Characterization of Deprotonated Constituents of Flavonoid-Rich Fraction from *Cosmos caudatus* Kunth. Leaves by UPLC-QToF-MS/MS

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Abstract

The flavonoid-rich fraction from the ethanolic extract of the leaves of Cosmos caudatus Kunth. (Kenikir) was analyzed by ultra-performance liquid chromatography-mass spectrometry with a quadrupole and time-of-flight analyzer featuring an electrospray ionization source (QToF-ESI-MS/MS). The deprotonated molecules $[M-H]^-$ were analyzed, and the flavonoids were tentatively annotated based on accurate mass and product-ion patterns. Chemical analysis of the fraction identified quercetin and three C-3 hydroxy group-substituted quercetin glycosides, namely quercetin 3-O-rutinoside (rutin), quercetin 3-O-β-D-glucopyranoside (isoquercitrin), and quercetin 3-O-arabinofuranoside. The results show that the fragmentation behavior of flavonol 3-O-glycosides can be analyzed based on the breakage of glycosidic link and interglycosidic linkages. Removal of the sugar moiety allows the distinctive product ions to be identified. Investigation of negative ion ESI-MS/MS spectra of flavonol O-glycosides allows their rapid characterization as flavonol 3-O-glycoside and enables their direct analysis in crude plant extracts. This study provides a detailed analysis of the MS/MS fragmentation pathways of 3-O-glycosylated flavonoids in a flavonoid-rich fraction of C. caudatus.

***Keywords**:* Cosmos caudatus, deprotonated molecules, flavonoid-rich fraction, quercetin 3-O-glycoside, UPLC-QToF-MS/MS

1. Introduction

The plant *Cosmos caudatus* originated in Latin America and has been established in Asia through the Philippines. *C. caudatus* is known by a variety of names in many countries, including "Ulam Raja" in Malaysia, "Cosmos" in the Philippines, "Dauruang-Pharma" in Thailand, and "Kenikir" among Indonesians (Cheng *et al*., 2015). In Southeast Asia, *C. caudatus* is frequently utilized as a traditional medicine thought to offer anti-aging properties and to make skin appear younger. Because of its distinct and alluring scent, which enhances the flavor of food, *C. caudatus* is frequently eaten raw in salads (Ahda *et al*., 2023).

According to pharmacological studies, *C. caudatus* possesses a high antioxidant capacity and a number of therapeutic properties, including antibacterial, antihypertensive, antidiabetic, and anti-inflammatory activities (Azwanida *et al*., 2020). To further understand the source of these bioactivities, different extraction methods were used to determine how they influence the plant's antioxidant potential. Among water and ethanol extracts prepared using three separate methods, ultrasonic extraction yielded a percentage antioxidant activity by the DPPH method comparable to that of ascorbic acid. Fifty percent of DPPH radicals were inhibited by 83.85 µg/mL of the water extract and by less than 50 µg/mL of the ethanol extract (Sia *et al*., 2020). Quercetin and its glycosides, are well-documented antioxidants due to their strong radical-scavenging ability and capacity to chelate metal ions (Qi *et al*., 2022). The presence of these flavonoids in *C. caudatus* may thus explain previously observed antioxidant and anti-inflammatory effects reported for the species (Ahda *et al*., 2023).

Chlorogenic, cryptochlorogenic acid, neochlorogenic acid, ferulic acid, caffeic acid, (+)-catechin, proanthocyanidins, and quercetin glycosides, including quercetin-3-*O*-arabinofuranoside, quercetin-3-O-glucoside, quercetin deoxyl-hexose, and quercetin-3-O-rhamnoside, have been identified in *C. caudatus* (Ahda *et al*., 2023). In earlier investigations, the TLC profile of the ethanol extract of kenikir leaves revealed isoquercitrin spots and a very dominant flavonoid content (Yesika *et al*., 2023). After dechlorophyllating the ethanol extract, a flavonoid-rich fraction is produced.

