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Identification of Flavonoids from Aerial Part of *Eleusine indica* Aqueous Extract through Tandem Mass Spectrometry Molecular Networking and Diagnostic Evidence Data Analyses

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Eleusine indica (Poaceae) is a perennial grass locally known as 'rumput sambau'. The aerial part of the plant has been used traditionally by the local people of Kadazandusun to treat ailments related to inflammation and immune-associated disorders. Previous biological activities particularly antioxidant, cytotoxic, and anti-inflammatory may suggest that the chemical components of *E. indica*, principally the flavonoids could modulate the immune response. However, study on the flavonoid's constituent in the aerial part of the plant is limited. Thus, this study aimed to identify the flavonoids component in the aqueous extract of *E. indica* aerial part through liquid chromatography tandem mass spectrometry-based molecular networking technique applying MZmine, Global Natural Product Social Molecular Networking (GNPS), and SIRIUS platforms. In addition, diagnostic evidence analysis was performed based on fragmentation role, local experience and literature data to increase the confidence level in the structural identification of the flavonoids. Five flavonoids belonging to the C-glycosyl group were putatively identified as vitexin (**1**), schaftoside (**2**), isoschaftoside (**3**), vicenin 2 (**4**), and vicenin X (**5**). Some of these flavonoids have been reported to exhibit immune-related biological activities which could provide scientific support to justify the plant's traditional use. This is the first report on the existence of vicenin 2 and vicenin X in the plant.

Keywords: Ethnomedicine; *Eleusine*; glycosyl flavonoid; LCMS; SIRIUS; MZmine

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Eleusine indica (L.) Gaertn (Poaceae), locally known as 'rumput sambau', is an annual weed that thrive in various habitats, including lawns, gardens, agricultural fields, roadsides, and disturbed areas [1]. This grass can be found in tropical and subtropical regions around the world [2]. The plant is used for traditional medicinal purposes such as to treat symptoms related to microbial infections, strained muscles, hemoptysis, and incidents of centipede or scorpion envenomation [3]. The aerial part of the plant has been used traditionally by the local people of Kadazandusun to treat symptoms related to flu viral infection [3]. Pharmacological studies have shown that this plant has diverse range of biological activities including antioxidant, antimicrobial, cytotoxic, anti-inflammatory, antidiabetic, antiplasmodial, and hepatoprotective [1, 2, 5, 6].

Several classes of phytochemicals have been reported from the plant including flavonoids, terpenes and a number of primary metabolites [1, 5, 7-10]. Flavonoids have been linked to a wide range of health-promoting effects and are essential components in numerous applications related to nutrition, pharmaceuticals, medicine, and cosmetics. This is due to their ability to act as antioxidants, reduce inflammation, prevent mutations, and inhibit carcinogenesis [11-15]. A perusal of the mentioned traditional usage indicates that *E. indica* has been used to treat several disorders related to immune diseases. The existence of flavonoids in *E. indica* may suggest the ability of this plant to modulate the immune response,

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which could be associated to its traditional uses. However, not many flavonoids have been reported from the plant particularly the part of interest, aerial. To date, only three flavonoids from the C-glycoside group have been identified from the aerial part of the plant namely schaftoside, isoschaftoside, and vitexin [10].

Identification of flavonoids in plant sample can be done through liquid chromatography coupled to mass spectrometry (LCMS) technique due to its high sensitivity, flexibility and capability to effectively separate and detect a wide range polarity of compounds [16, 17]. Tandem MS analysis is crucial for obtaining accurate structural information of both precursor and fragment ion data, which can be utilized for annotating, characterizing, and dereplication of compounds [18]. Data obtained from tandem LCMS can be integrated with metabolite annotation tools such as MZmine, Global Natural Social Molecular Networking (GNPS) and SIRIUS to assist in the structural identification of the flavonoids [19]. Molecular networking concept is based on mining and organization of tandem MS data into maps, which clusters similar compounds together. These clusters formed structural networks, allowing better visualization of similar classes of compounds [20]. Using this technique, the flavonoids identification will be inferred from the known previously reported structure in the same molecular network based on their common structural characteristics.

The present work aims to identify the flavonoids component in the aerial aqueous extract of *E. indica* through tandem LCMS-based molecular networking technique and diagnostic evidence analysis. This paper reports on the identification process of the flavonoids based on fragmentation role, local experience and literature data. In addition, discussion on the previously reported biological activities on the identified C-glycosyl flavonoids was also included to provide insight on their possible role in the plant traditional uses.

EXPERIMENTAL

Chemicals and Solvents

Methanol (MeOH) of HPLC and LCMS grades were purchased from Merck (Germany) and ultra-pure water (UPW) was from Sartorius.

Plant Materials and Extraction

The plant of *E. indica* (5 kg) was collected from Agriculture Research Centre Tuaran, Sabah. The plant specimen was identified by a certified botanist, Mr Johnny Gisil, and a voucher specimen with the code ZZ 001 was deposited at BORNEENSIS Herbarium, Institute for Tropical Biology and Conservation (BORH), Universiti Malaysia Sabah, Malaysia. The fresh sample was sorted into different parts including flower, leaf, aerial and root. The aerial part (375 g) was washed and cut into small pieces and ground with electrical grinder. The ground sample was then macerated in distilled water for 15 minutes at temperature of 50°C to mimic the traditional practice. The extract was filtered and subjected to freeze-drying method to sublimate the solvent. This yielded 7.8 g of crude extract, which was then stored at -4°C before analysis.

Sample Preparation for LCMS

Solid phase extraction (SPE) using Strata® C18E (55 µm, 70 A), Phenomenex cartridges (500 mg, 6 mL) were employed for sample clean-up and pre-concentration. To activate the cartridges, 6 mL UPW was used, followed by 6 mL methanol. Before loading a 2 mL crude extract (5 mg/mL), the cartridge was equilibrated with 6 mL of 95% MeOH at a constant flow rate. Elution was performed with 5 mL of 95% MeOH. The extract was dried using a vacuum concentrator. Then, 9 mg of the extract was dissolved in 40% of 1 mL MeOH and filtered through 0.22 µm syringe filters into a vial, capped, and subjected to LCMS analysis [17].

LCMS Analysis

LCMS analysis was performed using a Phenomenex reversed-phase luna omega polar C18 column (100 × 2.1 mm, 100 Å, 1.6 µm particle size). Mobile phase A was UPW with 0.1% formic acid (% v/v) and mobile phase B was LCMS grade MeOH with 0.1% formic acid (% v/v). A constant flow rate of 0.2 mL/min was used. The mobile phase gradient was: min 0; 9% B, min 5; 10% B, min 11; 20% B, min 25; 100% B, min 30; 100% B. The column was equilibrated with 91% mobile phase A for 15 min before the next injection. The column oven was set at 35 °C, and the full loop injection volume was set at 1 µL. The LCMS instrument used was a Thermo Scientific Vanquish Horizon UHPLC hyphenated with Orbitrap Fusion MS detector with electrospray ionization (ESI) in negative mode. The instrument was externally calibrated with Thermo Pierce calibration solution before LCMS runs. Full scan mode was used to record all the masses in the range of 100–1000 m/z. In addition to the full scan, data-dependent MS/MS fragmentation was recorded for the 5 tallest peaks on each spectral scan with various collision energies. The spectrum data obtained from the LCMS analysis were viewed on FreeStyle software. The data was further processed using several available platforms such as MZmine, GNPS, and SIRIUS.

