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Key words: *Morinda citrifolia*, hydrolysis, scopoletin, octanoic acid.

ABSTRACT

For facilitating the release of bionutrients from noni, 6 h of hydrolysis with 100 U/mL cellulase at 50°C and an additional 48 h of fermentation with *Pediococcus pentosaceus* BCRC 14053 at 37°C were performed. Increases in the extraction yields (87.8-607.8 mg/g) and the total phenolic content (2.02-7.45 mg/g) of various extracts were obtained, suggesting that bionutrients were released from noni during hydrolysis or fermentation. In hydrolyzed and/or additionally fermented samples, significant decreases in the half maximal inhibitory concentration (IC_{50}) against α-amylase (10.28-1.61 mg/mL) and α-glucosidase (46.47-3.73 mg/mL) were observed. Gas chromatography–mass spectrometry results revealed that the major components with these inhibition abilities were scopoletin and octanoic acid. Octanoic acid substantially inhibited both α-amylase and α-glucosidase, whereas scopoletin inhibited α-glucosidase with an IC_{50} of 9.7 μg/mL, which is much lower than that of acarbose (a positive control, 780.8 μg/mL).

I. INTRODUCTION

Morinda citrifolia (noni), growing in tropical and subtropical areas (Lin, 2013), is rich in polyphenols, flavonoids, phenolic acid (vanillic acid), vanillin, and iridoids (asperulosidic acid and deacetylasperulosidic acid) (Dussossoy et al., 2011; West et al., 2011). The quality of commercial noni fruit products varies significantly with geographical conditions (Deng et al., 2010). Their fruits, leaves, roots, stem, and bark are frequently used in folk medicines with effectiveness in minimizing the symptoms of lifestyle-related diseases such as hypertension, atherosclerosis, stroke and cancer in Polynesia, Tahiti, Southeast Asia, Australia, and Hawaii (Mandukhail, 2010). Diabetes is a major metabolic syndrome affecting approximately 200 million people worldwide (Hashim Hashim et al., 2013) and the number is expected to exceed 300 million globally by 2025 (Nguyen et al., 2013). Lee et al. (2012) observed the antidiabetic effect of noni fermented by *Cheonggukjang* on KK-Ay diabetic mice. A methanol extract of *M. citrifolia* revealed potential stimulatory effects on glucose uptake in 3T3-L1 adipocyte cells and inhibitory effects on protein tyrosine phosphatase 1B (Nguyen et al., 2013). The *n*-BuOH soluble phase of methanol extract of *M. citrifolia* roots significantly reduced the blood glucose levels of streptozotocin (STZ)-induced diabetic mice (Kamiya et al., 2008). A practical method for managing diabetes controls postprandial hyperglycemia by inhibiting carbohydrases present in the gastrointestinal tract (Oboh, 2012).

Cellulase plays important roles in the effective biological hydrolysis of cellulose (Perez et al., 2002). An extract of noni leaves hydrolyzed with cellulase from *Trichoderma reesei* and *Aspergillus niger* reduces the glucose levels of STZ-induced diabetic mice (Sugiarto, 2013). Lactic acid bacteria (LAB) improve the intestinal microflora and consequently reduce the risk of cancer (John et al., 2007; Zhang et al., 2014). In addition, LAB increase immunity and antimicrobial activity (Pawlus and Kinghorn, 2007; Gupta and Patel, 2013) and reduce oxidative

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stress (Wang et al., 2014). Fermented noni fruits promote the growth of Lactobacillus and Bifidobacterium and regulate intracellular oxidation and inflammation in Caco-2 cells (Huang et al., 2015). Moreover, fermenting noni juice by using *Bifidobacterium longum* and *Lactobacillus plantarum* increases its antioxidant capacity (Wang et al., 2009).

Most of the studies, thus far, have focused on identifying bio-nutrients in noni, their functions and structures, and processes of noni fruits, roots and leaves. Studies on facilitating the release of bionutrients from noni fruits, roots, and leaves are scarce. In this study, cellulase hydrolysis was used for facilitating the release of bionutrients from noni fruits. Furthermore, the resulting hydrolysate was fermented using *Pediococcus pentosaceus* BCRC 14053. In addition, the inhibition effects of released bio-nutrients on α-amylase and α-glucosidase were also examined. The major components of bio-nutrients in noni extracts were identified using gas chromatography-mass spectrometry (GC-MS), and their inhibiton effects on α-amylase and α-glucosidase were further studied for elucidating the constituents inhibiting α-amylase and α-glucosidase activities.