An essential method for screening and separating flavonoids is UPLC, a type of HPLC (Carvalho *et al*., 2022). Although mass spectrometry (MS) is frequently employed to detect these substances due its high ionic resolution

and selectivity, tandem mass spectrometry (MS/MS) with an electrospray ionization (ESI) interface is a useful method for metabolite identification. It has been widely employed in recent years for isomeric discrimination of glycosylated flavonoids and for structural identification (Vukics & Guttman, 2010). Research has indicated that the fragmentation pattern of O-glycosylated flavonoids is significantly influenced by the site of sugar substitution (Ablajan *et al*., 2006).

The relative abundances of radical aglycone ions, which are most noticeable for flavonol 3-O-glycosides, have been demonstrated to be influenced by the glycosylation position, which also significantly affects the fragmentation behaviour of flavonoid O-glycosides (Cuyckens & Claeys, 2005; Hvattum & Ekeberg, 2002). The formation of a radical aglycone ion $[Y_0 - H]^{-\bullet}$ from deprotonated flavonoid mono-*O*-glycoside using ESI-MS/MS and concluded that the nature and position of the sugar substitution affect the fragmentation of the radical aglycone ions. The formation of radical aglycone ions has also been explained in detail in the case of the isoflavone glycoside genistein 7-*O*-glucoside (March *et al*., 2004).

In the present study, we focus on the structural characterization and the determination of flavonoids in *C. caudatus*. Considering the biological significance and flavonoid content of the species, the aim of this study was to obtain product ion mass spectra for deprotonated flavonoid glycosides from the flavonoid-rich fraction of *C. caudatus* using UPLC-QToF-MS/MS.

2. Methodology

2.1 Plant Materials

Fresh leaves of *C. caudatus* were collected in Sungai Sapih, Padang, West Sumatra, Indonesia (0°53'01.3"S 100°23'30.8" E) and identified by biologist Dr. Nurainas from Herbarium ANDA, Andalas University.

2.2 Flavonoid-rich Fractionation

The ethanol extract of *C. caudatus* leaves was prepared according to the previously reported method (Yesika *et al*., 2023). Briefly, fresh leaves were dried at 60 °C and ground into powder. The powdered material (400 g) was

macerated with 4 L of 70% distilled ethanol (1:10, w/v) per extraction cycle. The extraction was performed for three cycles, and the collected macerates were combined and concentrated at 50 °C using a rotary evaporator (Rotavapor R-210 equipped with Heating Bath B-491 and Interface I-300, BÜCHI Labortechnik AG, Switzerland) to obtain a thick ethanol extract.

The flavonoid-rich fraction was prepared as previously reported (Arifa et al., 2025). Briefly, the ethanol extract of *C. caudatus* leaves (5 g) was subjected to solid–liquid fractionation with 50 mL of *n*-hexane per extraction cycle. The fractionation was repeated 15 times until the *n*-hexane fraction became clear. After separation of the *n*-hexane-soluble fraction, the remaining extract was subsequently subjected to solid–liquid fractionation with 50 mL of ethyl acetate per extraction cycle, following the same procedure for 15 cycles. The remaining extract was then dissolved in ethanol to obtain the ethanol fraction. The fractionation yielded 0.196 g (3.92%) of the *n*-hexane fraction, 0.237 g (4.74%) of the ethyl acetate fraction, and 4.567 g (91.34%) of the ethanol fraction. The total extract, n-hexane fraction, ethyl acetate fraction, and ethanol fraction were quantitatively analyzed by TLC densitometry (CAMAG, Muttenz, Switzerland) using ethyl acetate/distilled water/formic acid (10:1:1) as the solvent and visualized under a 366 nm UV lamp (UV Cabinet 4, CAMAG, Muttenz, Switzerland) after spraying with citroborate reagent and heating at 100ºC for 5 minutes to determine the isoquercitrin content. The ethanol fraction showed the highest isoquercitrin content (23.2 mg/g fraction) and was therefore selected as the flavonoid-rich fraction (Arifa *et al*., 2025).

