Natural Product Sciences
17(3) : 206-211 (2011)

Isolation of Prenylated Isoflavonoids from *Cudrania tricuspidata* Fruits that Inhibit A2E Photooxidation†

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Abstract – High-performance liquid chromatography coupled to an online $ABTS^+$-based assay (online HPLC-$ABTS^+$) system was used to determine the principal antioxidants in *Cudrania tricuspidata* fruits. Six prenylated isoflavonoids (**1** - **6**) were isolated from *C. tricuspidata* fruits according to the online HPLC-$ABTS^+$ system. The structures of isolated compounds, alpiniumisoflavone (**1**), 6,8-diprenylorobol (**2**), 6,8-diprenylgenistein (**3**), pomiferin (**4**), 4'-methylalpiniumisoflavone (**5**), and osajin (**6**) were identified by their retention time, UV spectra, ESI-MS, and NMR data. Among these compounds, 6,8-diprenylorobol (**2**) and pomiferin (**4**) reduced A2E photooxidation in a dose dependent manner.
Keywords – *Cudrania tricuspidata*, prenylated isoflavonoids, online HPLC-$ABTS^+$ system, A2E photooxidation, 6,8-diprenylorobol, pomiferin

Introduction

Age-related macular degeneration (AMD) is the major cause of irreversible blindness in developed countries. Cellular damage due to high levels of oxidative stress appears to be one of the main pathological explanations for age-related ocular diseases including AMD, and cellular accumulation of lipofuscin, a complex mixture of highly fluorescent retinoid and phosphoethanolamines, is considered to be a primary pathogenic biomarker of aging in the retinal pigment epithelium (RPE) (Bhosale *et al*., 2009). A2E, a bisretinoid, is one of the important constituents of lipofuscin in the RPE. The photosensitization of A2E leads to the generation of singlet oxygen, and reaction of the latter at carbon-carbon double bonds leads to A2E photooxidation. Ultimately, the photochemical events provoked by the irradiation of A2E in RPE cells initiates cell death (Sparrow *et al*., 2000; Ben-Shabat *et al*., 2002, Sparrow *et al*., 2002). Natural antioxidants, such as anthocyanins, carotenoids, vitamin E, and flavonoids, showed protective effects against A2E photooxidation and against A2E and light mediated-cell death in RPE cells (Sparrow *et al*., 2003; Jang *et al*., 2005; Laabich *et al*., 2007).

The cortex and root bark of *Cudrania tricuspidata* (carr.) Bur. (Moraceae) has been used as a traditional medicine for the treatment of neuritis and inflammation (Jung and Shin, 1990). Several bioactive prenylated flavonoids and xanthones have been isolated from the root bark of *C. tricuspidata* (Fujimoto *et al*., 1984; Hano *et al*., 1990a, b; Hano *et al*., 1991; Zou *et al*., 2005). The fruits of *C. tricuspidata* also contain carotenoids (Novruzov and Agamirov, 2002), prenylated isoflavonoids and benzylated flavonoids that inhibit inflammation and monoamine oxidase (Han *et al*., 2005; Han *et al*., 2009). Plant materials usually have hundreds of organic compounds with different types of structures. So, isolation and structural elucidation of bioactive compounds from natural products requires many chromatographic steps and takes a lot of time. Thus, numerous HPLC hyphenated analysis techniques, such as LC-MS and LC-NMR, have been developed. Recently, an online antioxidant screening system was developed using a post-column reaction with ABTS or DPPH reagents (Nuengchamnong *et al*., 2005; Stewart *et al*., 2005; Pérez-Bonilla *et al*., 2006; Kim *et al*., 2009).

This paper describes a method for the rapid detection of antioxidants in *C. tricuspidata* fruits using an online HPLC-$ABTS^+$ system and their ability to inhibit A2E photooxidation.