II. MATERIALS AND MATHODS

1. Preparation of Raw Material

Fresh noni, purchased from a noni farm in southern Taiwan, was homogenized using a homogenizer and used as raw material. Cellulase (30,000 U/g) was purchased from Kwang Hwa Trading Corp., Rd. Chilin, Taipei, Tawian. *A P. pentosaceus* BCRC 14053 strain was obtained from the Bioresource Collection and Research Center (BCRC) at the Food Industry Research and Development Institute, Rd. Shih-Pin, Hsinchu, Taiwan. De Man, Rogosa, and Sharpe (MRS) agar was purchased from Becton Dickinson and Company (Franklin Lakes, NJ, U.S.A.). The bacterial strain was stored at -80°C until use and was activated through cultivation in lactobacilli MRS broth for 24 h and transferred twice before use. Porcine pancreatic α-amylase (EC 3.2.1.1), *Saccharomyces cerevisiae* α-glucosidase (EC 3.2.1.20), and *p*-nitrophenyl-α-D-glucopyranoside were purchased from Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.

2. Preparation of Noni Hydrolysate and Fermented Products

For facilitating the release of bionutrients, 60 kg of homogenized noni (noni: water = 1:1) using a homogenizer (FD24-3S-6P, King Mech, Taipei, Taiwan), was hydrolyzed with 100 U/mL cellulase at 50°C for 6 h with shaking at 130 rpm. To the resulting hydrolysate, 1.5% glucose and 0.6% $CaCO_3$ were added. After we adjusted the pH to 6.5 by using $Ca(OH)_2$, the hydrolysate was fermented with 5% *P. pentosaceus* BCRC 14053 at 37°C for 48 h in a 100L Bioreactor (MGT-SV100, Micro Giant, Taichung, Taiwan). The hydrolysates and fermented product before freeze-dried (FD24-3S-6P, King Mech, Taipei, Taiwan) at -50°C for 2 days to remove the seeds and stored in an electronic dry cabinet before extraction.

3. Determination of Viable Microbial Counts

Viable microbial counts were measured for evaluating whether the noni slurry hydrolysate could be used by probiotics. Microbial growth during fermentation was measured using a plate count method with MRS agar, after we performed serial decimal dilutions from an initial 0.5 mL of sample in 4.5 mL of a sterile 0.85% NaCl solution. The plates were incubated at 37°C for 48 h and the viable probiotics were counted (Darmayanti et al., 2014).

4. Extraction of Morinda Citrifolia

The freeze-dried noni sample was mixed with solvents (water, methanol, and ethanol) in a 10-fold quantity and homogenized for 3 min in a refrigerator (approximately 4°C). The homogenized samples were placed in a hood with ventilation at 25°C for 4 h with shaking at 150 rpm. All resulted samples were centrifuged at 5000 *xg* for 20 min to remove insoluble components. The various resulting extracts were concentrated to a minimum volume (approximately 80 mL) by using a rotary evaporator in vacuum at 40°C and then freeze-dried. All freeze-dried extracts were stored in an electronic dryer with < 20% relative humidity before analyses.

5. Assays

The total phenolic content (TPC) and inhibitory abilities of various freeze-dried noni extracts against α-amylase and α-glucosidase were measured, and GC-MS assays of these extracts were performed.

1) Total Phenolic Content

The TPC was measured using the Folin-Ciocalteu method. To 1.25 mL of 10% Folin-Ciocalteu phenol reagent, 0.25 mL of sample and 1.25 mL of 7.5% Na_2CO_3 were added. After a 45-min reaction at 25°C in the dark, absorbance was measured at 765 nm. TPC was calculated according to the standard curve calibrated using gallic acid (Singleton et al., 1999).

2) Gas Chromatography–Mass Spectrometry

A GC-MS system, equipped with the Thermo Trace 1300 gas chromatograph, AI-1310 autosampler, and ISQ mass spectrometer, was used in a scanning range of 35-1000 m/z. The Rxi-5MS GC column measured 30 m × 0.25 mm (internal diameter) with a 0.1-μm film thickness. The oven temperature was increased from 50°C to 320°C at 10°C/min. When the temperature reached 310°C (injector temperature), 0.2 μL of extract was injected, and the flow rate of helium gas was set at 1.0 mL/min.

