# Food & Function

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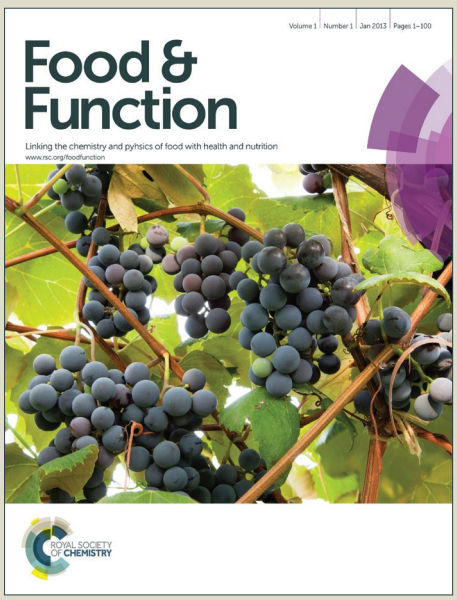


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Food & Function

ARTICLE

eceived 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

# Mechanistic evidence of *Passiflora edulis* (*Passifloraceae*) anxiolytic activity in relation to its metabolite fingerprint as revealed *via* LC-MS and chemometrics

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## Abstract

***Passiflora edulis* Sims *f. flavicarpa* along with several other plants belonging to the genus *Passiflora* have been reported as sedatives and for treatment or prevention of central disorders. This study evaluated the anxiolytic effect *P. edulis* ethanol extract and its fractions (*viz*. chloroform, ethyl acetate and butanol) using the elevated plus-maze model of anxiety and assessment of γ-aminobutyric acid levels. Results revealed that butanol and chloroform extracts exhibit the strongest effect followed by ethyl acetate suggesting that a combination of different classes of metabolites are likely to mediate for *P. edulis* anxiolytic effect in these fractions. To further pinpoint bioactive agent in fractions, ultra-performance liquid chromatography (UPLC) coupled to high resolution qTOF-MS was used for secondary metabolites profiling. A total of 65 metabolites were characterized including *O*-flavonoid, *C*-flavonoids, cyanogenic glycosides and fatty acids. Harman type alkaloids found in *P. incarnata* were not detected in *P. edulis* ethanol extract or any of its fractions suggesting that they do not mediate for its CNS modulating effects. Multivariate data analysis (PCA) was further applied to identify metabolite markers for active fractions and revealing for enrichment of flavonoid *C*-glycoside type in chloroform/ethyl acetate fractions versus exclusive presence of cyanogenic glycosides in its butanol fraction.**

**Keywords**: *P. edulis*, ultra-performance liquid chromatography (UPLC), chemometrics, anxiolytic

**Abbreviations**: Agl, Aglycon; AQF, aqueous mother liquor fraction; BF, butanol fraction; CF, chloroform fraction; DZ, diazepam; EAE, ethyl acetate fraction; EE, ethanol extract; GABA, γ-aminobutyric acid, $GABA_AR$: $GABA_A$ receptors, *P.*, *Passiflora*, PFF, petroleum ether fraction; OE%, percentage of open arms entries; OT%, percentage of time spent in open arms.

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Electronic Supplementary Information (ESI) available: Supplementary data associated with this article can be found, in the online version, at http://.....

## Introduction

The genus (*Passiflora*) contains *ca.* 400 species of flowering plants and is the largest in the family of *Passifloraceae*. The medical utility of very few *Passiflora* species has been scientifically elucidated. Passion extracts have been attributed to a myriad of pharmacological activities including anxiolytic, spasmolytic, hypnotic, sedative, narcotic and anodyne; and with aerial parts (stem and leaf) used traditionally to enhance the effects of mind-altering drugs.

The neuropharmacological effects of *Passiflora* spp., *i.e*., *Passiflora incarnata*,[1] *P. caerulea*,[2] and *P. edulis*,[3] is thought to be mostly mediated *via* its flavonoids content. Flavonoids are absorbed in the digestive tract, and then transported by the circulatory system to the brain, where they must pass the blood-brain barrier before exerting a CNS effect. Some natural flavones have been shown to possess selective affinity for the benzodiazepine binding site on the $GABA_A$ receptors ($GABA_AR$) in the brain. $GABA_AR$ is an ionotropic receptor and ligand-gated ion channel which binds to its endogenous ligand, γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system.[4] The first flavone described as a specific ligand for the benzodiazepine binding site was chrysin (5,7-dihydroxyflavone)[5] isolated from *Passiflora caerulea* and was found to be selective and competitive inhibitor of [3H] flunitrazepam binding to the benzodiazepine binding site. Luteolin is another flavonoid aglycon found in a wide variety of *Passiflora* spp. It has been reported to displace [3H]flunitrazepam from the benzodiazepine binding site *in vitro*, with low affinity, and has demonstrated anxiolytic-like effects when administered orally in mice. In addition, a commercial extract of *P. incarnata* enriched in *C*-glycosyl flavones demonstrated, *in vivo*, GABA mediated anxiolytic activity.[6] Based on structure-activity relationship studies, incorporation of electronegative groups into the *C*-6 and *C*-3′ on the flavone backbone was found to enhance its binding affinities for the benzodiazepine binding site.[7,8]

*P. edulis* is economically the most important species within the genus *Passiflora* being an edible variety most commonly known for its tasty fruit. There have been several reports on the neuropharmacological activities of *P. edulis*, *albeit*, experimental results from different research groups have brought some controversy on *P. edulis* potential anxiolytic effect. The work by Petry[9] revealed that *P. edulis* extract was active in rodent model of anxiety, as well as those by Coleta[10] and in opposite to Dhawan[11] findings that *P. edulis* was devoid of any anxiolytic activity. These results suggest for the need of more thorough investigations on *P. edulis* effect on the nervous system and or chemical profiling of its secondary products metabolome for a proof of action.

The present study aims at evaluating anxiolytic and sedative effects of *P. edulis* ethanol extract (EE) as well as its sub-fractions, *viz*. chloroform (CF), ethyl acetate (EAE), n-butanol (BF) so as to ascertain if this species could be used as a resource material of passion flower extract or an alternative to *P. incarnata* official drug. The behavioural effects in mice of *P. edulis* aerial parts crude ethanol extract and its sub-fractions were assayed using the elevated plus-maze model (EPM) in mice as one of the most popular behavioural tests for assessing anxiolytic drugs. γ-Aminobutyric acid (GABA) levels were also measured in treated animal brain versus control animals to determine whether GABA-ergic system mediates for *P. edulis* anxiolytic effect.

Furthermore, *P. edulis* crude ethanol extracts and its sub-fractions used for biological testing were subjected to comprehensive metabolites profiling using UPLC-high resolution qTOF-MS to reveal for its chemical composition. Different metabolites including several *C*-flavonoid glycosides were identified in *P. edulis* crude extract and sub-fractions in addition to cyanogenic glycosides and fatty acids. To define both metabolites similarities and differences among fractions and assist in pinpointing bioactive agents that could correlate with these effects, multivariate data analysis, principal component analysis (PCA) was adopted. Such a metabolomic approach could identify potential active anxiolytic agents in the different examined *P. edulis* extracts.

## Materials and Method

### 1. Plant material and chemicals

The aerial parts of *P. edulis* were collected in September 2012, from El-Orman Botanical Garden, Giza, Egypt and authenticated by Mrs Teresa Labib, Head of the Taxonomists at El-Orman Botanical Garden. A voucher specimen (26-12-2012) is deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Cairo University, Egypt. Solvents used for plant extraction; ethanol, petroleum ether, ethyl acetate, n-butanol were all of analytical grade and purchased from El-Gomhouria Co. (Cairo, Egypt). For the pharmacological assays; GABA was purchased from Sigma-Aldrich and diazepam from Memphis Pharmaceutical & Chemical Industry (Cairo, Egypt).

### 2. Animals

Male Swiss albino mice, weighing 20–35 g were bred at the Animal House of the college of Pharmacy, Cairo University, with free access to food and water in a temperature-controlled room (25 ± 2◦C) for at least 1 week prior to the behavioural experiments. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy Cairo University, Cairo, Egypt following the 18th WMA

General Assembly, Helsinki, June 1964 and updated by the 59thWMAGeneral Assembly, Seoul, October 2008. Each animal was used just once. The animals were assigned randomly to different treatment groups (n=5).

**3. Extraction and fractionation procedures**

1200 g of dried *P. edulis* aerial parts (leaf and stem) was extracted with ethanol–$H_2O$ (70:30) at room temp. The solvent was evaporated in vacuum and the residue of ethanol extract (EE, 70 g) was partitioned between petroleum ether-$H_2O$, followed by chloroform-$H_2O$, ethyl acetate-$H_2O$, and n-butanol-$H_2O$, in turn, to give petroleum ether fraction (40 g), chloroform fraction (1.3 g), ethyl acetate fraction (2 g) and n-butanol fraction (20 g).

