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HEPATOPROTECTIVE EFFECTS OF PHENOLIC GLYCOSIDES FROM THE METHANOL EXTRACT OF *PHYSALIS ANGULATA*

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ABSTRACT

By various chromatographic separations, four phenolic glycosides, methyl salicylate 2-*O*-triglycoside (**1**), icariside E_5 (**2**), quercetin 3-*O*-rutinoside (**3**), and isorhamnetin 3-*O*-rutinoside (**4**) were isolated from the methanol extract of the whole plant of *Physalis angulata*. Their structures were determined by 1D-, 2D-NMR spectroscopic analyses and by comparison with reported data. Compounds **3** exhibited significant hepatoprotective activity with an EC_{50} value of 17.08 µg/mL. This is the first report of **1-4** from *Physalis angulata.*

Keywords: Physalis angulata, phenolic glycoside, hepatoprotective effect.

1. INTRODUCTION

Physalis is a genus of herbaceous plants in the Solanaceae family. The genus comprises approximately 120 species, mainly distributed worldwide in tropical and temperate regions. As reported by Vo Van Chi [1], in Vietnam the genus *Physalis* includes 4 species: *P. angulata*, *P. alkekengi*, *P. peruviana* and *P. minima*. Most species of the genus *Physalis* have been used for a long time in the ethnomedical folk traditions of Asian and American populations to treat different illnesses, such as malarial, asthma, hepatitis, dermatitis, liver disorders, and as an antimycobacterial, anticancer, antileukemic, antipyretic, and immune-modulatory agent [2].

In term of liver disorder, 20 methanol extracts of Thai Binh's medicinal plants (*Annona glabra* (leave and fruit), *Annona squamosa, Michelia alba, Glinus oppositifolius, Hibiscus tiliaceus, Hibiscus rosa-sinensis, Euphorbia pulcherrima, Euphorbia hirta, Chenopodium ficifolium, Chenopodium ambrosioides, Suaeda maritima, Ricinus communis, Physalis angulata,*

Cudrania tricuspidata, Solanum nigrum, Datura metel, Solanum procumbens, Heliotropium indicum, Lactuca indica) were screened on the protective effects against hydrogen peroxide induced Balb/c mice hepatocytes damage. The results indicated that the methanol extract of *P. angulata* exhibited great protective activity, with an EC_{50} value of 56.0 ± 14.0 µg/mL. Thus, it was subjected to chemical study to clarify active constituents. Our results report four phenolic glycosides, methyl salicylate 2-*O*-triglycoside (**1**), icariside E_5 (**2**), quercetin 3-*O*-rutinoside (**3**) and isorhamnetin 3-*O*-rutinoside (**4**) from the water-soluble fraction of the methanol extract of *P. angulata*. This is the first report of **1**, **2** and **4** from this genus [2].

2. MATERIALS AND METHODS

2.1. Plant Material

Whole plant of *Physalis angulata* were collected at Thai Binh province, Vietnam, in August, 2015. Its scientific name was identified by Dr. Tran Thi Phuong Anh, Vietnam National Museum of Nature. A voucher specimen (TB14.2015) is deposited at the Herbarium of Institute of Marine Biochemistry, VAST.

2.2. General experimental procedures

The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer and TMS was used as an internal standard. Column chromatography was performed using a silica gel (Kieselgel 60, 70 - 230 mesh and 230 - 400 mesh, Merck, Whitehouse Station, NJ) or RP-18 resins (30 - 50 µm, Fuji silysia Chemical Ltd.), and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

