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APPENDICES

APPENDIX

APPENDIX A

List of the chemicals and materials used in the study

Chemicals/Materials	Source
Absolute ethanol	BDH, England
Ascorbic acid	Merck, Germany
ß-NADPH	Sigma-Aldrich, USA
Bovine serum albumin	Pierce, USA
2-butanol	BDH, England
Catechin	Sigma-Aldrich, USA
Chroloform	BDH, England
Cytochrome c	Sigma-Aldrich, USA
Deoxyribose	Sigma-Aldrich, USA
Diethyl ether	BDH, England
Dimethyl sulfoxide	Amresco, USA
Diphotassium hydrogen phosphate	Merck, Germany
Disodium hydrogen phosphate	BDH, England
5, 5'-dithiobis-(2-nitrobenzoic acid)	Sigma-Aldrich, USA
Dithiothreitol	Sigma-Aldrich, USA
2, 2-Diphenyl-1-picryhydrazyl (DPPH)	Sigma-Aldrich, USA

Ethylene diamine tetraacetic acid	Sigma-Aldrich, USA
Folin-Ciocalteau's phenol reagent	Fluka A.G., Switzerland
Glacial acetic acid	Carlo-Erba, Italy
Glucose-6-phosphate	Sigma-Aldrich, USA
Glucose-6-phosphate dehydrogenase	Sigma-Aldrich, USA
Glutathione reduced form	Wako, Japan
Glutathione oxidized form	Sigma-Aldrich, USA
Glutathione reductase	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
Hemin	Sigma-Aldrich, USA
Hydrochloric acid	Lab-Scan, Ireland
Hydrogen peroxide	Carlo-Erba, Italy
Magnesium chloride	APS Finechem, Australia
Malondialdehyde bis (dimethyl acetal)	Sigma-Aldrich, USA
Mayer's hematoxylin	Bio-Optica, Italy
Methanol	BDH, England
Phenobarbital	Wako, Japan
Phenylmethylsulphonyl fluoride	Sigma-Aldrich, USA
Potassium chloride	Carlo-Erba, Italy
Potassium dihydrogen phosphate	May and Baker, England
Potassium hydroxide	BDH, England

Skim milk	Merck, Germany
Sodium dihydrogen phosphate	Merck, Germany
Sodium bicarbonate	BDH, England
Sodium hydroxide	BDH, England
Tert-butyl hydroperoxide	Sigma-Aldrich, USA
Tricarboxylic acid	BDH, England
Triethanolamine	Sigma-Aldrich, USA
Tris base	Vivantis, Malaysia
Trolox	Sigma-Aldrich, USA
Thiobarbituric acid	Sigma-Aldrich, USA
Uric acid	Sigma-Aldrich, USA
Xanthine	Sigma-Aldrich, USA
Xanthine oxidase	Sigma-Aldrich, USA
Xylene	BDH, England

APPENDIX B

List of the instruments used in the study

Instruments	Model	Source
Autoclave	SS-245	Tomy Seiko, Japan
Centrifugator	PMC-060	Tomy Seiko, Japan
	22R D-78532	Mikro, Germany
Freezer (-86 °C)	0838	Forma Scientific, USA
Hotplate/ stirrer	HPMS	Whatman, USA
Microplate reader	MCC/340	ICN, Flow, USA
pH meter	320	Mettler Toledo, USA
Water bath	W-350	Mammert, Germany
Refrigerator	SR-F511	Sanya, Thailand
Ultracentrifugator	L-100 XP	Beckman Coulter
UV-Vis spectrophotometer	UV-1700	Shimazu, Japan
Vortex	G-560E	Scientific industries, USA

APPENDIX C

Reagents preparation

1. Folin reagent

Folin reagent was made by diluting Folin-Ciocalteu's phenol reagent in distilled water at equal volume.

2. 1% vanillin reagent

1.25 g of vanillin was dissolved in 250 ml of 4% HCl/MeOH.

4. DPPH solution

1.5 mg of DPPH was dissolved in 5 ml of Absolute ethanol

5. 120 mM KH_2PO_4 – KOH buffer pH 7.4

1.633 g of KH_2PO_4 was dissolved in 60 ml of distilled water, pH of solution was adjusted to 7.4 with conc. KOH followed by making total volume 100 ml.

6. Homogenizing buffer

KCl	11.5	g
EDTA * 2Na	0.37	g

Ingredients were dissolved in 1 liter of deionized water. After completely dissolved, 1 ml of 0.25 M PMSF (in ethanol) was added and adjust pH of solution to 7.4 with 1 N NaOH.

7. 0.1 M sodium phosphate buffer pH 7.0

0.2 M of NaH_2PO_4 (dissolving NaH_2PO_4 14.32 g in distilled water 200 ml)

0.2 M of Na_2HPO_4 (dissolving NaH_2PO_4 6.24 g in distilled water 200 ml)

Slowly add 0.2 M NaH_2PO_4 into 0.2 M Na_2HPO_4 until pH value reaches 7.0.