3) Qualitative and Quantitative Analysis by Using GC-MS

The phenolic compounds of various extracts were analyzed and identified by comparing their mass spectra with those of each reference compound in GC-MS libraries, namely the US National Institute of Standards and Technology and Wiley 10th edition libraries. Quantification was performed using Xcalibur 2.2 software (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

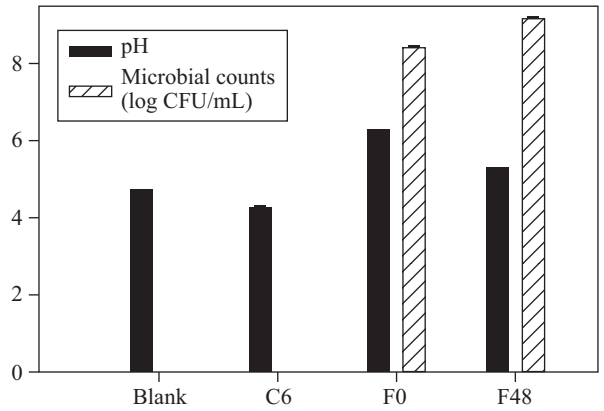

Fig. 1. Changes in pH and microbial counts of noni hydrolysate after 48 h fermentation by *Pediococcus pentosaceus* BCRC 14053. Blank, un-hydrolyzed sample; C6, 6 h hydrolysis with 100 U/mL cellulase; F0, 0 h fermented noni hydrolysate with 5% *Pediococcus pentosaceus* BCRC 14053; F48, 48 h fermented noni hydrolysate with 5% *Pediococcus pentosaceus* BCRC 14053.

4) Inhibition Assay for α-amylase

The α-amylase inhibitory activity of various extracts, scopoletin, and octanoic acid were determined by mixing 40 μL of sample with 200 μL of 1.0 U/mL α-amylase in 20 mM phosphate buffer (pH 6.9 with 0.006 M NaCl), whereas the sample in the control group was replaced with 20 mM phosphate buffer. After a 10-min reaction at 25°C, 400 μL of 0.25% starch in the same buffer was added. After another 10-min reaction at 25°C, 1.0 mL of a dinitrosalicylic reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH) was added. After a 5-min reaction at 100°C (for stopping the reaction), the absorbance was measured at 540 nm (A_{540}). Inhibitory activity against α-amylase (%) = [(A_{540} (control) − A_{540} (sample)]/A_{540} (control) × 100% (Kim et al., 2005; Dong et al., 2012). The IC50 was defined as the inhibitor concentration required for inhibiting 50% of α-amylase activity.

5) Inhibition Assay for α-glucosidase

The α-glucosidase inhibitory activity of various extracts, scopoletin, and octanoic acid was determined by mixing 60 μL of sample with 50 μL of 0.2 U/mL α-glucosidase in 0.1 M phosphate buffer (pH 6.8), whereas the sample in the control group was replaced with 0.1 M phosphate buffer. After a 10-min reaction at 37°C, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside in the same buffer (pH 6.8) was added. After another 20-min reaction at 37°C, the reaction was terminated using 160 μL of 0.2 M Na_2CO_3. The absorbance was measured at 405 nm (A_{405}). Inhibitory activity against α-glucosidase (%) = [(A_{405} (control) − A_{405} (sample)]/A_{405} (control) × 100% (Andrade-Cetto et al., 2008; Kwon et al., 2008). The IC50 was defined as the inhibitor concentration required for inhibiting 50% of α-glucosidase activity.

6) Statistical Analysis

The Duncan multiple range test was used for determining the significance of differences within treatments. For each treatment, 3 determinations were used, and mean values were calculated. $P < 0.05$ was considered statistically significant (Norušis, 1993).