2.3. Extraction and isolation

The dried and powdered whole plants of *Physalis angulata* (3.0 kg) were sonically extracted with methanol at 50 °C for three times (4.0 L each). After removal of the solvent, the methanol extract (110 g) was suspended in distilled water (1.5 L) and successively partitioned with dichloromethane and ethyl acetate (three time, 1.5 L each) to give corresponding soluble extracts, dichloromethane (PAD, 34.5 g), ethyl acetate (PAE, 3.6 g), and water soluble layers (PAW, 71.9 g). The water layer was then passed through Diaion HP-20 column chromatography, washed with distilled water and desorbed with methanol/water (25 %, 50 %, 75 % and 100 % volume of methanol, each 1.0 L, stepwise) to give four fractions PAW1−PAW4. Fraction PAW2 was chromatographed on a silica gel column, eluting with dichloromethane/methanol/water (3/1/0.1, v/v/v) to give two sub-fractions PAW2A-PAW2B. Sub-fraction PAW2A was firstly chromatographed on a reverse phase C_{18} column, eluting with methanol/water (2/1, v/v), and then further purified on a Sephadex LH-20 column, eluting with methanol/water (3/1, v/v) to obtain compound **2** (12.0 mg). Fractions PAW3A and PAW3B were obtained after CC of the sub-fraction PAW2A on a reverse phase C_{18} column, eluted with acetone/water (1/1, v/v). Fraction PAW3B was purified by a silica gel CC, eluting with ethyl acetate/methanol/water (15/1/0.05, v/v/v) to obtain compound **1** (9.0 mg). Fraction PAW4 (3.1 g) was separated by a silica gel CC, eluting with dichloromethane/ methanol/ water (2/1/0.1, v/v/v) to give two fractions PAW4A and PAW4B. Fraction PAW4A was chromatographed on a reverse phase C_{18} column, eluting with methanol/ water (1/2, v/v) and further purified by a silica gel CC, eluted

with dichloromethane/ methanol/ water (2/1/0.05, v/v/v) to obtain compounds **3** (19.0 mg) and **4** (12.0 mg).

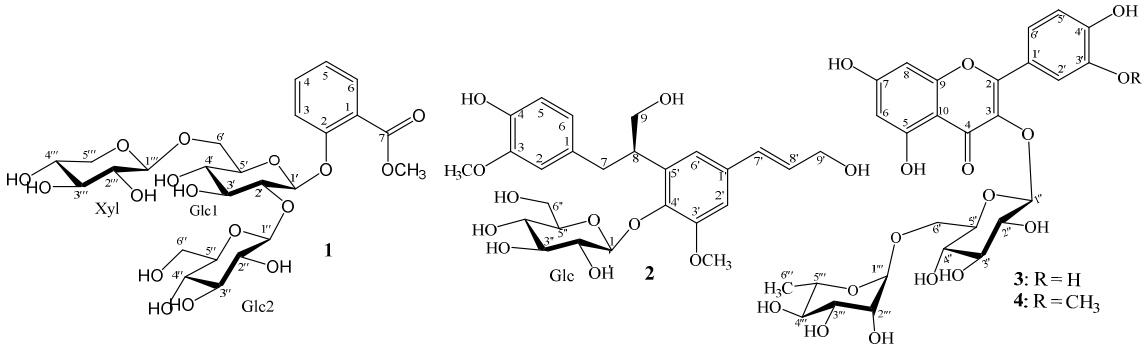

Figure 1. Chemical structures of compounds **1**-**4**.

Methyl salicylate 2-*O*-triglycoside (1): White, amorphous powder; MF: $C_{27}H_{28}N_2O_4$, MW: 444, ^{1}H (CD_3OD, 500 MHz)- and ^{13}C-NMR (CD_3OD, 125 MHz), see Table 1.

Icariside E_5 (2): Amorphous powder; MF: $C_{25}H_{26}N_2O_3$, MW: 402, ^{1}H (CD_3OD, 500 MHz)- and ^{13}C-NMR (CD_3OD, 125 MHz), see Table 1.