8. 0.1 M sodium phosphate buffer pH 7.5 with 5 mM EDTA

0.2 M of NaH_2PO_4 (dissolving NaH_2PO_4 14.32 g in distilled water 200 ml)

0.2 M of Na_2HPO_4 (dissolving NaH_2PO_4 6.24 g in distilled water 200 ml)

Add 0.2 M NaH_2PO_4 into 0.2 M Na_2HPO_4 until pH reached 7.5, and finally add 270 mM EDTA at ratio 3.7 ml:100 ml (EDTA:buffer). This buffer would be subsequently diluted 10-fold where reaction took place.

9. 10 mM 5, 5'-dithio-bis (2-nitrobenzoic acid)

10 mg of 5, 5'-dithio-bis (2-nitrobenzoic acid) was dissolved in 2.5 ml of 0.1 M phosphate buffer pH 7.5 containing 5 mM EDTA, followed by adding diluted phosphate buffer to final volume of 5 ml.

10. 1M Tris-HCl buffer pH 8.0 with 5 mM EDTA

Tris base	121.14	g
EDTA • 2Na	1.86	g

Buffer was made up by dissolving all ingredients in deionized water. After completely dissolved, pH of solution was adjusted to 8.0 with conc. HCl followed by making total volume 1 liter.

This buffer would be subsequently diluted 10-fold where reaction took place.

11. 0.1 M potassium phosphate buffer pH 7.0

0.2 M of KH_2PO_4 (dissolving KH_2PO_4 14.32 g in distilled water 200 ml)

0.2 M of K_2HPO_4 (dissolving K_2HPO_4 6.24 g in distilled water 200 ml)

Mixing 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 and pH of solution was adjusted to 7.0 with conc. HCl followed by making total volume 1 liter.

13. 100 mM potassium phosphate buffer pH 7.4 with $MgCl_2$

13.61 g of KH_2PO_4 was dissolved in 800 ml of deionized water. After completely dissolved, pH of solution was adjusted to 7.4 with conc. KOH followed by making total volume 1000 ml.

81.2 mg of $MgCl_2$ was dissolved in 200 ml of potassium phosphate buffer.

14. 0.05 M Tris-buffered saline pH 7.6

Tris	6.057 g
NaCl	8.77 g

Buffer was made up by dissolving all ingredients in distilled water. After completely dissolved, pH of solution was adjusted to 7.6 followed by making total volume 1 liter.

15. DAB

30-40 mg of DAB was dissolved in 150 ml of TBS. After completely dissolved, 150 µl of 30% H_2O_2 was added into solution.

APPENDIX D

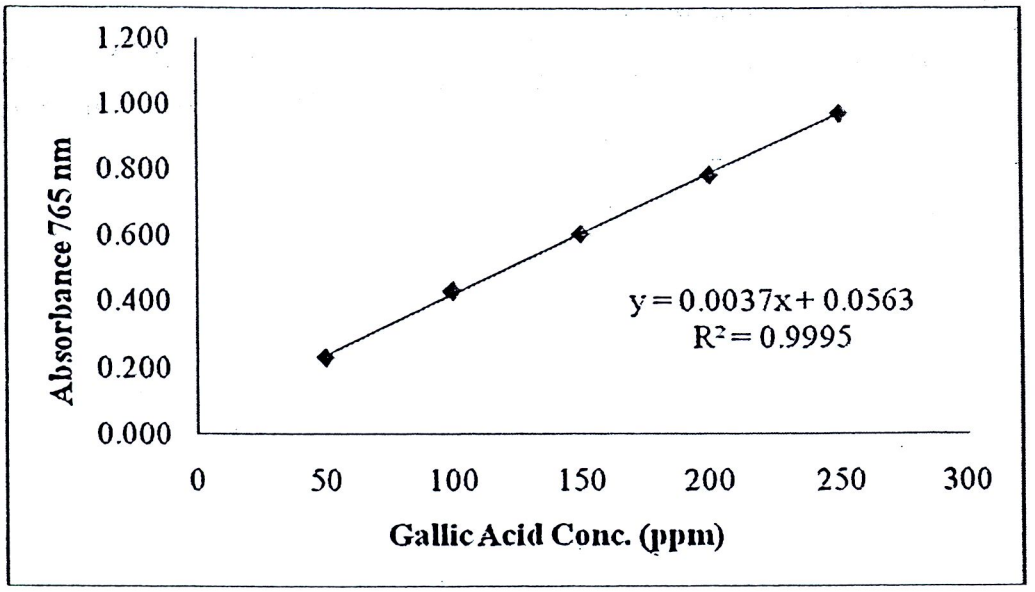

Figure S1 Concentration-response curve of total phenolic compounds.