**4. Drugs and treatment**

EE, CF, EAE and BF were uniformly suspended in distilled water by ultra-sonication with the addition of 1% Tween 80. Diazepam i.v. solution (DZ) was diluted with distilled water and used at a dose of 2 mg/kg, reference drug for anxiolytic and sedative tests. The trial animals received single administration of uniform suspension of the different test extracts and fractions. The negative control group received vehicle (distilled water containing Tween 80), the positive control group received DZ. All the treatments were orally administered (*p.o*) at a volume of 10 ml/kg. In the EPM tests, EE was given to mice at two doses, *viz.* 200 and 400 mg/kg, whereas CF, EAE, and BF were administered to mice at a single dose of 200 mg/kg.

**5. Pharmacological assays**

**Elevated plus-maze (EPM)**

The model is based on the natural aversion of rodents for open spaces and uses conflict between exploration and aversion to elevated open places and such model has been well validated for assessing both rats and mice.[12] The EPM is in the form of a 'plus' with two open elevated arms facing opposite to each other and separated by a central square and two arms of the same dimensions, but enclosed by walls. The maze is raised off the ground so that the open arms combine elements of unfamiliarity, openness and elevation (Suppl. Fig. 1S).

The apparatus is composed of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm) with 40 cm high wall arranged so that the arms of the same type are opposite to each other with a central square of 10 cm to form a plus sign. The apparatus is elevated to a height of 50 cm above floor level by a single central support. To facilitate adaptation to new surroundings, animals are transported to the laboratory at least 1 h prior to testing. At the start of the experiment, each mouse was placed individually at the centre of the maze facing an open arm and allowed for free exploration for a period of 5 min. The conventional spatial-temporal measures recorded were the numbers of all entries (all four paws on open or enclosed arms), and the percentage of open arms entries (OE%, 100 X open/total entries) and of the time spent in open arms (OT%, 100 X open/(open + enclosed)). After each test, the maze is carefully cleaned up with 10% ethanol.[13]

**Determination of brain neurotransmitter (GABA) level**

To assess for the possible involvement of the GABA-ergic system, brains of all animal groups, *viz*. negative control, positive drug control (DZ) and treatment groups (EE, CF, EAE and BF) were surgically isolated and evaluated for their GABA content. Each brain tissue was weighed and homogenized in 75% aqueous HPLC grade methanol (10% w/v) (Arafa *et al.*, 2010). The homogenate was spun at 4000 rpm for 10 min and the supernatant was dried using vacuum adjusted at 70 millitorr at room temperature and used for GABA determination. GABA was detected by High Performance Liquid Chromatography (HPLC) using the precolumn PITC derivatization technique according to the method of Heinrikson and Meredith (1984).[15] Standard GABA was prepared as previously mentioned in brain sample preparation. Quantification of GABA in HPLC chromatogram of samples was compared to that of the standard and determined as µg content per gram brain tissue (µg/g).

**Statistical analysis**

Data were analysed with PSPP, 0.8.2 for Windows (SPSS Inc., Chicago, IL). Data are expressed as mean ± S.E.M. of the groups. Differences between treatments were compared by one way ANOVA, followed by Least Significant Difference test (LSD test) for approximately normally distributed variables or non-parametric Mann-Whitney test. Differences between treated and control groups were considered statistically significant when $P \leq 0.05$ for one way NOVA and $P \leq 0.1$ for Mann-Whitney test (due to small sample size).

**6. High-resolution UPLC-PDA-qTOF-MS analysis**

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with an HSS T3 column (100 × 1.0 mm, particle size 1.8 µm; Waters) applying two elution binary gradients at a flow rate of 150 µL·min−1: (1) 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/ formic acid, 99.9/0.1 [v/v]); 1 to 16min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20min, isocratic 5% B. The second binary eluent (2) was composed of ammonium acetate 50mM buffer adjusted to pH 5(A) and 100% acetonitrile (B) using the same elution gradient as above. The injection volume was 3.1 µL (full loop injection).

Eluted compounds were detected from *m/z* 100 to 1000 using a MicroTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in both negative and positive ion modes using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 l ·min−1, 190°C; capillary, − 5500 V (+ 4000 V); end plate offset, − 500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; prepulse storage, 5 µs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 µL 10mM lithium formiate in isopropanol/water, 1/1 (v/v), at a gradient time of 18min using a diverter valve. Metabolites were characterized by their UV-vis spectra (220–600 nm), mass spectra, and comparison to our in-house database and reference literature.

**7. UPLC-MS data processing for multivariate analysis**

Relative metabolites quantification of *P. edulis* metabolite profiles derived using UPLC–MS was performed using XCMS data analysis software, which can be downloaded free as an R package from the Metlin Metabolite Database https://metlin.scripps.edu/download/ [16]. This software approach employs peak alignment, matching and comparison following the exact procedure described in Farag and Wessjohann.[17] Principal component analysis (PCA) was performed on the MS-scaled data using custom script under the R 2.9.2 environment. The PCA was run for obtaining a general overview of the variance of metabolites. Distance to the model (DModX) test was used to verify the presence of outliers and to evaluate whether a submitted sample fell within the model applicability domain.

## Result and Discussion

**Elevated plus-maze test**

Diazepam included in the elevated plus-maze test as a positive drug control induced a selective anxiolytic-like effect in mice characterized by a significant increase ($P \leq 0.1$) in the percentage of open arm entries, OE% and OT%, of the EPM, without changing the number of enclosed arm entries, compared to negative control (Suppl. Table 1 & Fig. 1) and in agreement with previous results.[18] Treatment with *P. edulis* ethanol extract (EE) at 200 and 400 mg/kg significantly increased the percentage of open arm entries, OE% and OT%, of mice demonstrating that EE of *P. edulis* exhibit anxiolytic-like effect. Additionally, marked decrease in the number of enclosed entries in the EE at 400 mg/kg treatment groups was observed, suggesting that EE reduce mice motor activity, namely, EE showed anxiolytic activity at lower dose and more of a sedative-like activity at a high dose. The administration of CF, EAE and BF at 200 mg/kg resulted in a significant increase in the number of open arm entries, OE% and OT%, without changing the number of enclosed arm entries, compared to negative control, suggesting for an anxiolytic effect of *P. edulis* fractions. However, no significant difference could be observed among treatment groups. BF was found to manifest the highest activity followed by EAE and CF. (Suppl. Table 1 & Fig. 1)

**Determination of brain amino acids (GABA) levels**

The results obtained after determination of brain GABA level (expressed as µg/g tissue) of the tested animals are expressed as mean ± S.E.M. and summarized in (Fig. 2). Following *P. edulis* extract and fractions administration, a significant increase in the concentration of GABA neurotransmitter at ($P \leq 0.05$) was revealed compared to the negative control, implying the involvement of GABA-ergic system in mediating for *P. edulis* anxiolytic-like effects, that is, these extracts might function as positive allosteric modulators of GABA and whose actions critically depend on the presence or absence of endogenous neurotransmitters. In details, CF and BF presented the highest effect at an oral dose of 200 mg/kg, followed by the EAE fraction. These results were consistent with those revealed from elevated plus maze model (Suppl. Table 1 & Fig. 1). Significant difference exists among each of the examined extract or fraction, *viz*. EE, CF, EAE, and BF when compared to negative control. *Albeit*, no significant difference could be observed among *P. edulis* treatment groups arguing that the anxiolytic activity in *P. edulis* is attributed to the synergistic effect of several active constituents (*i.e*., flavonoids) rather than a specific single chemical entity. Our results concur with Appel[19] findings in that CNS pharmacological effect of *P. incarnata* is mediated *via* modulation of the GABA system including affinity to $GABA_A$ and $GABA_B$ receptors, and effects on GABA uptake.

**Metabolites profiling in *P. edulis* crude ethanol extract and fractions**

To assess for metabolite composition among the different *P. edulis* examined fractions, non-targeted metabolites profiling was conducted for plant crude extracts and its sub-fractions using ultra-performance liquid chromatography coupled to photodiode array and high resolution qTOF-MS operated in both negative and positive ionisation modes.

Following the identification process, 65 chromatographic peaks were detected, of which 33 were tentatively assigned belonging to various metabolite classes. Metabolite assignments were made by comparing retention time, UV-Vis spectra and MS data (accurate mass, isotopic distribution and fragmentation pattern in both negative and positive

ionisation modes) and subsequent confirmation by comparison with literature data. The chromatographic and spectroscopic data are summarized in (Table 1 & 2).

Identified metabolites belonged to various classes, including *O/C* flavonoids, nitriles and fatty acids, with *C*-glycosylated flavones amounting for the most abundant class of secondary metabolites in *P. edulis* as many as 15 peaks were proposed to represent flavonoid conjugates. Compound abundance in this metabolite group is also visible from the total ion current chromatogram that is rich in the elution region ($t_R$ 170–350 s, Fig. 3A & B).

