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# Rapid screening and characterisation of antioxidants of *Cosmos caudatus* using liquid chromatography coupled with mass spectrometry

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**Abstract**

Ulam raja (*Cosmos caudatus*) is used traditionally for improving blood circulation. In this study, it was found that ulam raja had extremely high antioxidant capacity of about 2400 mg L-ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh sample. Antioxidant peaks in extract of ulam raja were firstly characterized using free radical spiking test through high performance liquid chromatography coupled with mass spectrometry (MS). Upon reaction with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals, intensities of antioxidant peaks will be significantly reduced. HPLC/$MS^n$ was further applied to elucidate the chemical structures of antioxidant peaks characterized in the spiking test. More than twenty antioxidants were identified in ulam raja, and their chemical structures were proposed. The major antioxidants in ulam raja were attributed to a number of proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neo-chlorogenic, crypto-chlorogenic acid and (+)-catching. High content of antioxidants antioxidants contained in ulam raja could be partly responsible for its ability to reduce oxidative stress.
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*Keywords:* Ulam raja; Antioxidants; Phenolic compounds; HPLC/MS

## 1. Introduction

Ulam raja (*Cosmos caudatus*) is an annual, short-lived, perennial, aromatic herb. It originated from tropical Central America and is now widespread in almost all tropical regions. Its young leaves are often eaten raw with chilli or coconut paste and are used in dishes such as kerabu. They are also used as an appetiser and food flavouring due to their unique taste and aroma. Several bioactive components in ulam raja have been reported. For instance, Ragasa et al. have reported several antimutagen and antifungal compounds from ulam raja, e.g. cotunolide, stigmasterol, lutein and 4,4′-bipyridine [1]; Zanariah et al. have reported protein and amino acid compositions of ulam raja [2]. Ulam raja is also recommended in the traditional medicine system for improving blood circulation, but the mechanism of its medicinal function is still not clear.

Clinical and epidemiological research support the hypothesis that consuming fruits and vegetables is beneficial in helping combat age-related diseases, several cancers and heart diseases [3–7]. This could be partly attributed to various antioxidants contained in them [3,4,8–14] which has the ability to scavenge free radicals and therefore reducing oxidative stress. Our preliminary screening indicated that ulam raja had extremely high antioxidant capacity, which may partly be responsible for some of its believed medicinal functions. It is important to look into the compounds in ulam raja that contribute to its high antioxidant capacity to help understand its ability to reduce oxidative stress. The main objective of this study was to screen antioxidants in ulam raja and further identify them using tandem mass spectrometry.

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## 2. Experimental section

### 2.1. Solvent extraction of antioxidants

Preliminary HPLC/MS analysis implied that proanthocyanidins and quercetin derivatives were major antioxidants of ulam raja. Results from initial solvent extraction at room temperature (∼30 °C) only showed ulam raja had an antioxidant capacity of 1200–1400 mg AEAC/100 g sample (wet basis). For the extraction of antioxidants from ulam raja, the leaves were dried at 40 °C in an oven with forced ventilation for 24 h. They were then homogenized and immediately extracted with boiling water, 50% aqueous ethanol at 80 °C and 50% aqueous acetone (w/v, 1:50) in sealed glass bottles at 80 °C for 45 min, respectively. The water content of the leaves was determined by freeze-drying. The extracts were centrifuged, and the supernatants were used directly for total antioxidant capacity (TAC) assay. Fifty percent of aqueous acetone at 80 °C for 45 min was found to give maximum antioxidant extraction. After 45 min, the extracts were rapidly cooled to room temperature using an ice bath, after which the extracts were centrifuged at 14,000 × *g* and the supernatant was used without further treatment for the determination of total antioxidant capacity and total phenolic content. A portion of the supernatant was also dried by rotary evaporation at 35 °C with a vacuum of 25 Torr, redissolved in 20% aqueous methanol and centrifuged again. The antioxidant capacity of the supernatant was measured. Less than 5% of TAC was lost after evaporation and reconstitution. The obtained solution was filtered with a 0.45 μm membrane filter and then used for HPLC/MS and HPLC/MS/MS analyses.

### 2.2. Total antioxidant capacity assay

The total antioxidant capacity (TAC) assay was carried out on the Ultraspec 3000 UV–vis Spectrophotometer (Pharmacia Biotech Ltd., Cambridge CB4 4FJ, England). The procedure was described by Leong and Shui [15] with the results expressed as mg/100 g L-ascorbic acid equivalent antioxidant capacity (AEAC). Briefly, the prepared $ABTS^{\bullet+}$ solution was diluted to obtain an initial absorbance of ∼1.6 at 730 nm (∼1.0 mM) with pH 4.5 HCl solution before use. Thirty microlitres of extract was added to 3 mL of $ABTS^{\bullet+}$ solution. The changes of absorbance after 60 min were recorded and used for TAC calculations.

### 2.3. Total phenolic content assay

Total phenolic contents (TPC) were determined using Folin-Ciocalteau reagents [16]. Briefly, 40 μL of plant extract or gallic acid standard was mixed with 1.8 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature (about 28 °C) for 5 min; 1.2 mL of sodium bicarbonate (7.5%) was added to the mixture. After 60 min at room temperature, the absorbance was measured at 765 nm. Forty microlitres of distilled water was used as a blank following the same procedure as above. Total phenolic contents of a biological sample was obtained by comparing the increase of absorbance upon addition of its extract of total phenolic contents with that of gallic acid. Results were expressed as mg/g gallic acid equivalents (GAE).

### 2.4. Characterisation and identification of antioxidants in salak by HPLC/ESI/MS and HPLC/ESI/$MS^n$

Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with TSP 4000 HPLC system, which includes UV6000LP PDA detector, P4000 quaternary pump and AS3000 autosampler was used. The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen is operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate. The full scan mass spectra from *m*/*z* 50–2000 were acquired both in positive and negative ion mode with a scan speed of 1 s per scan. Tandem mass spectrometry was performed using helium as the collision gas, operated at 0.8 mTorr.

The HPLC elution conditions were: flow rate = 500 μL $min^{-1}$; room temperature (around 27 °C); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50 to 90% B in 20 min and keeping constant for 10 min, from 90 to 20% B in 5 min, keeping constant at 20% for 10 min for reconditioning of the column. Mass spectra were recorded within 45 min.