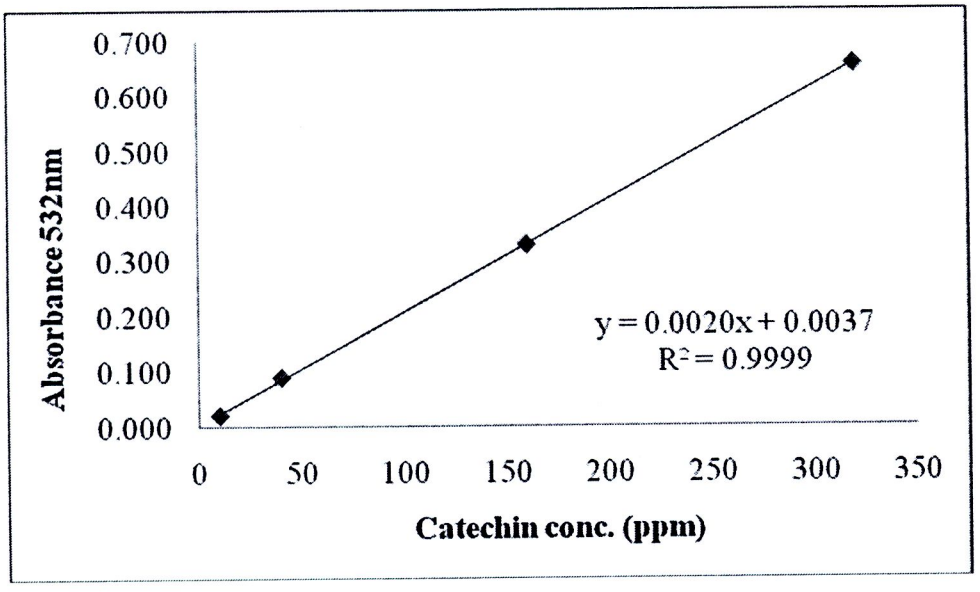

Figure S2 Concentration-response curve of total flavonoids.

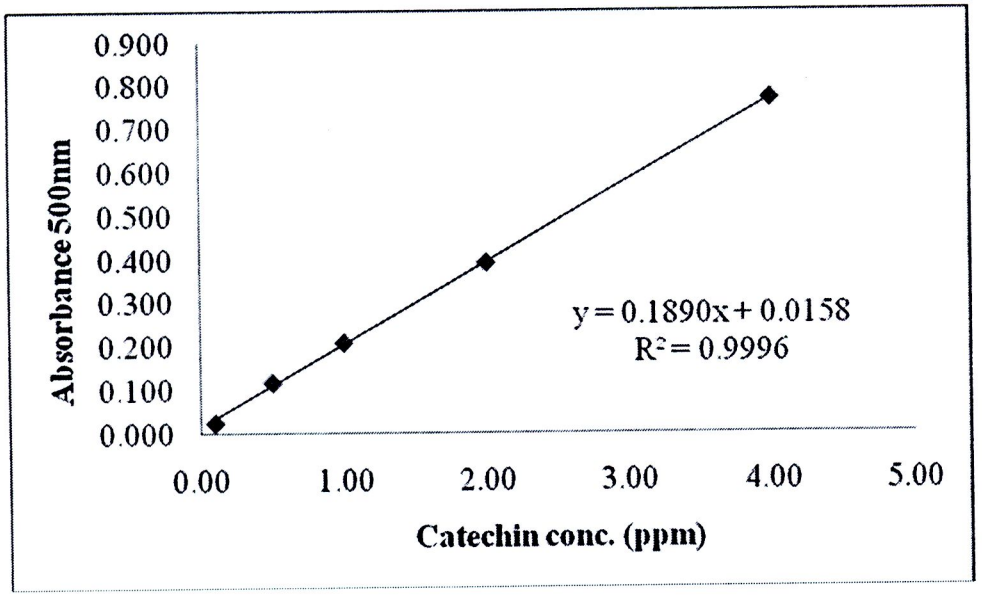

Figure S3 Concentration-response curve of condensed tannins.