***C/O*-Flavonoids**

Photodiode array detection (200-600 nm) provided an overview of the main flavonoid constituents in *P. edulis* with flavone glycosidic conjugates representing the major sub-class and found mainly in BF, with a UV maximum absorbance near 270 nm and a second max between 335-350 nm, MS/MS was performed to assist in structural elucidation, where the nature of sugars in *O*-glycosides could be revealed from elimination of the sugar residue, *i.e.*, 162 amu (hexose; glucose or galactose), 146 amu (deoxyhexose), 132 amu (pentose), or 130 (didexoyhexose) or from molecular ions.[20] The readily cleaved sugar moieties from aglycon infers *O*-type glycosides, nevertheless in *P. edulis* most flavonoid peaks showed intense molecular ion peaks with $[M-90-H]^-$ and $[M-120-H]^-$ fragments in $MS^n$ spectrum indicative of sugar cleavage of a *C*-glycoside and found in most identified flavone peaks. Fragmentation pathways most characteristic of *C*-glucosyl flavonoids include dehydration (-18 amu) and cross-ring cleavages (O,2 and O,3) of the sugar moiety, *i.e.*, (-120 amu) and (-90 amu) for *C*-hexosides and (-90 amu) and (-60 amu) for *C*-pentosides.[21,22]

In *P. edulis*, mono-*C* and di-*C*/glycosides identified were all flavone derivatives of apigenin and luteolin on the basis of UV-Vis spectra. The ions $[Agl+41/ Agl+71]^-$ in mono-*C* and $[Agl+83/ Agl+113]^-$ in di-*C* glycosides typify the aglycon (Agl), that is, $[311/341]^-$ and $[353/383]^-$ for apigenin mono-*C* and di-*C*-glycosides respectively, $[295, 325]^-$ and $[337, 367]^-$ for chrysin mono-C and di-*C*-glycosides respectively, and ions $[327/357]^-$ and $[369/399]^-$ for luteolin mono-*C* and di *C*-glycosides respectively.[23] Such ions were identified in apigenin containing peaks (5, 9, 12, 15, and 21), chrysin containing peaks (11, 17 and 18), and luteolin conjugates in peaks (3, 8, 10, 13, 16, 17, & 19), and of which peaks (9, 11, 13, 18, & 19) are first time to be reported in *P. edulis* leaves. The following outline conditions used to identify each of these unreported flavonoid peaks.

The ESI-MS spectrum of peak 5 exhibited a molecular ion $[M-H]^-$ at *m/z* 593.1651 $(C_{27}H_{29}O_{15})^-$ and product ions at *m/z* 575 $[M-18-H]^-$ $(-H_2O)$, 503 $[M-90-H]^-$, 473 $[M-120-H]^-$, 353 $[Agl+83]^-$, and 383 $[Agl+113]^-$, typical fragmentation pattern of flavone di-*C*-hexoside, and was identified as apigenin-6,8-*C*-di-glucoside, also known as vicenin-2.[24] Apigenin-6-*C*-monoglucoside was detected in peak 12 from its $[M-H]^-$ at *m/z* 431.0971 $(C_{21}H_{19}O_{10})^-$ and fragment ions at *m/z* 413 $[M-18-H]^-$, *m/z* 341 $[M-90-H]^-$, and a base peak at *m/z* 311 $[M-120-H]^-$, a 353 $[Agl+41]^-$, and 383 $[Agl+71]^-$typical fragmentation pattern of flavone mono-*C*-hexoside (Isovitexin) a widespread flavone conjugate in *Passiflora* spp.[25]

Peak 9 was characterized by an $[M-H]^-$ at *m/z* 563.1512 $(C_{26}H_{27}O_{14})^-$ and fragment ions in MS/MS spectrum appearing at *m/z* 545 $[M-18-H]^-$, *m/z* 473 $[M-90-H]^-$, and 443 $[M-120-H]^-$, typical fragmentation pattern of flavone-*C*-hexoside, in addition to a fragment at *m/z* 503 $[M-60-H]^-$ indicating the presence of extra *C*-linked pentose moiety. A base peak at *m/z* 459 [M-120-H] and the high abundance of the fragmentation peaks for the hexosyl unit relative to the pentosyl, suggested that a hexose at position 6 occurs in this compound. Consequently, this *C*-glycoside was identified as apigenin-6-*C*-glucoside-8-*C*-arabinoside, also known as schaftoside.[26]

Two apigenin isomer conjugates were found in peaks 11 and 15 with $[M-H]^-$ at *m/z* 577.1444 $(C_{27}H_{29}O_{14})^-$. For peak 11, MS/MS spectrum of deprotonated molecular ion produced ions at *m/z* 559 $[M-18-H]^-$, *m/z* 487 $[M-90-H]^-$, and a base peak at *m/z* 457.1 $[M-120-H]^-$, a fragmentation pattern of flavone-di-*C*-hexoside. This flavone was characterized as chrysin-6,8-di-*C*-hexoside. In contrast, peak 15 exhibited fragmentation pattern with an intense ion at *m/z* at 415 $[M-162-H]^-$ resulting from the loss of hexose moiety and suggesting for an *O*-glycosidic linkage, and a base peak at *m/z* 311 $[M-hexose-104-H]^-$ indicative of a *C*-deoxyhexose moiety. Accordingly peak 15 was identified as apigenin-*C*-deoxyhexoside-*O*-hexoside, previously reported in *P. edulis* leaf [27].

Peak 18 was characterized by a $[M-H]^-$ at *m/z* 561.1717 $(C_{27}H_{29}O_{13})^-$ and with product ions at *m/z* 543 $[M-18-H]^-$, 415 $[M-deoxyhexose-H]^-$, *m/z* 325 $[M-deoxyhexose-90-H]^-$, and a base peak at *m/z* 295 $[M-deoxyhexose-120-H]^-$, exhibiting a fragmentation pattern of flavone-*C,O*-di-glycoside which was characterized as chrysin-*C*-hexoside-*O*-deoxyhexoside.

Peak 21 with $[M-H]^-$ at *m/z* 415.1039 $(C_{21}H_{19}O_9)^-$ exhibited fragmentation pattern indicative of a mono-*C*-glucoside, that is, *m/z* 397 $[M-18-H]^-$, *m/z* 325 $[M-90-H]^-$ and *m/z* 295 $[M-120-H]^-$. The low abundance of *m/z* 397 $[M-18-H]^-$ suggests for an 8-*C*-rather than 6-*C*-linked sugar. Consequently peak

21 was identified as apigenin-8-*C*-deoxyhexoside, previously reported in *P. edulis*.[27]

Peak 3 exhibited an $[M-H]^-$ at *m/z* 609.1558 $(C_{27}H_{29}O_{16})^-$ and its $MS^n$ spectrum showed ions at *m/z* 489 $[M-120-H]^-$, *m/z* 519 $[M-90-H]^-$, 591 $[M-18-H]^-$corresponding to the fragmentation of a flavone-di-*C*-glycoside, and characterized as luteolin 6,8-di-*C*-glucoside (lucenin-2) previously isolated from the leaves of *P. edulis f. flavicarpa*.[18]

Other *C*-glycosides identified in *P. edulis* include luteolin-6-*C*-glucoside (isoorientin) peak 8, and luteolin-8-*C*-glucoside (orientin) peak 10, with $[M-H]^-$ of 447.1030 $(C_{21}H_{19}O_{11})^-$ and a base peak at *m/z* 327 $[M-120-H]^-$ characteristic for *C*-hexosides. Distinguish between both isomers was based on the high intensity of the ion *m/z* 429 $[M-18-H]^-$ in peak 8 indicating 6-*C*-hexosyl flavone (isoorientin), due to the formation of an additional hydrogen bond between 2"-hydroxy group of sugar and the aglycon 5- or 7-hydroxy group which confers additional rigidity.[22,28] Such ion showed less abundance in luteolin-8-*C*-glucoside (orientin), peak 10. Both compounds are of common occurrence in *Passiflora* spp.[25]

Peak 13 was characterized by an $[M-H]^-$ at *m/z* 563.1391 $(C_{26}H_{27}O_{14})^-$ and product ions at *m/z* 545 [M-18-H] and a base peak at *m/z* 459 $[M-104-H]^-$ indicating a 6-*C*-deoxyhexosyl moiety. Additional ion at *m/z* 503 $[M-60-H]^-$ characteristic for *C*-pentosides identified peak 13 as luteolin- 6-*C*-deoxyhexoside-8-*C*-pentoside.

