ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 8:1 (2014) 25-31

records of natural products

Two New Saccharide Fatty Acid Esters from the Fruit of *Morinda citrifolia* L. and Their ABTS Radical Scavenging Activities

Hong-Cai Zhang [1,†], Yu Wang [1,†], Chun-Miao Yu [2], Dong-Hua Yu [1], Ping-Ping Chen [1] and Shu-Min Liu [3*]

[1] *Academy of Traditional Chinese Medicine, Heilongjiang University of Chinese Medicine, 24 Heping Road, Harbin 150040, China,*

[2] *College of Pharmacy, Heilongjiang University of Chinese Medicine, 24 Heping Road, Harbin 150040, China,*

[3] *Drug Safety Evaluation Center, Heilongjiang University of Chinese Medicine, Harbin 150040, China*

(Received May 31, 2013; Revised September 23, 2013; Ocotober 02, 2013)

Abstract: Two new saccharide fatty acid esters (1) and (2), and six other compounds were isolated from the fruit of *Morinda citrifolia* L. (Rubiaceae) The structures were established as (2E)-oct-2-enoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside(1), (2E)-2,6-dimethyl -6-hydroxyl-oct-2,7-dienoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside(2), saccharumoside C(3), *O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranosides of 3-methyl-2-butenol(4), 3-methyl-but-2-en-1-yl β-D-glucopyranosyl (1→6)-β-D-glucopyranoside(5), benzyl-glucopyranoside(6), Hexyl-*O*-β-D-glucopyranoside(7), and caproic acid(8). Compound 1 and 2 showed moderate activity against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)) radical in concentration of 0.1-3.2 mg/mL.

Keywords: *Morinda citrifolia* L.; saccharide fatty acid ester; ABTS radical scavenging activity.

1. Introduction

Morinda citrifolia L. (Rubiaceae) is a popular traditional edible and medicinal plant in a wide range of tropical areas such as India, southern Asia, the Caribbean, and Polynesia. All parts of this plant including fruit, flower, leaf, bark, stem, and root have been used as food and medicine for more than 2000 years by Polynesian people [1]. *M. citrifolia* was used to treat many diseases including arthritis, infections, cancers, diabetes, inflammation and pains, etc [2, 3]. In tropical regions, its fruit (Noni) was used as food before the twentieth century. The first record was that Captain James Cook of the British Navy noted in the late 1700's that this fruit was eaten in Tahiti [4]. In the United States, 19 patents of the plant had been registered by the US Patent and Trademark Office since 1976 [5]. Noni juice had been accepted in the European Union as a kind of novel food. Recent researches confirmed that *M. citrifolia* displayed antibacterial, anticancer, analgesic, hypotensive, anti-inflammatory, and immune-enhancing activities[6-10].

* Corresponding author: E-Mail: keji-liu@163.com; Phone:086-451-82193278
† *These authors contributed equally to this work.*

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 11/18/2013 EISSN:1307-6167

To date, about 200 compounds have been isolated and identified from the *M. citrifolia*, including anthraquinones, flavonoids, coumarins, sterols, glycosides, iridoids, lignans, and triterpenoids [11]. Compounds such as scopoletin, rutin, ursolic acid, and damnacanthal were considered as the effective components of *M. citrifolia* [12, 13]. In our previous study, two new glycosides had been identified from the fruit of *M. citrifolia* [14]. Upon continuation, two new saccharide fatty acid esters together with 6 known compounds were isolated from *M. citrifolia* fruits. This paper presented herein the isolation, structural elucidation, and ABTS radical scavenging activities of these compounds.

2. Materials and methods

2.1. General

IR and NMR spectra were recorded on SHIMADZU FTIR-8400S and Bruker DPX 500 (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR), respectively. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard, and coupling constants are given in Hz. The HR-ESI-MS analyses were conducted on Waters LCT Premier XE TOF-MS. A DiamonsiI C_{18} (5μm, 4.6 mm×25 cm, Dikma, America) was employed for analytical HPLC (Waters, 2695-2998). A ZORBAX SB-C_{18} (5μm, 9.4 mm×25 cm, Agilent, America) was employed for Agilent 1100 preparative HPLC (G1315B UV-vis detector). Open column chromatography was carried out using silica gel (200-300 mesh, Qingdao Marine Chemical Co., Qingdao, China) and octadecyl silica gel (ODS, 50μm, Fuji, Japan) as stationary phases. TLC employed precoated Silica gel plates (5-7μm, Qingdao Marine). TLC Silica gel60 Rp-18 F_{254s} (Merck, Germany). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma, America). All the other reagents used in this study were of the highest available purity and were purchased from Sigma-Aldrich.