III. RESULTS AND DISCUSSION

1. pH and Microbial Counts

After 6 h of hydrolysis with 100 U/mL cellulase at 50°C, the pH slightly decreased from 4.71 to 4.26 (Fig. 1). We previously tested several probiotics, but none of them could grow in the noni slurry and its cellulase-hydrolyzed samples, except for *P. pentosaceus* BCRC 14053, which grew in noni hydrolysate with 0.6% $CaCO_3$ (Lee et al., 2015). Therefore, 0.6% $CaCO_3$ was added to the resulting hydrolysate. After 48 h of fermentation with *P. pentosaceus* BCRC 14053 at 37°C, LAB viable counts increased to 9.17 log CFU/mL, and the pH decreased to 5.27 (Fig. 1). This increase in LAB counts and the decrease in pH suggested that the noni hydrolysate could be used by *P. pentosaceus*. Although glucose is the main carbohydrate derived from cellulase hydrolysis and can be used by probiotics (Kimoto-Nira et al., 2010; Hernandez-Hernandez et al., 2012), the noni hydrolysate and commercial noni juice with no carbonates could not be used by the probiotics tested (Lee et al., 2015). Adding $CaCO_3$ in the noni hydrolysate was essential for successful LAB fermentation. MRS agar with 0.1% $CaCO_3$ substantially improved probiotic growth during *dongchimi* kimchi fermentation because of the buffering effect (Chae et al., 2009). Therefore, during LAB fermentation, probiotic growth is inhibited because of lactic acid. Undissociated lactic acid passes through the bacterial cytoplasmic membrane and dissociates inside the cell, causing cytoplasmic acidification and proton motive force failure, which consequently reduces the amount of energy required for cell growth (Markovic et al., 2011).

2. Effects of Hydrolysis and Fermentation on the Extraction Yield of Noni

Unhydrolyzed, cellulase-hydrolyzed, and additionally fermented noni were extracted using water, methanol, and ethanol. The yields of the water extracts of unhydrolyzed, hydrolyzed, and fermented products were 123.7, 607.8, and 561.1 mg/g, respectively (in dry base), while those of methanol and ethanol extracts were 160.3, 346.7, and 415.8 mg/g and 87.8, 124.0, and 111.6 mg/g, respectively (Table 1). Regardless of the solvent used for extraction, the extraction yields of hydrolyzed samples and fermented hydrolysates were much higher than those of unhydrolyzed samples (Table 1).

Cellulase plays a major role in the effective biological hydrolysis of cellulose into glucose through the synergistic actions of endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-D-glucosidase (EC 3.2.1.21) (Perez et al., 2002). The action mode of cellulases on polymers is either exo-cleavage or endocleavage, and all cellulases target the specific

Table 1. Extraction yield of *Morinda citrifolia* (noni) extracts.

	Extraction yield (mg/g)		
Extract solvent	Blank*	C6	F48
Water	123.7 ± 0.15a**	607.8 ± 0.07c	561.1 ± 0.69b
Methanol	160.3 ± 0.36a	346.7 ± 0.81b	415.8 ± 0.59c
Ethanol	87.8 ± 0.18a	124.0 ± 0.38c	111.6 ± 0.19b

*Blank: un-hydrolyzed sample; C6: 6 h hydrolysis with 100 U/mL cellulase; F48: 48 h fermented noni hydrolysate with 5% *Pediococcus pentosaceus* BCRC 14053.

**a~c: Values with different letters at the same row are differ significantly ($P < 0.05$).

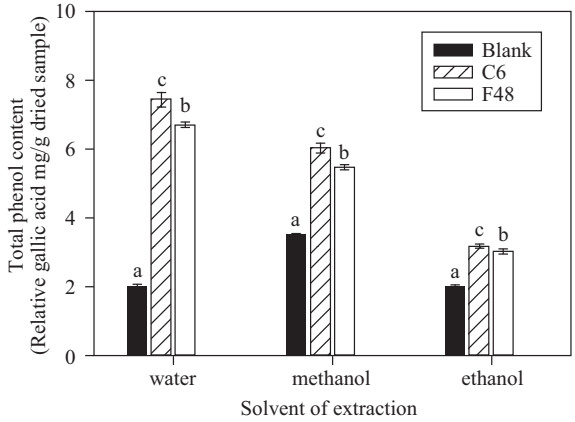

Fig. 2. Effects of the hydrolysis and fermentation on total phenolic content of noni. Blank, C6 and F48 refer to the footnote of Fig. 1. a~c: means with different extracts within the same solvent extraction are significantly different ($P < 0.05$).