Data Analysis

The chromatograms obtained through LCMS/MS were converted into MzML file using MSConvert 3.0 and processed using MZmine software version 3.47 for data pre-treatment. The resulting MS^2 feature data were exported to GNPS platform and SIRIUS software version 5.5.7 for creating a molecular networking and compound's identification [21, 22]. Data processing using SIRIUS platform was based on available

databases including Biocyc, CHEBI, COCONUT, HMDB, KEGG, KNApSAcK, MeSH, Natural Products, Plantcyc, Pubchem, PubMed, SuperNatural, ZINC bio and KEGG. The results were processed in the Cytoscape software to visualize molecular networking and identification of similarities of compound classes particularly flavonoids.

RESULTS AND DISCUSSION

Comprehensive high-resolution MS in a data-dependent full-scan acquisition method was developed to separate and detect the phytochemicals in the extract. The MS feature data for compounds are recognized by their measured mass to charge ratio (m/z), retention time (RT), and relative abundance. The feature data were pre-processed using MZmine to set noise level at 3E5 and combine isotope features. The data in MGF format file was then uploaded to Feature-Based Molecular Networking (FBMN) workflow, in GNPS. FBMN is advanced molecular networking analysis, facilitating the differentiation of isomers, the inclusion of proportional quantification, and integration of ion mobility data. The annotation in GNPS is based on MS^2 fragmentation similarity with the available library. While SIRIUS annotation is based on comparison of the experimental MS^1 spectra with the predicted isotopic pattern and determining the fragmentation spectra align with the candidate molecular formulas through the utilization of fragmentation trees [17]. SIRIUS incorporates additional algorithms and models, such as ZODIAC to enhance molecular formula prediction, CSI:FingerID for potential structure annotation, COSMIC for evaluating match confidence, and CANOPUS for putative chemical class identification. In addition, the molecular networking of flavonoids identified was visualized using Cytoscape 3.0 platform that linked compounds based on their spectral similarity. The flow of the work design for the flavonoid's identification is illustrated in Figure 1.

Molecular Networking

Off the 21 total compounds identified in the extract using GNPS platform, several clusters of compounds have been generated through the molecular networking (Figure 2). Since the focus of the present study was on the flavonoid's constituent, the compound's networking was designed to focus on flavonoids cluster. As a part of compound identification, a molecular interaction network (MN) of MS/MS data was established to find the nearest correlated compound classes to the two previously reported flavonoids in the plant, vitexin and schaftoside. Two cluster networks were found interconnecting the flavonoids and showing a strong relationship (cosine > 0.7), which confirmed a similar fragmentation pattern of C-glycosyl flavonoids. Only four flavonoids were managed to be annotated at RT 17.63, 17.12, 17.71 and 16.29 minutes (Table 1) based on this flavonoids cluster.

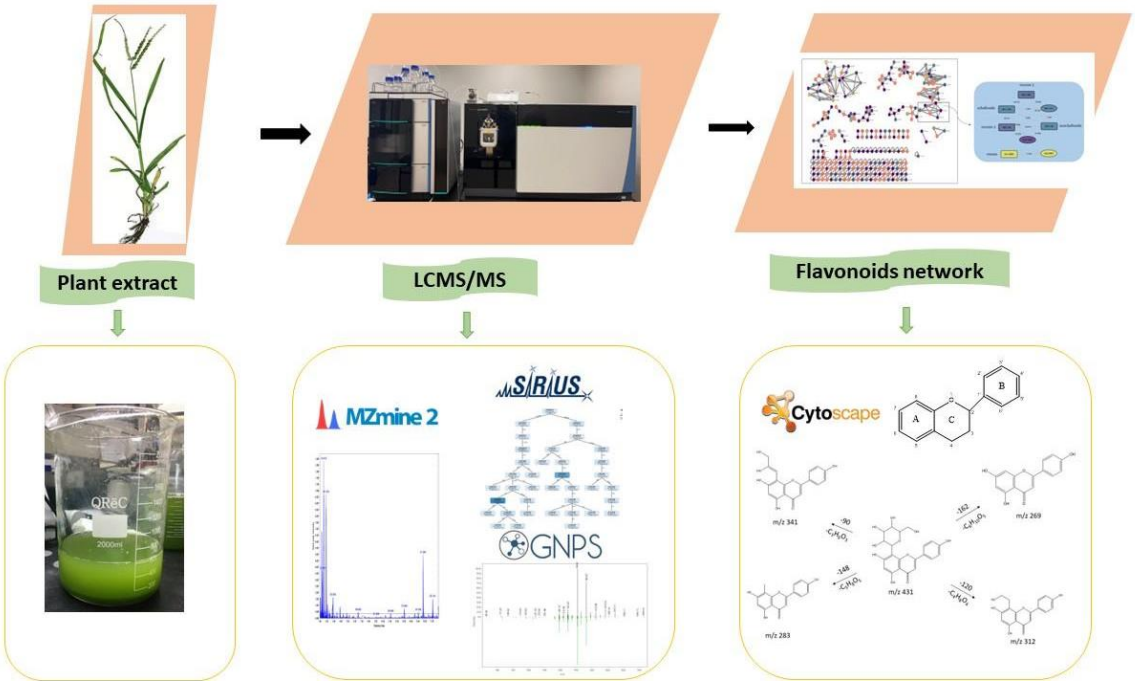


Figure 1. Infographic explaining the flavonoids identification workflow.

To support the flavonoid clustering, the data were further processed using SIRIUS platform for in silico MS/MS fragmentation pattern. A total of 279 features of compounds were processed using SIRIUS and five features were managed to be identified as C-glycosyl flavonoids base on their MS/MS fragmentation patterns that linked to the flavonoids network. They include the four previously identified flavonoids in GNPS platform and in addition one at RT 17.44 min (Table 2). Database identified this flavonoid as vicenin 2. However, the fragmentation pattern was slightly different from vicenin 2 in the base peak m/z value (Table 2) suggesting the spatial relationship in their molecular structure [22]. Thus, this C-glycosyl flavonoid was named vicenin X. In addition, there were three unidentified compounds which were also linked to the flavonoids network exhibiting molecular ion peaks at m/z 564.1417, 631.1259 and 432.0997. Due to the similarity of their spectra, additional analysis is required to identify these compounds which was done through further fragmentation process.

Analysis on the fragmentation pattern of the flavonoids is important to increase the confidence level in the structural identification. There are five level of metabolite identification confidence (MIC) proposed by Schrimpe-Rutledge [23]. Level 1, known as the highest identification level, confirms a structure based on at least two independent properties from a pure reference standard acquired using the same analytical conditions. When no reference standard is available but there is predictive or externally obtained structural evidence, such as MS/MS data showing fragments or neutral losses consistent with a specific structure, it falls under a putative identification (Level 2). Initial identifications (Level 3) are derived from accurate mass and isotopic distribution patterns, yielding possible structures through database searches. Molecular formula candidates (Level 4) and a deconvoluted experimental m/z value (Level 5) constitute the classifications for less confident molecular structural identification.

The GNPS and SIRIUS platforms will only provide initial ideas about the potential metabolites that could be found within the extract. Thus, applying a diagnostic fragmentation using MS^2 will give a confidence level 2 to all the identified flavonoids. To do this, some special structural characteristics need to be understood and for the C-Glycosyl flavonoids, they commonly have glycosyl groups attached at the C_6 or C_8 positions, or at both C_6 and C_8. Due to the instability of the C–C bond in flavonoid C-glycosides, the occurrence of glycosyl group ring-opening reactions takes place during the cleavage process [24]. This pattern can be seen in the following discussion on the diagnostic fragmentation pattern of each identified flavonoid.

