

Short Communication:

Analysis of detected metabolites compounds from the crude extract of Rimau Gerga Lebong oranges fruit (*Citrus reticulata* 'RGL') using LC-QTOF-MS/MS

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Abstract. Yunita E, Kurniati T, Rosa FL, Melati P, Lestari N. 2022. Short Communication: Analysis of detected metabolites compounds from the crude extract of Rimau Gerga Lebong oranges fruit (*Citrus reticulata* 'RGL') using LC-QTOF-MS/MS. *Biodiversitas* 23: 3778-3783. Rimau Gerga Lebong (RGL) oranges are the leading commodity in Bengkulu Province. However, the content of bioactive compounds in RGL oranges has not been widely reported. This study aims to identify the flavonoid, alkaloids, and tannins from the ethanol extract of RGL oranges. The results of further identification will be used as a reference in further research on molecular docking in various biological activities. Chemical compounds were identified using qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS/MS). The measurement of vitamin C levels was carried out using High-Performance Liquid Chromatography (HPLC). The results showed 25 identified compounds, consisting of 6 compounds belonging to the alkaloid group, 17 from the flavonoid group, and 2 from the tannin group. The alkaloid group compounds were 2 α , 3 β , 6 α -trihydroxy nortopane, 2 α , 3 β -dihydroxy nortopane, isosalsolin, 3 α -dihydrocadambine, chelidimerine, and oleracein D. The compounds from identified flavonoid group were chebuloside, neokurarinol, rutinose, flavonone, glucoside, licochalcone, viscumneose, and pinnatifinoside. The identified tannins are corilagin and moracin. These compounds that have been identified have biological activity potential as antioxidants, anti-asthma, anti-dementia, and anticancer. The vitamin C content of the ethanol extract of RGL oranges was 0.02 \pm 0.0001%. This vitamin C supports the antioxidant activity of RGL orange.

Keywords: Alkaloid, flavonoid, LC-QTOF-MS/MS, oranges, RGL, tannin

Abbreviations: RGL: Rimau Gerga Lebong; LC-QTOF-MS/MS: Qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry

INTRODUCTION

One of the typical oranges cultivated in Bengkulu Province is the Rimau Gerga Lebong (RGL) (*Citrus reticulata*) and which has better antioxidant activity than the kalamansi orange (Yunita et al. 2021). Oranges have been known to have various biological activities such as antioxidants (Wijaya et al. 2017; Yunita et al. 2021), antidiabetic, and antihypertensive (Alu'Datt et al. 2017). The RGL orange is a superior commodity from Bengkulu Province. It was designated as a national superior variety by the Ministry of Agriculture of the Republic of Indonesia in 2012 because it can bear fruit throughout the year with two harvests in one year (Astuti et al. 2018). In addition, RGL oranges are yellow-orange and are in great demand by local people (Siagian et al. 2021). This RGL orange development program is mainly located in PAL VIII Village, Bermani Ulu Raya Sub-district, Rejang Lebong District, with an area of 200 hectares.

However, no specific data is reported regarding the content of RGL bioactive compounds. Similar studies have identified bioactive compounds from Mandarin Oranges belonging to the same species as RGL (Wang et al. 2021). However, the results of the study showed that the typical compounds in each type of mandarin orange could be different even though they came from the same species (Wang et al. 2021). In addition, most of the reported data regarding the compounds present in the RGL citrus species are the result of HPLC, so it has not been possible to analyze large amounts of bioactive compounds simultaneously (Abeyasinghe et al. 2007; Mei et al. 2021).

Flavonoids, alkaloids, tannins, and saponins are phytochemical screening parameters for oranges (Roghini and Vijayalakshmi 2018). This study aims to identify and analyze the flavonoid compounds, alkaloids, tannins, and saponins in the ethanolic extract of RGL citrus fruit using LC-QTOF-MS/MS. In addition, this study also analyzed vitamin C content in the RGL orange extract using High-

Performance Liquid Chromatography (HPLC). This research is expected to provide information on the content of active compounds in RGL oranges from Bengkulu Province, Indonesia. Information on the content of RGL bioactive compounds is expected to expand the utilization of RGL oranges. In addition, further research with bioinformatics is needed as early identification of the role of these various compounds in biological systems.