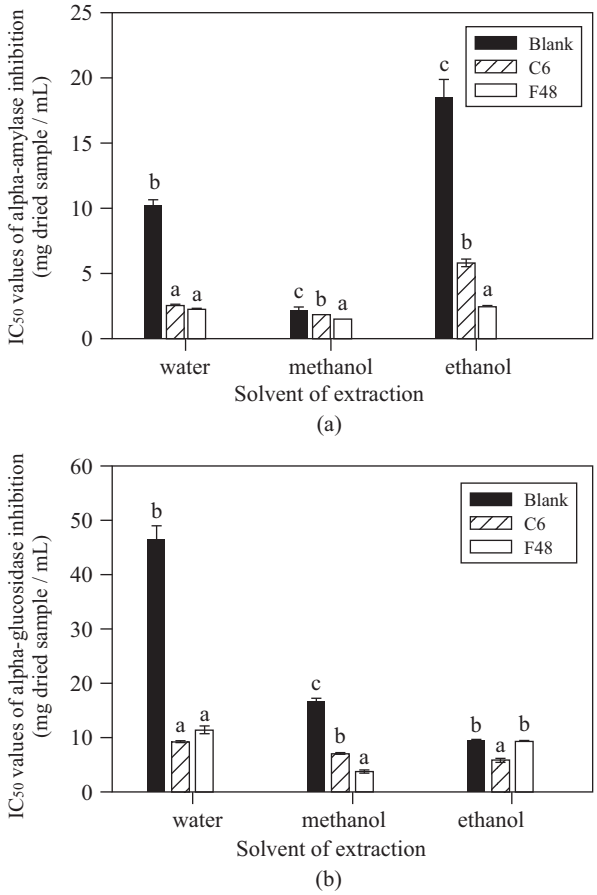

Fig. 3. Effects of hydrolysis and fermentation of noni on the inhibition (a) α-amylase, (b) α-glucosidase. Blank, C6 and F48 refer to the footnote of Fig. 1. a~c: means with different extracts within the same solvent extraction are significantly different ($P < 0.05$).

cleavage of β-1,4-glycosidic bonds (Wood and McCrae, 1979). The higher extraction yields of hydrolyzed samples and fermented hydrolysates than those of unhydrolyzed samples indicated that cell wall lysis occurs in noni fruits during cellulase hydrolysis, facilitating the release of these extracts.

3. Effects of Hydrolysis and Fermentation on Total Phenolic Content of Noni

The TPCs of water extracts of unhydrolyzed, hydrolyzed, and fermented samples were 2.02, 7.45, and 6.71 mg/g (in dry base), respectively, while those of methanol and ethanol extracts were 3.54, 6.03, 5.48 mg/g and 2.03, 3.18, and 3.03 mg/g, respectively (Fig. 2). Both hydrolysis and fermentation significantly increased the TPCs of the extracts. Although the TPC of a methanol extract of noni obtained by Krishnaiah et al. (2015) was higher than the TPC obtained in this study (4.3 mg/g vs. 3.54 mg/g), this might be due to the differences in harvest area and season. In the present study, significant increases in the TPCs of various extraction solvents after cellulase hydrolysis showed that cell wall disruption occurred during hydrolysis, releasing most bionutrients. By contrast, slight decreases in the TPCs might be attributed to the utilization by LAB (Fig. 2).

4. Effects of Hydrolysis and Fermentation on α-amylase and α-glucosidase Inhibition of Noni

For α-amylase, the IC_{50} of the water extracts of unhydrolyzed, hydrolyzed, and fermented products were 10.28, 2.62, and 2.35 mg/mL, respectively, while those of methanol and ethanol extracts were 2.32, 1.90, and 1.61 mg/mL and 18.53, 5.88, and 2.57 mg/mL, respectively (Fig. 3a). For α-glucosidase, the IC_{50} of the water extracts of unhydrolyzed, hydrolyzed, and fermented products were 46.47, 9.07, and 11.29 mg/mL, respectively, while those of methanol and ethanol extracts were 16.65, 6.94, and 3.73 mg/mL and 9.55, 5.62, and 9.27 mg/mL, respectively (Fig. 3b). The biological functional effects of polyphenols in fruits and vegetables fruits have long been studied. These compounds have inhibitory ability against carbohydrate hydrolyzing enzymes (Mai et al., 2007; Cheplick et al., 2010; Ranilla et al., 2010; Wongsa and Zamaludien, 2012).