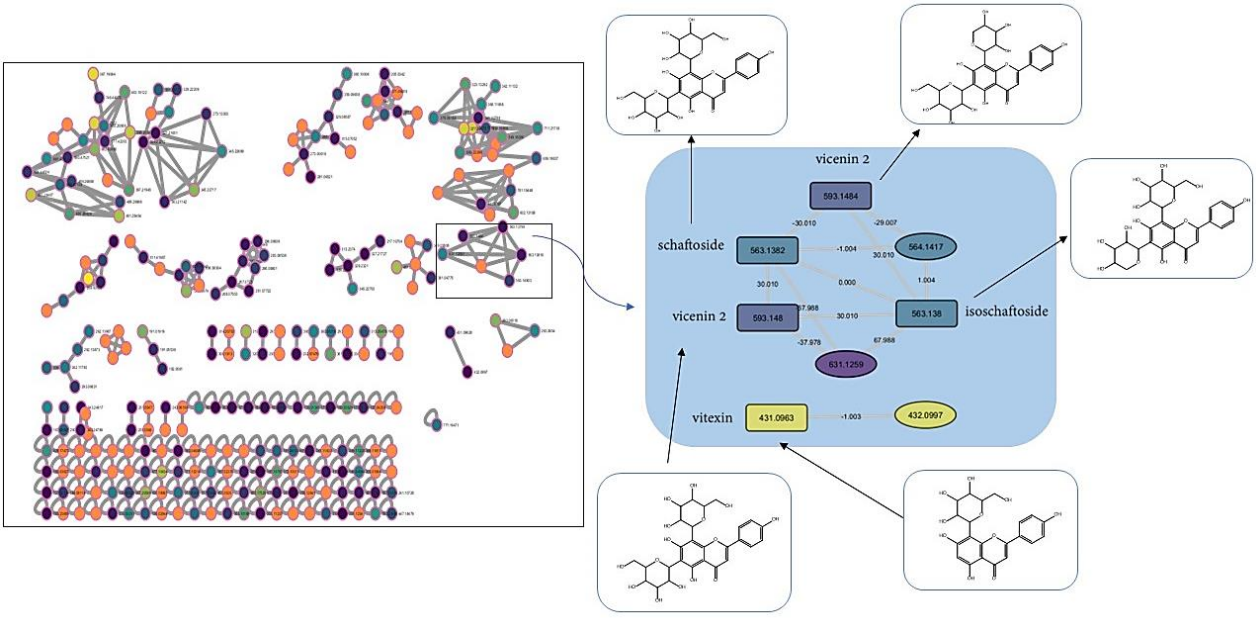


Figure 2. Flavonoids networking visualized using cytoscape software.

Table 1. Identified flavonoids in GNPS along with their specific features.

No.	Compound name	Cluster index	Library class	Cosine	MZ error (ppm)	Spec m/z / Library m/z $[M+H]^-$	RT (min)	Instrument	Ion mode	Database	Ion source
1.	Vitexin	292	Bronze	0.92	6.22954	431.099/ 431.096	17.63	qTof	Negative	Stephane GREFF	LC-ESI
2.	Schaftoside	282	Bronze	0.88	4.98564	563.138/ 563.138	17.12	ESI-QFT	Negative	Massbank	ESI
3.	Isoschaftoside	294	Bronze	0.80	5.31079	563.138/ 563.138	17.71	ESI-QFT	Negative	Massbank	ESI
4.	Vicenin 2	264	Bronze	0.78	13.4801	593.150/ 593.148	16.29	qTof	Negative	Desine	DI-ESI

Table 2. Identified flavonoids using SIRIUS along with their specific features.

No	Annotation (CSI: FingerID)	$[M+H]^-$	Fragment ions	RT (min)	Molecular formula	COSMIC	Classification	Smiles	Database Links
1.	Vitexin	431.099	269.0444, 283.0601*, 311.0551, 341.0658	17.65	$C_{21}H_{20}O_{10}$	0.233	Flavonoid-8-C-Glycoside	C1=CC(=CC=C1C2=CC(=O)C3=C(O2)C(=C(C=C3O)O)C4C(C(C(C(O4)CO)O)O)O)O	Biocyc, CHEBI, COCONUT, HMDB, KEGG, KNApSAcK, MeSH, Natural Products, NORMAN, Plantcyc, Pubchem, PubMed, SuperNatural, ZINC bio
2.	Schaftoside	563.1382	297.0748, 325.0696, 353.1646*, 413.0852, 425.0857, 443.1526, 473.1060, 503.1138	17.12	$C_{26}H_{28}O_{14}$	0.183	Flavonoid-8-C-Glycoside	C1C(C(C(C(O1)C2=C3C(=C(C(=C2O)C4C(C(C(C(O4)CO)O)O)O)O)C(=O)C=C(O3)C5=CC=C(C=C5)O)O)O)O	CHEBI, COCONUT, HMDB, KNApSAcK, MeSH, Natural Products, Pubchem, PubMed, SuperNatural, ZINC bio

3.	Isoschaftoside	563.1380	297.0751, 325.0698, 353.0645*, 413.0831, 425.0839, 443.0950, 473.1078	17.71	$C_{26}H_{28}O_{14}$	0.178	Flavonoid-8-C-Glycoside	C1C(C(C(C(O1)C2=C(C(=C3C(=C2O)C(=O)C=C(O3)C4=CC=C(C=C4)O)C5C(C(C(C(O5)CO)O)O)O)O)O)O)O	CHEBI, COCONUT, HMDB, KEGG, KNApSAcK, MeSH, Natural Products, Pubchem, PubMed, SuperNatural, ZINC bio
4.	Vicenin 2	593.1480	297.0754, 325.0689, 353.0644*, 383.0751, 413.0821, 473.1044	16.29	$C_{27}H_{30}O_{15}$	0.275	Flavonoid-8-C-Glycoside	C1=CC(=CC=C1C2=CC(=O)C3=C(C(=C(C(=C3O2)C4C(C(C(C(O4)CO)O)O)O)O)C5C(C(C(C(O5)CO)O)O)O)O)O	Biocyc, CHEBI, COCONUT, HMDB, KEGG, KNApSAcK, MeSH, Natural Products, Plantcyc, Pubchem, PubMed, SuperNatural, ZINC bio
5.	Vicenin X	593.1484	297.0754, 353.0654, 383.0751*, 413.0551, 473.1057, 503.1123	17.44	$C_{27}H_{30}O_{15}$	0.331	Flavonoid-8-C-Glycoside	C1=CC(=CC=C1C2=CC(=O)C3=C(C(=C(C(=C3O2)C4C(C(C(C(O4)CO)O)O)O)O)C5C(C(C(C(O5)CO)O)O)O)O)O	Biocyc, CHEBI, COCONUT, HMDB, KEGG, KNApSAcK, MeSH, Natural Products, Plantcyc, Pubchem, PubMed, SuperNatural, ZINC bio