2.3 UPLC-ESI-MS/MS

For UPLC-QToF-MS/MS analysis, 5 mg of the selected ethanol fraction was dissolved in 5 mL of methanol to obtain a final concentration of 1000 µg/mL. Investigations using high-resolution mass spectrometry were carried out with a mass spectrometer (Xevo G2-S QTof, Waters, USA) and an ultra-performance liquid chromatography (UPLC) unit (LC: ACQUITY UPLC H-Class System, Waters, USA) using a C18 column (1.8 µm, 2.1 x 100 mm, ACQUITY UPLC HSS, Waters, USA) with the column heater module at 50 °C and the sample compartment at 25 °C. The LC analysis applied two mobile phases: acetonitrile + 0.05% formic acid (B) and water + 5 mM ammonium formic (A). The gradient program was as follows: 0–2 min, 5% B; 2–3 min, 25% B; 3–14 min, 25–100% B; 14–15 min, 100% B; 15–19 min, 1-00-5% B, 19–23 min, 5% B, followed by re-equilibration to initial conditions for 3 min. The run time was 23 minutes, the injection volume was 5 µL (first

filtered via a 0.2 µm syringe filter), and the flow rate was 0.2 mL/min (gradient elution). Analysis was carried out using mass spectrometry (MS) and electrospray ionisation (ESI) in negative mode. Source and desolvation temperatures were 100 °C and 350 °C, respectively, and the mass range was 50–1200 *m/z*. The cone and desolvation gas flow rates were set at 0 and 793 L/h, respectively. The low collision energy was set at 4 eV, while the high collision energy was ramped from 25 to 70 eV. The UPLC-QToF-MS/MS analysis was performed as a single analytical injection. Since the study focused on qualitative MS/MS fragmentation analysis rather than quantitative comparison, no statistical analysis was applied to the LC-MS/MS data. Data collection, analysis, and instrument control were conducted using MassLynx software v.4.1 (2008) and MZmine 2.53 (2019) (Harahap *et al*., 2024).

3. Results and Discussion

Chromatographic profiling of the flavonoid-rich fraction of *C. caudatus* ethanol extract revealed four major peaks corresponding to flavonoids (Figure 1). The four major peaks were tentatively annotated as were quercetin 3-*O*-rutinoside (rutin), quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin), quercetin 3-*O*-arabinofuranoside, and quercetin, with retention times of 4.35, 4.83, 5.16, and 6,87 min, respectively (Table 1.). Tentative annotation was performed using MZmine 2.53 and MassLynx v.4.1. Molecular formulas were assigned based on the accurate *m/z* values of the deprotonated precursor ions $[M-H]^-$ and evaluated together with the observed product ions by comparison with previously reported quercetin derivatives (Fernández-Ochoa *et al*., 2022). Authentic reference standards were not analyzed under identical UPLC-QToF-MS/MS conditions; therefore, the compound assignments are reported as tentative annotations.

The chromatographic profile showed prominent signals corresponding to quercetin glycosides in the flavonoid-rich fraction of *C. caudatus*. The detection of rutin and isoquercitrin is consistent with previous phytochemical analyses of the species, in which these compounds were also reported as major phenolic constituents.

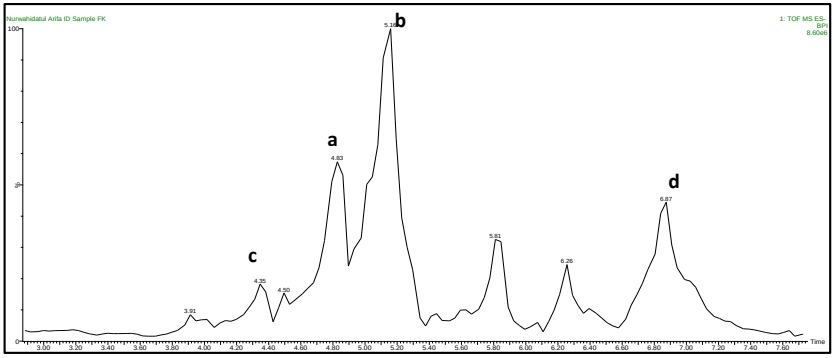


Figure 1. Chromatogram of flavonoid-rich fraction of *C. caudatus* leaves

Table 1. UPLC-QToF-MS/MS data identification of flavonoids in *C. caudatus* flavonoid-rich fraction