Quercetin 3-*O*-rutinoside (3): Pale yellow powder; MF: $C_{27}H_{30}O_{16}$, MW: 610. ^{1}H-NMR (CD_3OD, 500 MHz) δ_H (ppm): 6.18 (1H, s, H-6), 6.37 (1H, s, H-8), 7.66 (1H, s, H-2′), 6.86 (1H, d, *J* = 8.0 Hz, H-5′), 7.61 (1H, d, *J* = 8.0 Hz, H-6′), 5.05 (1H, d, *J* = 7.6 Hz, H-1″), 3.46 (1H, overlapped, H-2″), 3.41 (1H, overlapped, H-3″), 3.28 (1H, overlapped, H-4″), 3.34 (2H, m, H-5″, H_b -6″), 3.78 (1H, d, *J* = 10.0 Hz, H_a -6″), 4.49 (1H, s, H-1‴), 3.27 (1H, overlapped, H-2‴), 3. 53 (1H, overlapped, H-3‴), 3.62 (1H, overlapped, H-4‴), 3.43 (1H, overlapped, H-5‴), and 1.10 (3H, d, *J* = 6.0 Hz, H-6‴). ^{13}C-NMR (CD_3OD, 125 MHz) δ_C (ppm): 159.4 (C-2), 135.6 (C-3), 179.3 (C-4), 162.8 (C-5), 100.0 (C-6), 166.0 (C-7), 94.9 (C-8), 158.4 (C-9), 105.5 (C-10), 123.0 (C-1′), 116.0 (C-2′), 145.8 (C-3′), 149.8 (C-4′), 117.7 (C-5′), 123.5 (C-6′), 104.7 (C-1″), 75.6 (C-2″), 78.1 (C-3″), 71.3 (C-4″), 77.1 (C-5″), 68.6 (C-6″), 102.4 (C-1‴), 72.0 (C-2‴), 72.1 (C-3‴), 73.9 (C-4‴), 69.7 (C-5‴), and 17.9 (C-6‴).

Isorhamnetin 3-O-rutinoside (4): Pale yellow powder; MF: $C_{28}H_{32}O_{16}$, MW: 624. ^{1}H-NMR (CD_3OD, 500 MHz) δ_H (ppm): 6.16 (1H, s, H-6), 6.33 (1H, s, H-8), 7.96 (1H, s, H-2′), 6.91 (1H, d, J = 8.5 Hz, H-5′), 7.63 (1H, d, J = 8.5 Hz, H-6′), 3.96 (3H, s, OCH_3), 5.17 (1H, d, J = 7.5 Hz, H-1″), 3.42 (1H, overlapped, H-2″), 3.38 (1H, overlapped, H-3″), 3.27 (1H, overlapped, H-4″), 3.48 (1H, m, H-5″), 3.40 (1H, d, J = 11.0 Hz, H_a -6″, 3.83 (1H, d, J = 11.0 Hz, H_b -6″), 4.54 (1H, br s, H-1‴), 3.51 (1H dd, J = 3.5, 9.5 Hz, H-2‴), 3.64 (1H, overlapped, H-3‴), 3.29 (1H, overlapped, H-4‴), 3.46 (1H, m, H-5‴), and 1.13 (d, J = 6.5 Hz, H-6‴). ^{13}C-NMR (CD_3OD, 500 MHz) δ_C (ppm):158.4 (C-2), 135.3 (C-3), 178.8 (C-4), 162.7 (C-5), 101.2 (C-6), 169.9 (C-7), 95.8 (C-8), 158.8 (C-9), 104.6 (C-10), 122.9 (C-1′), 114.5 (C-2′), 148.4 (C-3′), 151.1 (C-4′), 116.2 (C-5′), 124.0 (C-6′), 56.7 (3′-OCH_3), 104.8 (C-1″), 75.9 (C-2″), 77.3 (C-3″), 71.6 (C-4″), 78.2 (C-5″), 68.6 (C-6″), 102.5 (C-1‴), 72.3 (C-2‴), 72.1 (C-3‴), 73.9 (C-4‴), 69.8 (C-5‴), and 17.9 (C-6‴).

2.4. Hepatocyte protection assay

The primary hepatocytes freshly isolated from BALB/c mice was seeded in to 96 well plate at the concentration of 1×10^4 cells per well. After 24 hours of growing in the incubator (5 % CO_2, 37 ^oC, 100 % humidity), cells were treated with different concentrations of testing compounds for following 2 hours. 100 µM H_2O_2 was then added per well and testing plate stayed for another 2 h. In order to determine the number of living hepatocytes after H_2O_2 exposure as well as the tested samples' protective activities, 50 µl of MTT (1mg/ml) was added into each well and incubated for 4 hours at the 37^{o}C. The supernatant was discarded and then 100 µl/well of DMSO 100% was added. The optical density (OD) values of formazan solution were determined by Microplate Reader at 492 nm. The survival percentage of hepatocytes was calculated as the following:

$$\% \text{ survival} = \frac{[OD(_{sample}) - OD(H_2O_2)] \times 100}{OD(_{cell}) - OD(H_2O_2)}$$