Peaks 16 and 19 MS spectra exhibited a deprotonated $[M-H]^-$ at *m/z* 431.1018 $(C_{21}H_{19}O_{10})^-$ and an intense peak at *m/z* 327 $[M-104-H]^-$ indicating a *C*-deoxyhexose moiety. Distinguish between both isomer peaks was based on mass loss $[M-18-H]^-$ very intense in compound 16 indicating 6-*C*- glycoside, *albeit* detected at low intensities in peak 19 indicating 8-*C*-glycoside, accordingly peak 16 was annotated as luteolin-6-*C*-quinovoside or luteolin-6-*C*-fucoside, previously reported in *P. edulis f. flavicarpa*, whereas peak 19 as luteolin-8-*C*-deoxyhexoside.[29]

Peak 17 exhibited an $[M-H]^-$ at *m/z* 415.1039 $(C_{21}H_{19}O_9)^-$ and its $MS^n$ spectrum showed ions at *m/z* 397 $[M-18-H]^-$, *m/z* 325 [M-90-H]- and a base peak at 295 $[M-120-H]^-$, a fragmentation pattern typical of flavone-*C*-hexoside. The low abundance of *m/z* 397 $[M-18-H]^-$ suggests 8-*C*-rather than 6-*C*-linkage, thus compound 17 was characterized as chrysin-6-*C*- hexoside.[30]

It should be noted that identified *C*-flavone conjugates were found most enriched in BF and EAE (Suppl. Fig. 2S) which both showed potent anxiolytic activity especially in BF, as previously described, and accordingly it is likely that flavone-*C*-glycosides enrichment in these fractions that might contribute significantly to the anxiolytic activity of *P. edulis.* This assumption is in accordance with previous studies on flavone-*C*-glycosides isolated from other plants. The work by de Oliveira[31] proved that vitexin, isovitexin and diosmetin-6-*C*-glucoside from *Erythrina falcate*, a known anxiolytic plant resulted in a significant retention of fear memory and could represent potential therapeutic approaches for the treatment of neurocognitive disorders. Standard orientin, isoorientin, vitexin and isovitexin were previously assessed for behavioural effects in mice and reported to exhibit anxiolytic activity in treated animal[32] and our results provide further evidence for their role in modulating CNS effects.

**Cyanogenic Glycosides**

Cyanogenesis is widespread in plants, but relatively few cyanogenic compounds have been isolated and characterized. In *P. edulis* crude ethanol extract (EE), four cyanogenic glycosides were identified in peaks (1, 2, 6 & 7) as evident from the loss of cyano moiety (-26 amu) in their tandem MS spectra. Peak 1 with a molecular ion of *m/z* 456.1499 $(C_{20}H_{26}NO_{11})^-$ and fragment ions at 431 derived from loss of cyano group $[M-26-H]^-$, 293 loss of glucose $[M-162-H]^-$ from the molecular ion, in addition to the fragment ion *m/z* at 269 $[M-(26+162)]^-$ was identified as mandelonitrile-*O*-di-glucoside (2-hydroxy-2-phenyl-acetonitrile-*O*-di-glucoside) previously isolated from *P. edulis*.[33] Likewise, peak 6 at *m/z* 440.1550 $(C_{20}H_{26}NO_{10})^-$ was identified as mandelonitrile-*O*-rutinoside exhibiting the same fragmentation pattern with product ions at *m/z* 415 $[M-CN-H]^-$, *m/z* 294 $[M-rhamnose-H]^-$ and 269 $[M-(rhamnose+CN)-H]^-$known to occur in *P. edulis*.[34] It should be noted that cyanogenic glycosides were found most enriched in BF among other fractions, considering its hydrophilic character.

For peaks 2 at *m/z* 502.1570 $(C_{21}H_{29}NO_{13})$- and 7 at *m/z* 486.1604 $(C_{21}H_{29}NO_{12})$-, both MS spectra showed the fragments characteristic for phenyl acetonitriles, *albeit,* with a mass difference of 46 amu compared to that in peaks 1 and 6 respectively, indicating extra methoxy (30 amu) and hydroxy (16 amu) groups. Peaks 2 and 7 were tentatively assigned as 2-(4'-methoxy-3'-hydroxy) derivatives of peaks 2 and 6 respectively, and are first time to be reported in the genus *Passiflora*. Whether cyanogenic glycosides can influence the anxiolytic effect of *P. edulis* C-flavonoids has yet to be fully examined.

**Fatty Acids**

In the second half of the chromatographic run (350–800 s), particularly in PEF, MS spectra of several unsaturated fatty acids, that is, oleic 64, linoleic 61, and linolenic acid 60 were

identified, as evident from high resolution mass at 281.2484, 279.2331 and 277.2172 with predicted molecular formulae of $(C_{18}H_{33}O_2)^-$, $(C_{18}H_{31}O_2)^-$, and $(C_{18}H_{29}O_2)^-$ respectively and with oleic acid being most abundant fatty acid. The abundance of such metabolites type in petroleum ether extract is expected considering its lipophilic nature, extracting mostly non polar metabolites. MS signals were also assigned for saturated fatty acids, that is, palmitic 63 and stearic acids 65, as evident from high resolution masses 255.2311 and 283.2484 with predicted molecular formulae of $(C_{16}H_{31}O_2)^-$ and $(C_{18}H_{35}O_2)^-$ respectively. Negative ionisation MS also revealed for several unexpected hydroxylated fatty acids, major peaks (26, 30, 29, 40, 48, & 59). In detail, peaks 40 and 48 showed a mass weight of 293.2120 amu and 295.2276, with predicted molecular formulae of $(C_{18}H_{29}O_3)^-$ and $(C_{18}H_{31}O_3)^-$ respectively. A mass difference of 2 amu between 40 and 48 is indicative of an extra double bond and they are tentatively identified as hydroxyoctadecatrienoic acid and hydroxyoctadecadienoic acid (oxylipids). Similarly peaks 26 and 29 showed a mass weight of and 329.2320 and 327.2167 amu, with predicted molecular formulae of $(C_{18}H_{33}O_5)^-$ and $(C_{18}H_{31}O_5)^-$ respectively, and with a mass difference of 2 amu suggestive of an extra double bond and they are tentatively identified as trihydroxyoctadecadienoic acid and trihydroxyoctadecaenoic acid. Other oxygenated fatty acid were detected in peaks 30 and 59 showing a mass weight of 287.2215 and 271.2275 amu with predicted molecular formulae of $(C_{16}H_{31}O_5)^-$ and $(C_{16}H_{31}O_4)^-$ respectively, and a loss of water molecule (18 amu), suggestive of an extra hydroxy group, and tentatively assigned as dihydroxyhexadecanoic and hydroxyhexadecaenoic acid. This is the first report for the presence of oxygenated fatty acids in *P. edulis* and suggests that UPLC–MS represents another platform for fatty acids profiling in *P. edulis*. There is an increasing interest in hydroxylated fatty acids, due to their anti-inflammatory, anti-microbial and cytotoxic activities.[35,36]

**Alkaloids**

Harmala indole alkaloids were reported to be present in small amounts in *Passiflora* spp.[25] Alkaloids show much higher response in positive versus negative ionisation which highlights the importance of acquiring MS data in both negative and positive ion modes. Nevertheless, no alkaloids were detected in the positive mode in any of the examined fractions suggesting that the anxiolytic activity of *P. edulis* is attributed to constituents other than the harmala alkaloids. It should be noted that standard harmine alkaloid analysed under same analytical condition was detected at a level of up to 1 ug/ml.

To the best of our knowledge, this UPLC-MS fingerprint provides the first comprehensive profile of secondary metabolites composition in *P. edulis* and help provide chemical based evidence for its potential anxiolytic effect.

**Quantification of flavonoids *via* UPLC–MS**

Luteolin and apigenin conjugates were quantified using calibration curves derived from MS detection of luteolin and apigenin standards, respectively. Standard for each was prepared at concentrations spanning from 0.1, 1, 10 and 100 ug/ml and assays were carried out from three independent biological replicates. The results revealed the enrichment of C-glycosyl flavones in *P. edulis* aerial parts. Details on the quantification of flavonoids are provided in Suppl. Table 2. Pharmacopoeias exclude *P. edulis* aerial portion as the resource of Passionflower extract *i.e.*, British Pharmacopoeia (2009) distinguish *P. incarnata* from *P. edulis*, as the latter lacks diglycosylflavones and isoorientin. Nevertheless, our results reveal that *P. edulis f. flavicarpa* indeed contains diglycosylflavones as evident in peaks (3, 5, 9, 11, 13, and 18) and isoorientin (8). Additionally, the anxiolytic effect observed in this study suggests that *P. edulis* could be considered as the resource of Passionflower extract.