†Dedicated to Professor Young Choong Kim of the Seoul National University for her leading works on Pharmacognosy.
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Experimental

General Experimental Procedures – HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Other solvents were obtained from Daejung Chemicals & Metals Co. Ltd (Siheung, Gyeonggi-do, Korea). The following reagents were used for radical scavenging assays. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and potassium persulfate were purchased from Sigma-Aldrich. A2E was synthesized as previous described (Parish *et al*., 1998). TLC was carried out on silica gel 60 F_{254} and RP-18 F_{254} plates (Merck, Germany). Column chromatography was performed over silica gel 60 (Merck, particle size 230 - 400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on a Varian 500 Unity Inova spectrometer (Varian, Palo Alto, USA) at 500 MHz and 125 MHz, respectively. ESI-MS was measured with an LCQ Fleet ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA).

Plant materials – The dried fruits of *C. tricuspidata* were purchased from Kyungdong oriental herbal market, Korea in November 2010. A voucher specimen was deposited in the KIST herbarium (No, KIST-CT-F001).

Online detection of radical scavengers – The detection of radical scavenging constituents in *C. tricuspidata* was carried out using an online HPLC-$ABTS^{+}$ system essentially as described by Stewart (Stewart *et al*., 2005). For an $ABTS^{+}$ reagent, 2 mM $ABTS^{+}$ stock solution containing 3.5 mM of potassium persulphate was prepared in distilled water and stored at room temperature for 14 hours in the dark to allow for stabilization of the radical. The working $ABTS^{+}$ reagent was prepared by diluting the stock 8-fold in water. The analytical column was a reversed-phase Atlantis dC18 column (3.0 × 150 mm, 3 μm, Waters). Injection volume was 10 μl and a linear gradient mobile phase system was adopted (50% to 100% acetonitrile in H_2O with 0.1% formic acid) with a run time of 30 minutes. The flow rate for the main analytical HPLC system was 0.3 ml/min and the reagent pump flow was adjusted to 0.15 ml/min. The respective retention times of the major compounds were 18.3, 18.6, 22.0, 24.2, 25.7, and 27.6 min at 280 nm; a negative trace at 734 nm indicated the antioxidant potential of the separated components.

Extraction and isolation – Components of the dried fruits (600g) were extracted at room temperature with methanol (1000 mL × 3) using an ultrasonic apparatus; they were then concentrated *in vacuo* to yield 150 g of crude extract. The concentrated residue was resuspended in 1000 mL water and was partitioned three times with 1000 mL of *n*-hexane, and methylene chloride; a total of 25 g of an *n*-hexane layer and 15 g of a methylene chloride layer was obtained. The *n*-hexane soluble fraction (20 g) was chromatographed on a silica gel (230 -400 mesh, 6 × 20 cm) column eluted with *n*-hexane-ethyl acetate (10 : 1 → 1 : 1, gradient system) to yield 13 subfractions (CTH-1 – CTH-13). CTH-8 was further purified over HPLC (20 × 250 mm, Hydrosphere C18, YMC, 75% acetonitrile, flow rate 10 ml/min) to yield 1.36 g of compound **1**, 1.53 g of compound **2** and 2.97 g of compound **3**. CTH-13 was further purified using HPLC (20 × 250 mm, Hydrosphere C18, YMC, 70% acetonitrile, flow rate 10 ml/min) to afford 53 mg of compound **4**. Compounds **5** and **6** were crystallized and purified from CTH-3 and CTH-4. The structural identification of isolated compounds (**1** - **6**) was carried out by ESI-MS, ^{1}H and ^{13}C NMR including 2D NMR (COSY, HSQC, and HMBC).

Alpinumisoflavone (1) – pale yellow needle; ESI-MS *m/z* 337 $[M+H]^{+}$; UV λ_{max} 281 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.1 (1H, s, 5-OH), 7.81 (1H, s, H-2), 6.34 (1H, s, H-8), 7.35 (2H, d, *J* = 8.6, H-2',H-6'), 6.84 (2H, d, *J* = 8.6, H-3', H-5'), 5.63 (H, d, *J* = 10.0, H-3"), 6.72 (1H, d, *J* = 10.0, H-4"), 1.47 (6H, s, H-2"-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.8 (C-2), 123.7 (C-3), 181.1 (C-4), 157.0 (C-5), 105.6 (C-6), 159.7 (C-7), 95.0 (C-8), 157.4 (C-9), 106.2 (C-10), 122.8 (C-1'), 130.4 (C-2', C-6'), 115.8 (C-3', C-5'), 156.2 (C-4'), 78.2 (C-2"), 128.3 (C-3"), 115.6 (C-4"), 28.4 (C-2"-CH_3).