4. RESULTS AND DISCUSSION

Compound **1** was obtained as a white amorphous powder. The ^{1}H-NMR of **1** showed the presence of four protons of a 1,2-disubstituted benzene ring at δ_H 7.79 (1H, d, *J* = 7.5 Hz), 7.46 (1H, t, *J* = 7.5 Hz), 7.33 (1H, d, *J* = 8.5 Hz), and 7.09 (1H, t, *J* = 7.5 Hz); and a methoxy signal at δ_H 3.89 (3H, s). Besides, three anomeric protons were observed at δ_H 5.34 (1H, d, *J* = 7.0 Hz), 4.86 (1H, overlapped), and 4.31 (1H, d, *J* = 7.0 Hz) suggesting the presence of a trisaccharide moiety. The ^{13}C-NMR and HSQC spectrum of **1** gave 25 carbon signals, including a carbonyl carbon at δ_C 167.9, six aromatic carbons at δ_C at 157.6 (C), 135.2 (CH), 132.4 (CH), 122.6 (CH), 121.2 (C), and 116.5 (CH); a methoxy at δ_C 52.6. The anomeric carbon signal at δ_C 105.3 and oxymethylene carbon at δ_C 66.8 suggested the presence of a xylopyranose. Besides, two anomeric carbon signals were observed at δ_C 99.6 and 104.3 together with an oximethine carbon at δ_C 82.9; and two oxymethylene carbons at δ_C 69.6 and 61.4 were indicative for the presence of the 2-*O*-*β*-D-glucopyranosyl-(1→2)-[*O*-*β*-D-xylopyranosyl-(1→6)]-*O*-*β*-D-glucopyranoside fragment. The NMR data of **1** were similar to methyl salicylate 2-*O*-triglycoside [3].

The structure of **1** was identified with the aid of HSQC and HMBC spectra (Fig. 2). HMBC correlations between anomeric protons H-1′ (δ_H 5.34, Glc1) and C-1 (δ_C 157.5); H-1″ (δ_H 4.86, Glc2) and C-2′ (δ_C 82.9); H-1□″ (δ_H 4.31, Xyl) and C-6′ (δ_C 69.6) were indicative for the 2-*O*-*β*-D-glucopyranosyl-(1→2)- [*O*-*β*-D-xylopyranosyl-(1→6)]-*O*-*β*-D-glucopyranoside structure; and the position of methoxy at C-7 was confirmed by HMBC correlation between methoxy proton (δ_H 3.89) and C-7 (δ_C 167.9). Based on above evidence, the structure of **1** was established as methyl salicylate 2-*O*-triglycoside [3].

Compound **2** was obtained as amorphous powder. The ^{1}H-NMR of **2** showed two protons of a double bond (*trans* configuration) at δ_H 6.58 (1H, d, *J* = 16.0 Hz) and 6.33 (1H, dt, *J* = 5.5, 16.0 Hz); three protons of a 1,3,4-trisubstituted aromatic ring at δ_H 6.60 (1H, d, *J* = 1.5 Hz), 6.57 (1H, br s), and 6.50 (1H, dd, *J* = 1.5, 8.0 Hz); two other singlet aromatic protons at δ_H 6.95 (1H, br s), and 6.94 (1H, brs); two methoxy protons at δ_H 3.85 (3H, s) and 3.71 (3H, s). The signal of an anomeric proton at δ_H 4.70 (1H, d, *J* = 7.0 Hz) suggested the presence of one sugar moiety.