Identification of Anthocyanins in Ma-kiang by Liquid Chromatography-Electrospray-Mass Spectrometry (LC-ESI-MS)

1. Methodology

1.1 Anthocynin extraction

Each 0.5 gram of the aqueous extracts of *C. nervosum* from 2 lots (lot 1 for peotocol I and lot 2 for protocol II) was added into a bottle containing 25 ml of 0.1 % formic acid in methanol. After being shaken well for 60 min, the mixture was filtered and the methanolic solution was subjected to solvent evaporation under reduced pressure at temperature below 40° C until its volume was reduced to 2.0 ml

1.2 Anthocyanin separation conditions

Apparatus: An HPLC system (Agilent Technologies, USA) consisting of an automatic sample injector (G1329A), a vacuum solvent degassing unit (G1322A), a quaternary high pressure gradient pump (G1312A), and a photodiode array absorbance detection system (G1315A) was utilized for the separation and analysis of anthocyanins in the *C. nervosum* extracts. A 2.1 × 150 mm Halo column (Agilent Technologies, USA) with a particle size of 2.7 μm was used. The mobile phase consisted of water containing 0.5% formic acid (solvent A) and methanol (solvent B), with gradient elution started at 85:15 (A:B). The flow rate was 0.1 ml/min. The HPLC effluent was passed through the photodiode array detector (PAD), which was set to scan at a wavelength between 200 and 800 nm and monitor at wavelengths of 254 and 520 nm before it was directed into the electrospray ion source of the LC-MS system.

1.3 Electrospray mass spectrometry conditions

In this experiment, the Agilent HPLC system (Agilent Technologies, USA) was used to separate components in the extracts. After separation, the HPLC effluent was delivered into a single quadrupole mass spectrometer (Model G 1946 A, Agilent Technologies, USA) via an orthogonal API-electrospray interface, where it was

nebulized by a nitrogen gas stream. This nitrogen gas was also used as drying gas. The API-electrospray operating parameters were optimized in order to obtain the highest sensitivity for the detection of anthocyanins in the extracts. The optimum electrospray ionization (ESI) conditions were as follows: ionization mode, positive; nebulizer pressure, 32 psi; drying gas flow rate, 10 L/min; drying gas temperature, 350 °C; and capillary voltage, 4000 V. Helium was used as a collision gas and a fragmentor voltage of 130 V was used for the CID. The quadrupole temperature was 100 °C and the electron multiplier voltage was 2650 V.

1.5 Determination of the relative contents of anthocyanins in extracts

Relative contents of anthocyanins in the extracts of *C. nervosum* of the two cultivars were obtained by using LC-ESI-MS technique. Naphtholphthlein was used as the internal standard that was added to the sample extracts at concentration of 25 μg/ml. Peak area normalization was utilized to determine the relative contents of the individual anthocyanin components. Three replicates of each sample extract were analysed by LC-ESI-MS. The contents of anthocyanins in extracts of the two cultivars of *C. nervosum* (1, 2) were determined as the average values.

2. Results

2.1 Identification of anthocyanins by LC-DAD

The UV-Vis spectra of anthocyanins can give information on the nature of the aglycone, glycosylation pattern, and possibility of acylation. Single wavelength detectors can selective monitor anthocyanins between 520 and 546 nm, where no other plant phenolics show absorption at these wavelengths. Figure S4 shows the LC-DAD chromatograms at wavelength 520 nm of extracts from *C. nervosum* (1, 2) which contain the peaks corresponding to anthocyanin components. UV-Vis spectra of these anthocyanin peaks are shown in Figure S5.