By using GC-MS, octanoic acid and scopoletin were identified as major bionutrients in all of the noni extracts (Figs. 4 and 5). The IC_{50} values of octanoic acid were 4.29 and 4.09 mg/mL for inhibiting α-amylase and α-glucosidase activities,

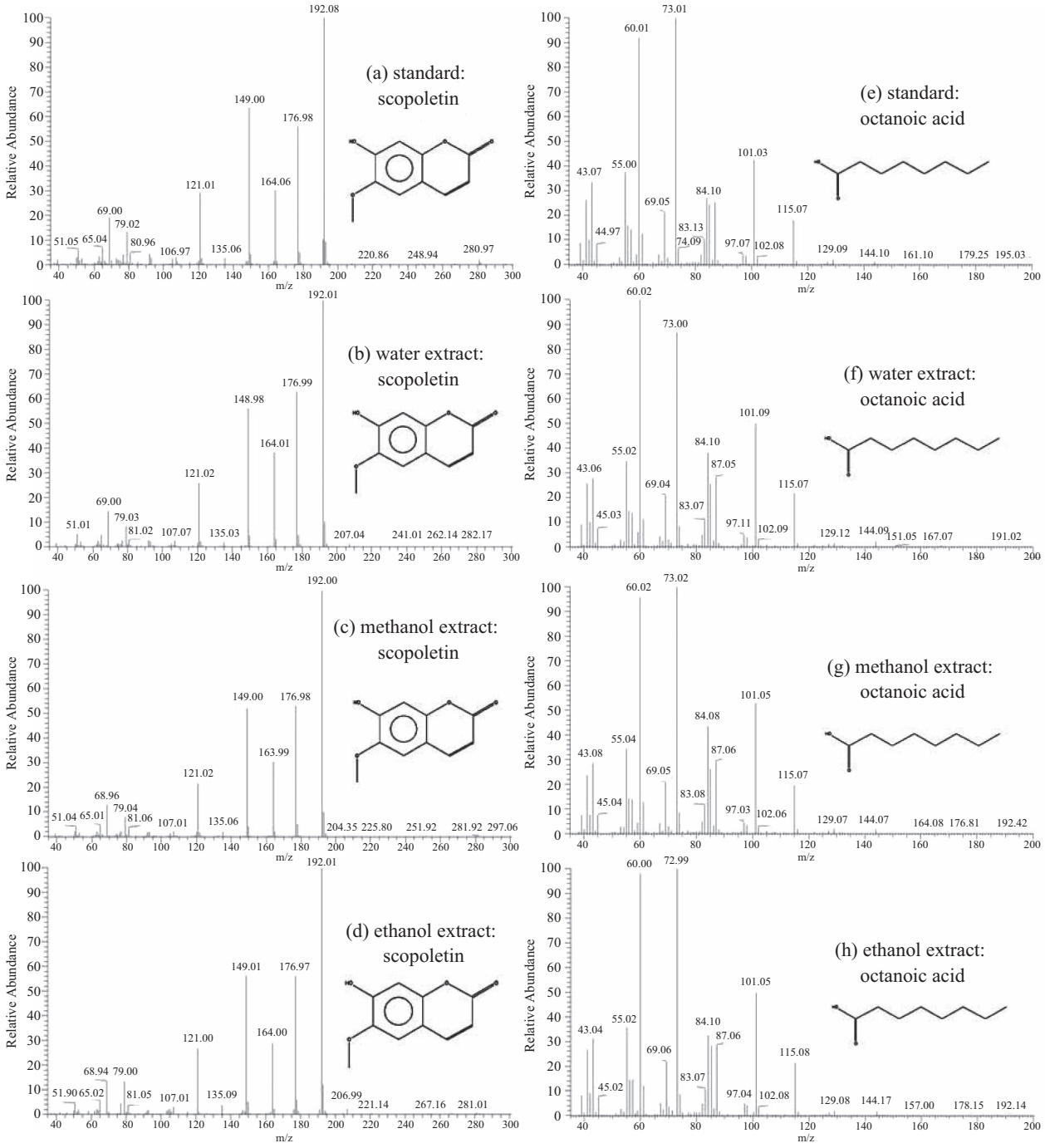

Fig. 4. GC-MS chromatograms with qualitative of noni extract on scopoletin and octanoic acid.

respectively (Table 2). Scopoletin could not inhibit α-amylase activity, but considerably inhibited α-glucosidase activity. The IC_{50} of scopoletin was 9.7 μg/mL, which was considerably lower than that of the positive control acarbose (780.8 μg/mL), an anti-diabetic drug (Table 2). This result showed that the inhibitory ability of scopoletin was almost 80-fold higher than that of acarbose. Scopoletin also showed anti-hyperglycemic activity in STZ-induced diabetic rats (Verma et al., 2013).