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid radical cations ($ABTS^{\bullet+}$) were generated using 7.4 mM of ABTS and 2.45 mM of potassium persulfate. The reaction mixture, which served as a stock solution, was kept at room temperature overnight for the reaction to complete. The stock was diluted with HCl solution at pH 4.5 accordingly to consume all reactable antioxidants, i.e. excess amount of $ABTS^{\bullet+}$ was added. One millilitre of extract and 2 mL of $ABTS^{\bullet+}$ stock solution were mixed to react for 1 h and then passed through 0.45 μm filter and injected for HPLC assay. A blank of the extract with water was used as a control.

For characterised antioxidant peaks, the collision energy was set from 50 to 80% to obtain fragment ions from their corresponding parent ions.

## 3. Results and discussions

### 3.1. Total antioxidant capacity and total phenolic content of ulam raja

Table 1 shows the effects of solvents on the extraction efficiency of antioxidants and polyphenolic compounds. The acetone/water extract system gave the highest extraction efficiency both for antioxidant (AEAC >2500 mg per 100 g fresh sample) and for total phenolic content (GAE >1200 mg per 100 g fresh sample). The moisture content of leaves was found to be 82.1%.

Table 1
Antioxidant capacity and phenolic content of ulam raja under various extraction conditions

| Extraction solvent | Water | Water:ethanol (1:1) | Water:acetone(1:1) |
|---|---|---|---|
| AEAC (mg/100g, wet basis)[a] | 1436.2 ± 23.6 | 1870.3 ± 154.3 | 2511.7 ± 285.4 |
| GAE (mg/100g, wet basis)[a] | 844.8 ± 19.7 | 1144.6 ± 56.6 | 1274.3 ± 98.3 |

[a] Mean ± S.D. ($n \geq 3$).

Previously, we reported the total antioxidant capacity of a number of fruits in Singapore markets [15], and showed that the AEAC values for most fruits were lower than 200 mg AEAC per 100 g of fresh sample. Even for strawberry, a fruit that is often regarded as having very high TAC, its AEAC value was found to be 500 mg per 100 g of fresh sample. Surprisingly here, it was found that ulam raja showed extremely high antioxidant capacity with AEAC value of 2500 mg per 100 g of fresh sample, which was close to that of ciku fruit, and certainly much higher than any of the AEAC values for the vegetables we have tested (data not shown). The extremely high antioxidant capacity of ulam raja may be mainly, or at least partly, responsible for its medicinal uses. In our opinion, it is of critical importance to further study the compounds in ulam raja which contribute to such a high antioxidant capacity, as such will help understand its antioxidant protection mechanism and further explore its medicinal uses, especially in its ability to reduce oxidative stress.

### 3.2. Free radical active components in ulam raja

Various separation techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis have been widely used for the identification of naturally occurring antioxidants in plants. Several HPLC methods, based on online-detection of some antioxidants by post-column reaction of eluates with free radicals, have been reported to be successfully applied to identify and quantify antioxidants in certain biological samples [17–20]. These methods could be used for rapid screening and even quantifying antioxidants provided that the reaction between antioxidants and free radicals are fast and their

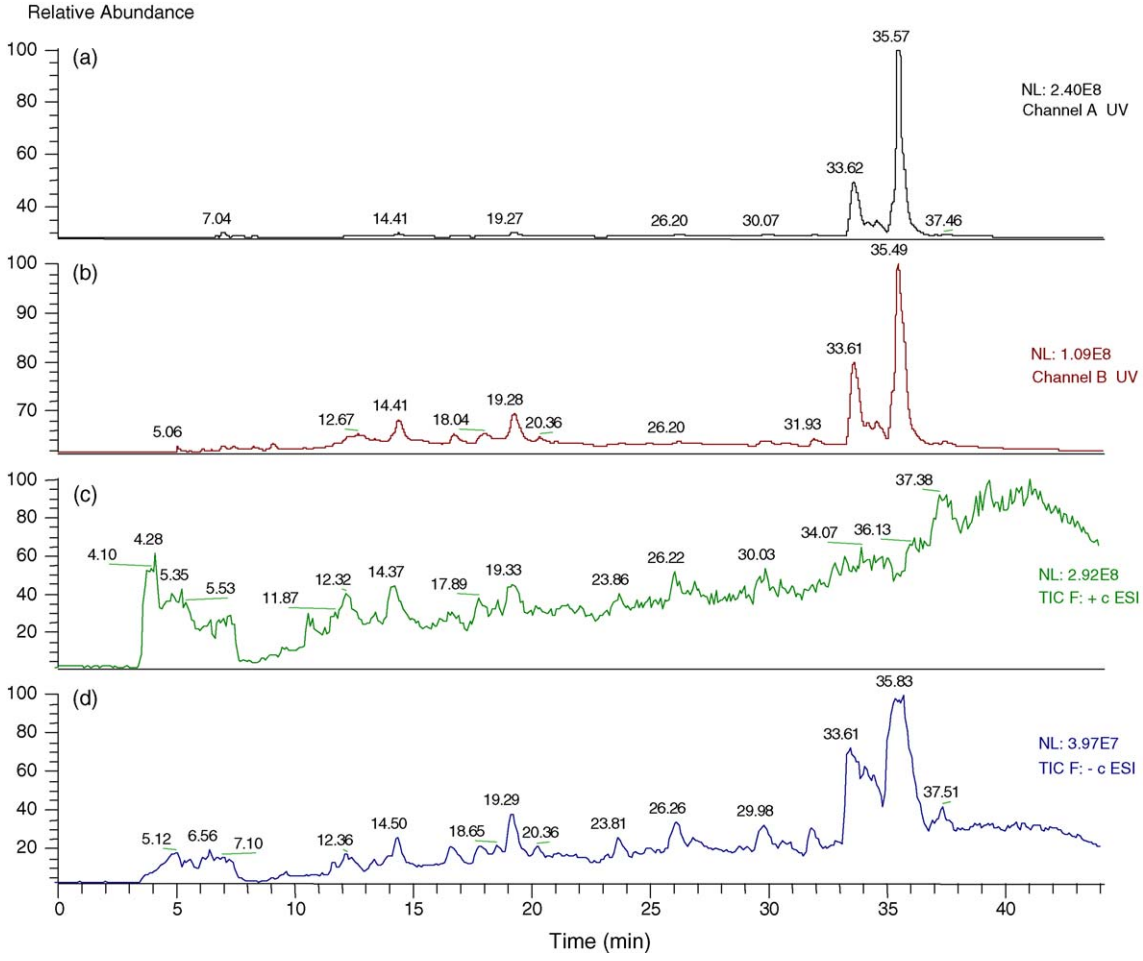


Fig. 1. HPLC chromatograms of ulam raja extract (a) UV at 250 nm; (b) UV at 280 nm; (c) TIC at positive mode; (d) TIC at negative mode.