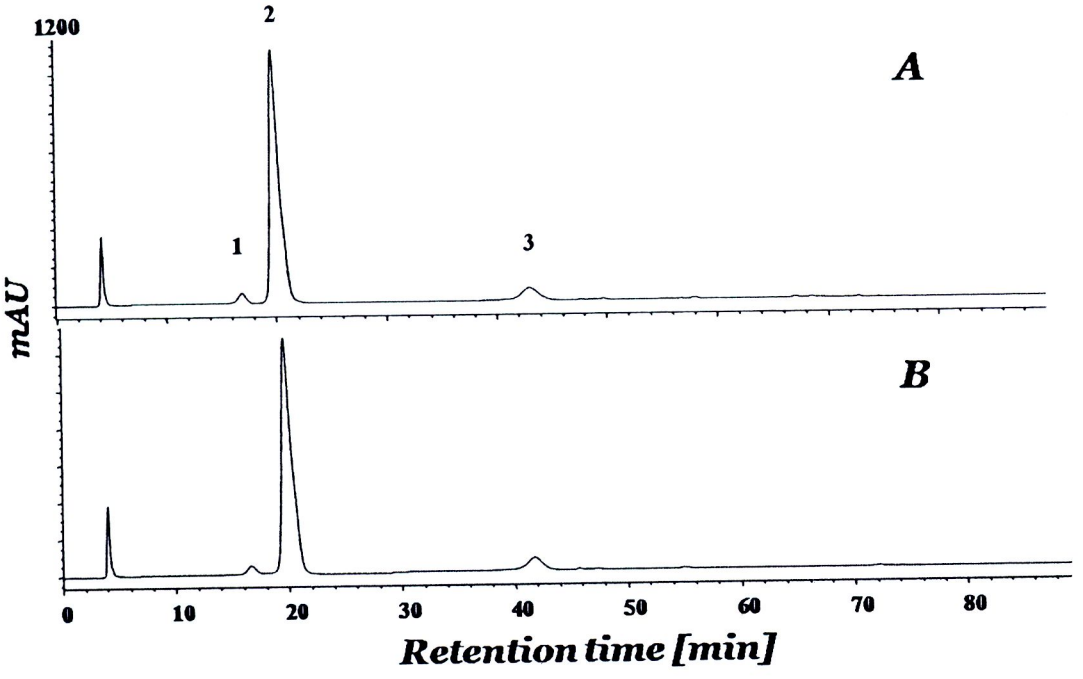

Figure S4 Chromatograms obtained by LC-DAD at wavelength 520 nm of extracts from *C. nervosum* (1, 2); (A) *C. nervosum* 1 (MK-1) (B) *C. nervosum* 2 (MK-2)

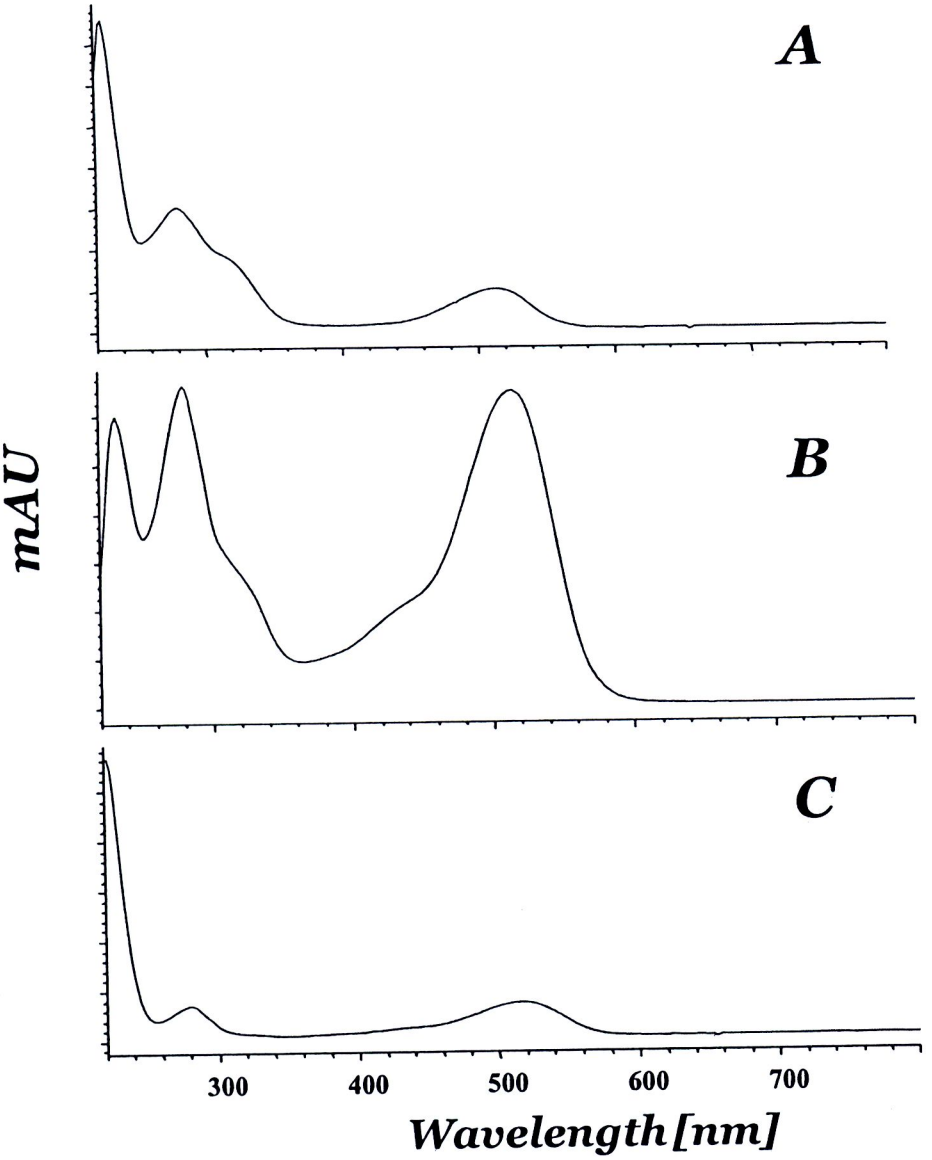

Figure S5 UV-Vis spectra of the peaks shown in Figure 21 obtained by LC-DAD of an extract from *C. nervosum* (1) (A) Peak 1, (B) Peak 2, and (C) Peak 3