2.2. ABTS scavenging assay

The radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\bullet+}$) was produced by oxidation of ABTS according to the literature procedure [15]. The $ABTS^{\bullet+}$radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating for 16 h in the dark at room temperature until the reaction was complete and the absorbance was stable. The absorbance of the $ABTS^{\bullet+}$solution was equilibrated to 0.70 (± 0.02) by diluting with water at room temperature, then 1 ml was mixed with 10 μl of the test sample (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) and the absorbance was measured at 734 nm after 6 min. All experiments were repeated three times. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of samples. Vitamin C (ascorbic acid) was used as positive control.

2.3. Plant material

The fresh fruits of Morinda citrifolia L. were collected from Hainan Province of China, in July 2010. The voucher specimen (20100720) was deposited in Heilongjiang University of Chinese Medicine, Harbin, China.

2.4. Extraction and Isolation

The fresh fruits of *M. citrifolia* L (24.0kg) were extracted with 95% EtOH (4×10 L) to afford the EtOH extract (12.6kg) which was suspended in water (25 L) then and extracted with petroleum ether, $CHCl_3$, EtOAc and *n*-butanol (3×25L), respectively. Solvent was removed to obtained petroleum ether fraction (19.3g), $CHCl_3$ fraction (5.6g), EtOAc fraction (5.1g) and *n*-butanol fraction (140.8g). The *n*-butanol fraction (140.8g) was subjected to silica gel column with a stepwise gradient $CHCl_3$-MeOH (30:1; 20:1; 15:1; 10:1; 7:1; 5:1; 3:1, v/v), gave seven fractions (I-VII). Fraction IV (15.6g) was subjected to silica gel column with $CHCl_3$-MeOH (20:1, 15:1, v/v) to afford two sub-fractions, IV_1-IV_2. Sub-fraction IV_2 (12.4g) was subjected to ODS column chromatography with

MeOH/H_2O (1:9→1:0) and finally purified by preparative HPLC on a ZORBAX SB-C_{18} column with MeOH/H_2O (35:65) to afford **8** (8.9mg, t_R=27min), **3** (16.3mg, t_R=39 min). Fraction V (11.4g) was subjected to silica gel column with $CHCl_3$-MeOH (10:1, 7:1, v/v) to afford three sub-fractions, V_1-V_3. Sub-fraction V_3 (4.8g) afforded was subjected to ODS column chromatography with MeOH/H_2O (1:9→1:0) and finally purified by preparative HPLC on a ZORBAX SB-C_{18} column with MeOH/H_2O (30:70) to afford **7** (17.2mg, t_R =27min), **4** (13.6mg, t_R=31 min), **1** (15.1mg, t_R =37min). Fraction VI (23.3 g) was subjected to silica gel column which was eluted with $CHCl_3$-MeOH (7:1, 5:1, v/v) to afford three sub-fractions, VI_1-VI_3. Sub-fraction VI_2 (12.7g) afforded was subjected to ODS column chromatography with MeOH/H_2O (1:9→1:0) and finally purified by preparative HPLC on a ZORBAX SB-C_{18} column with MeOH/H_2O (25:75) to afford **5** (8.7mg, t_R=34 min), **2** (14.8mg, t_R =41min), **6** (19.5mg, t_R =45min).

(2E)-oct-2-enoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (**1**). White amorphous powder, mp. 141–143°C. IR (KBr): 3400.1, 1700.5, 1472.1, 980.3 cm^{-1}. HR- ESI-MS *m/z* 481.2300 $[M-H]^-$ (calc. $C_{22}H_{34}O_{13}$, 481.2291); ^1H and ^{13}C-NMR (CD_3OD) data are showed in Table 1.

(2E)-2,6-dimethyl-6-hydroxyl-oct-2,7-dienoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (**2**). White amorphous powder, mp. 79–82°C. IR (KBr): 3401.2, 2950.3, 1751.4, 1057.8 cm^{-1}. HR-ESI-MS *m/z* 523.2082 $[M-H]^-$ (calc. $C_{16}H_{30}O_{10}$, 523.2096); ^1H and ^{13}C-NMR (CD_3OD) data are showed in Table 1.