The ^{13}C-NMR and HSQC spectrum of **2** revealed 26 carbons, including two olefin carbons at δ_C 131.5 and 129.7; six carbons of a β-*D*-glucose moiety at δ_C 105.4, 76.0, 77.9, 71.3, 78.1, and 62.5; a methine carbon at δ_C 42.8; two oxymethylenes and a methylene at δ_C 66.8, 63.7 and 39.2; two methoxy carbons at δ_C 56.4 and 56.3; and 12 carbons of two aromatic rings including 7

quaternary carbons and 5 methine carbons. The NMR data of **2** were very similar to those of ircariside E_5 [4]. The HMBC correlations (Fig. 2) from H-2 (δ_H 6.57) and H-5 (δ_H 6.60) to C-1 (δ_C 133.2), C-3 (δ_C 148.4), and C-4 (δ_C 145.4); from H-6 (δ_H 5.50) to C-4 (δ_C 145.4) and C-7 (δ_C 39.2); and from methoxy proton (δ_H 3.71) and C-3 (δ_C 148.4) confirmed the position of methoxy group at C-3. The HMBC correlations between H-9′ (δ_H 4.25) and C-7′ (δ_C 131.5)/ C-8′ (δ_C 129.7); H-7′ (δ_H 6.58) and C-2′ (δ_C 109.1)/ C-6′ (δ_C 119.2); H-2′ (δ_H 6.95)/H-6′ (δ_H 6.94)/H-1″ (δ_H 4.70, Glc) and C-4′ (δ_C 145.0); and between methoxy proton (δ_H 3.85) and C-3′ (δ_C 153.5) confirmed the position of the double bond at C-7′, glucose moiety at C-4′, and methoxy group at C-3′, respectively. Base on above evidence and comparison with the reported data, the chemical structure of **2** was identified as icariside E_5 (Fig. 1).

Compounds **3** and **4** were identified as quercetin 3-*O*-rutinoside [5] and isorhamnetin 3-*O*-rutinoside [6], respectively, based on spectral evidences, which were in agreement with those of the reported data in literature.

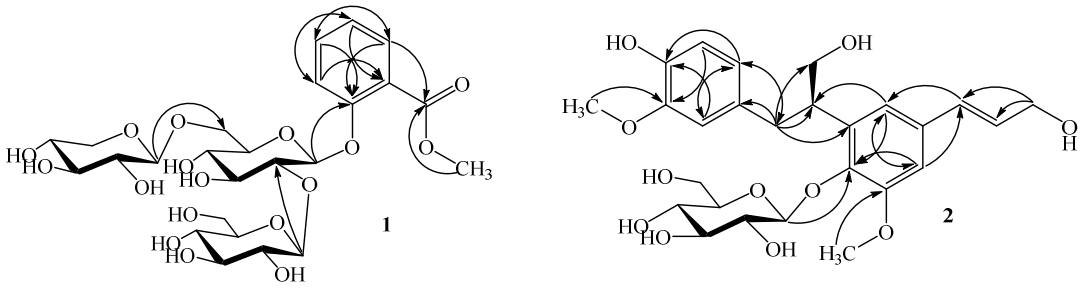

Figure 2. Key HMBC correlations of **1** and **2**.

Table 1. ^{1}H- and ^{13}C-NMR data for **1**-**2** and reference compounds.

No.		1		No.		2	
	$^{\#}\delta_C$ [d]	δ_C [a,b]	δ_H [a,c] (mult., *J* in Hz)		$^{\$}\delta_C$ [a]	δ_C [a,b]	δ_H [a,c] (mult., *J* in Hz)
1	121.0	121.2	-	1	133.2	133.2	-
2	157.4	157.5	-	2	116.0	115.7	6.57 (br s)
3	116.4	116.5	7.33 (d, 8.5)	3	148.3	148.4	-
4	134.1	135.2	7.46 (t, 7.5)	4	145.3	145.4	-
5	121.5	122.6	7.09 (t, 7.5)	5	114.1	113.8	6.60 (d, 1.5)
6	131.6	132.4	7.79 (d, 7.5)	6	122.8	122.6	6.50 (dd, 1.5, 8.0)
7	166.3	167.9	-	7	39.1	39.2	2.74 (dd, 9.5, 14.0) 2.99 (dd, 6.0, 14.0)
7-OMe	52.0	52.6	3.89 (s)	8	43.0	42.8	3.98 (m)
		3-O-Glucosyl		9	66.5	66.8	3.70 (m) 3.80 (d, 11.0)
1'	100.0	99.6	5.34 (d, 7.0)	1'	135.4	135.4	-
2'	82.7	82.9	3.85*	2'	108.7	109.1	6.95 (br s)
3'	77.8	77.4	3.72 (t, 8.5)	3'	153.4	153.5	-
4'	71.1	70.86	3.48*	4'	145.0	145.0	-
5'	77.5	77.6	3.99*	5'	139.0	139.0	-
6'	69.5	69.6	3.78 (dd, 1.5, 11.5) 4.11 (d, 11.5)	6'	118.7	119.2	6.94 (br s)