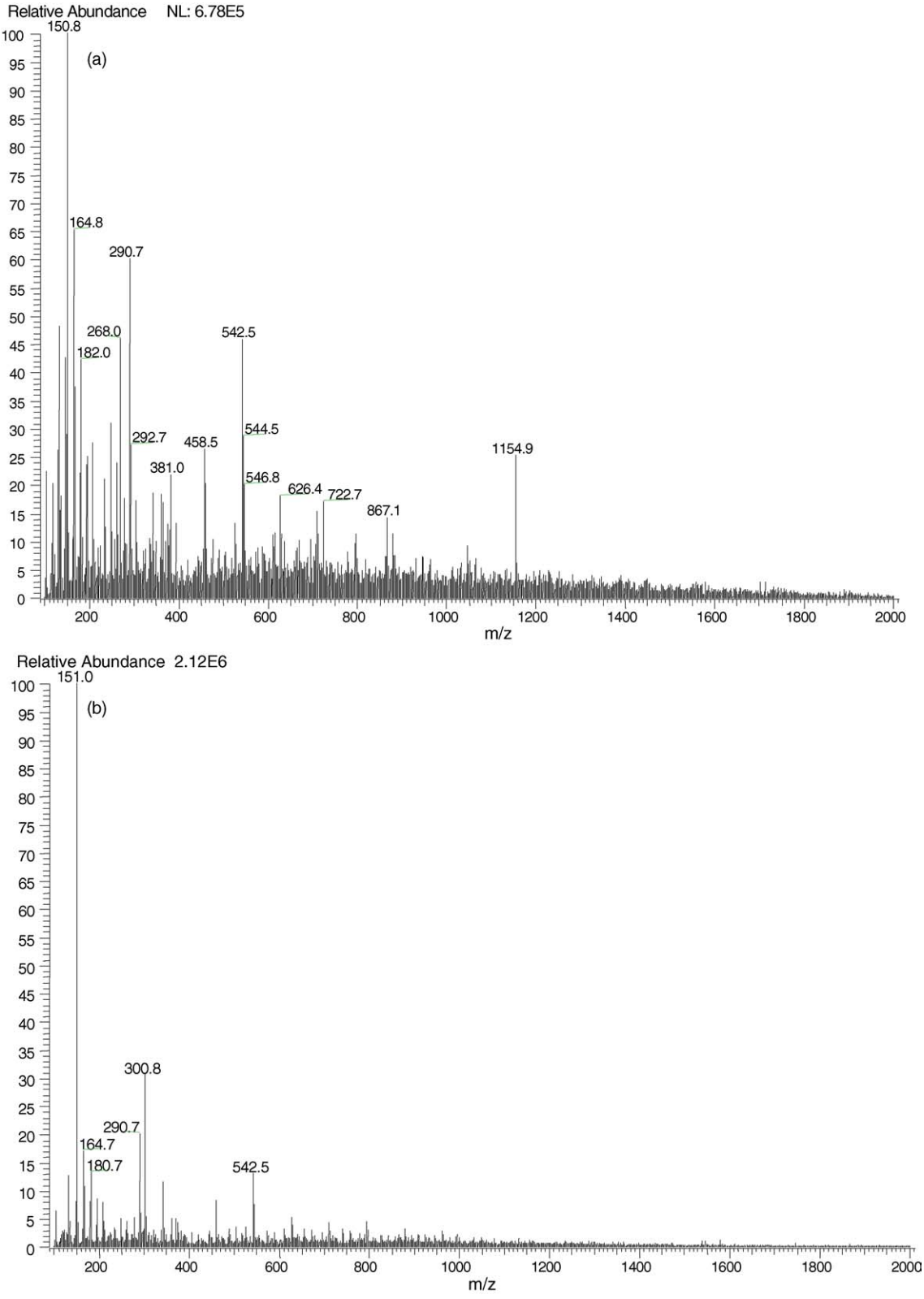


Fig. 2. Positive ESI/MS of elution period 1 (0–10 min). (a): Sample with water; (b): sample with $ABTS^{\bullet+}$.

stoichiometry is known. In many cases, further purification and structural identification are still needed to be carried out instead of using various authentic standards. Recently, we developed two approaches for the identification of major antioxidants in biological samples using a free radical spiking test using HPLC with UV or DAD detection [21,22]. However, one disadvantage of these approaches is that it may not be applicable when antioxidant peaks are not sensitive to UV or DAD detectors.

The coupling of chromatographic techniques and mass spectrometry (MS) has overcome the main analytical problem of traditional detectors associated with GC and HPLC, i.e. the lack of structural information. Antioxidants are usually found as complex mixtures in plant extracts and the