Table 1. NMR data of **1** and **2** in CD_3OD (δ in ppm, *J* in Hz, recorded at 500 MHz and 125 MHz, respectively).

No.	**1**		**2**	
	δ_C (DEPT)	δ_H (*J*, Hz)	δ_C (DEPT)	δ_H (*J*, Hz)
1	166.5 (C)		167.8 (C)	
2	121.8 (CH)	5.80 d (15.7)	128.2 (C)	
3	153.1 (CH)	7.02 dt (15.7,7.0)	145.9 (CH)	6.84 dd (8.7,1.1)
4	33.3 (CH_2)	2.15 brdd (13.9,7.0)	24.7 (CH_2)	2.16 m
5	28.8 (CH_2)	1.25 m	41.6 (CH_2)	1.52 m
6	32.5 (CH_2)	1.25 m	73.6 (C)	
7	23.5 (CH_2)	1.40 brt (7.4)	145.9 (CH)	5.82 dd (17.4,10.8)
8	14.3 (CH_3)	0.83 t (6.9)	112.5 (CH_2)	5.13 dd (17.4,1.4) 4.96 dd (10.8,1.4)
9			12.4 (CH_3)	1.75 s
10			27.9 (CH_3)	1.18 s
1'	94.3 (CH)	5.52 d (6.3)	94.5 (CH)	5.54 d (7.7)
2'	82.7 (CH)		82.5 (CH)	
3'	77.7 (CH)		77.7 (CH)	
4'	71.2 (CH)		71.2 (CH)	
5'	77.9 (CH)		77.8 (CH)	
6'	62.4 (CH_2)		62.4 (CH_2)	
1"	105.6 (CH)	4.45 d (7.8)	105.5 (CH)	4.46 d (7.8)
2"	75.9 (CH)		76.0 (CH)	
3"	78.7 (CH)		78.0 (CH)	
4"	70.8 (CH)		70.8 (CH)	
5"	77.8 (CH_2)		78.6 (CH)	
6"	62.3 (CH_2)		62.4 (CH_2)	

3. Results and Discussion

By repeated silica gel column chromatography, ODS column chromatography, and preparative HPLC, we achieved: (2E)-oct-2-enoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside (1); (2E)-2,6-dimethyl -6-hydroxyl-oct-2,7-dienoate-2-O-β-D-glucopyranosyl-β-D-glucopyranoside (2); saccharumoside C(3) [16]; O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranosides of 3-methyl-2-butenol (4) [17]; 3-methyl-but-2-en-1-yl β-D-gluco-pyranosyl (1→6)-β-D-glucopyranoside(5) [18]; benzyl-glucopyranoside(6) [19]; Hexyl-O-β-D-glucopyranoside (7) [20]; and caproic acid(8). The structures of all compounds are showed in Fig.1.