MATERIALS AND METHODS

Plant Materials, sample preparation, and extraction

The mature RGL citrus fruits were collected from Rimbo Penadang Sub-district, Lebong District, Bengkulu Province, Indonesia in October 2019 with a medium maturity level. The oranges are immediately taken to the laboratory and dried without being exposed to direct sunlight. After that, 45 g of dried citrus was macerated using 96% ethanol at room temperature. The maceration process was carried out for 5x24 hours. Finally, the ethanol extract was concentrated using a rotary evaporator. The concentrated extract was then directly examined by LC-QToF-MS/MS and HPLC for measuring vitamin C levels.

Identification of metabolite compounds and measurement of vitamin C levels

Bioactive compounds were identified at the PT Saraswanti Indo Genetech Laboratory, Bogor, Indonesia, with standardized tests from the laboratory (Qiao et al. 2013) Contract No. SIG.Mark.R.X.2019.018559. Chemical compounds in RGL oranges were identified using LC-QToF-MS/MS, Acquity I Class-Waters-Waters Xevo G2S QTOF-Waters. The compounds were identified using two types of ESI-MS ionization modes, namely positive and negative ion modes, to determine the group of compounds, i.e., alkaloids, flavonoids, tannins, and saponins. The standard chemical compounds were biotin at 1 mg/L and chloramphenicol at 1 ppm. The biotin standard was prepared by pipetting 25 μ L of 1000 mg/L biotin standard and dissolved with distilled water to a volume of 25 mL in a volumetric flask. Standard chloramphenicol 1 ppm was prepared by pipetting 25 μ L of standard chloramphenicol 1000 mg/L and dissolved with distilled water to a volume of 25 mL.

0.1 g of sample was added with methanol until it reached a volume of 10 mL, homogenized, filtered with a 0.22 μ m GHP/PTFE membrane filter, then injected into the UPLC system using a C18 column. The LC setting condition used is Column C18. The column temperature was 40°C, while the autosampler temperature was 15°C. The sample was injected at a volume of 10 μ L. The mobile phase used was 0.1% acetonitrile formic acid and 0.1% formic acid in aquabidest. The flow rate used is 0.6 mL/min, Gradient. The MS settings used are ToF-MS^E operation mode, ionization ESI (-)/ESI (+), and the acquisition range was 50-1200 Da.

Vitamin C levels were measured at the Laboratory of the Department of Food Science and Technology, Faculty of Agricultural Technology, IPB University. A total of 1 g

of RGL orange extract was analyzed with the Luna 5u C18 (2) 100A (4.6 X 250 mm) HPLC system, using a UV-Vis PDA detector at a wavelength of 242 nm. The mobile phase was methanol pro-HPLC. The flow rate used in this study was 1 mL/minute. The test was carried out twice.

Data analysis

The identification of chemical compounds from natural ingredients using LCMS/MS-QTOF is carried out with UNIFI software which already has a mass spectrum library of chemical compounds from the Waters database. UNIFI software identified the mass spectrum of the compound in the sample by matching it with the mass spectrum in the library. The criteria for the active substances to be identified are: (i) Mass error of analyte reading ≤ 5 ppm error; (ii) Isotope match MZ RMS PPM ≤ 6 ppm and Isotope match MZ RMS % ≤ 10 %; (iii) Analyte intensity 300; and (iv) There is one fraction with brake value < 4 in Fragment match elucidation system.

RESULTS AND DISCUSSION

Identified bioactive compounds in RGL orange extract

The analysis of RGL orange extract showed the presence of bioactive compounds from the alkaloids, flavonoids, and tannins group, but saponins were not detected in the examined RGL extracts. Figure 1 shows the number of chemical compounds identified in each group. Flavonoids have the most variation. A previous metabolomics study of *Citrus reticulata* fruit from China showed that the highest number of bioactive components were flavones (143 compounds), followed by alkaloids (40 compounds) and flavonoids (28 compounds) (Wang et al. 2020). It indicated that the bioactive compounds of *C. reticulata* from different planting locations might differ from the same species (Abeyasinghe et al. 2007; Wang et al. 2019; Wang et al. 2020). The difference in secondary metabolites could be due to genetic variations, availability of nutrients, and environmental conditions where they are grown (Anggraheni 2021; Siagian et al. 2021). In addition, the difference in the number of identified compounds may be influenced by the sensitivity of the LC-QTOF-MS/MS used. The higher the sensitivity of the equipment used, the more compounds that can be detected. In this study, the Acquity I Class-Waters-Waters Xevo G2S QTOF-Waters equipment can detect up to 1 ppm of sample concentration with a mass error of analyte reading of less than 5 ppm. Differences in mass spectrum libraries in different equipment for detecting compounds also underlie differences in the number of detected compounds.