6,8-Diprenylorobol (2) – pale yellow needle; ESI-MS *m/z* 423 $[M+H]^{+}$, 421 $[M-H]^{-}$; UV λ_{max} 272 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.1 (1H, s, 5-OH), 7.87 (1H, s, H-2), 6.95 (1H, d, *J* = 1.1, H-2'), 6.80 (1H, d, *J* = 8.1, H-5'), 6.73 (1H, dd, *J* = 8.1, 1.1, H-6'), 3.43 (2H, m, H-1"), 5.23 (1H, m, H-2"), 1.83 (3H,s, H-4"), 1.75 (3H, s, H-5"), 3.45, (2H, m, H-1'"), 5.21 (1H, m, H-2'"), 1.73, (3H, d, *J* = 0.9, H-4'"), 1.82 (3H, d, *J* = 0.9, H-5'"); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.4 (C-2), 123.6 (C-3), 181.9 (C-4), 157.4 (C-5), 110.6 (C-6), 160.0 (C-7), 105.7 (C-8), 163.6 (C-9), 105.8 (C-10), 122.8 (C-1'), 116.6 (C-2'), 144.1 (C-3'), 144.8 (C-4'), 115.6 (C-5'), 121.4 (C-6'), 21.8 (C-1"), 121.4 (C-2"), 135.6 (C-3"), 18.1 (C-4"), 26.0 (C-5"), 21.8 (C-1'"), 121.5 (C-2'"), 134.5 (C-3'"), 25.9 (C-4'"), 18.0 (C-5'").

6,8-Diprenylgenistein (3) – pale yellow needle; ESI-MS *m/z* 407[$M+H]^{+}$, 405 $[M-H]^{-}$; UV λ_{max} 270 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.1 (1H, s, 5-OH), 7.82 (1H, s, H-2), 7.26 (2H, d, *J* = 8.5, H-2',H-6'), 6.77 (2H, d, *J* = 8.5, H-3', H-5'), 3.40 (2H, m, H-1"), 5.18 (1H, m, H-2"), 1.76 (3H, d, *J* = 1.1, H-4"), 1.69 (3H, d, *J* = 1.1, H-5"), 3.40, (2H, m, H-1'"), 5.14 (1H, m, H-2'"), 1.66, (3H,

d, J = 0.7, H-4'"), 1.75 (3H, d, J = 0.7, H-5'"); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.8 (C-2), 123.4 (C-3), 181.6 (C-4), 157.6 (C-5), 110.4 (C-6), 159.7 (C-7), 105.4 (C-8), 153.5 (C-9), 105.9 (C-10), 122.7 (C-1',), 130.4 (C-2', C-6'), 115.8 (C-3', C-5'), 156.6 (C-4'), 21.8 (C-1"), 121.4 (C-2"), 135.6 (C-3"), 18.0 (C-4"), 26.0 (C-5"), 21.8 (C-1'"), 121.7 (C-2'"), 134.2 (C-3'"), 25.9 (C-4'"), 18.1 (C-5'").