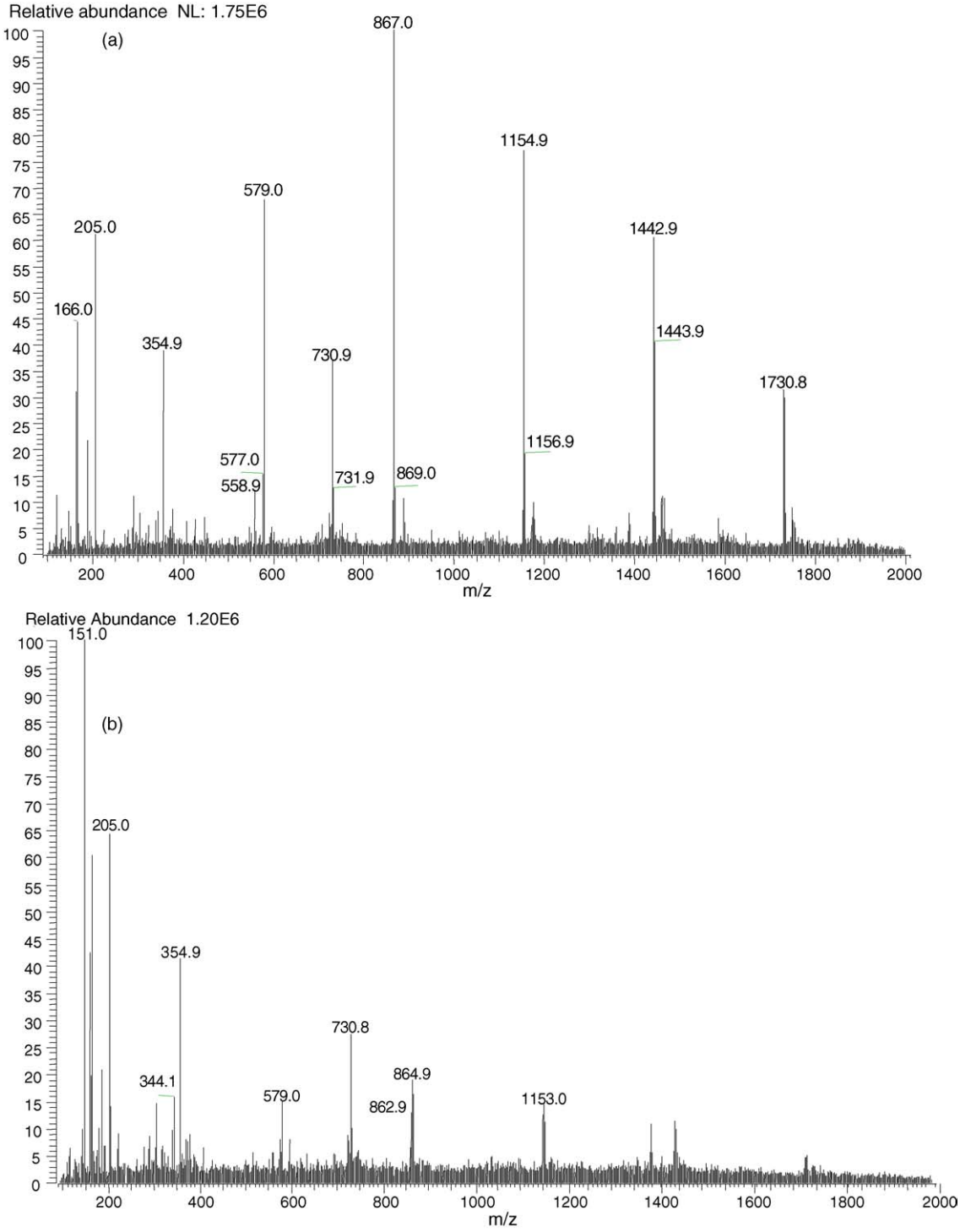


Fig. 3. Positive ESI/MS profiles of elution period 2 (10–20 min). (a) Sample with water; (b) sample with $ABTS^{\bullet+}$.

composition changes according to the plant examined. In such cases, hyphenated techniques are usually needed for the analysis of the extracts. Among these, HPLC/MS represents a rapid and reliable technique to analyze these non-volatile antioxidants. For instance, HPLC/MS is widely applied for characterizing antioxidants in biological samples [23–34]. In this study, we use a readily available free radical and MS as tools to directly screen and characterize antioxidants in ulam raja. Basically, a reaction between an antioxidant and a free radical results in the oxidation of the antioxidant. This may involve the transfer of hydrogen atom to the free radical, especially in the case of polyphenolic antioxidants. Once a

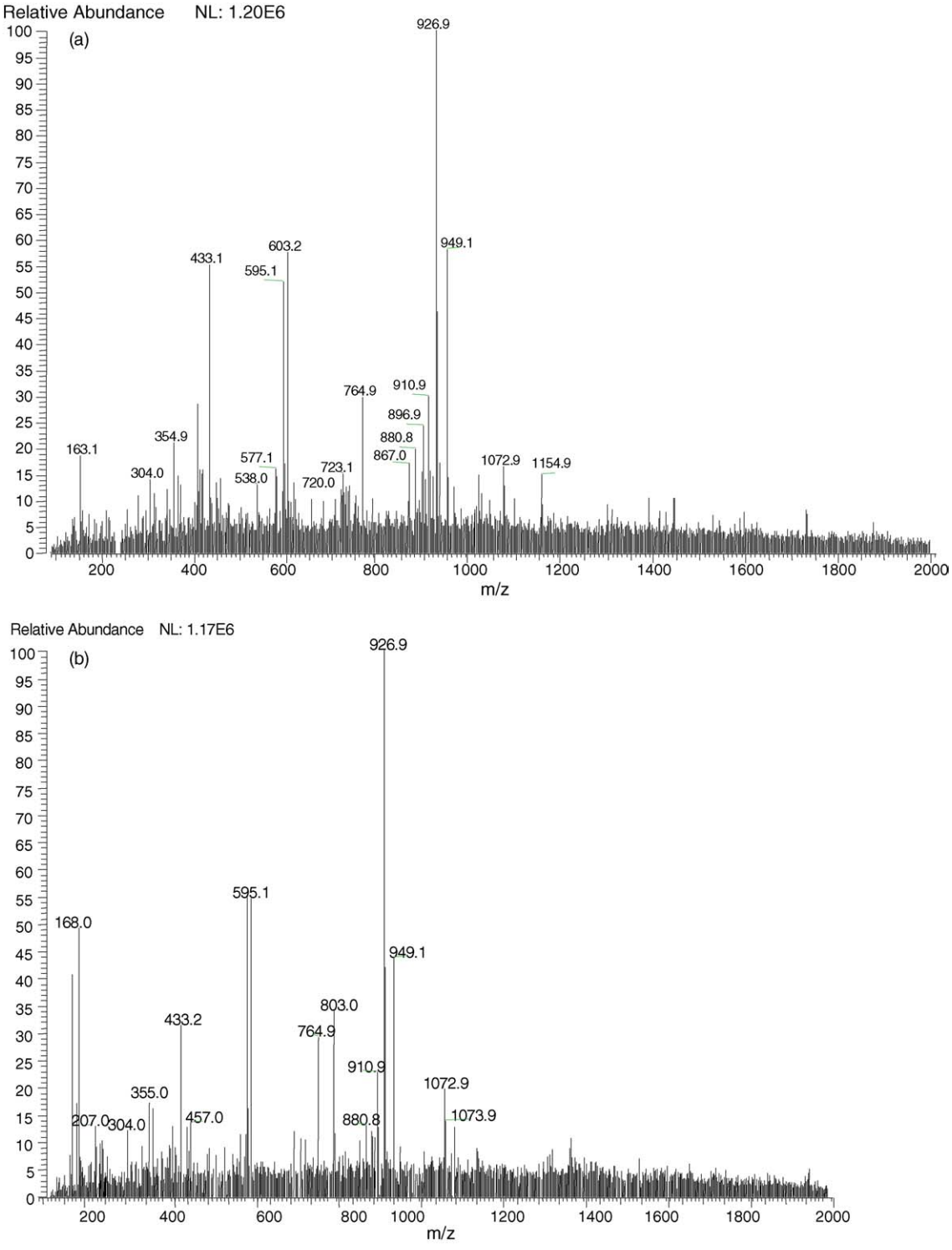


Fig. 4. Positive ESI/MS profiles of elution period 3 (20–30 min). (a) Sample with water; (b) sample with $ABTS^{\bullet+}$.