		2″-O-Glucosyl		7'	131.5	131.5	6.58 (d, 16.0)
1"	105.7	104.3	4.86*	8'	129.6	129.7	6.33 (dt, 5.5, 16.0)
2"	76.8	76.0	3.23 (m)	9'	63.5	63.7	4.25 (dd, 1.0, 6.0)
3"	78.1	77.1	3.16*	3-OMe	56.0	56.3	3.71 (s)
4"	71.2	70.6	3.40*	3'-OMe	55.9	56.4	3.85 (s)
5"	78.3	77.3	3.14 (m)			4′-O-Glucosyl	
6"	62.3	61.4	3.17 (d, 11.5) 3.50*	1"	106.0	105.4	4.70 (d, 7.0)
		6″-O-Xylosyl		2"	75.7	76.0	3.48 (dd, 7.0, 8.5)
1‴	105.9	105.3	4.31 (d, 7.0)	3"	77.5	77.9	3.43 (t, 8.5)
2‴	75.0	75.0	3.19 (d, 2.5)	4"	71.0	71.3	3.39 (t, 8.5)
3‴	78.1	77.4	3.39*	5"	77.8	78.1	3.13 (m)
4‴	70.8	71.1	3.51*	6"	62.3	62.5	3.68* 3.79 (d, 12.0)
5‴	67.1	66.8	3.10 (t, 11.5) 3.83 (dd, 5.0, 11.5)				

Measured in [a] CD_3OD, [b] 125 MHz, [c] 500 MHz, [d] pyridine. [#] δ_C of methyl salicylate 2-O-triglycosides [3], *[$] δ_C of icariside E_5* [4], **overlapped signal.*

All compounds were evaluated for their protective effects on H_2O_2 induced Balb/c mice hepatocytes injury. Briefly, hepatocytes were isolated from the livers of healthy Balb/c mice. The cells were cultured, treated with the compounds and H_2O_2. The damaged cells were measured via MTT assay and the protective effects of the samples were calculated by comparing cell viability with the vehicles (Table 2).

Table 2. Hepatoprotecive effects of compounds **1-4**.

Concentration (µg/ml)	**1**	**2**	**3**	**4**	**Curcumin**
100	5.19	8.65	42.12	4.22	62.14
20	13.57	13.73	52.14	0.71	39.29
4	11.03	10.08	28.33	-	18.33
0.8	-	-	7.86	-	4.52
EC_{50}	**>100**	**>100**	**17.08**	**>100**	**4.56**

(-) Hepatoprotective effects were not observed.

In comparison with positive control, curcumin (EC_{50} 4.6 ± 0.3 µg/mL), compound **3** exhibited significant protective activity (EC_{50} 17.08 µg/mL), while others were inactive (EC_{50} > 100 µg/mL). The results suggest that the free hydroxyl group in position C-3′ of compound **3** may play an important role in its hepatoprotective activity.

4. CONCLUSIONS

By various chromatographic separations, four phenolic glycosides, methyl salicylate 2-*O*-triglycoside (**1**), icariside E_5 (**2**), quercetin 3-*O*-rutinoside (**3**), and isorhamnetin 3-*O*-rutinoside (**4**) were isolated from the methanol extract of the whole medicinal plant *Physalis angulata* collected in Thai Binh province, Vietnam. Their structures were determined by 1D-, 2D-NMR spectroscopic analyses and by comparison with reported data. Compounds **3** exhibited

significant hepatoprotective activity with an EC_{50} value of 17.08 µg/mL. This is the first report of **1**-**4** from *Physalis angulata.*

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