*Base peak

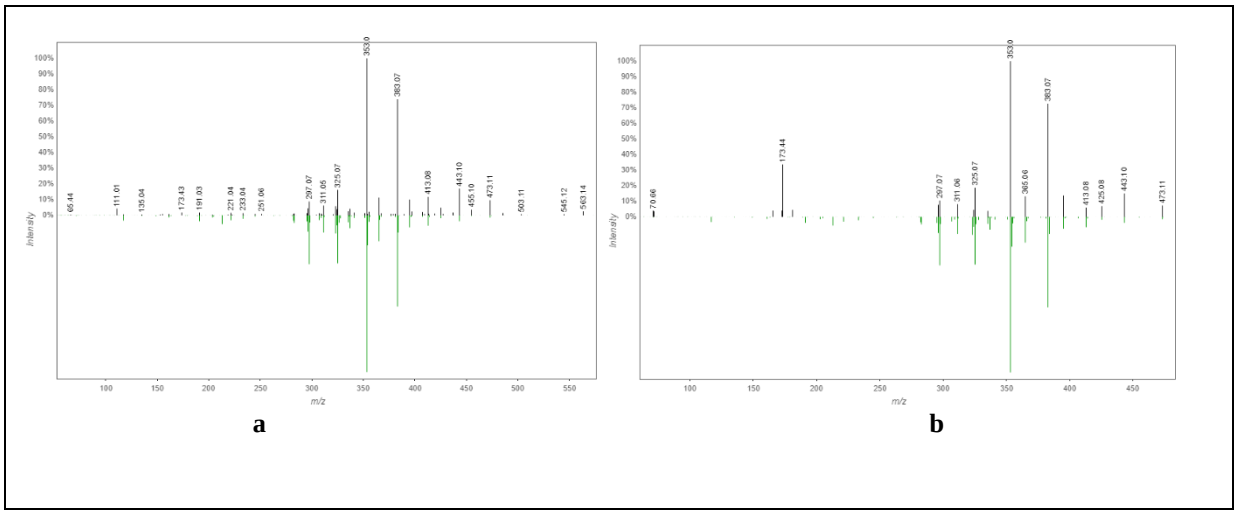
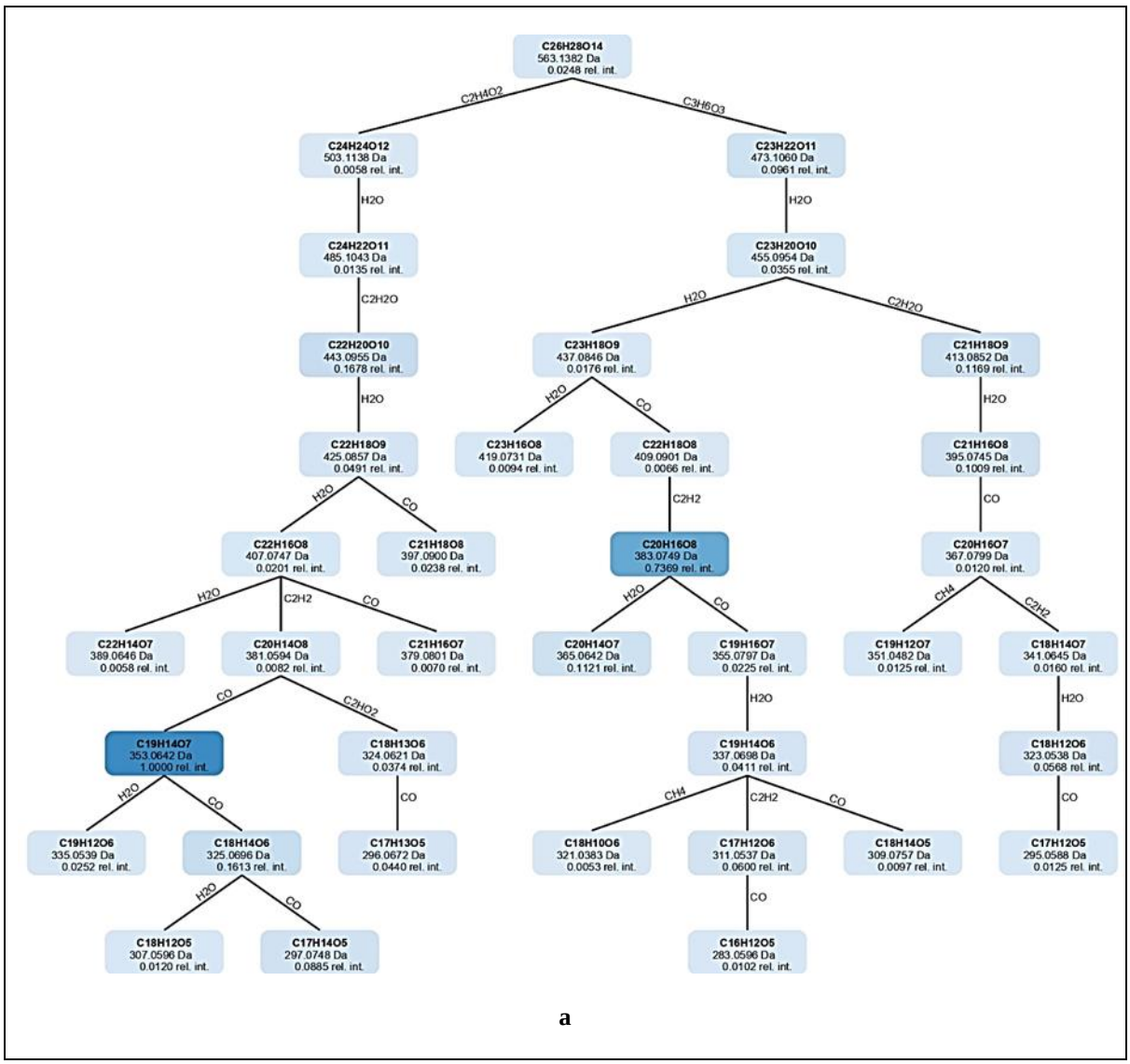


Figure 3. Mirror spectral match of schaftoside (**a**) and isoschaftoside (**b**).



