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Extraction and Characterization of Antioxidant Compositions From Fermented Fruit Juice of *Morinda citrifolia* (Noni)

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Abstract

Extraction and characterization of antioxidative compositions from the extracts of fermented Xisha Noni (*Morinda citrifolia* L.) juice were studied. The antioxidative constituents of 184.6 g freeze-dried extracts of naturally fermented Xisha Noni juice were isolated successfully by petroleum ether, EtOAc and n-BuOH solvents, and the antioxidative effects were measured according to scavenging activity against hydroxyl generated in Fenton reaction system and superoxide anion radicals in pyrogallol autoxidation system. The EtOAc extract exhibited most significantly higher ($P<0.01$) antioxidative activity than mannitol or vitamin C, while the petroleum ether and n-BuOH extracts showed lower activities compared to mannitol. Three antioxidant phenolic compounds, isoscopoletin, aesculetin and 3,3′,4′,5,7-pentahydroxyflavone (quercetin) were isolated from the EtOAc extract by several chromatography techniques for the first time. The results suggest that several compounds, in particular, the phenolic compounds, contribute separately or synergistically to the antioxidative activity of fermented Noni fruit juice.

Key words: antioxidant activity, *Morinda citrifolia* L., fermented fruit juice

INTRODUCTION

Oxidative stress, commonly occurred in living organisms, is involved in the pathology of cancer, arteriosclerosis, malaria, and rheumatoid arthritis, and could play a role in neurodegenerative diseases and ageing processes. It has been demonstrated that many eatable vegetables, fruits, and food contained various bioactive compositions against the oxidative stress, which were attributed to vitamin C, vitamin E, α-tocopherol, β-carotene, and polyphenolic compounds (Moure *et al.* 2001). Therefore, searching natural antioxidants from food and plants, particularly from folk medicinal plants, is receiving increasing attention throughout the world.

Morinda citrifolia L. (Noni), a shrub originated in tropical Asia or Polynesia, has been extensively used in folk medicine and as a dye in Asian countries. The roots, stems, bark, leaves, flowers, and fruits of Noni plant are all involved in various combinations in almost 40 known and recorded herbal remedies. Particularly, the fruit juice of Noni plant is in high demand in alternative medicine for different kinds of illnesses such as arthritis, diabetes, high blood pressure, muscle aches and pains, menstrual difficulties, headaches, heart disease, AIDS, cancers, gastric ulcers, sprains, mental depression, senility, poor digestion, atherosclerosis, blood vessel problems, and drug addiction (Kamiya *et al.* 2004; Wang *et al.* 2002). Therefore, one of the

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challenges in recent years was to process Noni fruit juice to make a more modern drug from a traditional product (Chunhieng *et al.* 2005). A number of *in vitro* biological activities have been reported, such as angiogenesis inhibition, antioxidant, cyclooxygenases-1 and -2 inhibition, and tyrosine kinase inhibition, but most of them have only been tested with crude extracts or fractions of Noni, and the compound(s) responsible for the biological activities have not been fully determined, except for two compounds, neolignan, americanin A, which were recently identified in n-BuOH-soluble partition of the MeOH extract of Noni fruits (Su *et al.* 2005; Zin *et al.* 2002).

It is well known that the quality of the herb and the nutritional components contained in plants are directly related to the soil in which they grow. Any factor such as the terrain, the weather, geographic location and so on exerting influence on soil conditions may affect what are contained in the plants. In the present study, we focused on isolation of the natural products with scavenging capacities towards free radicals from the naturally fermented fruit juice of Xisha Noni, which is a wild species of Noni found in Xisha Island of China.

MATERIALS AND METHODS

Materials

Silica (200-300 mesh) and Sephadex LH-20 were purchased from Chemical and Industrial Factory of Nankai University, China.