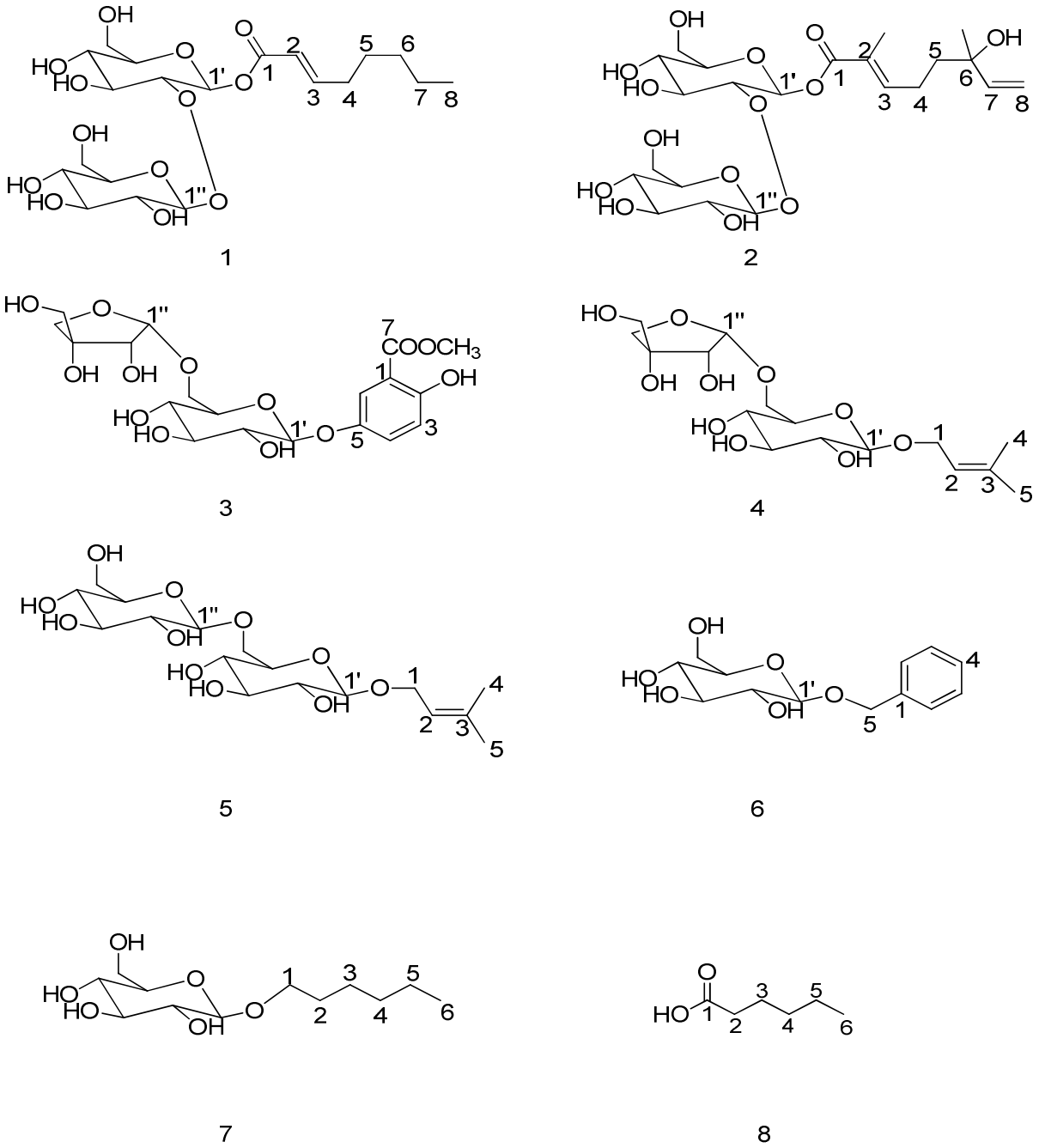

Figure 1. Structures of compounds **1**-**8**

3.1 Structure determination of two new saccharide fatty acid esters

Compound **1** was obtained as a white powder (15.1mg). The molecular formula was determined to be $C_{20}H_{34}O_{13}$ by the HR-FAB-MS 481.2300 $[M-H]^-$. The configuration of the sugar unit was assigned after hydrolysis with 1 N HCl. The hydrolysate was trimethylsilylated, and GC retention times of sugar were compared with those of authentic sugar samples prepared in the same manner[21]. The result of GC showed that Acid hydrolysis of **1** only gave D-glucose. The ^1H and ^{13}C NMR spectra of **1** demonstrated a alkenoic acid ester moiety and two glucose groups. The alkenoic acid ester moiety was supported by ^1H NMR signals at δ_H7.02 (1H, dt, J=15.7, 7.0Hz), 5.80 (1H, d, J=15.7Hz), 2.15 (2H, brdd, J=13.9, 7.0Hz), 1.40 (1H, brt, J=7.4Hz), 1.25(4H, m) and 0.83 (3H, t, J=6.9Hz) and by ^{13}C NMR signals at δ_C166.5(s), 121.8(d), 153.1(d), 33.3(t), 28.8(t), 32.5(t), 23.5(t), and 14.3(q). A combination of the COSY, HSQC, and HMBC data allowed assignment of the ^{13}C NMR signals from

the disaccharide. The characteristic features of the two glucose moieties appeared in the ^{13}C NMR spectra, which exhibited at δ_C94.3(d), 82.7(d), 77.7(d), 71.2(d), 77.9(d), and 62.4(t) for the first glucose and signals at δ_C105.6(d), 75.9(d), 78.7(d), 70.8(d), 77.8(d), and 62.3(t) for the second glucose [22]. ^{13}C NMR spectrum revealed corresponding signals for anomeric protons signals (δ_H5.52 (1H, d, J = 6.3 Hz) and 4.45(1H, d, J = 7.8 Hz)) and carbons signals (δ_C94.3(d) and 105.6(d)), it inferred that the β configuration was exist in the disaccharide moiety. From the HMBC studies, anomeric proton δ_H4.45 (H-1") was found to be linked to C-2' (δ_C82.7), that is, terminal glucose was connected to the inner glucose. The linkage between the fatty acid ester moiety and the disaccharide was also confirmed by the HMBC data (anomeric proton δ_H5.52 (H-1') and δ_C166.5 (C-1)). Important HMBC interactions of compound **1** are shown in Fig. 2. On the basis of the above data, the structure of **1** was deduced to be (2E)-oct-2-enoate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside, a new saccharide fatty acid esters.