**Multivariate data analysis of *P. edulis* fractions UPLC–MS dataset**

UPLC-MS and multivariate data analyses were further used to identify potential active anxiolytic agents in the different examined *P. edulis* fractions likely to mediate for observed effects. Such a metabolomic approach has been proven effective to expedite bioactive drug discovery in complex plant extracts.[37] Multivariate statistical analysis (principal component analysis, PCA) is commonly used to define both similarities and differences among fractions in an untargeted approach. Although differences between fractions were apparent by visual examination of the UPLC-MS traces (Suppl. Fig. 2S), multivariate data analysis was adopted for fractions classification in a more holistic way using (PCA) to explore the relative variability within the different samples. Principal component analysis is an unsupervised clustering method requiring no knowledge of the data set and acts to reduce the dimensionality of multivariate data while preserving variance within the data.[38] From the crude ethanol extract and 5 fractions of *P. edulis* extract a total of 1626 mass signals were extracted by XCMS from the UPLC-MS data set acquired in negative ionisation mode. The main principal component (PC) to differentiate between fractions (PC1) accounts for 39 % of variance and PC2 for 31%. The PC1 plotted against PC2 score plot (Fig. 4A) shows that four major distinct clusters are formed, corresponding to the four fractions examined. PEF is positioned on the right-hand side

of the plot (positive PC1 values), whereas BF and aqueous mother liquor (AQF) samples are located on the far left-hand side (negative PC1 values), other sample for CF, EAE and EE are spread in between. Discrimination of CF and EAE samples from EE ones is also possible along PC2. Such samples segregation is clearly dependent on solvent polarity effect with lipophilic fractions (BF and AQF) joined together versus hydrophilic ones. The separation observed in PCA can be explained in terms of the compounds identified, using the loading plots (Fig. 4B) contributing peaks and their assignments could be made. Two major groups stand out in this plot. The first corresponds to the MS signals for mandelonitrile glycosides in peaks (1, 2, 6, & 7) and chrysin-6,8-*C*-di-hexoside 11 contributing negatively to PC1 and more enriched in BF. The second, from MS signals assigned to the unsaturated fatty acids namely linolenic 60 and linoleic acid 61 having a positive effect on PC1 and contributed the most to the discrimination of PEF. Examination of the PC2 loadings plot revealed that variables referred to oxygenated fatty acids namely, trihydroxyoctadecadienoic acid 26 and trihydroxyoctadecaenoic acid 29 contributed the most to the discrimination of CF from EAE, latter being distinctly rich in flavone mono-*C*-glycosides, *i.e.*, isovitexin 12 and orientin 10).

## Conclusions

In conclusion, this work represents the first attempt to rationalize using modern metabolites profiling for *P. edulis* use as a lead drug in the treatment of CNS disorders, well reported within that genus. Using the described UPLC-qTOF-MS/MS fingerprinting method, a total of 33 different metabolites including 12 *C*- and 4 *C,O*- flavone conjugates were annotated in *P. edulis* crude extract and its sub-fractions. Flavone and its 6/8 substituted derivatives have been shown to possess affinity for the $GABA_AR$ and to exhibit anxiolytic or sedative activity in other plants *i.e.*, *Scutellaria baicalensis*, *Leptospermum scoparium*, and *Valeriana wallichii*.[39] The metabolomic approach describe herein using UHPLC–PDA-ESI-MS data and multivariate data analysis helps reveal the major secondary metabolites that contribute to the discrimination of samples. A range of *C*-flavonoid glycosides were documented from the leaves of *P. edulis*., providing a valuable basis for the search for bioactive CNS drugs in that plant group and allowing the specific search for promising species and ecotypes for particular compounds or groups of compounds. Attempt was made to correlate between individual flavonoids (Table 1) and the measured activities to assist in pinpointing active agents likely to mediate for these effects in *P. edulis*. However, no correlation could be found for either assay, suggesting for additively or synergistic effect of several entities that may eventually be more relevant for the anxiolytic effects of *P. edulis* than just the high concentrations enrichment of certain C-flavonoid entity. Nevertheless, it should be noted that these findings are just a hint and would have to be followed by more detailed studies of *P. edulis* extracts in vivo assessing other anxiolytic treatment models, or ideally by studies on single, isolated compounds to be conclusive. Chemical profiling *via* UPLC-MS revealed that enrichment of di-*C*-glycosides versus mono-*C*-glycosides in BF and EAE that are likely to mediate for observed anxiolytic effect in these matrices. Whether other constituents found in *P. edulis* BF *i.e.*, cyanogenic glycosides can additively or synergistically enhance the anxiolytic effect has yet to be examined. Our results present one more evidence for the efficacy and complementarity of UPL*C*-MS metabolomics in the field of drug discovery to speed up natural product research, thereby helping to reduce the gap that has opened to modern drug discovery demands because of too lengthy discovery and development times when starting from natural resources.

## Acknowledgements

We are thankful to Dr. Juergen Schmidt, Leibniz institute of Plant Biochemistry, Halle Saale for assistance with the UPLC-MS analysis. Dr. Mohamed Ali Farag acknowledges the funding received by Science and Technology Development fund STDF, Egypt (grant number 12594).

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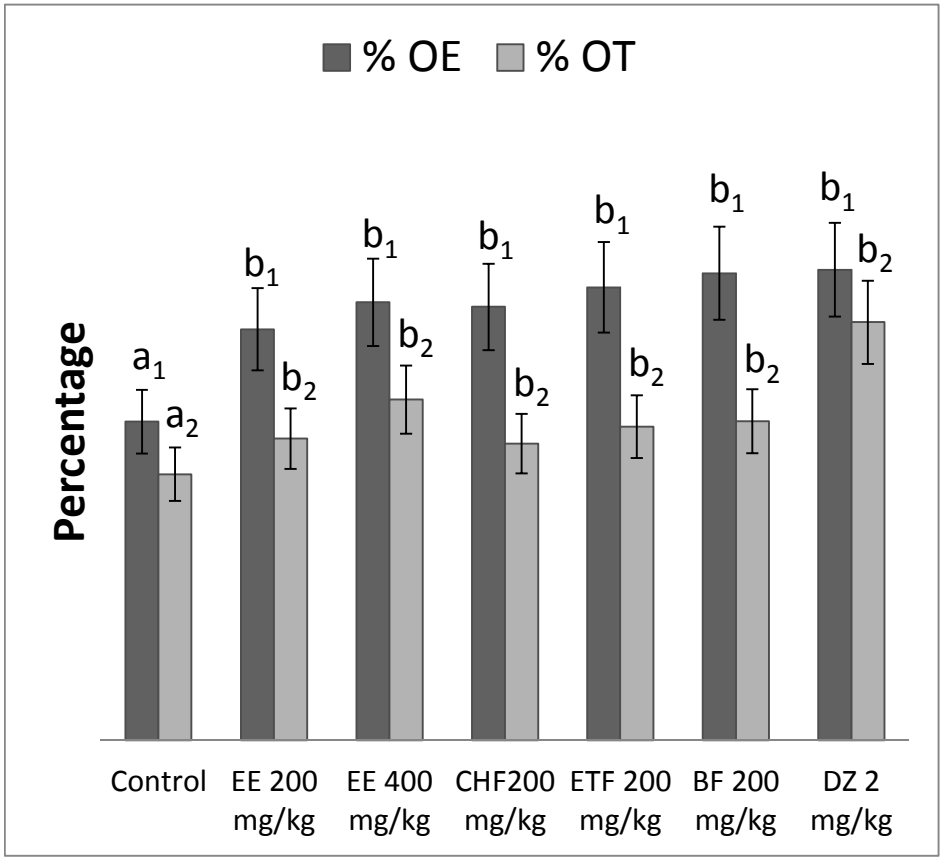


**Fig. 1** Effects of ethanolic extract of *P. edulis* and its fractions on the percentage of open arms entries (OE%), and of the time spent in open arms (OT%) in the elevated plus-maze. (EE) ethanolic extract, (CHF) chloroform, (EAF) ethyl acetate, (BF) butanol (DZ) diazepam. Results are expressed and mean ± S.E.M. (n = 5). $P \leq 0.1$, compared with control groups (Mann-Whitney test). Different letters indicate significant differences between treatments.