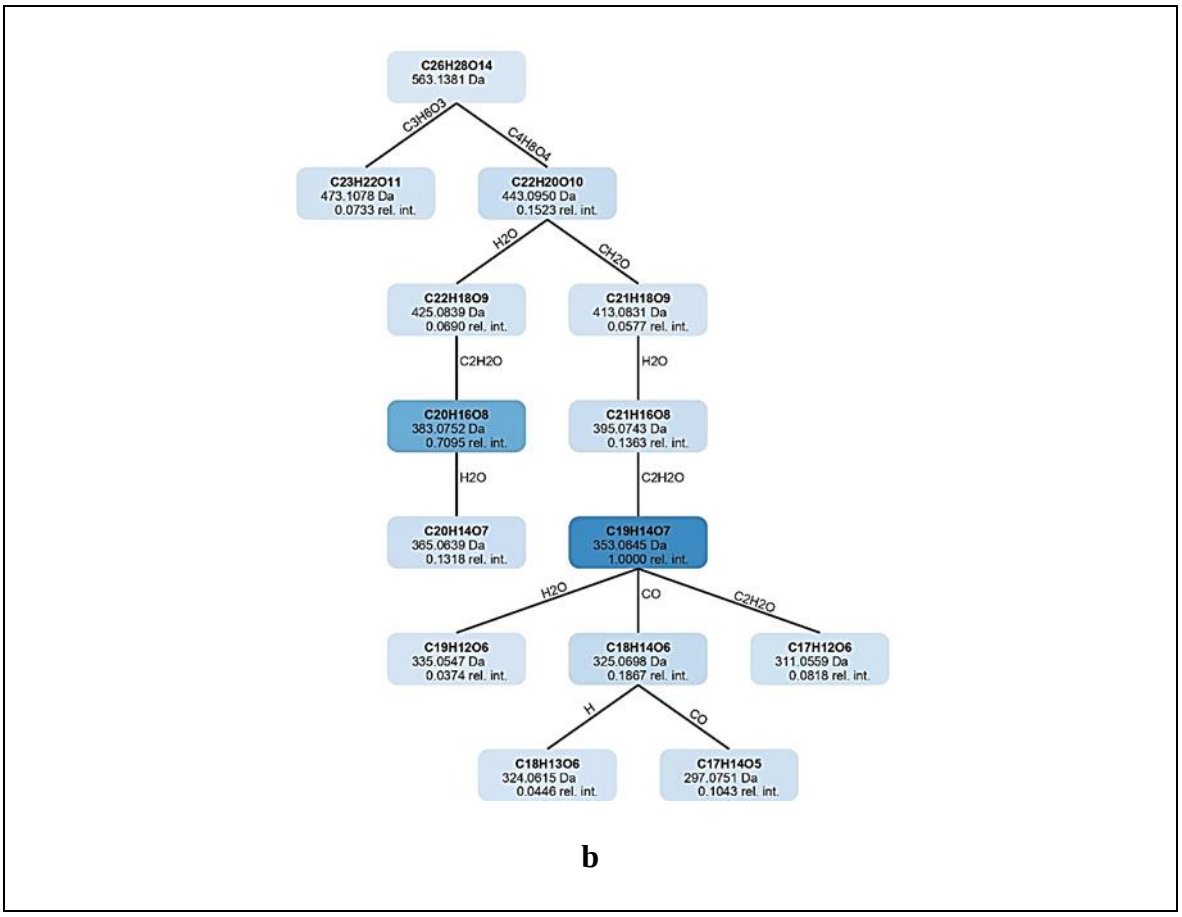


Figure 4. Fragmentation trees of schaftoside (**a**) and isoschaftoside (**b**).

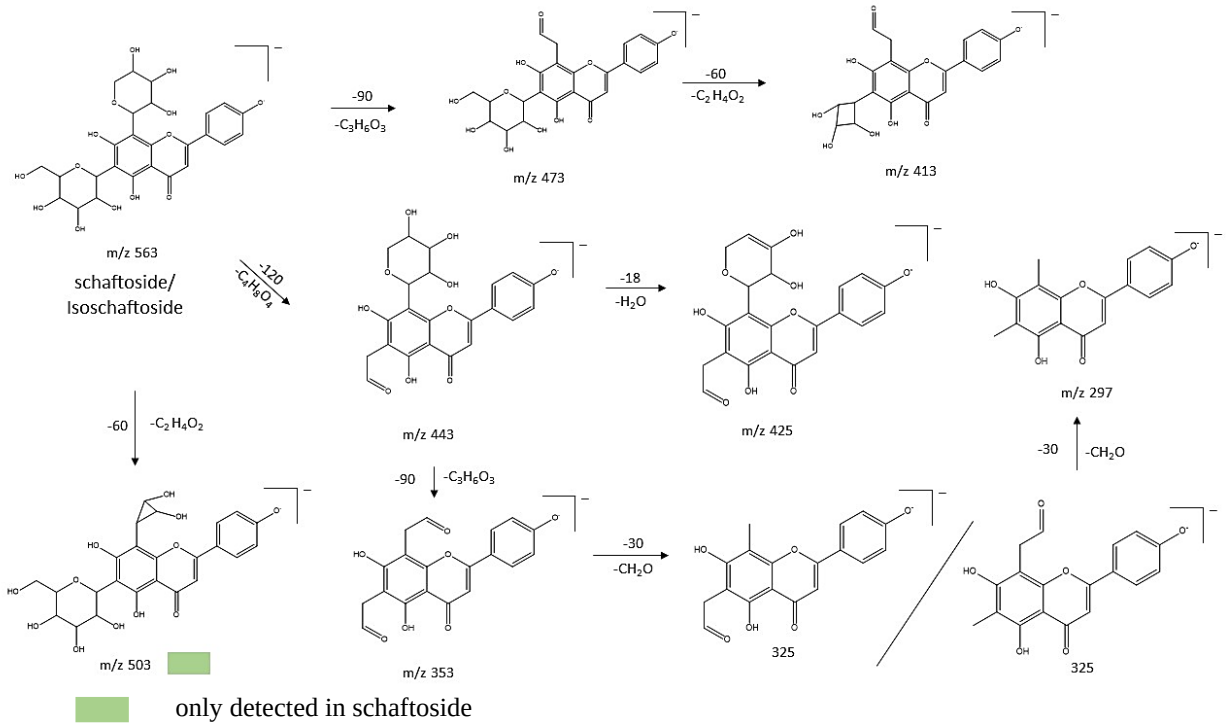


Figure 5. Proposed fragmentation patterns for schaftoside and isoschaftoside.

Diagnostic Evidence Analysis

Schaftoside and Isoschaftoside

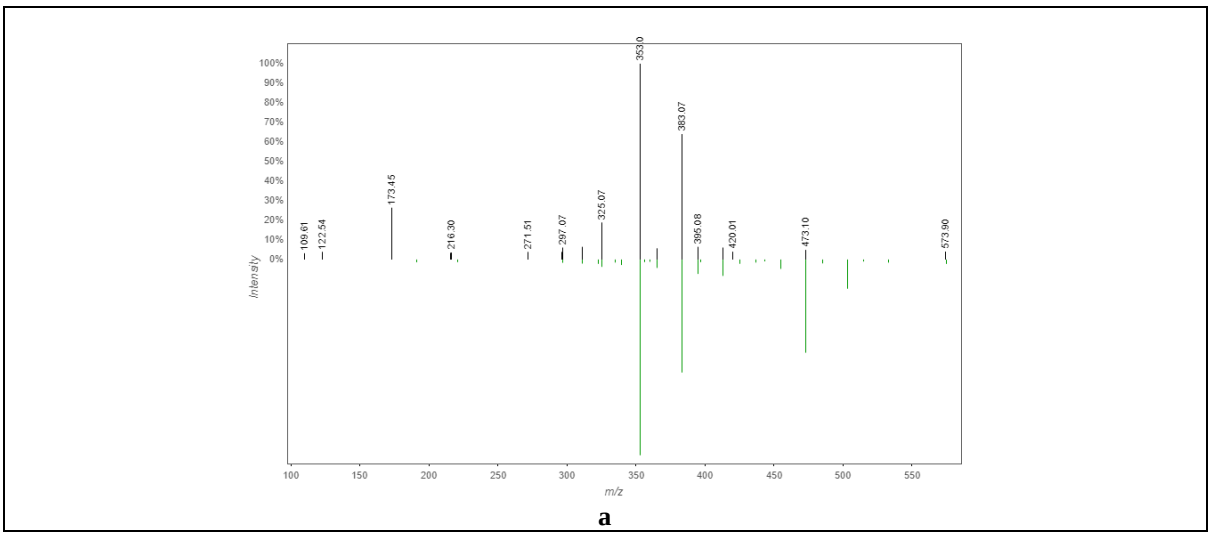
Schaftoside was eluted slightly earlier at 17.12 min compared to isoschaftoside at 17.71 min which is in line with a previous study [10]. Schaftoside had been reported from aerial part of *E. indica* species by De Melo and co-workers [5]. Isoschaftoside has also been isolated as the constituents of the aerial part of *E. indica*, and interestingly the experimental condition was closer to the present work which was isolated from aqueous extract using C-18 column acquired through electron spray ionization (ESI) LCMS system [10].