Extraction and isolation

184.6 grams of freeze-dried extracts of freshly fermented Xisha Noni fruit juice provided by Professor Zheng Xueqin, Noni Bio-Engineering Co. of Hainan, China, were kept in 4°C for 3 days and then fully dissolved in 500 mL of distilled water, which was equally distributed into 3 sphericity separatory funnels (capacity: 1 000 mL). Add three-fold volume of petroleum ether (about 500 mL) into each funnel, shake it with hands for 10 min, then keep it at room temperature for a half hour. After that, the clear solvent was evaporated off under reduced pressure with rotary evaporator (China National Medical Equipment Industry Corporation, Shanghai, China), and this process was carried out for several times until almost nothing could be extracted from the solvent, to yield the petroleum ether extract (1.2 g). The aqueous phase was extracted successively with EtOAc and n-BuOH to yield 3.9 and 15.9 g respectively. The remaining H_2O soluble phase was concentrated under reduced pressure to yield black hydrophilic extract (148.8 g).

The petroleum ether soluble phase, EtOAc-soluble phase, and n-BuOH soluble phase were chromatographed on silica gel column (200-300 mesh) eluting with petroleum ether:acetone or $CHCl_3$:MeOH (1:0-0:1) to give 7, 10, and 4 fractions, respectively. Three fractions (Fe-3, Fe-4, and Fe-6) with scavenging activity against hydroxyl radicals from EtOAc extract were subjected to chromatography on Sephadex LH-20 eluting with $CHCl_3$:MeOH (1:20) for several times, to give compounds 1, 2, and 3, respectively.

For structure elucidation of the acquired chemicals, melting points were determined on a Boetius micromelting point apparatus, and were uncorrected. All NMR spectra were collected on a Bruker DRX 500 spectrometer, and the chemical shifts were expressed in δ (p.p.m.) relative to $SiMe_4$ with coupling constants *J* in Hz. Electron ionization-mass spectrometry (EI-MS) were taken on a ZAB-HS mass spectrometer. All chemicals used in the study were of analytical grade.

Identifications of metabolites 1-3

Isoscopoletin (compound 1) White amorphous powder, mp. 185-187°C. ^{1}H NMR date (Acetone-d^6, 500 MHz): 3.89 (s, H_3CO-7), 6.16 (d, 9.6 Hz, H-3), 6.78 (s, H-8), 7.18 (s, H-5), 7.83 (d, 9.6 Hz, H-4), 8.84 (br s, HO-6). EI-MS: *m/z* 192 $[M]^+$ (100), 177 (55), 164 (20), 149 (38), 121 (17), 79 (12), 69 (40).
Aesculetin (compound 2) Colorless crystals, mp. 268-270°C. ^{1}H NMR date (CD_3OD, 500 MHz): 6.18 (d, 9.5 Hz, H-3), 6.75 (s, H-8), 6.94 (s, H-5), 7.78 (d, 9.5 Hz, H-4). EI-MS: *m/z* 178 $[M]^+$ (100), 150 (68).
Quercetin (compound 3) Yellow needles, mp. 300-302°C. ^{1}H NMR date (DMSO-*d*6, 500 MHz): 6.18 (d, 2.0 Hz, H-6), 6.39 (d, 2.0 Hz, H-8), 6.88 (d, 8.5 Hz, H-5′), 7.52 (dd, 2.2 Hz, 8.5 Hz, H-6′), 7.66 (d, 2.2 Hz, H-

2′), 12.40 (s, HO-5). EI-MS: *m/z* 302 $[M]^+$ (100), 301 (23), 273 (7), 153 (3), 137 (3).

Determination of scavenging capacity towards free radicals

Hydroxyl radicals were generated by a Fenton reaction system and the scavenging capacity towards the hydroxyl radicals was measured by a modified method (Kong *et al.* 2001) that was summarized in Table 1. Basically, mix phosphate buffer, extracts in different concentrations, EDTA, $FeCl_3$, and safranine T together, then add H_2O_2 to start reaction in a water bath at 37°C for 30 min. The absorbance of the solution was measured at 520 nm with a spectrophotometer. Mannitol was used as positive control and two negative controls (A_0, A_2) were applied as well. The scavenging capacity towards hydroxyl radical was evaluated with the inhibition percentage of safranine T oxidation by hydroxyl radicals, which was calculated according to the following formula: scavenging percentage = $(A_1 - A_0) \times 100 / (A_2 - A_0)$.