2.2 Identification of anthocyanins by LC-MS

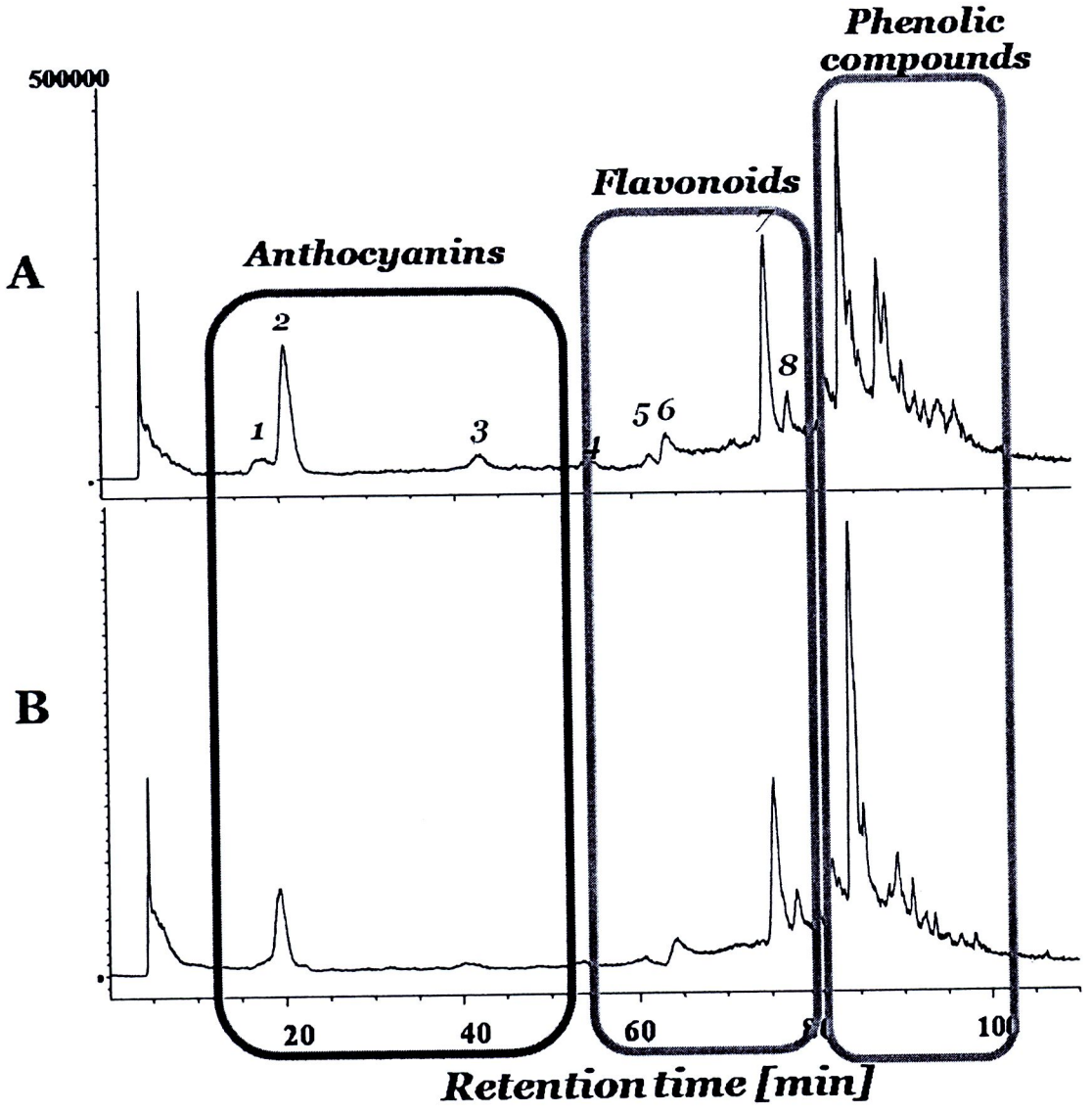

Figure S6 Chromatograms obtained by LC-ESI-MS of the extracts from; (A) *C. nervosum* (MK 1) (B) *C. nervosum* 2 (MK 2)