The octanoic acid and scopoletin contents of various extracts significantly increased after cellulase hydrolysis and additional LAB fermentation (Fig. 6). A slight decrease in scopoletin content after LAB fermentation was observed (Fig. 6b) and may be attributed to phenol consumption by LAB. Othman et al. (2008) reported that probiotics use total phenols during fermentation. According to the data obtained, considerable amounts of octanoic acid and scopoletin were released after cellulase hydrolysis and additional LAB fermentation

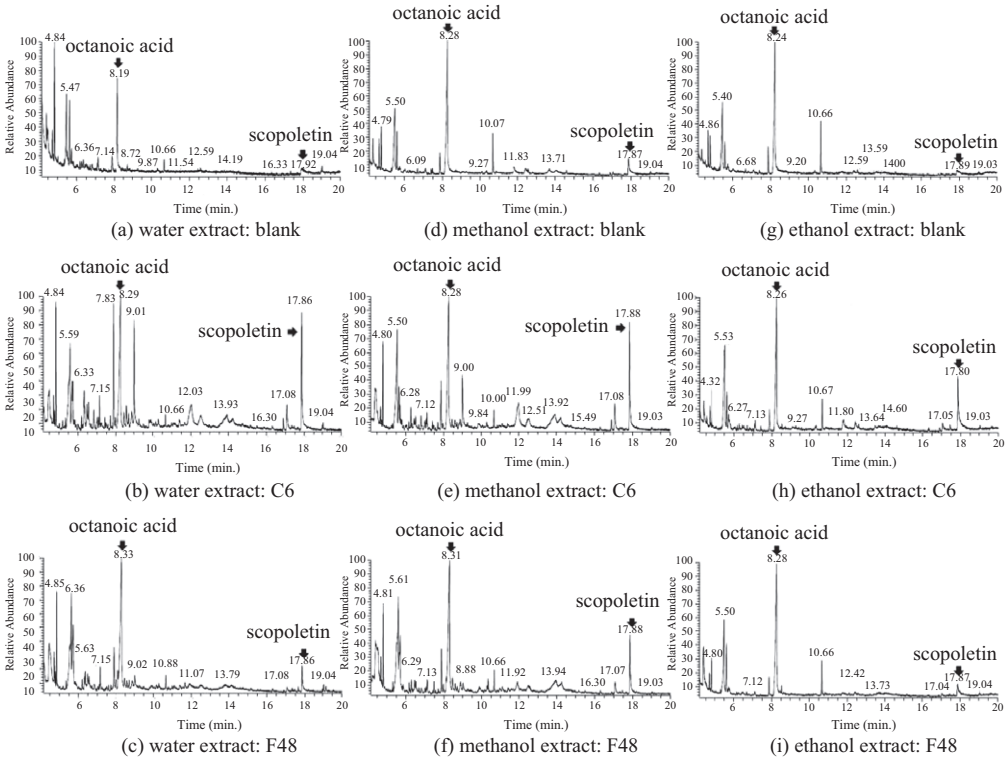

Fig. 5. GC-MS chromatograms of various extracts of noni with 4 fold dilution. Major changes were identified as scopoletin and octanoic acid. (a) water extract-blank, (b) water extract-C6, (c) water extract-F48, (d) methanol extract-blank, (e) methanol extract-C6, (f) methanol extract-F48, (g) ethanol extract-blank, (h) ethanol extract-C6, (i) ethanol extract-F48. Blank, un-hydrolyzed sample; C6, 6 h hydrolysis with 100 U/mL cellulase; F48, 48 h fermented noni hydrolysate with 5% *Pediococcus pentosaceus* BCRC 14053.

Table 2. Inhibitory activity (IC_{50}) of scopoletin and octanoic acid on α-amylase and α-glucosidase.