Compound **2** was obtained as a white powder (14.8mg). The molecular formula was deduced from the HR-FAB-MS 523.2082 $[M-H]^-$ and the ^{13}C NMR data to be $C_{22}H_{36}O_{14}$. The ^1H and ^{13}C NMR spectra of **2** demonstrated a aglycon was (2E)-2,6-dimethyl-6-hydroxyl-2,7-octadienoic acid and two glucose groups. The alkenoic acid ester moiety was supported by ^1H NMR signals at δH6.84 (1H, dd, J=8.7, 1.1Hz), 5.82(1H, dd, J=17.4, 10.8Hz), 5.54(1H, d, J=7.7Hz), 5.13(1H, dd, J=17.4, 1.4Hz), 4.96(2H, dd, J=10.8, 1.4Hz), 4.46(1H, d, J=7.8Hz), 2.16(2H, m), 1.75(3H, S), 1.52(2H, m), 1.18(3H, s) and by ^{13}C NMR signals at δC167.8 (s), 145.9(×2)(d), 128.2(s), 112.5(t), 73.6(s), 41.6(t), 27.9(q), 24.7(t), and 12.4(q). A combination of the COSY, HSQC, and HMBC data allowed assignment of the ^{13}C NMR signals from the disaccharide. The characteristic features of the two glucose moieties appeared in the ^{13}C NMR spectra, which exhibited at δC94.5(d), 82.7(d), 77.7(d), 71.2(d), 77.8(d), and 62.4(t) for the first glucose and signals at δC105.5(d), 76.0(d), 78.0(d), 70.8(d), 78.6(d), and 62.4(t) for the second glucose [23]. The ^1H NMR signals at δH5.54 (1H, d, J = 7.7 Hz) and 4.46 (1H, d, J = 7.8 Hz), and the ^{13}C NMR signals at δC 94.5(d) and 105.5(d) indicated the presence of anomeric protons and carbons in the disaccharide moiety that had a β configuration according to the coupling constant. The HMBC correlation between anomeric proton δH4.46 (H-1") and δC82.5 (C-2') connected the terminal glucose to the inner glucose. The linkage between the fatty acid ester moiety and the disaccharide was also established by the HMBC correlation between anomeric proton δH5.54 (H-1') and δC167.8 (C-1). Important HMBC interactions of compound **2** are shown in Fig. 2. On the basis of the above data, the structure of **2** was deduced to be (2E)-2,6-dimethyl-6-hydroxyl-oct-2,7-die-noate-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside, a new saccharide fatty acid esters.

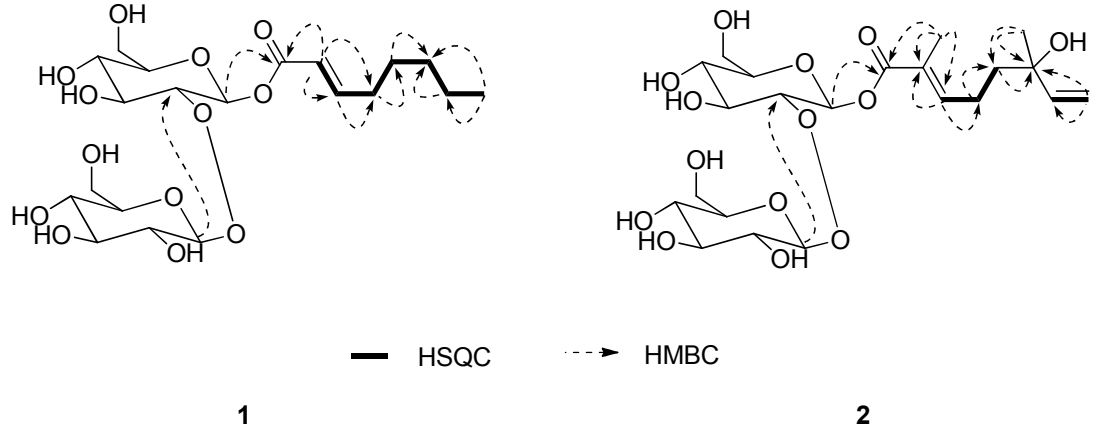

Figure 2. Key ^1H-^1H HSQC and HMBC correlations of compounds **1** and **2**

3.2 ABTS radical scavenging activity

The clearance compound 1 and 2 showed the dependency of concentration in 0.1-3.2mg/ml. The ABTS radical scavenging activity is showed in Fig.3.