Alkaloid contents in RGL oranges

Alkaloids are basic substances and contain heterocyclic nitrogen. Alkaloids are synthesized from amino acid derivatives. Therefore, alkaloids could be toxic components that protect plants from insects and other animals, neutralizing components of toxins for the plant body, growth regulators, and growth factors. Besides, alkaloids are a group of compounds that may act as antioxidants

(Dalimunthe et al. 2018). In the alkaloid group, the compounds found were 2 α , 3 β , 6 α -trihydroxy nortopane; 2 α , 3 β -dihydroxy nortopane; isossaline; 3 α -dihydrocadambine; Chelidimerine, and Oleracein D. Four compounds were identified under positive ESI conditions while 2 other compounds were identified under negative ESI conditions. Figure 2 shows the chromatogram of alkaloids in the positive ESI mode, and Figure 3 shows the chromatogram of the alkaloids in the negative ESI mode.

Analysis of the alkaloid group showed that 6 compounds were identified. A study by Fomani et al. (2016) showed similar results: *Citrus reticulata* has alkaloids that could act as oxidative burst inhibition, cytotoxicity, and antibacterial activity. Oleracin, one of the alkaloid compounds, has antidiabetic potential (Roozi et al. 2021). Further studies are needed to identify other compounds that may be identified in a wider spectrum of metabolomics studies. The alkaloid group of compounds isolated from Mandarin *C. reticulata* is also reported to have anti-asthmatic activity (Wang et al. 2021). In addition, the alkaloid group of compounds has also been reported to have cytotoxic activity on five human tumor cell lines (Ye

et al. 2021). Thus, the compounds identified in RGL oranges will likely be investigated further for their biological activities.

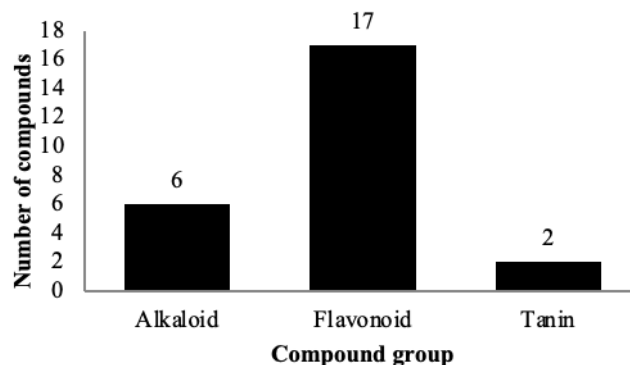


Figure 1. Number of compounds identified in the group of alkaloids, flavonoids, and tannins

