activity, indicating that the inhibitory potential for α-glucosidase and α-amylase may be associated with the total phenolic and flavonoid contents. The methanolic fraction also displayed greater by the tyrosinase inhibitor than other fractions. However, the inhibition activities for the α-glucosidase, α-amylase, and tyrosinase of the purified fraction were lower than those of the standard inhibitors. Moreover, these fractions showed no cytotoxicity in Human skin keratinocyte cells at the lower concentration at 0.8 mg/mL. From these results, the *Morinda citrifolia* unripe fruits are a good source of the total phenolic and flavonoid contents, antioxidant, and α-glucosidase inhibitor activities.

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