The superoxide anion radicals were generated by a described pyrogallol autoxidation system (Chen *et al.* 2004; Jiao *et al.* 2005). A volume of 1.31 mL of potassium phosphate buffer solution (100 mmol L^{-1}, pH=8.0) was mixed with 0.01 mL of distilled water and 0.06 mL of pyrogallol solution (50 mmol L^{-1} of pyrogallol in 10 mmol L^{-1} of HCl) in an eppendof tube. The mixture was incubated at 25°C for 30 s and then the absorbance was measured at 420 nm in every 30 s up to 2 min, which denotes the speed of pyrogallol autoxidation along with time increase. The inhibition of autoxidation speed by the samples was measured by adding 0.01 mL of sample (5 mg mL^{-1} in distilled water) in the reaction system to replace 0.01 mL of the distilled water. A blank control was obtained by adding 0.01 mL of each sample into a volume of 1.37 mL of potassium phosphate buffer solution so as to eliminate the possible bias caused by the sample color self. The same concentration of vitamin C was used as a positive control.

Table 1 Fenton reaction system used in measurement of the scavenging capacity towards the hydroxyl radicals

Agents	Concentration	A_1 (mL)	A_0 (mL)	A_2 (mL)	Positive control (mL)
Phosphate buffer	50 mmol L^{-1}	0.4	0.4	0.4	0.4
EDTA	1.04 mmol L^{-1}	0.03	0.03		0.03
$FeCl_3$	1.0 mmol L^{-1}	0.02	0.02		0.02
Safranine T	40 μg mL^{-1}	0.30	0.30	0.30	0.30
Extracts or mannitol	36, 72, and 108 μg mL^{-1}	0.43			0.43
H_2O_2	10 mmol L^{-1}	0.20	0.20	0.20	0.20
H_2O			0.43		
H_2O				0.48	

Statistical analysis

All experiments were conducted in triplicate, and statistical analysis was done according to the software Prism (2000) user's guides. The data were presented as mean ± sd. Determination of significant differences of the means between samples and positive control was performed by *T*-test.

RESULTS

Extraction and fractionation of antioxidative compounds

Petroleum ether, EtOAc, and n-BuOH were used in succession to extract the antioxidative constituents from fermented Xisha Noni fruit juice to get 1.2, 3.9, and 15.9 g, respectively. Only a small portion (11.4%) of the fruit juice could be extracted with the given 3 organic solvents, but the majority hydrophilic compositions (about 80.6%) couldn't.

The extract of petroleum ether was subjected to chromatography on silica gel column eluting with petroleum ether:acetone (1:0-0:1) to give 7 fractions (Fp-1, 560 mg; Fp-2, 133 mg; Fp-3, 14 mg; Fp-4, 40 mg; Fp-5, 40 mg; Fp-6, 34 mg; Fp-7, 158 mg). The EtOAc and n-BuOH extracts were separated with similar procedure and eluted with $CHCl_3$:MeOH (1:0 - 0:1) to give 10 fractions (Fe-1, 13 mg; Fe-2, 28 mg; Fe-3, 257 mg; Fe-4, 619 mg; Fe-5, 148 mg; Fe-6, 163 mg; Fe-7, 1 370 mg; Fe-8, 291 mg; Fe-9, 90 mg;

Fe-10, 216 mg) and 4 fractions (Fb-1, 739 mg; Fb-2, 839 mg; Fb-3, 2 763 mg; Fb-4, 10 373 mg), respectively. Fe-3, Fe-4, and Fe-6 from EtOAc extract, which have strong scavenging activity against hydroxyl radicals, were chromatographed on Sephadex LH-20 eluting with $CHCl_3$:MeOH (1:10) for several times, and gave metabolites 1 (104.1 mg), 2 (154.7 mg), and 3 (2.1 mg).

A careful comparison of the ^1H NMR and mass spectra of 1, 2, and 3 with those in literature readily allowed their identification as isoscopoletin (Lee *et al.* 2001), aesculetin (Zhao *et al.* 2002), and quercetin (Miyazawa and Hisama 2003), respectively (Fig.1).