Retention time	Molecular Formula	Experimental $[M-H]^-$	Calculated $[M-H]^-$	Mass error (ppm)	Product ion	Compound	Reference
4.35	$C_{27}H_{30}O_{16}$	609.1459	609.1461	-0.34	463.0875; 431.1979; 301.0343; 300.0282	Quercetin 3-O-rutinoside (rutin)	Mustafa *et al.*, 2022
4.83	$C_{21}H_{20}O_{12}$	463.0874	463.0882	-1.73	433.0778; 301.0341; 300.0279	Quercetin 3-O-β-D-glucopyranoside (isoquercitrin)	Ivanova *et al.*, 2024
5.16	$C_{20}H_{18}O_{11}$	433.0771	433.0776	-1.24	301.0339; 300.0272	Quercetin 3-*O*-arabinofuranoside	Mustafa *et al.*, 2022;
6.87	$C_{15}H_{10}O_7$	301.0350	301.0354	-1.25	285.0405; 266.1030	Quercetin	Chen *et al.*, 2012

The UPLC-QToF-MS/MS spectra of the tentatively annotated compounds are shown in Figure 2-3. Each flavonoid exhibited characteristic deprotonated moleculer ions $[M-H]^-$ and diagnostic product ions consistent with the fragmentation patterns of quercetin glycosylation at the 3-O position.

Isoquercitrin (*m/z* 463.0874) generated $[Y_0 - H]^{-\bullet}$ and Y_0^- ions at *m/z* 301.0434 and 300.0279 , respectively, corresponding to the cleavage of the 3-*O*-β-glucopyranoside bond. Quercetin 3-*O*-arabinofuranoside (*m/z* 433.0771) produced similar $[Y_0 - H]^{-\bullet}$ and Y_0^- ions at *m/z* 301.0339 and 300.0272. Meanwhile, rutin (*m/z* 609.1459) displayed sequential fragmentation yielding Y_1^- (*m/z* 463.0875), $[Y_0 - H]^{-\bullet}$ (*m/z* 301.0343), and Y_0^- (*m/z* 300.0282), indicating the loss of rhamnose (146 Da) and glucose (162 Da) moieties

typical of diglycosides. These results support the tentative annotation of the compounds as quercetin 3-*O*-glycosides and provide detailed insight into their MS/MS fragmentation behavior, including the proposed fragmentation pathway of isoquercitrin.

Fragmentation nomenclature followed the schemes proposed by Mabry *et al*. (1970), refined by Ma *et al*. (1997) and expanded by Domon and Cotello (1988). In this system, ions retaining charges on the carbohydrate moiety are donated A_i, B_i, and C_i, while aglycon-containing ions are designated X_j, Y_j, and Z_j. The number 0 refers to the glycosidic bond directly attached on the aglycone (Scheme 1).

The neutral loss of 3-O glycosyl residues accounts for the Y_0^- ions. Through homolytic breakage of the 3-O glycosidic link, the glycosyl radical is eliminated, forming the $[Y_0 - H]^{-\bullet}$ ion. The $[M - H]^-$ spectra in Figures 2a, 2b, and 2c show comparable levels of aglycone ion and radical aglycone ion relative to the Y_0^- product ion (Wojakowska *et al*., 2013). For the three quercetin-3-O-glycosides, the aglycone produced $[Y_0 - H]^{-\bullet}$ and Y_0^- at *m/z* 300/301. Together with the aglycone ion (Y_0), most of the quercetin-3-O-glycosides generated a large number of radical aglycone ions ($[Y_0 - H]^{-\bullet}$). High-accuracy QToF-MS analysis was used to support the proposed elemental composition of the $[Y_0 - H]^{-\bullet}$ product ions for the three glycosides.