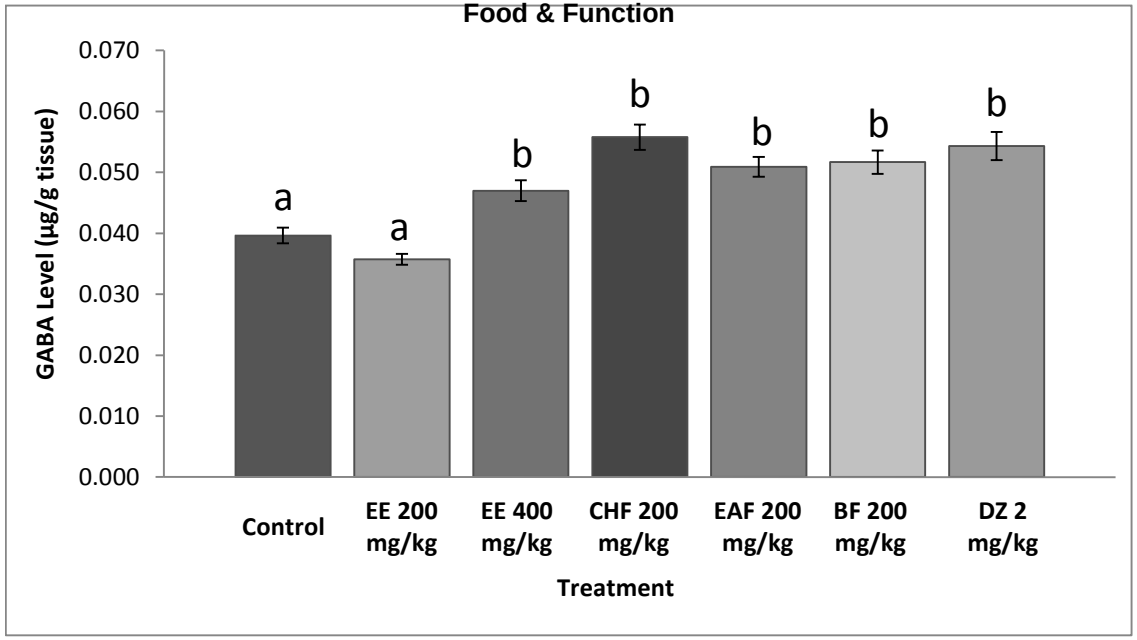


**Fig. 2** Effects of ethanolic extract of *P. edulis* and its fractions on GABA concentration expressed as µg/g of brain tissue. (EE) ethanolic extract, (CHF) chloroform, (EAF) ethyl acetate, (BF) butanol (DZ) diazepam. Results are expressed and mean ± S.E.M. (n = 5). $P \leq 0.05$, compared with control groups (one-way ANOVA followed by Least Significant Difference test (LSD test)). Different letters indicate significant differences between treatments.

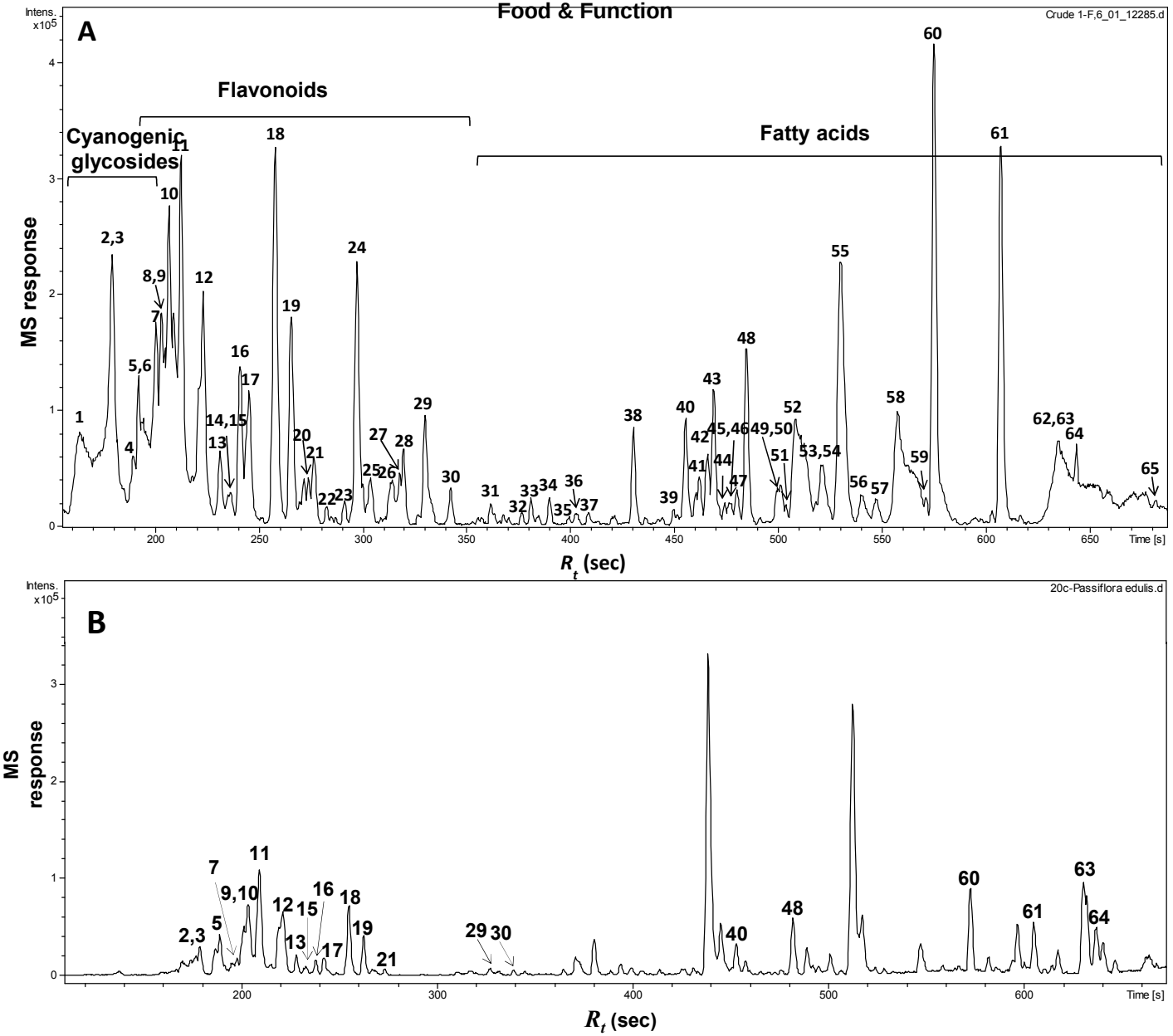


**Fig. 3** Representative UPLC-MS trace of *P. edulis* alcohol extract in in negative ion mode (A) and positive ion mode (B), spectrum is characterized by 2 main regions: (50-200 sec) with peaks principally due to cyanogenic glycosides, region (250-350) for flavonoids and region (350-700) for fatty acids. Peak numbers follow those listed in (**Table 2**) for metabolite identification using UPLC-PDA-MS