Peak 1 Cyanidin-3,5-diglucoside

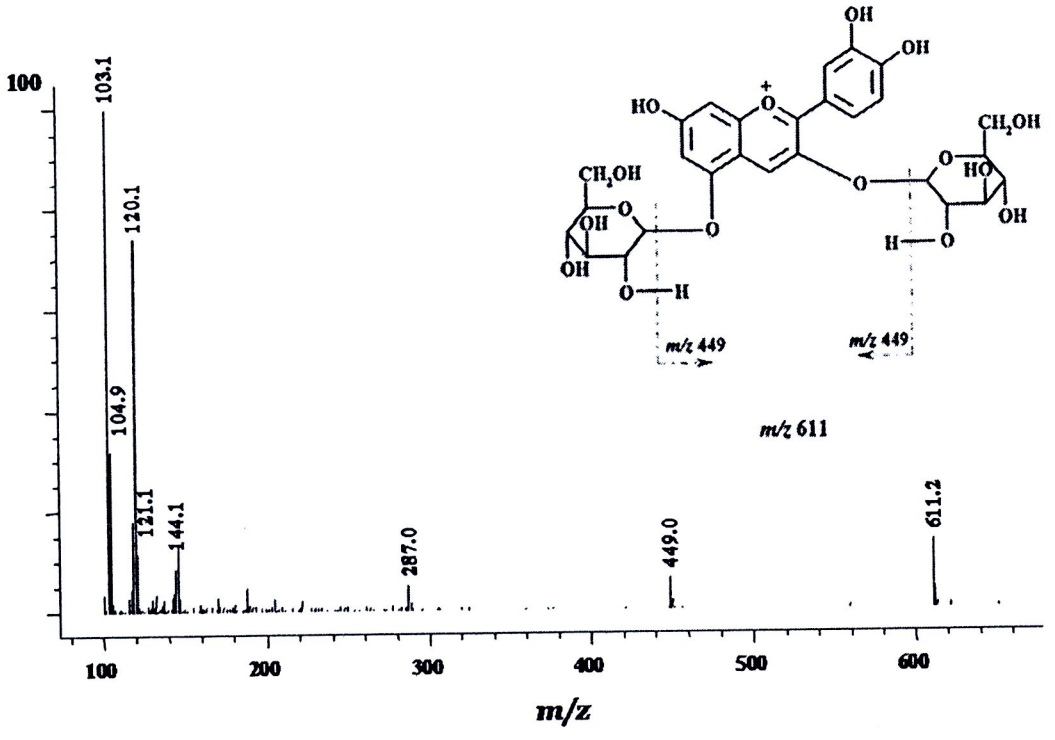

Figure S7 Full scan ESI-MS spectrum of component at retention time 17.80 (Peak number 1)

Mass spectrum of component at retention time 17.80 min (peak 1) obtained from LC-ESI-MS is shown in Figure S7. The molecular cation $[M]^+$ is at m/z 611. This molecular ion has mass equal to that of cyanidin diglucoside (287+162+162 Da). The full scan ESI-MS spectrum also provides corresponding fragment ions at m/z 287 and 449. Fragmentation of the molecular ion which resulted in the production of m/z 449 is corresponded to the loss of a glucose unit and m/z 287 by the loss of two glucose units (162+162 Da) indicating that this anthocyanin is cyanidin-3,5-diglucoside. The presence of fragment ion at *m/z* 449 reveals that the two glucose units are linked to the aglycone at different positions, normally at 3 and 5, and are not joined together as a 3-diglucoside. Regardless of the relatively low intensity, these fragment and molecular ions form a pattern well matched with that of cyanidin-3,5-diglucoside reported previously (Tian et al, 2005).