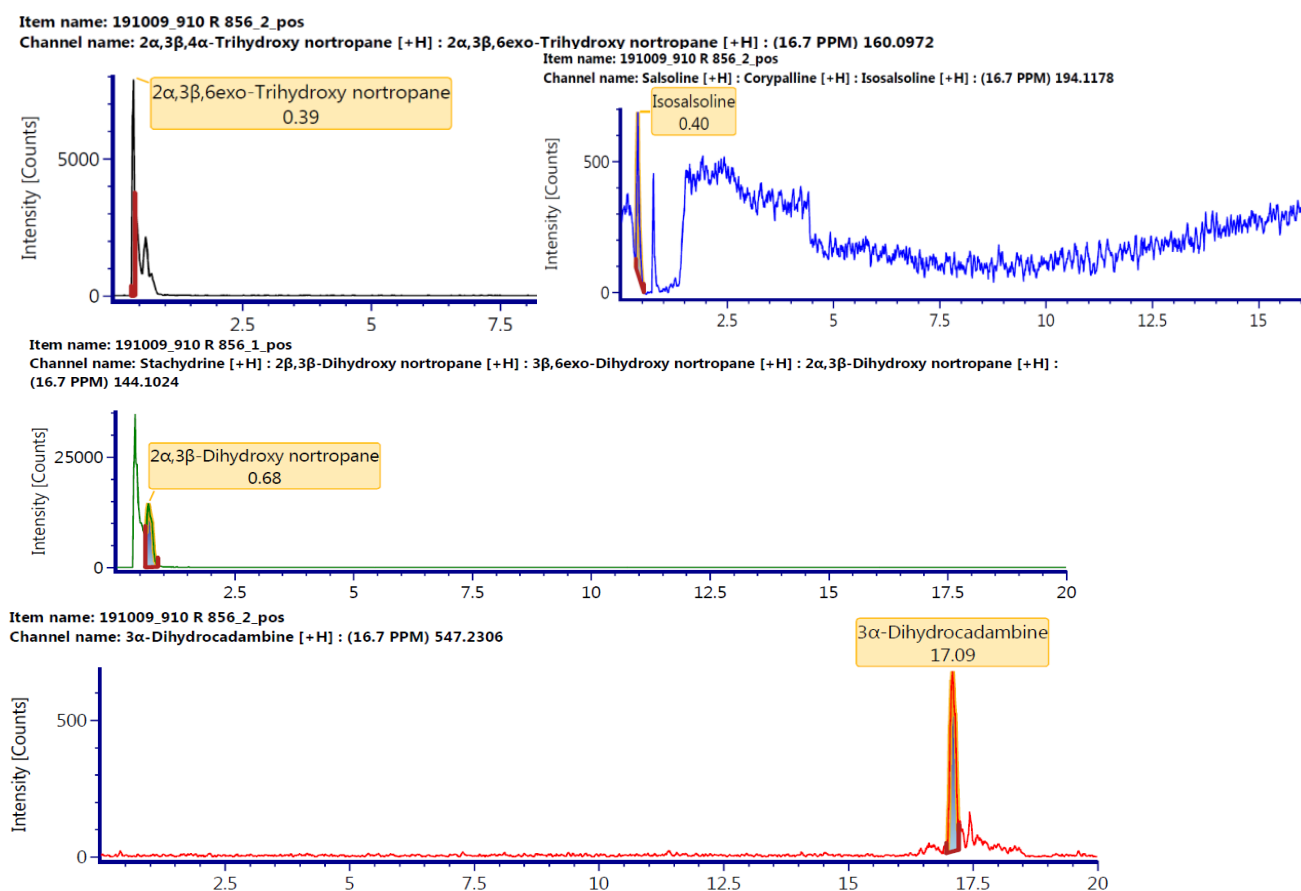


Figure 2. Chromatogram of alkaloid compounds in RGL oranges on positive ESI. The x-axis represents the retention time [min]

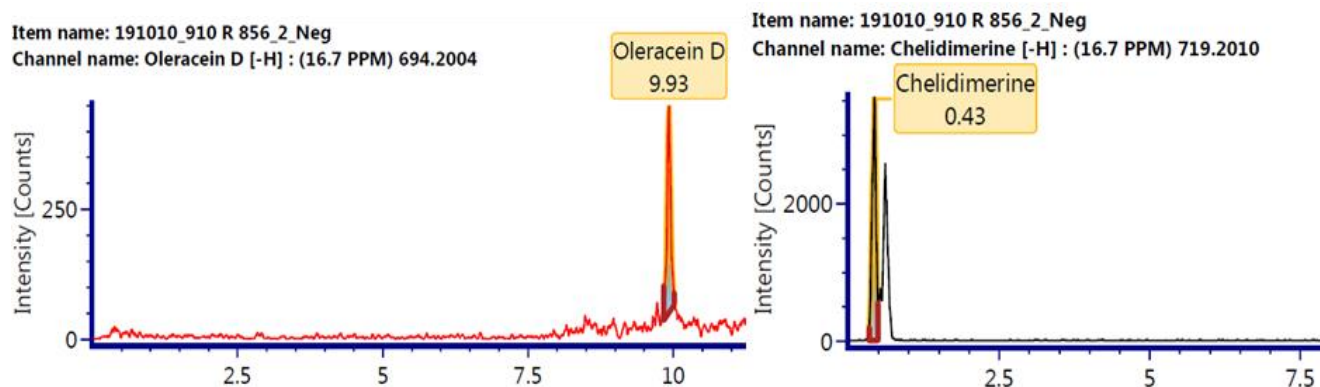


Figure 3. Chromatogram of alkaloid compounds in RGL oranges on negative ESI. The x-axis represents the retention time [min]

Flavonoid contents and vitamin C concentration in RGL oranges

Flavonoids are a group of secondary metabolites in plants that are found in many tissues. Flavonoids are included in the group of phenolic compounds with a C₆-C₃-C₆ chemical structure. Flavonoids have an aromatic ring A in their structure, an aromatic ring B, and a middle ring of the molecule in the form of a heterocyclic structure. The heterocyclic ring contains oxidized oxygen. The highest number of detected compounds in this study was in the flavonoid group, i.e., 17 compounds. The major phenolic compounds in oranges are flavonoids. Table 1 shows the details of the flavonoid compounds identified from the ethanolic extract of RGL citrus fruit.