Because of the pharmaceutical and nutritional benefits of Noni plant, many studies have been focused on the isolation and identification of the natural bioactive products from this plant. To date, the major chemical constituents of Noni plant have been found to be

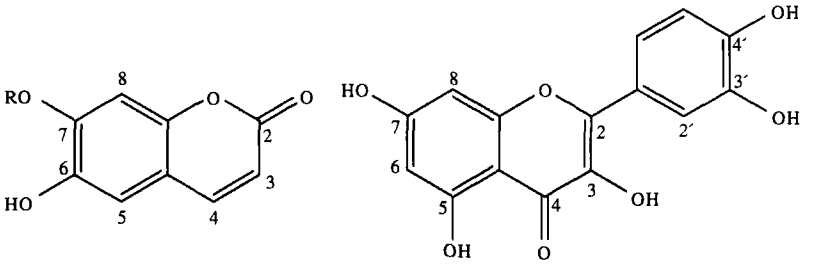

Fig. 1 Chemical structures of compounds 1, 2, and 3 isolated from EtOAc extract of Xisha Noni fruit juice.

anthraquinones, flavonol glycosides, iridoid glycosides, lipid glycosides, and triterpenoids (Su *et al.* 2005). However, to our knowledge, the phenolic compounds, isoscopolectin, aesculetin, and quercetin, were isolated for the first time from the naturally fermented fruit juice of *M. citrifolia*.

Scavenging activity against hydroxyl radicals

Mannitol, a well known antioxidant, was used as a positive control in this experiment. The scavenging capacity of mannitol towards hydroxyl radical was significantly correlated with the concentrations ranging from 30 to 120 μg mL^{-1} ($r^2 = 0.98$). Therefore, all the samples screened for scavenging capacity towards free radicals were carried out at the final concentrations of 36, 72, and 108 μg mL^{-1}, respectively. Result demonstrated that the majority of antioxidative constituents in Xisha Noni fruit juice was in EtOAc extract and its scavenging activity towards hydroxyl radicals was about 2-fold higher than that of mannitol ($P < 0.01$) at the same concentration. Petroleum ether and n-BuOH extracts also showed strong scavenging capacity towards free radicals at high concentration. In addition, the scavenging effects

Table 2 Scavenging percentage of various fruit extracts of Xisha Noni to hydroxyl radicals (%)

Extract	Concentrations (g mL^{-1})		
	36.2	72.5	108.8
Mannitol	12.0±1.7	20.7±4.2	30.8±5.9
Freeze-dried extract	1.35±0.21**	3.0±0.4**	6.1±0.7**
Petroleum ether extract	6.1±0.1**	20.1±2.3	31.9±2.3
EtOAc extract	21.6±2.2**	39.3±3.2**	70.3±6.1**
n-BuOH extract	8.3±4.0*	14.1±2.8*	26.0±4.5

* and ** indicate significant difference between the extracts and mannitol at 0.05 and 0.01 level by *T*-test.

of the test extracts were dose dependent. The scavenging percentage of EtOAc extract reached up to (21.6 ± 2.2)%, (39.3 ± 3.2)%, and (70.3 ± 6.1)% at concentrations of 36, 72, and 108 μg mL^{-1} respectively (Table 2).

The lower scavenging activity of n-BuOH extract could be compensated by the higher amount of extract (8.6% to the total extract), which was 4-fold more than that of EtOAc extract (2.1% to the total extract). Generally, the crude extracts obtained with the three organic solvents seemed to share similar scavenging capacity towards hydroxyl radicals. However, the most part of aqueous extract, about 80.6% of the total extract were demonstrated to have less or even none scavenging activity. Therefore, except for the loss (about 8%) during solvent extraction, there only about 11% constituents in the fermented Noni fruit juice could be

extracted with petroleum ether, EtOAc or n-BuOH and the majority hydrophilic compositions couldn't.

According to the scavenging capacity towards hydroxyl radicals, the antioxidative constituents in petroleum ether extract were subjected to chromatograph on silica gel columns eluting with petroleum ether:acetone to give seven fractions (Fp-1-Fp-7). As showed in Fig.2, Fp-2, about 7.4% of the total extract, exhibited the highest activity about 2-fold higher than that of mannitol ($P<0.01$). Moreover, Fp-1, Fp-5, and Fp-7 also demonstrated some degrees of scavenging activity, but significantly less than that of mannitol ($P<0.01$).

Similar procedure was carried out for the separation of antioxidative constituents from EtOAc and n-BuOH extracts. Based on TLC (thin layer chromatography) analysis, 10 fractions were obtained from EtOAc extract and 4 fractions from n-BuOH extract over silica gel column eluting with $CHCl_3$:MeOH (1:0-0:1), and their scavenging capacity towards hydroxyl radicals were showed in Figs.3 and 4.