The mirror spectral match between the experimental and library of schaftoside (**a**) and isoschaftoside (**b**) obtained in GNPS platform were very close (Figure 3). The only different was observed at their low molecular weight ion peak region where the former exhibits fragmentation ion peaks at m/z 251.06, 233.04, 221.04, 191.03, 135.04, 11.01 and 65.44, while the latter only have three ion peaks at m/z 191.03, 173.44 and 70.66. This suggesting slightly different fragmentation ion stability. This is further supported by the fragmentation tree predicted with SIRIUS platform where both flavonoids exhibited different fragmentation pathways based on MS^2 as shown in Figure 4.

Figure 5 shows the proposed diagnostic fragmentation patterns for schaftoside and isoschaftoside with characteristics fragment ions of McLafferty rearrangement and hexose ring-opening reaction [25]. Initially, the parent ion at *ca.* m/z 563 ($C_{26}H_{28}O_{14}$) of both isomers was fragmented into two molecular ions, first the losses of an arabinose molecule of $[M\text{-}H\text{-}90(C_3H_6O_3)]^-$ and McLafferty rearrangement process producing a molecular ion at *ca.* m/z 473 ($C_{23}H_{22}O_{11}$). Further fragmentation observed losses of ethene-diol ion $[M\text{-}H\text{-}90(C_3H_6O_3)\text{-}60(C_2H_4O_2)]^-$ and rearrangement of four-member ring producing molecular ion of *ca.* m/z 413 ($C_{21}H_{18}O_9$). The parent ion also loses a glucose unit $[M\text{-}H\text{-}120(C_4H_8O_4)]^-$ and McLafferty rearrangement producing a molecular ion of *ca.* m/z 443 ($C_{22}H_{20}O_{10}$). The molecular ion was further fragmented into two other molecular ions: $[M\text{-}H\text{-}120(C_4H_8O_4)\text{-}H_2O]^-$ at *ca.* m/z 425 ($C_{22}H_{18}O_9$), and $[M\text{-}H\text{-}120(C_4H_8O_4)\text{-}90(C_3H_6O_3)]^-$ at *ca.* m/z 353 ($C_{19}H_{14}O_7$) forming the base peaks for both flavonoids. The base peak was further fragmented by loss of $[M\text{-}H\text{-}120(C_4H_8O_4)\text{-}90(C_3H_6O_3)\text{-}30(CH_2O)]^-$ forming molecular ion of $C_{18}H_{12}O_6$ at *ca.* m/z 325 followed by fragmentation of $[M\text{-}H\text{-}120(C_4H_8O_4)\text{-}90(C_3H_6O_3)\text{-}30(CH_2O)\text{-}30(CH_2O)]^-$ producing molecular ion of at *ca.* m/z 297 ($C_{17}H_{10}O_5$). The major difference between the two flavonoids is the molecular fragment ion appeared at *ca.* m/z 503 only for schaftoside that is due to the removal of ethene-diol ion $[M\text{-}H\text{-}60(C_2H_4O_2)]^-$ and rearrangement of the its arabinose moiety to a 3-member ring fraction.

Vicenin 2 and vicenin X

Vicenin 2 was eluted earlier compared to vicenin X at 16.29 min and 17.44 min, respectively. This indicates that the two flavonoids have different polarities and this probably happened due to their stereoisomerism nature [26, 27]. However, vicenin X was not detected in GNPS indicating the platform limitation compared to SIRIUS. Worth mentioning that this is the first report on the identification of vicenin from *E. indica*. Figure 6(a) shows close mirror MS^2 fragments (at m/z 473.10, 395.08, 383.07,353, 325.07, and 297.07) spectral match between the experimental and library data of vicenin 2 through analysis on GNPS platform. While, Figure 6(b & c) displays the fragmentation trees of vicenin 2 and vicenin X predicted through SIRIUS platform. Similar to those earlier discussed for schaftoside and isoschaftoside, these two flavonoids also exhibited different MS^2 fragmentation pathways evident for their stereoisomerism nature [17].



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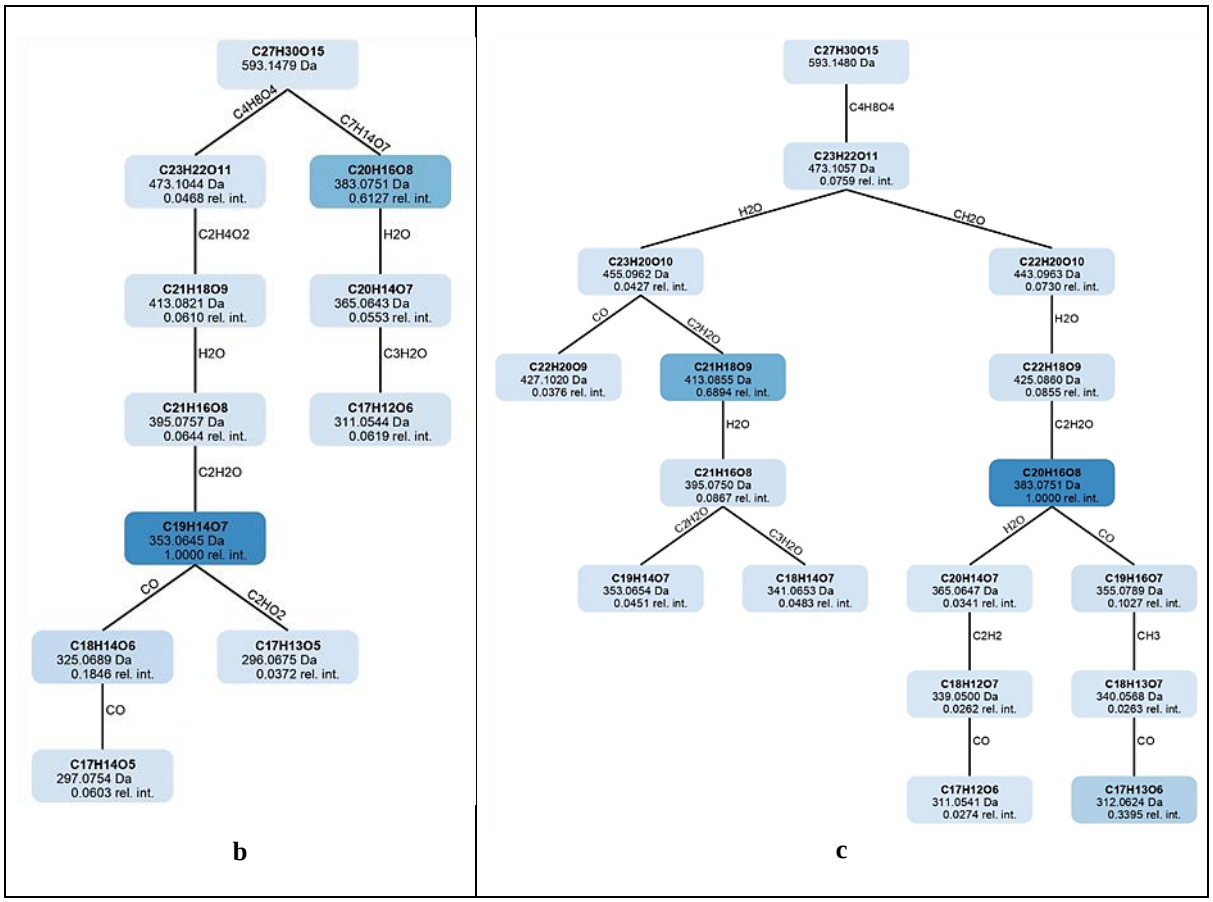