In this study, the flavonoid group (including compounds from the flavone group) had a great variety of compounds. A total of 10 compounds were identified under positive ESI conditions, and 8 compounds were identified under negative ESI conditions. Hesperidin was identified on positive and negative ESIs. Flavonoids have antioxidant activity by donating hydrogen atoms. In addition, flavonoid compounds are usually able to chelate metals. It is in line with previous studies that have reported the same citrus species in different planting areas (Wang et al. 2020). Flavonoid compounds have their main role as antioxidants due mainly to the structure phenolic hydroxyl group attached to the ring structure of flavonoids (Chen et al. 2020). Antioxidants prevent oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, or chelating metal ions (Abeyasinghe et al. 2007; Guimarães et al. 2010; Chen et al. 2020; Kadhon et al. 2020). A previous study by Yunita et al. (2021) showed that the antioxidant activity of RGL oranges was better than that of kalamansi oranges from Bengkulu Province (*Citrus microcarpa*). It may be due to many types of identified flavonoid compounds attributed to their antioxidant activity.

The concentration of vitamin C in RGL oranges in this study was 0.02±0.0001%. Vitamin C acts as an antioxidant (Sir et al. 2018) by donating an electron and neutralizing free radicals, and inhibit lipid peroxidation, and preventing cell damage. This data is in line with the previous research (Sir et al. 2018).

Tannin and saponin contents of RGL oranges

Tannins are secondary metabolites of phenolic compounds with molecular sizes of 500 Da to 3000 Da. Tannins mainly play a role in plant defense mechanisms. Tannins usually are water-soluble and form complexes with protein molecules. Tannins are commonly found in plant vacuoles (Hassanpour et al. 2011). Identification of the tannin group in this research showed the presence of two compounds, i.e., Corilagin on a positive ESI and moracin A on a negative ESI (Figure 4). Corilagin was detected at a retention time (rt) of 0.44 min, whereas moracin was detected at an rt of 16.45 min. A previous study showed that corilagin was analyzed in silico, is considered an inhibitor of pancreatic lipase enzymes, and could be used to treat obese patients (Hairulazam et al. 2021). Furthermore, Moracin is reported to inhibit the activity of the secretase enzyme; therefore, it can inhibit the synthesis of amyloid in Alzheimer's and dementia conditions (Murata 2019). However, further studies are needed to identify other compounds in broader metabolomics studies.

Saponins are amphipathic glycosides that foam when shaken vigorously. The molecular structure of the saponin group consists of a glycoside chain composed of sugars that bind to the aglycon (Herawati et al. 2020). There were no compounds from the saponin group in the ethanol extract of the RGL citrus. A previous study by Salman et al. (2014) showed that *Citrus reticulata* did not contain saponin; however, several citrus species contain saponins, including *Citrus aurantifolia*, *C. grandis*, *C. limon*, *C. paradisi*, and *C. reticulata* from China (Ezeabara 2014).

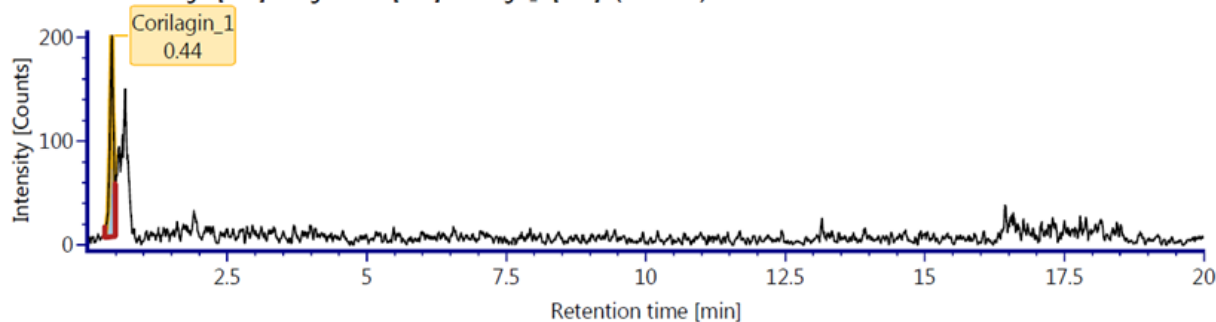
In conclusion, analysis of the ethanolic extract of RGL citrus fruit by LC-MS/MS/QTOF showed the presence of 6 compounds from the alkaloid group, 17 from the flavonoid group, and 2 tannin compounds it did not contain compounds from the saponin group. In addition, the ethanolic extract of RGL citrus fruit also contained vitamin C at the concentration of 0.02±0.0001%. These components will likely be investigated further so RGL oranges can be utilized more optimally.