Pomiferin (4) – pale yellow powder; ESI-MS *m/z* 421 $[M + H]^+$; UV λ_{max} 273 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.9 (1H, 5-OH), 7.80 (1H, s, H-2), 6.91 (1H, d, J = 1.1, H-2'), 6.73 (1H, d, J = 7.1, H-5'), 6.68 (1H, dd, J = 7.1, 1.1, H-6'), 3.32 (2H, d, J = 7.0, H-1"), 5.16 (1H, m, H-2"), 1.76 (3H, s, H-4"), 1.63 (3H, s, H-5"), 5.56 (H, d, J = 10.0, H-3'"), 6.65 (1H, d, J =10.0, H-4'"), 1.41 (6H, s, H-2'"-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.6 (C-2), 123.8 (C-3), 181.2 (C-4), 159.3 (C-5), 113.2 (C-6), 157.4 (C-7), 100.9 (C-8), 150.4 (C-9), 105.6 (C-10), 122.8 (C-1'), 116.6 (C-2'), 144.1 (C-3'), 144.8 (C-4'), 115.6 (C-5'), 121.8 (C-6'), 21.6 (C-1"), 121.9 (C-2"), 131.6 (C-3"), 18.1 (C-4"), 26.2 (C-5"), 77.8 (C-2'"), 127.4 (C-3'"), 115.2 (C-4'"), 28.3 (C-2'"-CH_3).

4'-*O*-methylalpinumisoflavone (5) – pale yellow needle; ESI-MS *m/z* 351 $[M + H]^+$, 349 $[M-H]^-$; UV λ_{max} 282 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.2 (1H, s, 5-OH), 7.82 (1H, s, H-2), 6.33 (1H, s, H-8), 7.45 (2H, d, J = 8.6, H-2', H-6'), 6.98 (2H, d, J = 8.6, H-3', H-5'), 5.62 (H, d, J = 10.1, H-3"), 6.73 (1H, d, J =10.1, H-4"), 3.84 (3H, s, H-4'-OCH_3), 1.47 (6H, s, H-2"-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.6 (C-2), 123.6 (C-3), 181.0 (C-4), 157.1 (C-5), 105.7 (C-6), 159.7 (C-7), 95.0 (C-8), 157.3 (C-9), 106.2 (C-10), 123.1 (C-1',), 130.3 (C-2', C-6'), 114.2 (C-3', C-5'), 159.9 (C-4'), 55.5 (C-4'-OCH_3), 78.2 (C-2"), 128.3 (C-3"), 115.6 (C-4"), 28.4 (C-2"-CH_3).

Osajin (6) – pale yellow needle; ESI-MS *m/z* 405 $[M + H]^+$; UV λ_{max} 272 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.1 (1H, s, 5-OH), 7.86 (1H, s, H-2), 7.33 (2H, d, J = 8.5, H-2', H- H-6'), 6.83 (2H, d, J = 8.5, H-3', H-5'), 3.35 (2H, d, J = 7.3, H-1"), 5.24 (1H, m, H-2"), 1.81 (3H, s, H-4"), 1.68 (3H, s, H-5"), 5.59 (H, d, J = 9.9, H-3'"), 6.70 (1H, d, J = 9.9, H-4'"), 1.48 (6H, s, H-2'"-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.4 (C-2), 123.7 (C-3), 181.1 (C-4), 159.3 (C-5), 113.0 (C-6), 157.40 (C-7), 100.8 (C-8), 150.6 (C-9), 105.6 (C-10), 123.0 (C-1'), 130.5 (C-2', C-6'), 115.8 (C-3', C-5'), 155.0 (C-4'), 21.4 (C-1"), 122.0 (C-2"), 131.7 (C-3"), 18.0 (C-4"), 26.0 (C-5"), 77.9 (C-2'"), 127.3 (C-3'"), 115.1 (C-4'"), 28.3 (C-2'"-CH_3).

Photooxidation of A2E – To study the effects of isolated prenylated isoflavonoids (**1 - 6**) on the photooxidation of A2E, 200 μM A2E in 0.1 ml dimethyl sulfoxide (DMSO) was added to 0.2 ml of water with and without compounds **1 - 6** at 50, 100 and 200 μM concentrations. The mixture was subsequently illuminated at 450 nm (4.5 mW cm^{-2}, 5 min). For quantification of A2E, an Agilent 1200 system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector and operated with Agilent ChemStation software was used with an Atlantis dC18 column (3.0 × 150 mm, 3 μm, Waters) and an acetonitrile/water (containing 0.1% trifluoroacetic acid) gradient: 85 - 100% (0 - 10 min), with a flow rate of 0.5 ml/min and monitoring at 430 nm. Injection volumes were 20 μL, and peak area was expressed as percent of control (untreated sample). Samples in each experiment were assayed in triplicate.