reaction occurred, the molecular weight of products will most likely be different from unreacted antioxidants. Based on this, free radical scavenging compounds could be monitored by observing the difference of peak intensity of individual ions between reactants and reaction products using a mass spectrometry detector. Even if the reactants and products are of the same molecular weights, by combining mass spectrometry with HPLC, they can still be characterized as they should

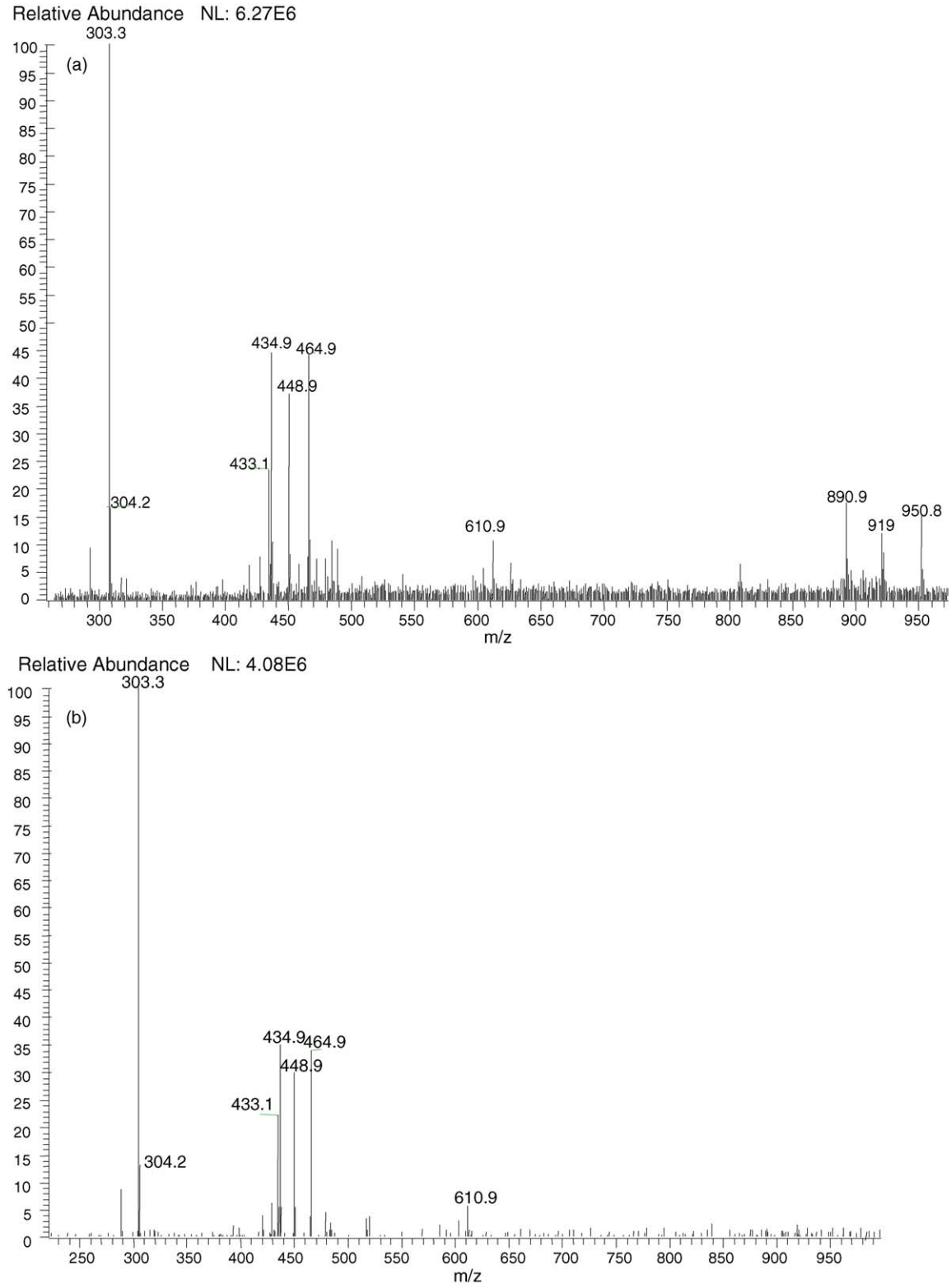


Fig. 5. Positive ESI/MS profiles of elution period 4 (30–40 min). (a) Sample with water; (b) sample with $ABTS^{\bullet+}$.

be eluted at different retention times due to their structural differences. Therefore, HPLC coupled with a mass spectrometer detector is an ideal tool for characterizing free radical active compounds.

Fig. 1 shows HPLC chromatograms of UV and mass spectral data, respectively. Although chromatograms from UV detection shows good peak shapes, total ion chromatograms (TICs) would be more useful as they contain information on structure and recognize more substances than those from UV detectors in cases where coeluates exist. To investigate which peaks contained antioxidants, the intensities of individual ion peaks at four elution periods were compared before and after reacting with $ABTS^{\bullet+}$. Figs. 2–5 shows the intensities of individual peaks of both control and reaction products at elution periods 1 (0–10 min), 2 (10–20 min), 3 (10–20 min) and 4 (30–40 min), respectively.

At elution period 1, only the intensity of ion peaks at *m/z* 1155 was significantly reduced from $1.76 \times 10^5$ ($6.78 \times 10^6 \times 26\%$) in Fig. 2(a) to less than $4.2 \times 10^4$ ($2.12 \times 10^6 \times 2\%$) in Fig. 2(b) while no obvious decrease for other ion peaks was observed. Extract ion chromatogram of *m/z* 1155 in the control and reaction solution were compared to characterise antioxidant peaks.