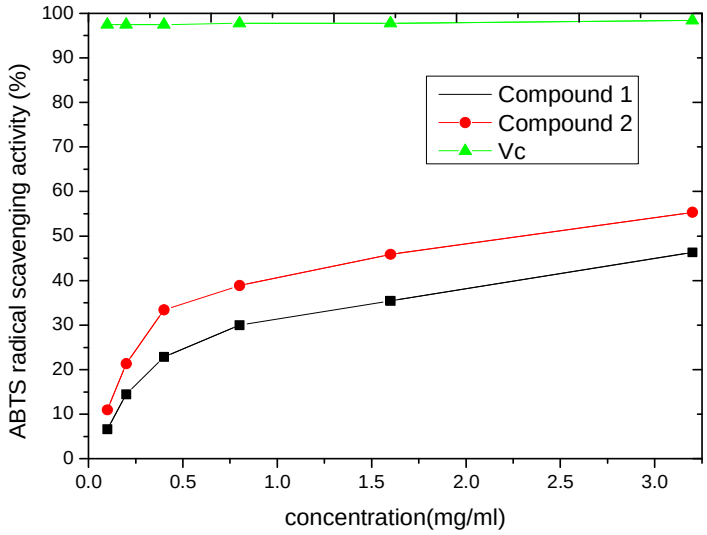

Figure 3. ABTS radical scavenging activity of compound **1, 2** and **vitamin C**

4. Conclusion

In this study, eight compounds were isolated and identified, of which compound 1 and 2 were new compounds. Compound 1 and 2 were evaluated for their ability of ABTS radical scavenging, and compound 2 showed stronger ability than that of compound 1. According to previous literature, the mechanism of radical scavenging is additional reaction between radicals and double bonds of compound [24]. So we surmised that the feasible mechanism of radical scavenging of compound 1 and 2 was also the additional reaction between radicals and double bonds. Some of remaining compounds also had the double bonds, so they could be potential effective antioxidant constituents of *M. citrifolia* . Separating and identifying of all these compounds provided a theoretical basis for the exploitation of the *M. citrifolia* antioxidants.

Reference

[1] A. Dixon, H. McMillen and N. Etkin (1999). Ferment this: The transformation of Noni, a traditional polynesian medicine (*Morinda Citrifolia*, Rubiaceae), *Econ.Bot*. **53**, 51-68.

[2] W. McClatchey (2002). From Polynesian healers to health food stores: changing perspectives of *Morinda citrifolia* (Rubiaceae), *Integr. Cancer. Ther.* **1**, 110-120; discussion 120.

[3] Y. Chan-Blanco, F. Vaillant, A. Mercedes Perez, M. Reynes, J. M. Brillouet and P. Brat (2006). The noni fruit (*Morinda citrifolia* L.): A review of agricultural research, nutritional and therapeutic properties, *J. Food. Compos. Anal*. **19**, 645-654.

[4] M. Y. Wang, B. J. West, C. J. Jensen, D. Nowicki, C. Su and A. K. Palu (2002). *Morinda citrifolia* (Noni): a literature review and recent advances in Noni research, *Acta. Pharmacol. Sin*. **23**, 1127-1141.

[5] USPTO (2005). Patent Full-Text and Image Database. Patents (*Morinda citrifolia*), *Retrieved January 17, 2005 from the World Wide Web: http://patft.uspto.gov/netacgi/nph*.

[6] N. Atkinson (1956). Antibacterial substances from flowering plants. 3. Antibacterial activity of dried Australian plants by a rapid direct plate test, *Aust. J. Exp. Biol. Med. Sci.* **34**, 17.