Table 1. Identification of flavonoid compounds in the ethanolic extract of RGL citrus fruit

RT (min)	Molecular formula	Adducts	Number of high energy fragments identified	Isotope MTCH intensity RMS percent	Compound name
10.01	C ₂₂ H ₂₄ O ₁₀	+H	1	8.79	(2S)-5,7-dihydroxy-6-methoxy-flavanone-7-O-β-D glucopyranoside
10.01	C ₁₆ H ₁₄ O ₆	+H	1	6.83	3,7-dihydroxy-3-(3,4-dihydroxy benzyl)-chroman-4-one
10.01	C ₂₈ H ₃₄ O ₁₅	+H, +Na, +K	3	3.32	Hesperidin
9.93		-H	2	2.69	
16.66	C ₁₉ H ₁₈ O ₆	+H, +Na	1	7.49	4',5,6,7-tetramethoxyflavone
16.66	C ₂₁ H ₂₂ O ₈	+H, +Na	1	4.97	3',4',5',5',7,8-hexamethoxyflavone
16.67	C ₂₂ H ₂₄ O ₉	+H	1	15.38	3,5,6,7,8,3',4'-heptemethoxyflavone
16.86	C ₃₆ H ₅₈ O ₁₁	+H	6	26.53	Chebuloide II
17.17	C ₂₇ H ₃₄ O ₇	+H	2	11.45	Neokurarinol
18.28	C ₂₂ H ₂₃ ClO ₁₁	+Na	1	50.04	Peonidin 3-glucoside
18.44	C ₂₁ H ₂₁ ClO ₁₁	+Na	2	83.51	Cyanidin 3-glucoside
0.42	C ₂₅ H ₂₆ O ₁₃	-H	13	165.51	ViscumneosideII
0.49	C ₂₃ H ₂₀ O ₁₀	-H	1	52.83	Pinnatifinoside C
9.48	C ₂₈ H ₃₂ O ₁₆	-H	1	66.76	Isorhamnetin-3-O-β-rutinoside_1
9.48	C ₂₇ H ₃₀ O ₁₄	-H	1	171.71	Patuletin-7-O-(6''-isobutryl)-glucoside
12.27	C ₂₈ H ₃₄ O ₁₄	-H	2	6.32	Isosakuranetin-7-rutinoside
14.00	C ₁₆ H ₁₄ O ₆	-H	1	6.71	3',4',5-Trihydroxy-7-methoxyflavanone
16.45	C ₁₆ H ₁₄ O ₅	-H	1	5.54	Licochalcone B

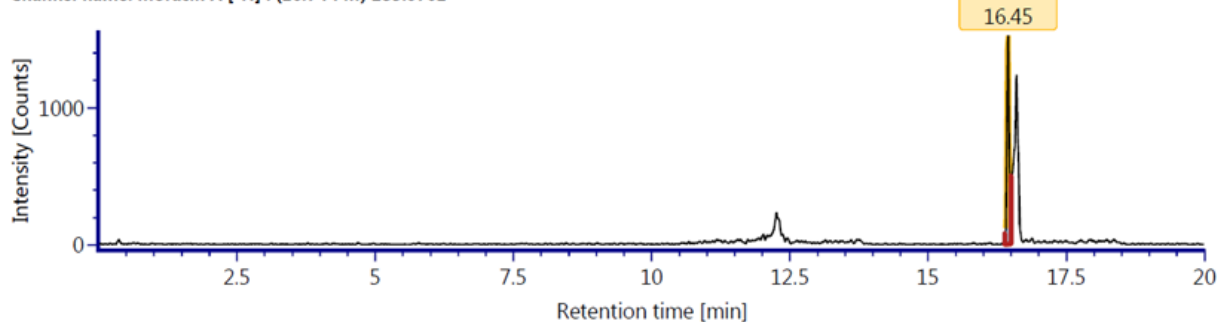
Item name: 191009_910 R 856_1_pos

Channel name: Corilagin [+Na] : Sanguin H-4 [+Na] : Corilagin_1 [+Na] : (16.7 PPM) 657.0704



Item name: 191010_910 R 856_2_Neg

Channel name: Moracin A [-H] : (16.7 PPM) 285.0762

**Figure 4.** Tannin compounds identified in positive and negative ESI mode

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