Figure 6. Mirror spectral match (**a**) and fragmentation tree of vicenin 2 (**b**) and vicenin X (**c**)

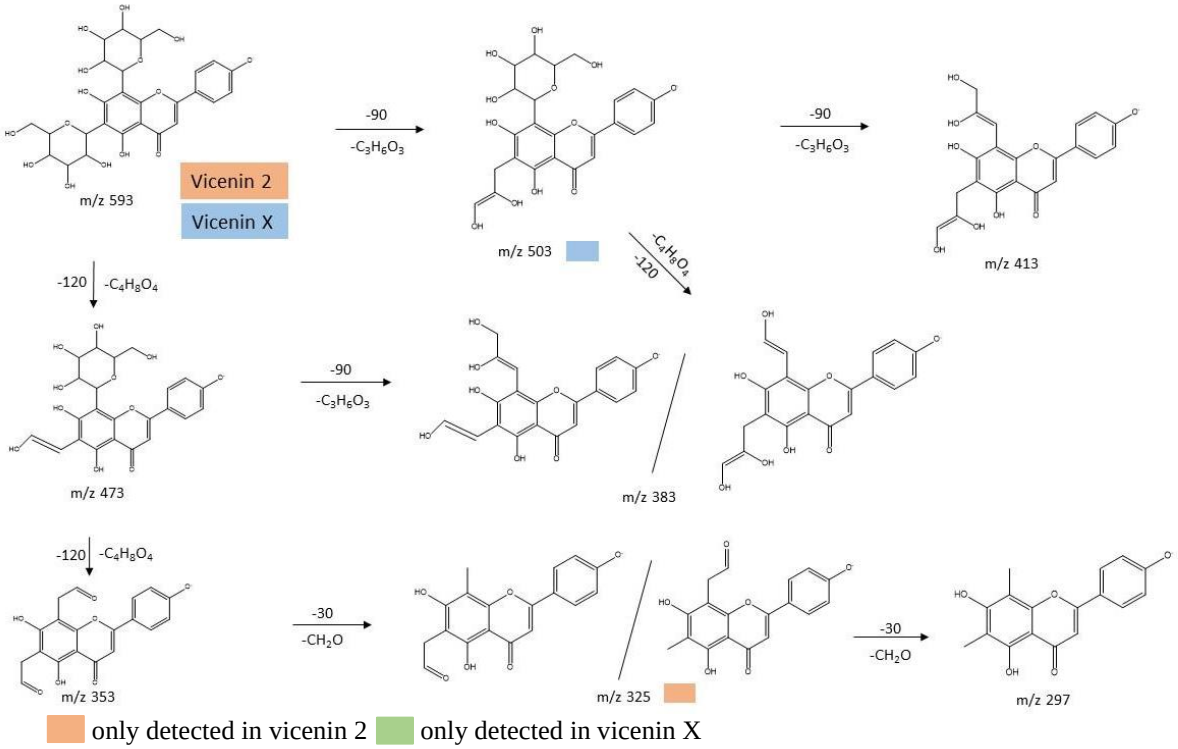

Figure 7. Proposed fragmentation patterns for vicenin 2 and vicenin X.

Diagnostic fragmentation analysis on vicenin 2 and vicenin X (Figure 7) shows the parent molecular ion peak of both flavonoids at *ca*. m/z 593 ($C_{27}H_{30}O_{15}$) fragmented by the loss of glucose unit [M-H-120 ($C_4H_8O_4$)]$^-$ and McLafferty rearrangement producing molecular ion peak at *ca*. m/z 473 ($C_{23}H_{22}O_{11}$). The molecular ion was further fragmented into two other molecular ions: loss of glucose unit [M-H-120($C_4H_8O_4$)-120($C_4H_8O_4$)]$^-$ forming molecular ion at *ca*. m/z 353 ($C_{18}H_{10}O_5$), and the loss of arabinose molecule [M-H-120($C_4H_8O_4$)-90($C_3H_6O_3$)]$^-$ producing molecular ion at *ca*. at m/z 383 ($C_{20}H_{16}O_8$) that could give two different isomers. The difference between vicenin 2 and vicenin X is the former exhibited a base peak at m/z 353.0644 while the latter at m/z 383.0751. In addition, vicenin X has molecular fragment ion appear at m/z 503.1123 due to the removal of arabinose ion of [M-H-90($C_3H_6O_3$)]$^-$.