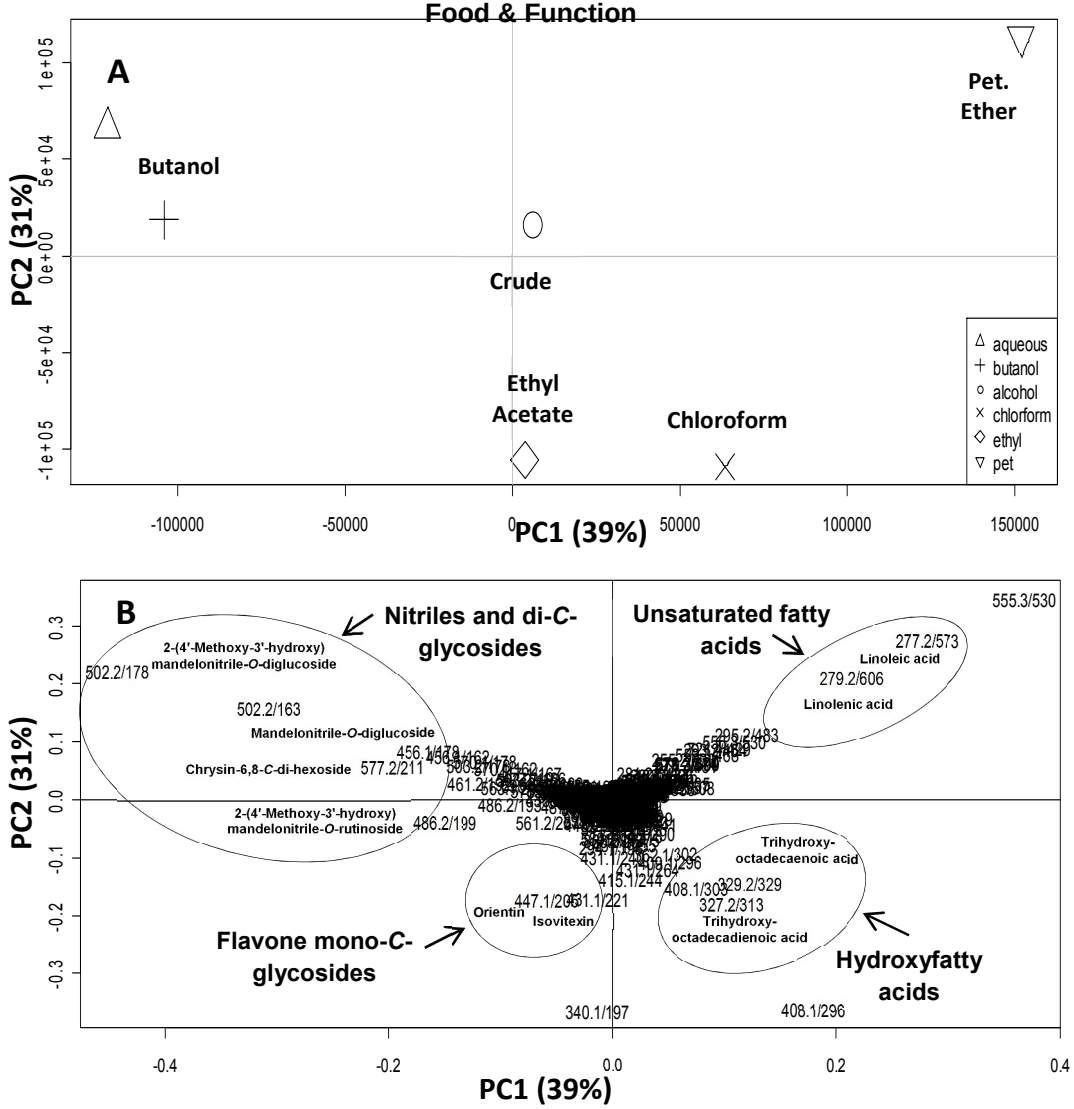


**Fig. 4** UPLC-qTOF-MS (m/z 100-1000) principal component analyses of the different *P. edulis* extract and fractions. The metabolome clusters are located at the distinct positions described by two vectors of principal component 1 (PC1 = 39%) and principal component 2 (PC2 = 31%). (A) Score Plot of PC1 vs PC2 scores. (B) Loading plot for PC1 and PC2 with contributing mass peaks and their assignments, with each metabolite denoted by its mass/$R_t$ (sec) pair. It should be noted that ellipses do not denote statistical significance, but are rather for better visibility of clusters as discussed.

**Table 1 Metabolites identified in *P. edulis* ethanol extract using UPLC–PDA–MS in negative ionization mode**

| Peak No. | $M^-$ | $R_t$(s) | UV | Molecular Formula | Error | $MS/MS^-$ | Identification |
|---|---|---|---|---|---|---|---|
| **1** | 456.1499 | 162 | 212, 269 | $C_{20}H_{26}NO_{11}^-$ | 1.6 | 269, 293, 325, 285, 431 | Mandelonitrile-*O*-di-glucoside (Amygdalin) |
| **2** | 502.1570 | 178 | 212, 269, 338 | $C_{21}H_{28}NO_{13}^-$ | 0.2 | 179, 315, 325, 341, 285, 477 | 2-(4'-Methoxy-3'-hydroxy)-mandelonitrile-*O*-di-glucoside |
| **3** | 609.1558 | 180 | 214, 269, 346 | $C_{27}H_{29}O_{16}^-$ | 0.8 | 369, 399, 447, 489, 519, 591 | Luteolin-6,8-*C*-di-glucoside (Lucenin-2) |
| **4** | 461.1668 | 190 | 214, 270, 337 | $C_{20}H_{29}O_{12}^-$ | 0.8 | 294, 340, 403, 415, 435 | Tyrosol-*O*- di-hexoside |
| **5** | 593.1651 | 191 | 214, 270, 335 | $C_{27}H_{29}O_{15}^-$ | 1.2 | 353, 383, 473, 503, 575 | Apigenin-6,8-*C*-di-glucoside (Vicenin-2) |
| **6** | 440.1550 | 193 | 214, 269, 234 | $C_{20}H_{26}NO_{10}^-$ | 2.0 | 269, 294, 415, 435 | Mandelonitrile-*O*-rutinoside |
| **7** | 486.1604 | 199 | 213, 270, 337 | $C_{21}H_{28}NO_{12}^-$ | 0.7 | 294, 315, 340, 461 | 2-(4'-Methoxy-3'-hydroxy)-mandilonitrile-*O*-rutinoside |
| **8** | 447.1030 | 202 | 212, 269, 349 | $C_{21}H_{19}O_{11}^-$ | 0.2 | 327, 357, 369, 285, 545 | Luteolin-6-*C*-glucoside (Isoorientin) |
| **9** | 563.1512 | 203 | 211, 270, 349 | $C_{26}H_{27}O_{14}^-$ | 1.3 | 443, 473, 383, 503, 487, 547 | Apigenin-6-*C*-glucoside-8-*C*-arabinoside (Schaftoside) |
| **10** | 447.1030 | 205 | 212, 269, 348 | $C_{21}H_{19}O_{11}^-$ | 0.8 | 327, 357, 285, 545 | Luteolin-8-*C*-glucoside (Orientin) |
| **11** | 577.1444 | 211 | 214, 270, 343 | $C_{27}H_{29}O_{14}^-$ | 3.4 | 457, 487, 559, 311, 473 | Chrysin-6,8-*C*-di-hexoside |
| **12** | 431.0971 | 221 | 214, 270, 333 | $C_{21}H_{19}O_{10}^-$ | 1.0 | 311, 341, 353, 383, 413 | Apigenin-6-*C*-glucoside (Isovitexin) |
| **13** | 563.1391 | 230 | 215, 270, 338 | $C_{26}H_{27}O_{14}^-$ | 3.1 | 459, 473, 545, 369, 443, 503 | Luteolin-6-*C*-deoxyhexoside-8-*C*-pentoside |
| **14** | 561.1717 | 234 | 211, 270, 321 | $C_{27}H_{29}O_{13}^-$ | 0.8 | 269, 311, 415, 547 | Apigenin-*C*-pentoside-*O*-deoxyhexoside-methyl ether |

| Peak No. | $M^-$ | $R_t$(s) | UV | Molecular Formula | Error | $MS/MS^-$ | Identification |
|---|---|---|---|---|---|---|---|
| **15** | 577.1444 | 235 | 215 | $C_{27}H_{29}O_{14}^-$ | 2.6 | 311, 269, 415, 457, 559, 369 | Apigenin-*C*-deoxyhexoside-*O*-hexoside |
| **16** | 431.1018 | 240 | 212, 270, 347 | $C_{21}H_{19}O_{10}^-$ | 2.7 | 285, 327, 357, 413, 311, 341 | Luteolin-6-*C*-quinovoside/fucoside |
| **17** | 415.1039 | 244 | 214, 270, 315 | $C_{21}H_{19}O_9^-$ | 2.8 | 295, 325, 357, 397, 327, 415 | Chrysin-6-*C*-hexoside |
| **18** | 561.1717 | 257 | 215, 270, 325 | $C_{27}H_{29}O_{13}^-$ | 2.4 | 295, 457, 543, 441 | Chrysin-*C*-hexoside-*O*-deoxyhexoside |
| **19** | 431.1128 | 264 | 211, 269, 347 | $C_{21}H_{19}O_{10}^-$ | 1.0 | 285, 327, 357, 311, 413 | Luteolin-8-*C*-deoxyhexoside |
| **20** | 607.1675 | 273 | 212, 269, 341 | $C_{28}H_{31}O_{15}^-$ | 1.1 | 362, 408, 493 | Unknown |
| **21** | 415.1039 | 276 | 217, 269, 330 | $C_{21}H_{19}O_9^-$ | 1.6 | 269, 294, 311, 341, 397 | Apigenin-8-*C*-deoxyhexoside |
| **22** | 905.2747 | 282 | 217, 271 | $C_{38}H_{63}O_1^{-}$ | 3.8 | 545, 743, 811, 859 | Unknown |
| **23** | 383.1335 | 290 | 216 | $C_{18}H_{23}O_9^-$ | 3.1 | 180, 249, 317 | Unknown |
| **24** | 362.1285 | 296 | 208, 220 | $C_{18}H_{20}NO_7^-$ | 3.6 | 225, 256, 294, 408(formic acid adduct | Mandelonitrile-*O*-butenoylglucoside |
| **25** | 743.4332 | 299 | 217, 270, 346 | $C_{38}H_{63}O_{14}^-$ | 3.9 | 415, 561, 643 | Unknown |
| **26** | 327.2167 | 313 | 209, 274 | $C_{18}H_{31}O_5^-$ | 2.6 | 113, 215, 257, 269, 301 | Trihydroxy-octadecadienoic acid ($C_{18:2}$) |
| **27** | 889.4931 | 317 | 218 | $C_{44}H_{73}O_{18}^-$ | 1.4 | 395, 467, 765, 809, 843 | Unknown |
| **28** | 741.4039 | 319 | 217, 270, 323 | $C_{38}H_{61}O_{14}^-$ | 3.1 | 327, 399, 697 | Unknown |
| **29** | 329.2320 | 329 | 215, 276 | $C_{18}H_{33}O_5^-$ | 1.2 | 183, 227, 249, 259, 309 | Trihydroxy-octadecaenoic acid ($C_{18:1}$) |