Peak 2: Cyanidin-3-glucoside

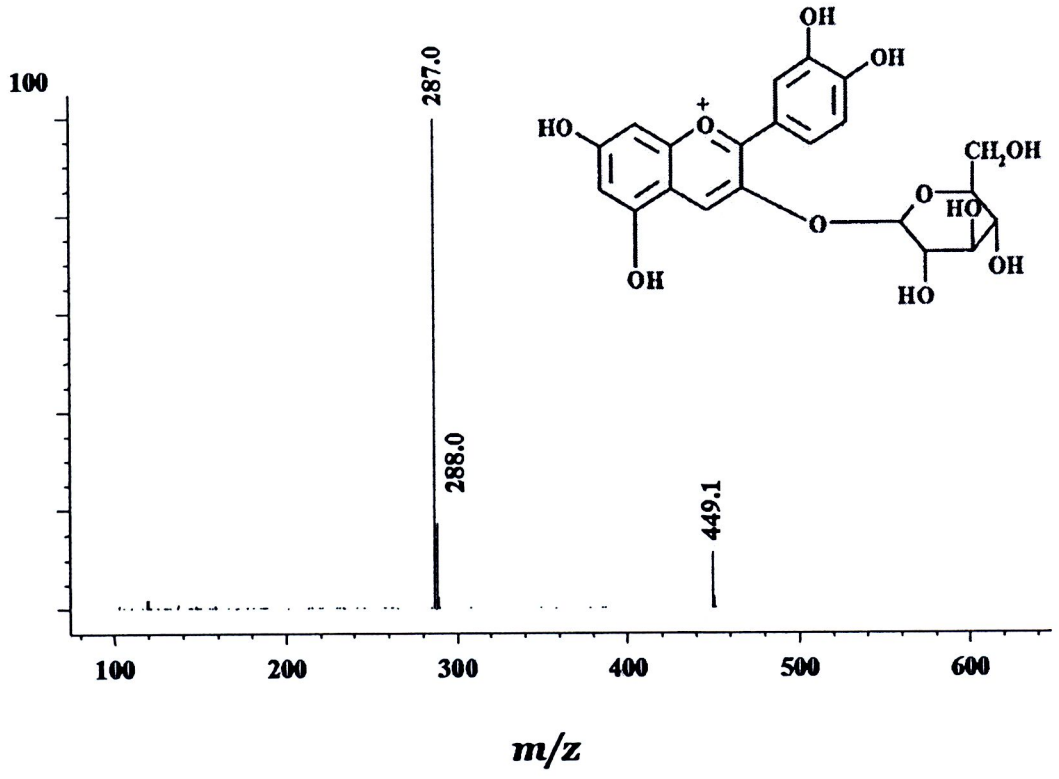

Figure S8 Full scan ESI-MS spectrum of component at retention time 20.83 (Peak number 2)

Full scan ESI-MS spectrum obtained from LC-ESI-MS of the peak at retention time 20.83 min is shown in Figure S8. The fragment ion at m/z 287 indicates the presence of cyanidin aglycone. The molecular ion at m/z 449 is identified as cyanidin glucoside by the mass difference of 162 Da equal to the loss of a glucose unit. However, the full-scan ESI-MS spectrum of the peak at retention time 42.82 min (peak 3) shown in Figure S9 provides the same pattern of mass spectrum. Due to the high stability and high abundance in nature of cyanidin-3-glucoside compared to those of cyanidin-5-glucoside, the anthocyanins at peak 2 and 3 are assigned as cyanidin-3-glucoside and cyanidin-5-glucoside, respectively.

Peak 3: Cyanidin-5-glucoside

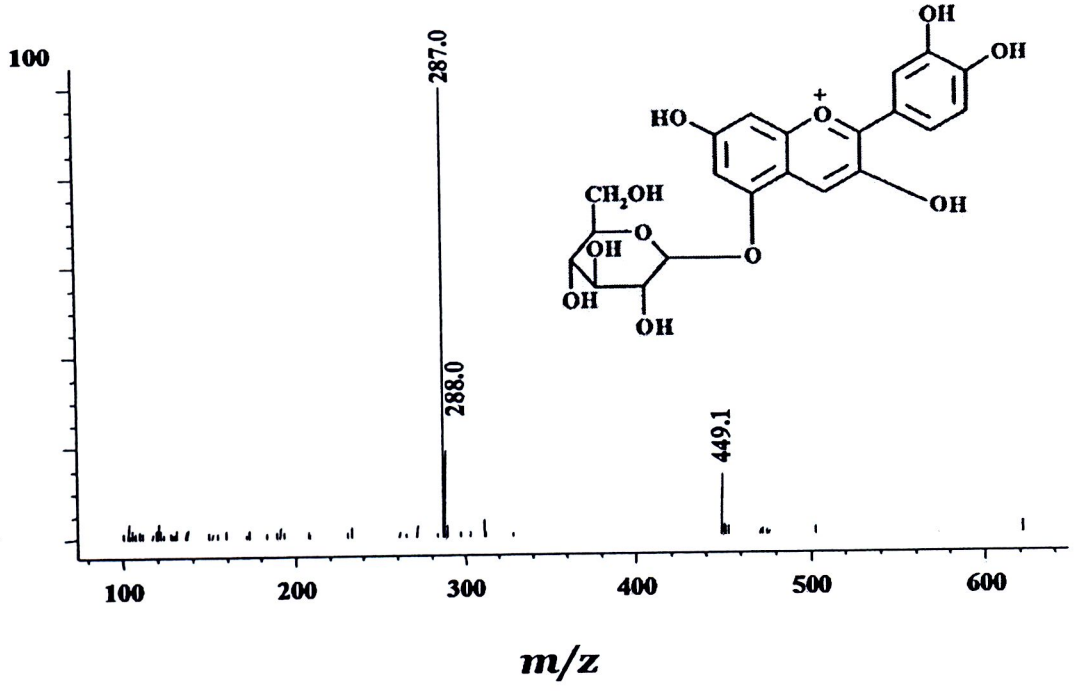

Figure S9 Full scan ESI-MS spectrum of component at retention time 42.82 (Peak number 3)

Table S1 Structural assignment of anthocyanins in *C. nervosum* extracts.

Peak No	RT (min)	Assignment compounds	$[M]^+$	ESI-MS spectrum	Peak area ratio		Relative content among the overall components (%)	
					MK 1	MK2	MK 1	MK2
1	17.55	Cyanidin-3-5-diglucoside	611	287 449 611	0.3318	0.0679	0.15	0.05
2	20.66	Cyanidin-3-glucoside	449	287 449	21.2754	10.4945	9.72	7.38
3	42.82	Cyanidin-5-glucoside	449	287 449	13.9365	1.4858	6.37	1.05

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