As showed in Fig.3, the scavenging activity of Fe-3, Fe-4 and Fe-6 from EtOAc extract against hydroxyl radicals were significantly higher than that of mannitol ($P<0.05$). Further study confirmed that the antioxidant compositions in Fe-3, Fe-4, and Fe-6 were phenolic substances, isoscopoletin (1), aesculetin (2), and quercetin (3) (Fig.1), which showed similar scavenging activity of 35.2, 32.7, and 38.1%, respectively, against hydroxyl radicals, about 3-fold higher than that of mannitol at concentrations of 36 g mL^{-1} *in vitro*.

Except for Fb-2 and Fb-4, Hydroxyl radical scavenging activity of Fb-1 and Fb-4 from n-BuOH extract was not significantly different from that of mannitol ($P>0.05$), which indicated that n-BuOH extract from Noni fruit juice did have antioxidative constituents (Fig.4). Therefore, it would be worthwhile to investigate the chemical nature of antioxidant compositions in n-BuOH extract in the future.

Scavenging activity against superoxide anion radicals

A series of fermented fruit juice extracts of Xisha Noni were evaluated on their scavenging activity against superoxide anion radicals in the pyrogallol autoxidation system. However, it seemed that the inhibition effects of all the extracts were negligible (< 10%) at given concentration of 36.2 μg mL^{-1} compared with that of vitamin C (85%), and this was also true even with the fractions obtained from further separation with chromatography on silica gel column or Sephadex LH-20. Isoscopoletin, which was isolated from EtOAc extract, however, showed 19.1% of scavenging activity against the superoxide radicals at the same concentration.

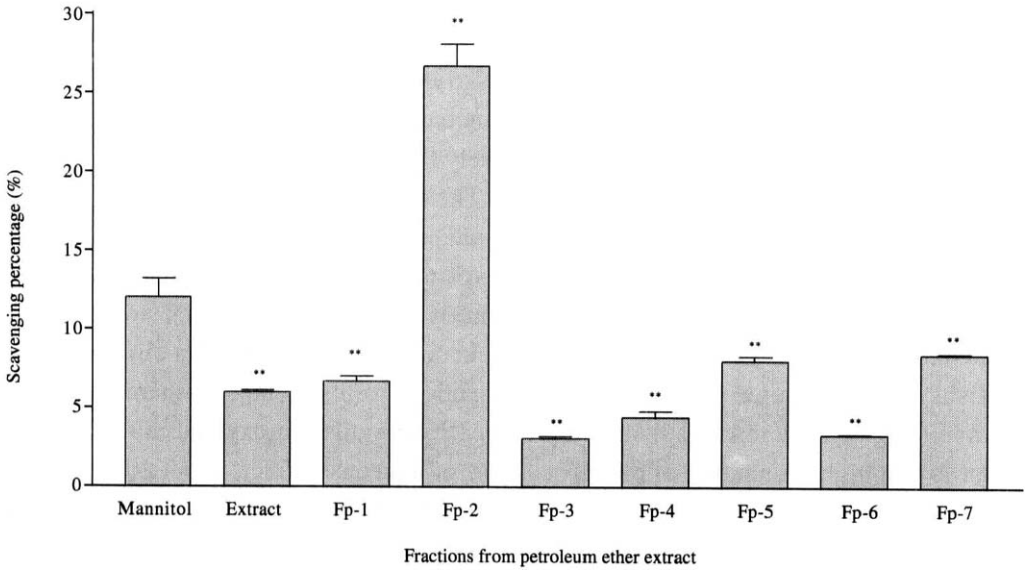

Fig. 2 Hydroxyl radicals scavenging activity in the fractions of petroleum ether extract of Xisha Noni fruit juice. * and ** indicate significant difference between the fractions and mannitol at 0.05 and 0.01 level by *T*-test. The same as below.