| Peak No. | M$^-$ | $R_t$(s) | UV | Molecular Formula | Error | MS/MS$^-$ | Identification |
|---|---|---|---|---|---|---|---|
| **30** | 287.2215 | 342 | 218 | $C_{16}H_{31}O_4^-$ | 4.6 | 175, 241, 248, 251, 256 | Dihydroxy-hexadecanoic acid ($C_{16:0}$) |
| **31** | 727.4248 | 361 | 218 | $C_{45}H_{59}O_8^-$ | 4.0 | 249, 317, 475, 533, 667 | Unknown |
| **32** | 253.0497 | 376 | 218 | $C_{15}H_9O_4^-$ | 4.3 | 175, 181, 242, 249 | 4',7-dihydroxyflavone (Chrysin)/ 5,7-Dihydroxyneoflavone (Serratin) |
| **33** | 311.1854 | 380 | 217 | $C_{17}H_{27}O_5^-$ | 2.7 | 175, 249 | Unknown |
| **34** | 293.1761 | 389 | 218 | $C_{17}H_{25}O_4^-$ | 2.0 | 175, 236, 243 | Unknown |
| **35** | 487.3400 | 398 | 220 | $C_{30}H_{47}O_5^-$ | 5.6 | 207, 250, 265, 309, 355 | Unknown |
| **36** | 357.2634 | 402 | 220 | $C_{20}H_{37}O_5^-$ | 3.3 | 113, 175, 249, 309 | Unknown |
| **37** | 883.4150 | 408 | 220 | $C_{65}H_{55}O_3^-$ | 5.0 | 113, 175, 249, 309 | Unknown |
| **38** | 721.3617 | 429 | 220 | $C_{34}H_{57}O_{16}^-$ | 0.2 | 649, 675 | Unknown |
| **39** | 313.0775 | 449 | 217 | $C_{10}H_{17}O_{11}^-$ | 0.5 | 113, 175, 249 | Unknown |
| **40** | 293.2120 | 454 | 220 | $C_{18}H_{29}O_3^-$ | 0.7 | 275 | Hydroxy-octadecatrienoic acid ($C_{18:3}$) |
| **41** | 577.1444 | 461 | 220 | $C_{30}H_{41}O_{11}^-$ | 4.9 | 361, 505, 551, 564 | Unknown |
| **42** | 265.1483 | 465 | 220 | $C_{12}H_{25}O_4S^-$ | 2.2 | 243, 249, 253 | Unknown |
| **43** | 559.3105 | 468 | 221 | $C_{28}H_{47}O_{11}^-$ | 2.2 | 297, 339,487, 513 | Unknown |
| **44** | 699.3792 | 473 | 220 | $C_{28}H_{47}O_{11}^-$ | 6.0 | 291, 297, 559, 595, 653 | Unknown |

| Peak No. | M$^-$ | $R_t$(s) | UV | Molecular Formula | Error | MS/MS$^-$ | Identification |
|---|---|---|---|---|---|---|---|
| **45** | 595.2880 | 475 | 220 | $C_{41}H_{39}O_4^-$ | 3.6 | 297, 365, 452, 559 | Unknown |
| **46** | 297.1522 | 476 | 220 | $C_{19}H_{21}O_3^-$ | 9.4 | 147, 175, 249, 265 | Unknown |
| **47** | 540.3291 | 479 | 220 | $C_{39}H_{42}NO^-$ | 3.3 | 295, 363, 417, 480 | Unknown |
| **48** | 295.2276 | 483 | 220 | $C_{18}H_{31}O_3^-$ | 0.3 | 221, 277 | Hydroxy-octadecadienoic acid ($C_{18:2}$) |
| **49** | 561.3255 | 498 | 220 | $C_{28}H_{49}O_{11}^-$ | 5.0 | 249, 311, 471, 539 | Unknown |
| **50** | 571.2870 | 499 | 220 | $C_{39}H_{39}O_4^-$ | 4.4 | 311, 471, 515, 561 | Unknown |
| **51** | 579.2829 | 500 | 220 | $C_{30}H_{43}O_{11}^-$ | 4.1 | 293, 561 | Unknown |
| **52** | 311.1679 | 506 | 220 | $C_{13}H_{27}O_8^-$ | 9.0 | 293, 297 | Unknown |
| **53** | 555.2819 | 517 | 220 | $C_{28}H_{43}O_{11}^-$ | 1.7 | 395, 419, 433, 507 | Unknown |
| **54** | 311.1679 | 520 | 220 | $C_{13}H_{27}O_8^-$ | 9.0 | 165, 249 | Unknown |
| **55** | 555.2829 | 530 | 220 | $C_{28}H_{43}O_{11}^-$ $C_{39}H_{37}O_3^-$ | 1.7 | 433 | Unknown |
| **56** | 353.1998 | 539 | 220 | $C_{19}H_{29}O_6^-$ | 8.4 | 285, 325, | Unknown |
| **57** | 483.2716 | 546 | 220 | $C_{36}H_{35}O^-$ | 5.6 | 325, 393 | Unknown |
| **58** | 325.1838 | 556 | 220 | $C_{14}H_{29}O_8^-$ | 7.6 | 293 | Unknown |
| **59** | 271.2275 | 570 | 220 | $C_{16}H_{31}O_3^-$ | 0.5 | 113, 265 | Hydroxy-hexadecanoic acid ($C_{16:0}$) |

| Peak No. | $M^-$ | $R_t$(s) | UV | Molecular Formula | Error | $MS/MS^-$ | Identification |
|---|---|---|---|---|---|---|---|
| **60** | 277.2172 | 574 | 220 | $C_{18}H_{29}O_2^-$ | 0.5 | 211, 251 | Linolenic acid ($C_{18:3}$) |
| **61** | 279.2331 | 606 | 220 | $C_{18}H_{31}O_2^-$ | 2.9 | 171, 211 | Linoleic acid ($C_{18:2}$) |
| **62** | 339.1995 | 633 | 220 | $C_{22}H_{27}O_3^-$ | 8.8 | 181, 323 | Unknown |
| **63** | 255.2311 | 633 | 220 | $C_{16}H_{31}O_2^-$ | 0.7 | 239 | Palmitic acid ($C_{16:0}$) |
| **64** | 281.2484 | 642 | 220 | $C_{18}H_{33}O_2^-$ | 0.6 | 171, 183, 239 | Oleic acid ($C_{16:1}$) |
| **65** | 283.2484 | 680 | 220 | $C_{18}H_{35}O_2^-$ | 5.7 | 113, 165, 183, 223, 239 | Stearic acid ($C_{18:0}$) |

**Table 2 $MS^n$ Data of mono-*C*- and di-*C*-glycosylflavone derivatines in *P. edulis***

| Peak No. | $M^-$ | Identification | $(M-18)^-$ | $(M-90)^-$ | $(M-120)^-$ | $(M-60)^-$ | $(M-104)^-$ |
|---|---|---|---|---|---|---|---|
| 3 | 609.1558 | Luteolin-6,8-*C*-di-glucoside (Lucenin-2) | + | + | ++ | - | - |
| 5 | 593.1651 | Apigenin-6,8-*C*-diglucoside (Vicenin-2) | + | + | ++ | - | - |
| 8 | 447.1030 | Luteolin-6-*C*-glucoside (Isoorientin) | + | + | ++ | - | - |
| 9 | 563.1512 | Apigenin-6-*C*-glucoside-8-*C*-arabinoside (Schaftoside) | + | + | ++ | + | - |
| 10 | 447.1030 | Luteolin-8-*C*-glucoside (Orientin) | - | + | ++ | - | - |
| 11 | 577.1444 | Chrysin-6,8-*C*-di-hexoside | + | + | ++ | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 12 | 431.0971 | Apigenin-6-*C*-glucoside (Isovitexin) | + | + | ++ | - | - |
| 13 | 563.1391 | Luteolin-6-*C*-rhamnoside-8-*C*-pentoside | + | + | - | + | ++ |
| 15 | 577.1444 | Apigenin-*C*-deoxyhexoside-*O*-hexoside | + | - | - | - | ++ [1] |
| 16 | 431.1018 | Luteolin-6-*C*-deoxyhexoside | + | - | - | - | + |
| 17 | 415.1039 | Chrysin-6-*C*-hexoside | + | + | ++ | - | - |
| 18 | 561.1717 | Chrysin-*C*-hexoside-*O*-deoxyhexoside | + [2] | + [3] | ++ [4] | - | + |
| 19 | 431.1128 | Luteolin-8-*C*-deoxyhexoside | + | - | + | - | ++ |
| 21 | 415.1039 | Apigenin-8-C-deoxyhexoside | + | - | - | - | + |

++ Base Peak, [1] $(M\text{-hexose-}104)^-$, [2] $(M\text{-deoxyhexose-}18)^-$, [3] $(M\text{- deoxyhexose }-90)^-$, [4] $(M\text{- deoxyhexose }-120)^-$