Fig. 3 shows that, at elution period 2, the intensity of ion peaks at *m/z* 579, 867, 1155, 1443 and 1731 were significantly reduced from $1.19 \times 10^6$ ($1.75 \times 10^6 \times 68\%$), $1.75 \times 10^6$, $1.37 \times 10^6$ ($1.75 \times 10^6 \times 78\%$), $1.09 \times 10^6$ ($1.75 \times 10^6 \times 62\%$) and $0.56 \times 10^5$ ($1.75 \times 10^6 \times 32\%$), respectively to less than $2.4 \times 10^5$ ($1.20 \times 10^6 \times 20\%$) for all ion peaks. Two ion peaks at *m/z* 355 and 731 were found to be reduced from $7 \times 10^5$ ($1.75 \times 10^6 \times 40\%$), and $6.6 \times 10^5$ ($1.75 \times 10^6 \times 38\%$) to $5 \times 10^5$ ($1.20 \times 10^6 \times 42\%$) and $3.4 \times 10^5$ ($1.20 \times 10^6 \times 28\%$), respectively. Therefore, extract ion chromatogram at *m/z* 355, 731, 579, 1155, 1443 and 1721 in control and reaction solution were compared to identify antioxidant peaks.

Intensities of ion peaks from extract at elution period 3, i.e. *m/z* 927, 949, 765, 603 and 595 were almost not reduced after reaction with free radicals (Fig. 4). However, intensities of ion peaks at *m/z* 433 and 355 were reduced. Extract ion chromatogram of ions at *m/z* 433 and 355 in the control and reaction solution were compared to identify antioxidant peaks.

Fig. 5 shows that the intensity of ion peaks at *m/z* 303, 465, 435, 449, 433, 611 and 891, 919 and 951 were significantly reduced after reaction with free radicals at elution period 4. Therefore, extract ion chromatogram at *m/z* 303, 465, 435, 449, 433, 611 and 891, 919 and 951 in the control and reaction solution were compared to identify antioxidant peaks.

Table 2 shows the profiles of antioxidant peaks in ulam raja extract, which were characterised from extracted ion chromatograms of ulam raja extract and reaction solution extract with free radicals. Other ions, which had the same signal of *m/z* as some of the antioxidant peaks, were not listed in the table as their intensities were not reduced and therefore were not characterized as antioxidants. Other than those antioxidant ion peaks listed in Table 2, several other ion peaks at *m/z* 731 (Fig. 3), 891, 919 and 951(Fig. 5) were also identified as antioxidant peaks. However, they arose from $[M + M + Na]^+$ of other antioxidant peaks at *m/z* 355 $[M + H]^+$, 435 $[M + H]^+$, 449 $[M + H]^+$ and 465 $[M + H]^+$, respectively.

### 3.3. Identification of antioxidants in ulam raja using $HPLC/MS^n$

Initial total ion chromatograms (TICs) of ulam raja extract, which were obtained by HPLC–ESI-MS at both positive and negative modes, indicated that compounds **U1–3** have a molecular weight of 578 Da. As their parent ion $[M + H]^+$ at *m/z* 579 gave similar fragment ions as that by proanthocyanidin dimers of star fruit [10], they were assigned as three isomers of proanthocyanidin dimers.

Similarly, compounds **U4–7** had a molecular weight of 866 Da, and their parent ions $[M + H]^+$ at *m/z* 867 gave similar fragment ions to that of proanthocyanidin trimers found in star fruit [22], Therefore, compounds **U4**–7 were assigned to be four proanthocyanidin trimers. Similarly, compounds **U8–11** were assigned to be four tetramers. Compounds **U12–15** had a molecular weight of 1443 Da and were identified to be four pentamers. Compounds **U16** and **U17** had a molecular weight of 1730 Da and were identified as two hexamers of catechin. Compound **U18** had a molecular weight of 290 Da. Compound **U18** was assigned to be (+)catechin by spiking and comparing its CID spectra with standard. Fig. 6 shows possible chemical structure of compounds **U1–18** with basic unit of (+/−)-catechin/epicatechin through C4–C6 or C4–C8 interflavanic linkages.

Compounds **U19–21** have the same molecular weight of 354 Da. They also showed identical $[M - H]^-$ ions at similar

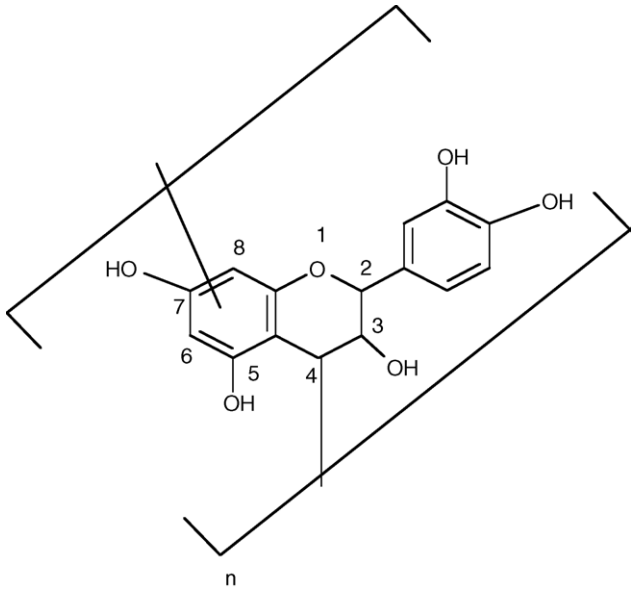


Fig. 6. Chemical structure of compounds **U1–U18** ($n$ = 1–6) with basic unit of (+/−)catechin/epicatechin.