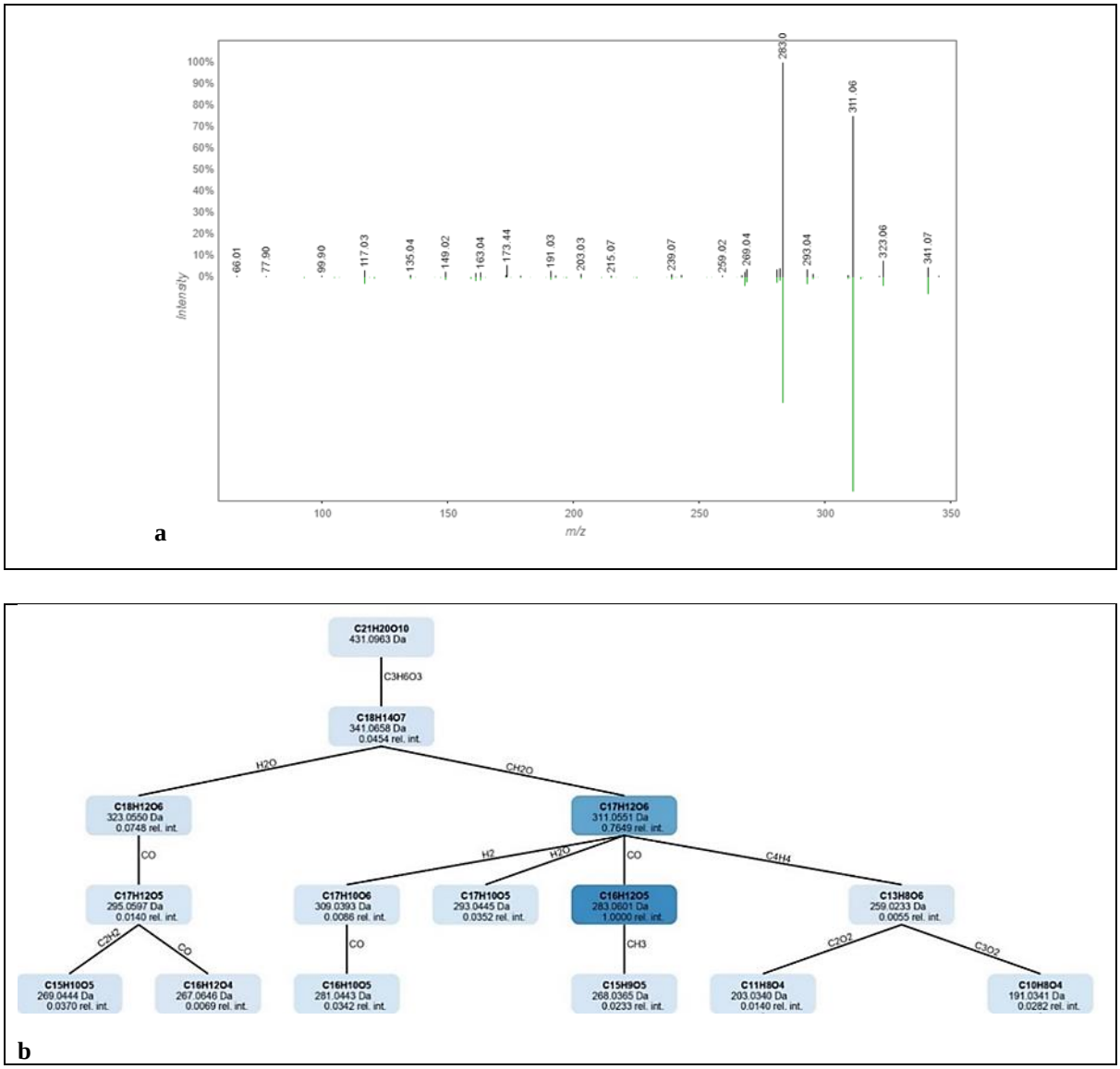

Figure 8. Mirror spectral match (**a**) and fragmentation tree (**b**) of vitexin

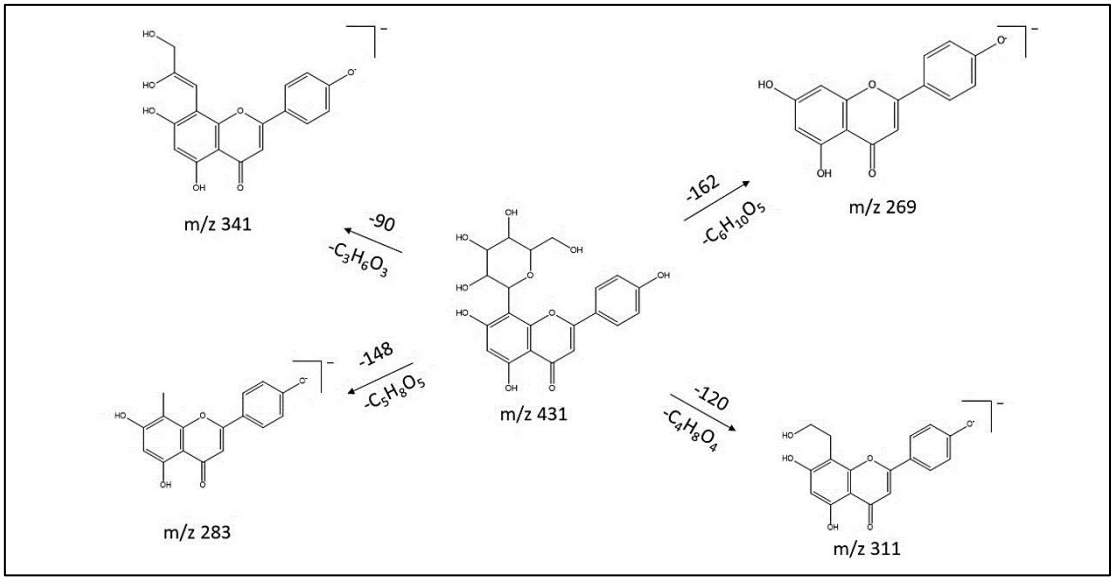

Figure 9. Proposed fragmentation pattern for vitexin

Vitexin

Vitexin is known as a simple C-glycosyl flavonoid with glycosyl group attached at C_8 positions. This flavonoid had been isolated from the aerial part of *E. indica* [5]. Figure 8(a) shows a mirror spectral match between the experimental and library of vitexin. A close match of MS^2 fragments of this compound is at m/z 341.07, 323.06, 311.06 293.04, 283, and 269.04. To further confirm the level of confidence in structural identification, a diagnostic fragmentation of vitexin was developed and proposed as in Figure 8(b) and Figure 9. As shown in Figure 9, the parent molecular ion peak at m/z 431.099 ($C_{21}H_{20}O_{10}$) was fragmented into four molecular ions: 1) loss of an arabinose molecule [M-H-90($C_3H_6O_3$)]$^-$ and McLafferty rearrangement producing molecular ion m/z 341.0658 ($C_{18}H_{14}O_7$), 2) loss of glucose unit [M-H-120($C_4H_8O_4$)]$^-$ giving rise to the molecular ion at m/z 311.0551 ($C_{17}H_{12}O_6$), 3) loss of benzyl moiety [M-H-148($C_5H_8O_5$)]$^-$ forming molecular ion m/z 283.0601 ($C_{16}H_{12}O_5$), and 4) the loss of glucose molecules [M-H-162($C_6H_{10}O_5$)]$^-$ producing molecular ion m/z 269.0444 ($C_{15}H_{10}O_5$).

Biological Activities of C-glycosyl Flavonoids

As has been mentioned earlier, the aerial part of *E. indica* infused with rice is used by the people of Kadazandusun at Sabah, Malaysia for the treatment of symptoms related to flu and this is believed to be due to the immunomodulatory property executed by its flavonoids content. To further support this inference, a literature search was conducted to gather information on the immunomodulatory ability of C-glycosyl group of flavonoids.

Previous studies have shown that C-glycosyl flavonoids possess pharmacological activities including hepatic protection against D-galactosamine (D-GalN)-induced hepatitis, carbon tetrachloride (CCl_4)-induced hepatic fibrosis, and nonalcoholic fatty liver disease (NAFLD) [11]. Furthermore, C-glycosyl flavonoids can mitigate $AlCl_3$-induced neurotoxicity in rats by inhibiting oxidative stress and inflammatory markers [12]. Proteomics analysis and cytokine assay revealed that schaftoside can regulate the immune response due to the inflammation of host cells infected with SARS-CoV-2 [13]. This compound has also been reported to exhibit an anti-inflammatory effect on lipopolysaccharide (LPS)-induced acute lung injury mice model [13].

Interestingly, schaftoside and vitexin isolated from the aqueous aerial extract of *E. indica* possessed an anti-inflammatory effect on mouse lung inflammation [5]. This study shows that at the tested concentration of 0.4 µg/mL, schaftoside and vitexin can inhibit 62% and 80%, respectively, neutrophil recruitment in mice exposed to LPS aerosols. The authors also found that the lung anti-inflammation activity was in a dose-dependent manner. Another study found that isoschaftoside could inhibits the activation of ERK1/2/mTOR and HIF-1α-mediated metabolic reprogramming caused by LPS resulting in the reduction of iNOS, COX-2, IL-1β, and TNF-α expression in BV2 microglial cells [14]. Separately, vicenin 2 was also found to possess anti-inflammatory potential [28] and anti-tumor by inhibiting prostate cells [29]. This concludes C-glycosyl flavonoids have various immune response activities supporting their possible role in the plant traditional practice.

CONCLUSION

Five flavonoids belonging to the C-glycosyl group namely vitexin, schaftoside, isoschaftoside, vicenin 2, and vicenin X had been putatively identified (with confidence level 2) through molecular networking

cluster, fragmentation similarity and in silico fragmentation pathways. This is the first report on the identification of vicenin 2 and vicenin X in this species. Interestingly, the identified flavonoids had been reported to exhibit immune response-related biological activities that may suggest their role in the plant's traditional uses. It is suggested to isolate and elucidate the absolute structure of vicenin X and evaluate the extract and the flavonoids on immune-modulatory assays.

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