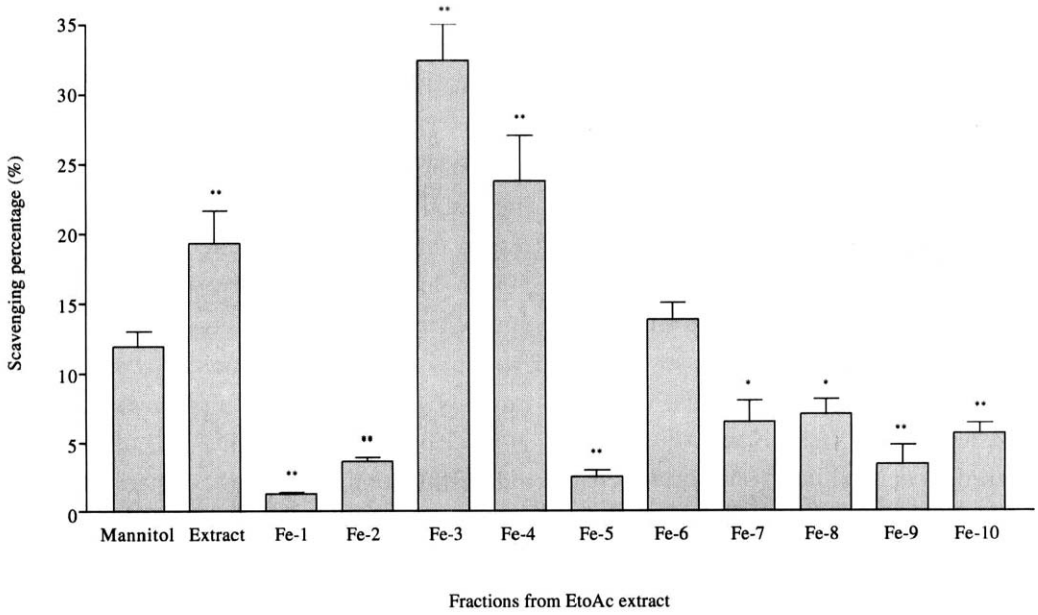

Fig. 3 Hydroxyl radicals scavenging activity in the fractions of EtOAc extract of Xisha Noni fruit juice.

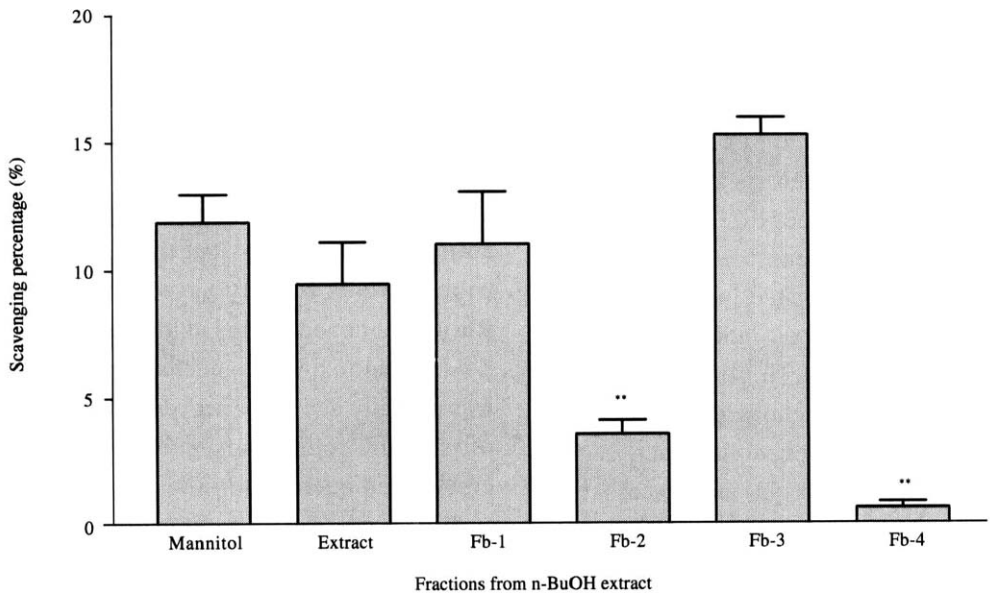

Fig. 4 Hydroxyl radicals scavenging activity in the fractions of n-BuOH extract of Xisha Noni fruit juice.

DISCUSSION

Hydroxyl radical and superoxide anion are the most reactive free radicals and play an important role in the formation of other reactive oxygen species that result in different biological damages in living systems (Wang *et al.* 2002; Subramaniam *et al.* 2003). It is well known that these radicals can be scavenged by some natural plant products (Moure *et al.* 2001; Zin *et al.* 2002; Jiao *et al.* 2005).