Table 2
Positive and negative ions and their corresponding CID ions of antioxidants

| Rt (min) | ESI-MS, *m*/z | | Major CID ion peaks of $[M \pm H]^{+/-}$ |
|---|---|---|---|
| | Mode | $[M \pm H]^{+/-}$ | |
| 12.70, 16.85, 19.51 (**U1**–**3**) | + | 579 | 427, 409, 291, 289, etc. |
| | – | 577 | Not investigated |
| 12.39, 13.64, 16.87, 18.70 (**U4**–**7**) | + | 867 | 715, 697, 579, 577, 427, 409, etc. |
| | – | 865 | Not investigated |
| 9.88, 11.94, 18.86 (**U8**–**11**) | + | 1155 | 1003, 985, 867, 865, 715, 697, 579, 577, 427, etc. |
| | – | 1153 | Not investigated |
| 11.49, 12.21, 15.34, 19.31(**U12**–**15**) | + | 1443 | 1291, 1273, 1155, 1153, 867, 865, 579, 577, etc. |
| | – | 1441 | Not investigated |
| 12.21, 13.10 (**U16**, **U17**) | + | 1731 | 1579, 1561, 1443, 1441, 1155, 1153, 867, etc. |
| | – | 1729 | Not investigated |
| 16.96 (**U18**) | + | 291 | 139, 123, 165, etc. |
| | – | 289 | Not investigated |
| 14.56 (**U19**) | + | 355 | 163, etc. |
| | – | 353 | 191, 179, 135 |
| 19.48 (**U20**) | + | 355 | 163, etc. |
| | – | 353 | 191,179, 135, etc. |
| 20.73 (**U21**) | + | 355 | 163, etc. |
| | – | 353 | 191, 179, 173, 135, etc. |
| 30.02 (**U22**) | + | 433 | 367, 397, 415, 379, 337, 313, 283, 271 |
| | – | 431 | 311, etc. |
| 32.17 (**U23**) | + | 433 | 367, 397, 415, 379, 337, 313, 283, 271 |
| | – | 431 | 311, etc. |
| 37.66 (**U24**) | + | 433 | 367, 397, 415, 379, 337, 313, 283, 271 |
| | – | 431 | 311, etc. |
| 33.68 (**U25**) | + | 611 | 465, 303 |
| | – | 609 | 463, 301 |
| 35.36 (**U26**) | + | 465 | 303 |
| | – | 463 | 301 |
| 35.82 (**U27**) | + | 435 | 303 |
| | – | 433 | 301 |
| 33.56 (**U28**) | + | 449 | 303 |
| | – | 447 | 301 |

fragmentation patterns with ions at *m*/*z* 191 [quinic acid–H]$^-$ and 179 [caffeic acid–H]$^-$, which were generated by cleavage of the ester bond and found to be consistent with the structures of caffeoylquinic acid derivatives (Fig. 7). Compound **U20** was assigned to be chlorogenic acid (5-*O*-caffeoyl quinic acid) by spiking and comparing its CID spectra with an authentic standard. Compounds **U19** and **U21** were most likely naturally occurring isomers of chlorogenic acid, i.e. neochlorogenic acid and cryptochlorogenic acid. According to their elution order, neochlorogenic acid was eluted prior to cryptochlorogenic acid [35]. Therefore, compounds **U19** and **U21** were assigned as neochlorogenic acid and cryptochlorogenic acid, respectively. As shown in Fig. 7, the intensity patterns of these characteristic fragments for compound **U21** were substantially different, e.g. the base peak for compounds **U19** and **U20** was the ion at *m*/*z* 191, and for compound **U21** that at *m*/*z* 179 or 173. These results are consistent with those obtained by Carini et al. [35].

Both ion peaks at *m*/*z* 731 and 355 at positive mode and ion peaks at 707 and 353 at negative mode were found to be eluted simultaneously. Ion peak at *m*/*z* 731 and 707 arose from $[M + M + Na]^+$ and $[M + M - H]^-$, respectively. Therefore, ion peaks at *m*/*z* 731 actually arose from chlorogenic acids and its isomers.

Compounds **U22**–**24** had a molecular weight of 432 Da. Their parent ions $[M + H]^+$ at *m*/*z* 433 and $[M - H]^-$ at *m*/*z* 431 gave daughter ions at *m*/*z* 313 and 311, respectively. Their chemical structures need to be further investigated.

Compound **U25** had a molecular weight of 610 Da. The parent ion $[M + H]^+$ at *m*/*z* 611 gave daughter ion at *m*/*z* 465 and 303. The fragmentation pattern was the same as that of quercetin-hexose-deoxylhexose, e.g. quercetin