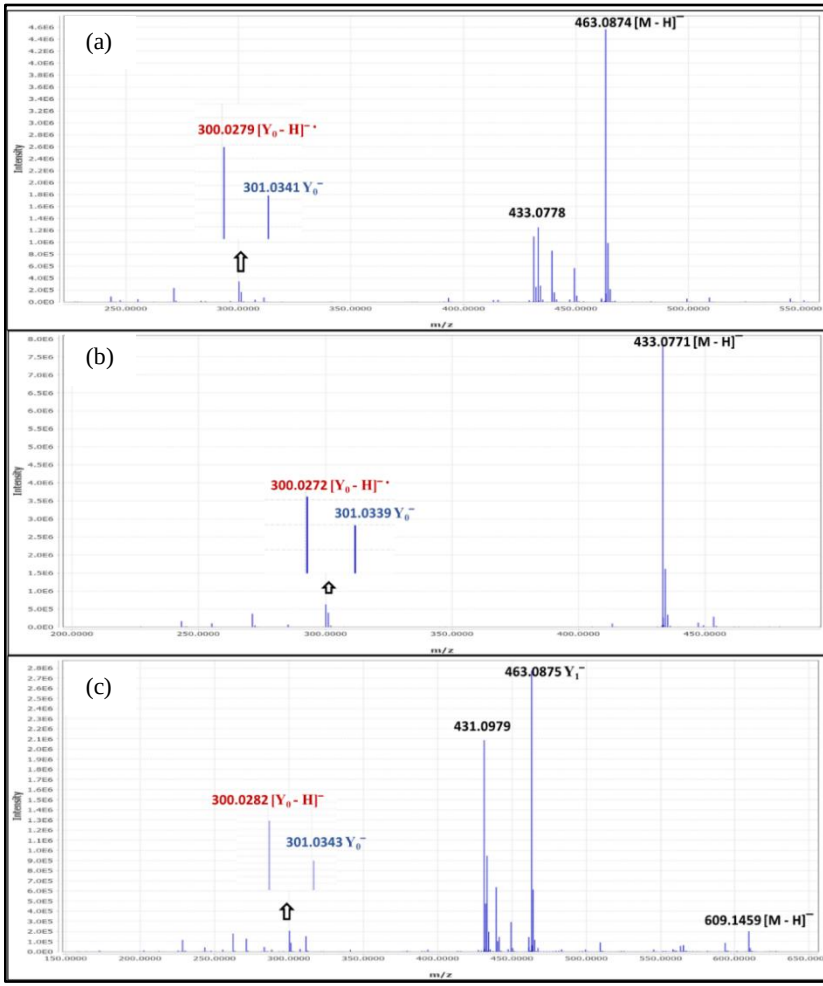


Figure 2. **[M − H]⁻** ion spectra of compounds in negative mode: deprotonated quercetin 3-*O*-β-D-glucopyranoside (*m/z* 463) (a); deprotonated quercetin 3-*O*-arabinofuranoside (*m/z* 433) (b); and deprotonated quercetin 3-*O*-rutinoside (*m/z* 609) (c)

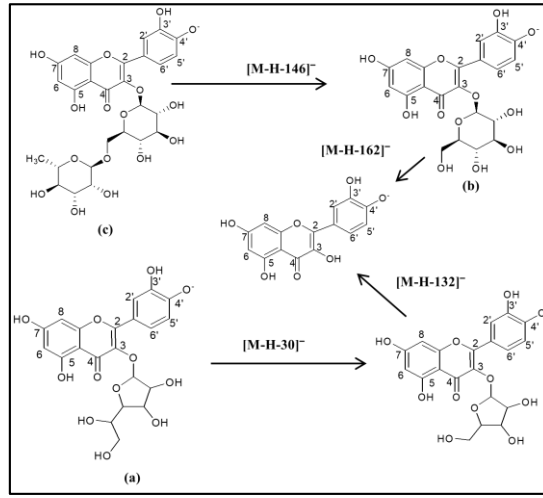
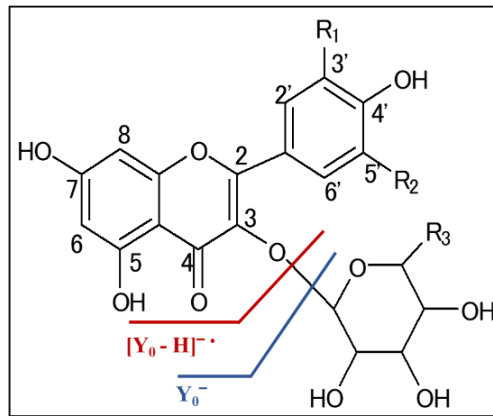


Figure 3. Fragmentation of quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) (a), quercetin 3-*O*-arabinofuranoside (b), quercetin 3-*O*-rutinoside (rutin) (c), and quercetin (d)