Compounds	IC_{50} (mg/mL)	
	α-Amylase	α-Glucosidase
Acarbose*	0.0018 ± 0.00	0.7808 ± 0.01
Scopoletin	ND**	0.0097 ± 0.00
Octanoic acid	4.2849 ± 0.24	4.0847 ± 0.10

*Acarbose, positive control.
**ND, not detected.

(Fig. 6). Because of their low IC_{50} values, hydrolyzed and additionally fermented noni fruits may replace acarbose.

5. Qualitative and Quantitative Analysis of Various Noni Extracts by Using GC-MS

Two components, octanoic acid and scopoletin, of various noni extracts (water, methanol, and ethanol extracts) were identified and quantified using GC-MS (Figs. 4 and 5). Fig. 6 shows the amounts of octanoic acid and scopoletin after the quantitative results of GC-MS were calculated. The octanoic acid contents of water extracts of unhydrolyzed, hydrolyzed, and fermented products were 1.74, 6.70, and 7.59 mg/g (in dry base), respectively, and those of methanol and ethanol extracts were 4.52, 6.71, and 7.90 mg/g and 3.47, 4.41, and 5.15 mg/g

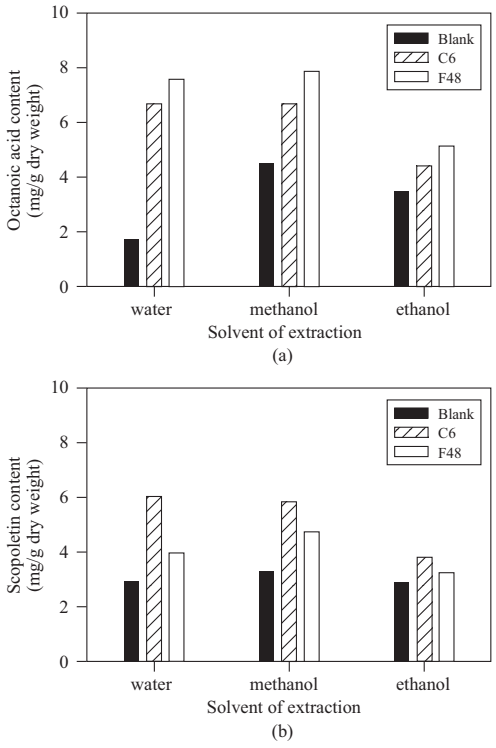

Fig. 6. Quantitative ingredients in various extracts of noni samples (a) octanoic acid, (b) scopoletin. Blank, C6 and F48 refer to the footnote of Fig. 1.

(in dry base), respectively (Fig. 6(a)). Pino et al. (2009) used diethyl ether/pentane (2:1 v/v) for extracting octanoic acid and obtained octanoic acid at approximately 3.06 mg/g (in dry base) from edible noni fruits. However, in this study, the octanoic acid content of various extracts after cellulase hydrolysis or LAB fermentation was almost 1.5-2.5-fold higher than that obtained by Pino et al. (2009).

The scopoletin contents of water extracts of unhydrolyzed, hydrolyzed, and fermented products were 2.94, 6.04, and 3.97 mg/g (in dry base), respectively, while those of methanol and ethanol extracts of unhydrolyzed, hydrolyzed, and fermented products were 3.33, 5.84, and 4.74 mg/g and 2.92, 3.83, and 3.26 mg/g (in dry base), respectively (Fig. 6(b)). The scopoletin content of various extracts in this study was much higher than that of a boiled water extract of noni powder [0.44 μg/g (in dry base)] obtained by Prapaitrakool and Itharat (2010) (Fig. 6(b)). However, Pandy et al. (2014) obtained a higher scopoletin content (18.9 mg/g) in a methanol extract of noni than we did. This difference may be attributed to differences in the extraction time (20 h vs. 4 h) and species.

CONCLUSION

The present study results revealed that cellulase hydrolysis and *P. pentosaceus* fermentation facilitated bionutrient release from noni fruits. Increases in extraction yields and TPCs were obtained after hydrolysis and LAB fermentation, consequently increasing the inhibitory abilities against α-amylase and α-glucosidase. The major components in bionutrients that caused α-amylase and α-glucosidase inhibition were octanoic acid and scopoletin. The obtained data suggest that cellulase hydrolysis and additionally fermented noni hydrolysates can be processed into nutritional supplements with antihypoglycemic properties.

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