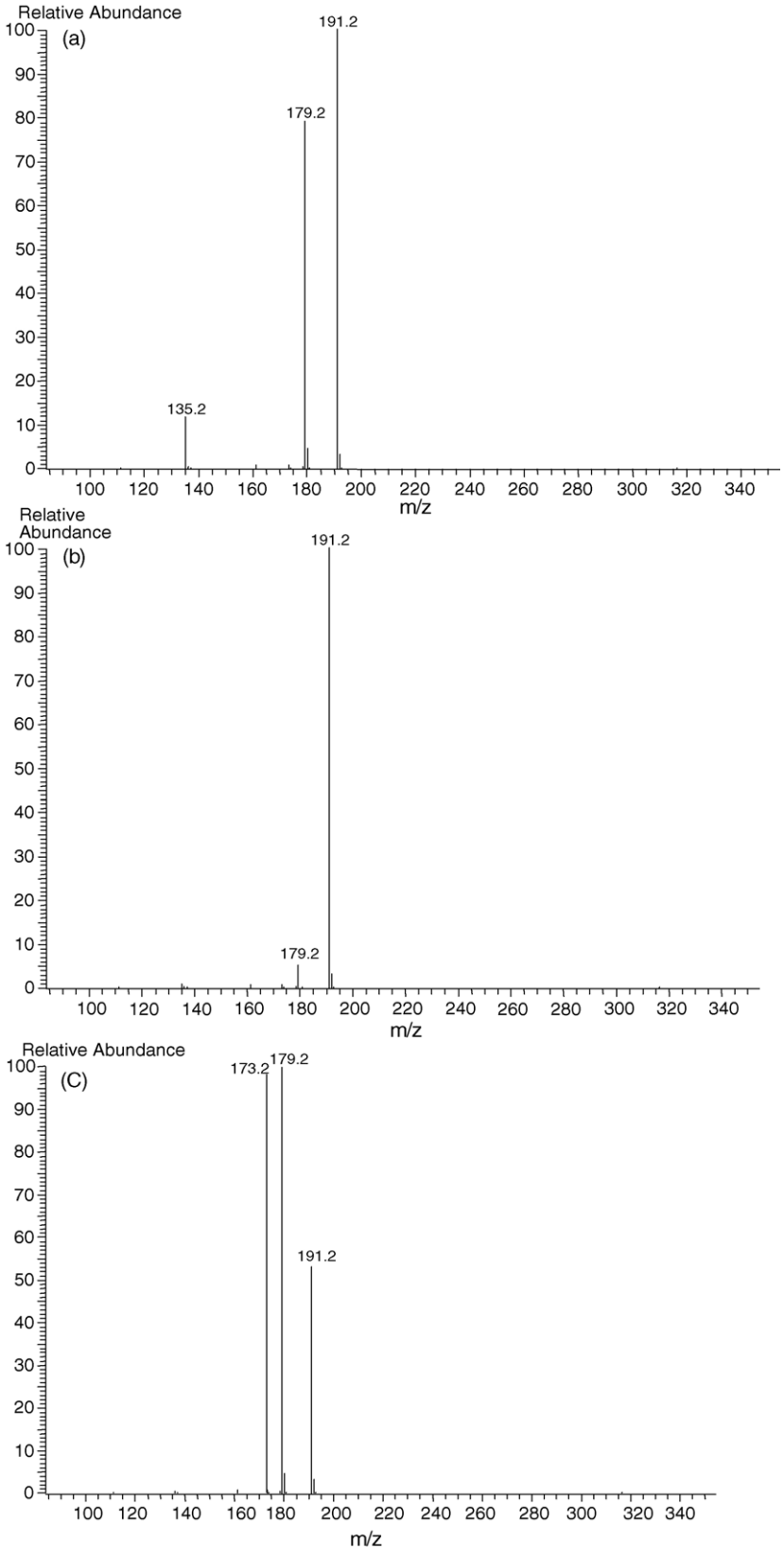


Fig. 7. CID spectra compounds **19**–**21** from parent ions at *m*/*z* 355. (a), Compound **19**. (b), compound **20** and (c), compound 21.

rutinoside[36,37]. Compound **U26** had a molecular weight of 464 Da. Its CID spectrum indicated it was a quercetin hexose. Compound **U26** was assigned to be quercetin 3-*O*-glucoside by spiking and comparing its CID spectra with pure compounds isolated from lady's finger [21].

Compound U27 had a molecular weight of 434 Da. Its parent ions $[M+H]^+$ at *m*/*z* 435 and $[M-H]^-$ at *m*/*z* 433 gave daughter ions at *m*/*z* 303 and 301, respectively. Its CID spectra indicated that compound **U27** were a quercetin pentose. Compound **U28** had a molecular weight of 448 Da. Its parent ions $[M+H]^+$ at *m*/*z* 449 and $[M-H]^-$ at *m*/*z* 447 gave daughter ions at *m*/*z* 303 and 301, respectively. Its CID spectra indicated that compound **U28** was a quercetin deoxylhexose.

Except for those compounds **U18–U21** and **U26**, the chemical structures and stereochemistry of other compounds still need to be elucidated by comparison with real standards or isolation of individual compounds for NMR assay.

It should be noted that only $ABTS^{\bullet+}$ was used for this study, other free radical, e.g. $DPPH^{\bullet}$ could also be used as tools for screening antioxidants. However, according to our recent studies on kinetics of a number of antioxidants, which include a variety of phenolic compounds, L-ascorbic acid, Vitamin E and some amino acids, its reaction with $ABTS^{\bullet+}$ is more reactive. In addition, $ABTS^{\bullet+}$ also shows higher stoichiometry with most antioxidants than $DPPH^{\bullet}$ [38].

While the approach utilized in this work was successfully applied to identify a number of antioxidants through free radical spiking tests, we failed to identify the possible reaction products of antioxidants with free radicals, even when some pure antioxidants were used to react with $ABTS^{\bullet+}$ or $DPPH^{\bullet}$. This might be due to poor ionization of formed products in selected analytical conditions. Peaks at *m*/*z* 865, 1153, etc. might arise from doubly-linked proanthocyanidins (type A) (Fig. 3). As there are no significant changes before and after mixing for these compounds, they are not studied in detail.

### 3.4. Potential beneficial effects of consuming ulam raja

Antioxidant treatments are thought to offset radical damage to biomolecules, thereby slowing or delaying the onset of the diseases by preventing oxidative stress. Phenolic compounds, as major natural antioxidants of many fruits and vegetables, are currently the focus of nutritional and therapeutic interest. Foods and beverages rich in phenolic compounds have been associated with decreased risk of age-related diseases in some epidemiologic studies [39–47].

Ulam raja, as reported above, contained a variety of phenolic compounds, e.g. proanthocyanidins, chlorogenic acids, quecertin and its derivatives. These natural compounds have been reported to be excellent antioxidants and be related to their various biological functions. Pycnogenol, as one of the best researched natural products, is an extract from French maritime pine bark (PBE). It is a highly characterised mixture of certain polyphenolic compounds, which comprises 80–85 wt.% of proanthocyanidins. Pycnogenol have been reported and claimed for its health effects [48–52]. Pycnogenol also corrects dangerous blood clotting tendencies that trigger heart attacks and strokes [53,54]. Proanthocyanidins from grape seeds is suggested to be useful in preventing or treating cardiovascular diseases [55]. Chlorogenic, as a natural antioxidants, is able to chelate iron [56] and protect against oxidative stress [57] and DNA damage [58]. Quercetin, the main representative of the flavonol, found in high concentration in onions, apple, red wine, Ginkgo biloba, influences some carcinnogenosis markers [59–66]. Quercetin and its derivatives, as the principal polyphenol of Ginkgo biloba, might be partly responsible for its medicinal function of decrease in blood pressure [59]. Ulam raja, rich in proanthocyanidins, chlorogenic acids and quercetin and its derivatives, might also have these biological functions. Its traditional usage for blood circulation could be due to these similar compounds also contained in herbs as described above. While ulam raja is believed to have traditional medicinal functions by the local population in improving blood circulation, further direct scientific study need to be carried out to confirm the claims. As different classes of antioxidants coexist in ulam raja, they may work synergistically. It is reasonable that ulam raja may have more biological functions than orally-transferred.

## 4. Conclusions

Antioxidants in ulam raja were firstly characterized using high performance liquid chromatography and mass spectrometry (HPLC/MS) and HPLC/MS/MS. A number of antioxidants were identified for the first time in ulam raja, and their chemical structures were proposed. The major antioxidants in ulam raja were attributed to a number of proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neochlorogenic, cryptochlorogenic acid and (+)catechin. Existence of a wide variety of antioxidants in ulam raja might mainly contribute to its ability to reduce oxidative stress.

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