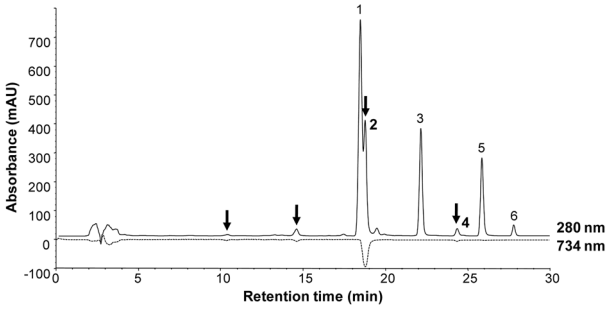

Fig. 1. Online HPLC-ABTS$^+$ analysis of a methanol extract of *C. tricuspidata* fruits. Crude extract analyzed by gradient reverse-phase HPLC with a DAD detector at 280 nm (positive trace) prior to reaction with ABTS$^+$ radicals and analysis of antioxidant potential at 734 nm (negative trace). The following peaks showed radical scavenging activity: (**1**) alpiniumisoflavone; (**2**) 6,8-diprenylorobol; (**3**) 6,8-diprenylgenistein, (**4**) pomiferin, (**5**) 4'-methylalpiniumisoflavone, and (**6**) osajin.

Results and Discussion

C. tricuspidata extract was analyzed using gradient reverse-phase HPLC with a DAD at 280 nm (positive trace) prior to reaction with the ABTS$^+$ radical and analysis of the antioxidant potential at 734 nm (negative trace). The ABTS$^+$-based radical scavenging activity profile showed that four peaks exhibited antioxidant activities (Fig. 1). Without the online HPLC assay system, peak **4** would have been easily overlooked because it was small. The peak for this compound was negligible in the HPLC chromatogram at 280 nm, but in the online assay system, it was larger and very active. As shown in Fig. 1, the online ABTS assay system is an efficient method for purifying antioxidants based on activity-guided chromatographic separation and isolation from natural resources. According to the online ABTS$^+$ assay, radical scavenging compounds (compounds **2** and **4**) were isolated first. Six prenylated isoflavonoids (Fig. 2) were isolated; They

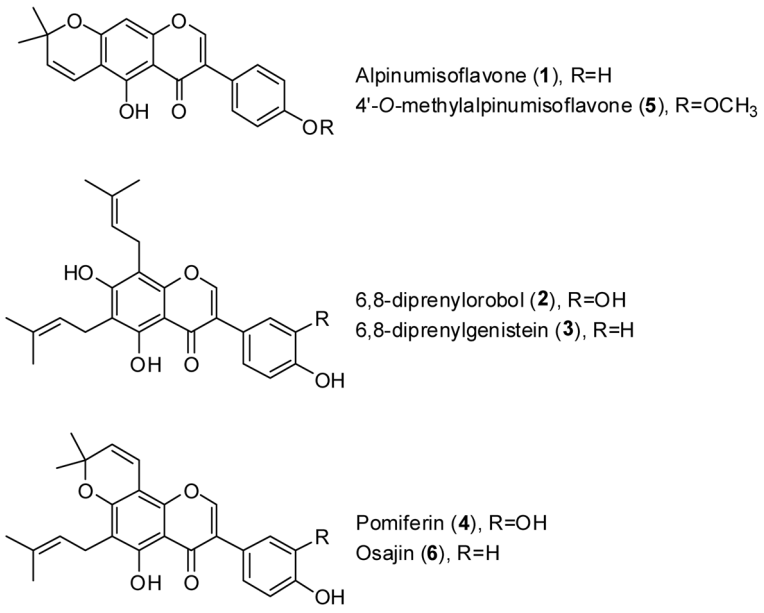

Fig. 2. Chemical structures of isolated compounds from *C. tricuspidata* fruits.