It was noted that Noni plant including leaf, fruit and root has antioxidative activities, however, the polar and non-polar components in Noni root exhibited appreciably different antioxidative activities, and compounds that contribute to antioxidative activity of Noni fruits are probably non-polar in nature (Zin *et al.* 2002, 2006). Results obtained in this study demonstrated that the possible mechanism of the antioxidative compounds in

the EtOAc extract of the fermented Noni fruit juice was partially contributed to their scavenging capacity towards hydroxyl radicals. Moreover, substances responsible for scavenging the hydroxyl radical in Noni plant must consist of a complex of compounds because many fractions in the EtOAc extract showed scavenging activities. In addition, three natural phenolic components, isoscopoletin, aesculetin and quercetin in EtOAc extract of fermented Noni fruit juice have been identified for the first time to posses scavenging capacity towards hydroxyl radicals, which indicated that the fermented Xisha Noni fruit juice could be considered as antioxidant food to prevent oxidation related diseases. Further work is required to characterize the antioxidative constituents in the remaining fractions of petroleum ether and n-BuOH extracts.

Phenolic substances, common natural phytochemicals in fruits and leafy vegetables, have been tested to possess antioxidant activities by donating hydrogen atoms to free radicals. Studies have shown that high consumption of fruits and vegetables containing phenolic antioxidants inhibits the oxidation of LDL, and thus slower the process of atherosclerosis and also reduce the risk of cancer and many other diseases (Zin *et al.* 2006). Isoscopoletin and quercetin, widely distributed phenolic compounds, were isolated in the fermented Noni fruit juice for the first time, indicating that the antioxidant properties of Xisha Noni fruit juice may possibly be attributed to the phenolic compounds. In fact, some of the antioxidative phenolics, such as glycosides (rutin and asperulosidic acid) and trisaccharide fatty acid ester [2,6-di-O-(b-D-glucopyranosyl)-1-O-octanoyl- b-D-glucopyranose] as well as neolignan and americanin A have been isolated from the n-BuOH extracts obtained from the ethanol extracts of the fruits of Noni (Su *et al.* 2005; Wang *et al.* 1999). However, the antioxidant mechanisms of each phenolic compound may be different, and the synergistic effect of many compounds cannot be excluded in the fruit juice of Xisha Noni, because most natural antioxidative compounds often work synergistically with each other against the free radical attack.

Quercetin, one of the most abundant flavonoids in human diets such as red wine, green tea, onions, apples, and vegetables, has been known to have various bioactivities, such as anti-ulcer, anti-allergy, anti-viral, immunomodulating activities and inhibition of lipid peroxidation (Subramaniam *et al.* 2003; Volonte *et al.* 2002). The mechanism of quercetin as an antioxitant against lipid peroxidation has been proved by scavenging reactive oxygen species and cheating metal ions responsible for the generation of ROS (Yamamoto *et al.* 1999). In the present study, we demonstrated that the antioxitant activity of quercetin was partly resulted from his scavenging capacity towards the hydroxyl radicals generated in the Fenton reaction system *in vitro*. Isoscopoletin, another phenolic compound and a demethylation product of coumarin, is naturally occurring in roots and barks of plants. However, we have not found any report regarding its scavenging capacity towards both hydroxyl radicals and superoxide anion radicals so far. Because of its strong antioxidant activity, isoscopoletin may be developed as a potential natural radical scavenger to prevent oxidation related diseases.

CONCLUSION

It was noted that Noni fruit juice exhibited appreciable antioxidative activity, but the compounds that contribute to this activity have not been well studied. Results obtained in this study demonstrated that the possible antioxidative mechanism of Noni fruit juice was partially due to the scavenging capacity of a group of phenolic compounds, such as isoscopoletin, aesculetin and quercetin in the EtOAc extract, towards hydroxyl radicals. In addition, the petroleum ether or n-BuOH extracts also showed similar scavenging activity towards hydroxyl radical as mannitol. These results indicated that the fermented Xisha Noni fruit juice could be considered as an antioxidant food to prevent oxidation related diseases. Further work is required to characterize the antioxidative constituents in the remaining fractions of petroleum ether and n-BuOH extracts.

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