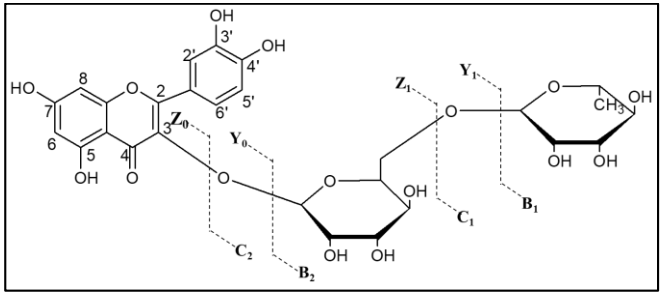
Scheme 1. Flavonol 3-*O*-glycoside fragmentation nomenclature for $[M - H]^-$

Along with glycosyl group removal, the flavonol-3-O glucoside can be identified through the glycan sequence of various sugar units. Diglycoside negative ion MS/MS spectra frequently do not reveal substantial Y_1^-, like rutin (quercetin-3-O-rutinoside) (Vukics & Guttman, 2010). However, the Y_1 and/or Z_1 ions can be used to characterize interglycosidic linkages, as shown with the molecule rutin (quercetin-3-O-rutinoside) in Scheme 2 (Ferreres *et al.*, 2004).

The fragmentation behavior and retention order observed here are broadly consistent with those reported in earlier and recent analyses of *C. caudatus* and related flavonoid-rich plants (Chen *et al*., 2012; Ivanova *et al*., 2024; Mustafa *et al*., 2022). However, the present study demonstrated a higher relative abundance of parent ions compared to UHPLC-Q Exactive Orbitrap-HRMS analyses (Ouyang *et al*., 2024), indicating improved ionization efficiency and sensitivity of the UPLC-QToF-MS/MS platform used.

Furthermore, the retention time of rutin (4.35 min) was slightly shorter than that reported by Ivanova *et al*. (2024) (4.52 min), possibly due to differences in column polarity or solvent gradient composition (Ivanova *et al*., 2024). The more pronounced $[Y_0 - H]^{-\bullet}$ ion observed in the current work also implies stronger fragmentation stability of quercetin glycosides under the applied ionization conditions. These subtle variations emphasize that both instrumental configuration and extraction method can significantly influence the fragmentation efficiency and relative abundance of glycosidic product ions.

Comparative glycoside analysis further reveals that diglycosides such as rutin produce a wider range of diagnostic ions (Y_1^-, Z_1^-) than monoglycosides (Ferreres *et al*., 2004; Vukics & Guttman, 2010). The consistent detection of $[Y_0 - H]^{-\bullet}$ and Y_0^-across all quercetin derivatives supports the proposed fragmentation interpretation and is consistent with 3-*O*-glycosylation as a prominent modification pattern in *C. caudatus* leaves.



Scheme 2. Nomenclature for glycosidic elimination in flavonoid O-glycoside fragments, illustrated with rutin (quercetin-3-O-rutinoside)

These findings expand the existing chemical profile of *C. caudatus*, highlighting quercetin glycosides as the principal bioactive constituents. The high spectral resolution achieved by UPLC-QToF-MS/MS facilitated detailed

characterization of the MS/MS fragmentation behavior of the tentatively annotated flavonoids. Future studies could benefit from comparing seasonal or geographical variations in *C. caudatus* flavonoid composition, or from analyzing related *Cosmos* species to assess the stability of these fragmentation patterns under differing experimental conditions.

4. Conclusions and Recommendations

By fractionating the ethanol extract to obtain a flavonoid-rich fraction, interference from other extract components was reduced, facilitating the analysis of flavonoids in *C. caudatus*. Flavonol 3-*O*-glycosides, particularly quercetin 3-*O*-glycosides, were tentatively annotated in the flavonoid-rich fraction. The three quercetin 3-*O*-glycosides exhibited characteristic losses of glycosyl residues and glycosyl radicals, producing distinct product-ion profiles. Product ions associated with interglycosidic cleavage of the flavonol 3-*O*-diglycoside were also characterized. This study provides detailed MS/MS fragmentation data and proposed fragmentation pathways based on high-resolution QToF-MS/MS analysis. The comparative analysis of quercetin-3-O-glycosides provides further insight into glycosidic bond cleavage and product-ion formation in flavonoid glycosides from *C. caudatus*.

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