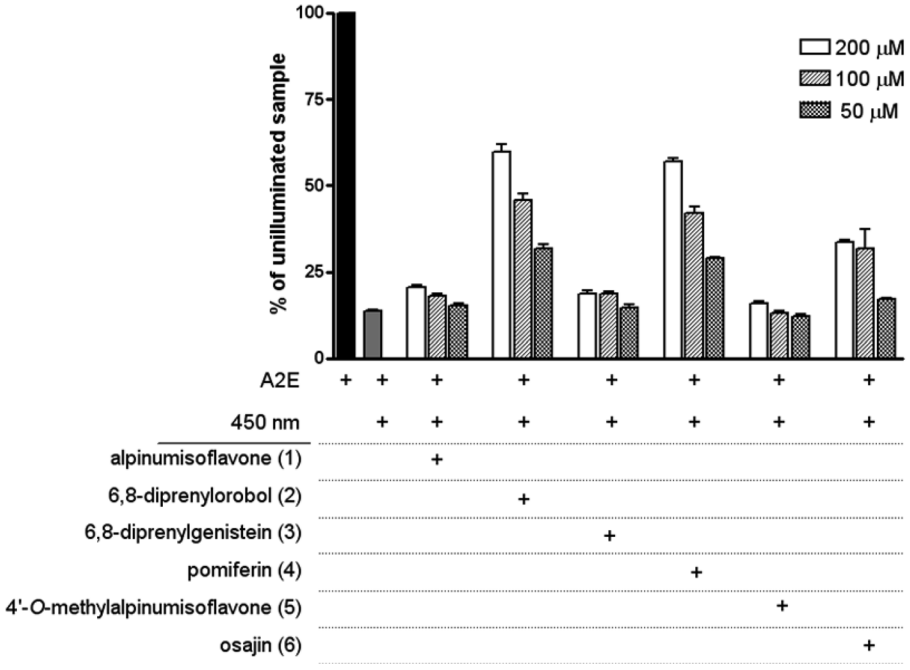

Fig. 3. 6,8-diprenylorobol (**2**) and pomiferin (**4**) reduce A2E photooxidation. The photooxidation of A2E is reected in the reduced content of A2E in a sample after 450 nm illumination. The loss of A2E was attenuated in the presence of antioxidant prenylated isoflavonoids (**2** and **4**) at the indicated concentrations. Means ± SEM of four experiments. The symbol (+) indicates the presence of blue light (450 nm) or compounds.

were identified as alpinium isoflavone (**1**), 6,8-diprenylorobol (**2**), 6,8-diprenylgenistein (**3**), pomiferin (**4**), 4'-methylalpiniumisoflavone (**5**), and osajin (**6**) by comparing ESI-MS, and NMR data with literature values (Singhal *et al*., 1980; Olivares *et al*., 1982; Monache *et al*., 1994).

Compounds **1** - **6** were tested for their effects on A2E photooxidation. Of the six compounds, 6,8-diprenylorobol (**2**) and pomiferin (**4**) reduced A2E photooxidation (Fig. 3). The photooxidation of A2E is reflected in the reduced content of A2E in a sample after 450 nm illumination. Loss of A2E was diminished in the presence of