[7] C. Su, M. Wang, D. Nowicki, J. Jensen and G. Anderson (2001). Selective COX-2 inhibition of *Morinda citrifolia* (Noni) in vitro. The proceedings of the Eicosanoids and other bioactive lipids in cancer, inflammation and related disease, in *The 7th Annual Conference*, pp 14-17.

[8] S. Basar, K. Uhlenhut, P. Högger, F. Schöne and J. Westendorf (2010). Analgesic and antiinflammatory activity of *Morinda citrifolia* L.(Noni) fruit, *Phytother. Res*. **24**, 38-42.

[9] A. K. Palu, A. H. Kim, B. J. West, S. Deng, J. Jensen and L. White (2008). The effects of *Morinda citrifolia* L.(noni) on the immune system: Its molecular mechanisms of action, *J. Ethnopharmacol.* **115**, 502-506.

[10] S. Harada, W. Hamabe, K. Kamiya, T. Satake and S. Tokuyama (2009). Protective effect of *Morinda citrifolia* on the ischemic neuronal damage, *Yakugaku. Zasshi.* **40**, 203-207.
[11] O. Potterat and M. Hamburger (2007). *Morinda citrifolia* (Noni) fruit-phytochemistry, pharmacology, safety, *Planta. Med.* **73**, 191-199.
[12] A. D. Pawlus, B. N. Su, W. J. Keller and A. D. Kinghorn (2005). An Anthraquinone with Potent Quinone Reductase-Inducing Activity and Other Constituents of the Fruits of *Morinda citrifolia* (Noni), *J. Nat. Prod.* **68**, 1720-1722.
[13] B. Dinda, S. Debnath and Y. Harigaya (2007). Naturally occurring secoiridoids and bioactivity of naturally occurring iridoids and secoiridoids. A review, Part 2, *Chem. Pharm. Bull.* **55**, 689-728.
[14] M. X. Hu, H. C. Zhang, Y. Wang, S. M. Liu and L. Liu (2012). Two New Glycosides from the Fruits of *Morinda citrifolia* L, *Molecules* **17**, 12651-12656.
[15] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free. Radical. Bio. Med. **26**, 1231-1237.
[16] T. Yuan, C. Wan, A. González-Sarrías, V. Kandhi, N. B. Cech and N. P. Seeram (2011). Phenolic Glycosides from Sugar Maple (*Acer saccharum*) Bark, *J. Nat. Prod.* **74**, 2472-2476.
[17] R. Baltenweck-Guyot, J. M. Trendel, P. Albrecht and A. Schaeffer (1997). New hemiterpene glycosides in Vitis vinifera wine, *J. Nat. Prod.* **60**, 1326-1327.
[18] K. Machida, E. Matsuoka and M. Kikuchi (2009). Five new glycosides from *Hypericum erectum* Thunb, *J. Nat. Med.* **63**, 223-226.
[19] T. Kanchanapoom and S. Ruchirawat (2007). Megastigmane glucoside from *Asystasia gangetica* (L.) T. Anderson, *J. Nat. Med.* **61**, 430-433.
[20] J. Kitajima, T. Ishikawa, Y. Tanak, M. Ono, Y. Ito and T. Nohara (1998). Water-soluble constituents of fennel. V. 1) Glycosides of aromatic compounds, *Chem. Pharm. Bull.* **46**, 1587-1590.
[21] G. Cioffi, F. Dal Piaz, P. De Caprariis, R. Sanogo, S. Marzocco, G. Autore and N. De Tommasi (2006). Antiproliferative Triterpene Saponins from *Entada africana*, *J. Nat. Prod.* **69**, 1323-1329.
[22] T. Akihisa, K. Seino, E. Kaneko, K. Watanabe, S. Tochizawa and M. Fukatsu (2010). Melanogenesis inhibitory activities of iridoid-, hemiterpene-, and fatty acid-glycosides from the fruits of *Morinda citrifolia* (Noni), *J. Oleo. Sci.* **59**, 49-57.
[23] K. Yoshikawa, Y. Suzaki, M. Tanaka, S. Arihara and S. Nigam (1997). Three acylated saponins and a related compound from *Pithecellobium dulce*, *J. Nat. Prod.* **60**, 1269-1274.
[24] M. Srinivasan, A. R. Sudheer, K. R. Pillai, P. R. Kumar, P. Sudhakaran and V. Menon (2006). Influence of ferulic acid on γ-radiation induced DNA damage, lipid peroxidation and antioxidant status in primary culture of isolated rat hepatocytes, *Toxicology.* **228**, 249-258.