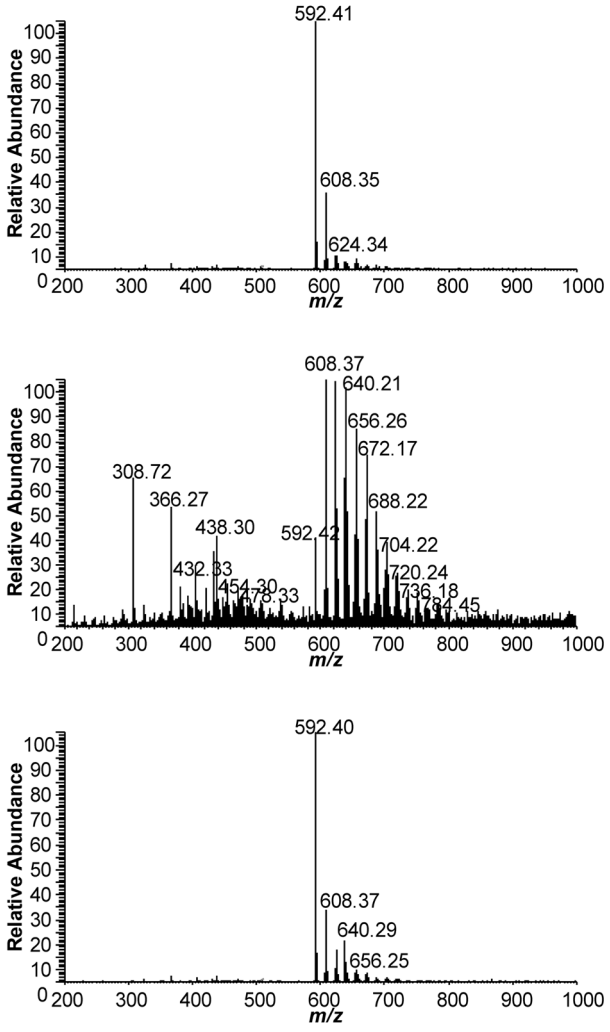

Fig. 4. Photooxidation of A2E and its reduction by 6,8-diprenylorobol (**2**). ESI-MS of non-irradiated A2E (200 μM) **(A),** A2E exposed to blue light (450 nm) **(B)**, and A2E irradiated with blue light in the presence of 6,8-diprenylorobol (200 μM) **(C)**. The molecular ion peak at a mass-to-charge (*m/z)* ratio of 592 corresponds to a molecular mass of A2E. A2E photooxidation in the irradiated samples is evidenced by the presence of additional higher molecular weight peaks: $[M+16]^+$ or $[M+32]^+$ etc. Illumination in the presence of 6,8-diprenylorobol (**2**) reduces the formation of these photooxidation products.

antioxidant prenylated isoflavonoids (**2** and **4**) at the indicated concentrations. Protection against A2E photooxidation by 6,8-diprenylorobol (**2**) and pomiferin (**4**) was apparent when samples were analysed by HPLC to monitor the consumption of A2E as photooxidation proceeded Thus, irradiation of A2E in the absence of 6,8-diprenylorobol (**2**) and pomiferin (**4**) caused a substantial decrease in the absorbance of the A2E peak such that levels were only ~14% of non-irradiated controls. Conversely, addition of 6,8-diprenylorobol (**2**) and pomiferin (**4**) (50, 100 or 200 μM) attenuated the loss of A2E, an effect that was indicative of the suppression of A2E photooxidation. Specifically, levels of A2E irradiated in the presence of 6,8-diprenylorobol (**2**) and pomiferin (**4**) were 4-fold higher than in the absence of these compounds. ESI-MS spectra of non-irradiated A2E, irradiated A2E with blue light (450 nm), and A2E irradiated with blue light are shown in Fig. 4. A molecular ion peak for A2E was found at *m/z* 592 and oxidation products of A2E ion peaks were at *m/z* 608, 624, 640, 688 etc. Protection of 6,8-diprenylorobol (**2**) against A2E photooxidation was confirmed by the absence of molecular ion peaks at *m/z* 672, 688 and 704 and by a reduced intensity of molecular ion peaks (e.g. *m/z* 608, 624, and 640) (Fig. 4). These results show that 6,8-diprenylorobol (**2**) and pomiferin (**4**) from *C. tricuspidata* fruits possess free radical scavenging activities and protect against A2E photooxidation.

After monitoring free radical scavenging activity using HPLC coupled to an online $ABTS^+$-based assay system, six prenylated isoflavonoids were isolated from *C. tricuspidata* fruits. The structures of the isolated compounds were alpiniumisoflavone (**1**), 6,8-diprenylorobol (**2**), 6,8-diprenylgenistein (**3**), pomiferin (**4**), 4'-methylalpiniumisoflavone (**5**), and osajin (**6**). Among these compounds, 6,8-diprenylorobol (**2**) and pomiferin (**4**) reduced A2E photooxidation in a dose dependent manner.

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Received July 20, 2011
Revised August 16, 2011
Accepted August 20, 2011