

Sweet Flag (*Acorus calamus* L.) Rhizomes Chromatography Column Isolates Potential as Rich Antioxidant Compounds

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Abstract

Plant biodiversity in Indonesia is the prospective solution to overcome the top issues in the pharmacy sector, such as the unavailability of local materials in the purposing of independently manufacturing pharmaceutical products. One of plants that can be used for raw materials in the biopharmaceuticals is a sweet flag (*Acorus calamus* L.), which is rich in bioactive compounds such as flavonoids, alkaloids, phenolic compounds, tannins, steroids, saponins, glycosides, and terpenoids. Flavonoids are the majority bioactive compounds in sweet flag rhizomes, which leads this study to identify the flavonoids as antioxidant agents for biopharmaceutical product development later. This study determined the antioxidant activity of sweet flag rhizomes as its crude extract, ethyl acetate fraction, and column isolate. Based on phytochemical tests, column isolates were identified only to contain flavonoid compounds. Liquid chromatography Mass Spectrometry (LC-MS) in ethyl acetate fraction identified isorhamnetin, quercetin, kaempferol, genistein, and glycitein. Additionally, this study aims to show that column isolate has the highest purity level with the least inhibitory concentration of 50% (IC₅₀) and the strongest antioxidant activity.

Keywords: Jeringau Rhizome, Acorus calamus, Column Isolate, Flavonoids, Antioxidants

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Abstrak (Indonesian)

Keanekaragaman hayati tumbuhan di Indonesia merupakan solusi prospektif untuk mengatasi permasalahan utama di sektor farmasi yaitu tidak tersedianya bahan lokal dalam pembuatan produk farmasi secara mandiri. Salah satu tanaman yang dapat dimanfaatkan sebagai bahan baku biofarmasi adalah tanaman Jeringau (*Acorus calamus* L.) yang kaya akan senyawa bioaktif seperti flavonoid, alkaloid, senyawa fenolik, tanin, steroid, saponin, glikosida dan terpenoid. Flavonoid merupakan senyawa bioaktif terbanyak pada rimpang Jeringau, sehingga penelitian ini bertujuan untuk mengidentifikasi senyawa flavonoid sebagai agen antioksidan untuk pengembangan produk biofarmasi nantinya. Penelitian ini mengetahui aktivitas antioksidan rimpang Jeringau sebagai ekstrak kasar, fraksi etil asetat, dan isolat kolom. Berdasarkan uji fitokimia, isolat kolom teridentifikasi hanya mengandung senyawa flavonoid. Kromatografi Cair Spektrometri Massa (LC-MS) pada fraksi etil asetat teridentifikasi isorhamnetin, kuersetin, kaempferol, genistein, dan glisitin. Selain itu, penelitian ini bertujuan untuk mendapatkan data bahwa isolat kolom mempunyai tingkat kemurnian paling tinggi dengan konsentrasi hambat paling kecil 50% (IC₅₀) dan aktivitas antioksidan paling kuat.

Kata Kunci: Rimpang Jeringau, Acorus calamus, Isolat Kolom, Flavonoid, Antioksidan

INTRODUCTION

Indonesia has the main issue on the pharmacy products supply chain which costs extra for production

sectors. This concern is regarding the unavailability of primary raw materials for pharmaceutical goals. On the other hand, our country has a biodiversity of plants

with many medicinal compounds that still need to be fully explored. One of them is *Acorus calamus* L., an Araceae family called 'Jeringau' in Indonesia, while globally, it is known as 'sweet flag'. It is a semiaquatic herb with rhizomes.

Furthermore, this plant contains a few active constituents named α -asarone (phenylpropanoid (E)-1,2,4-trimethoxy-5-(1-propen-1-yl)-benzene) and β -asarone (phenylpropanoid (Z)-1,2,4-trimethoxy-5-(1-propen-1-yl)-benzene), which is found in the essential oils of leaves, roots and rhizomes [1]. Moreover, the quantity of these active constituents relies on botanical variety. This is a good reason why this study must provide the scientific data of its potential active constituents for the sweet flag in Indonesia's land.

Sweet flag is frequently used as the traditional medicine against invertebrate pests in essential oil (EO) forms due to its physicochemical properties and aromatic and volatile liquid. The chemical composition of the EO from sweet flag rhizomes is α -asarone, β -asarone, methyl isoeugenol, and methyl eugenol, which for more than 50% is the highest constituent is α -asarone. In addition, it is also known as food preservation related to the secondary metabolite compounds that can act as insecticidal and antimicrobial. For instance, it indicated that it has an insecticidal effect against *Sitophilus zeamais*, *Rhyzopertha dominica*, *Tribolium castaneum*, etc. On the other side, the antimicrobial activity against a few pathogen bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*, which zone of inhibition has positive response as the concentration increases [2].

The main bioactive compounds in the sweet flag are flavonoid, monoterpene, sesquiterpene, quinone, and phenylpropanoid. Recent studies found that the ethanol extract of this plant has antiproliferative and immunosuppressor effects against the growth of murine and human cell lines. Also, it can potentially to inhibit mitogen-induced proliferation of peripheral blood mononuclear cells and the generation of interleukin (IL-12) and tumor necrosis factor (TNF- α). Other studies evaluated that it can repair the hepatic enzymes in acetaminophen-induced liver damage and reduce free radical-induced oxidative stress [3]. Concerning the health benefits discovered by the latest studies, we aim to study further flavonoid compounds in the sweet flag rhizomes with ethanol extraction methodology.

The article review suggested that some research has been done and concluded that *Acorus* L. has ten flavonoids identified as mostly in flavonoid glycoside components. Apart from it, they also identified 12

amino acids such as tryptophan, aspartic acid, phenylalanine, lysine, norvaline, alanine, arginine, asparagine, threonine, proline, tyrosine, and glutamic acid [4]. Some of these amino acids are potentially good as a precursor for flavonoid biosynthesis.

According to recent studies of *Acorus calamus* L., this study aims to identify the effectivity of the variants of the sweet flag in Semarang City, Indonesia, using the column chromatography isolation method, ethanol extraction, and partition. Physicochemical properties for the bioactive compounds in the secondary metabolite of the sweet flag are determined by multisets of analysis using instruments such as Fourier transform infrared (FTIR) spectroscopy, spectrophotometry UV-Vis, and liquid chromatography-mass spectrometry (LC-MS).

MATERIALS AND METHODS

Materials

The primary materials used in this research were sweet flag rhizomes from Semarang, Central Java. For chemical reagents, we used ethanol (70%), ethyl acetate, *n*-hexane, methanol, distilled water, silica gel sodium bicarbonate (NaHCO₃), hydrochloric acid (HCl) 37%, magnesium (Mg) powder, iron (III) chloride (FeCl₃) 1%, chloroform, acetic anhydrous, concentrated H₂SO₄, potassium bromide (KBr), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) product from Sigma-Aldrich, Dragendroff and Mayer reagent product from Merck.

Ultrasonic-assisted extraction (UAE)

Ultrasonic-assisted extraction (UAE) was performed with 50 g of sample added with 500 mL of 70% ethanol with a sample and solvent ratio of 1:10 (w/v). It was extracted using ultrasonic waves with a wave of 47 kHz within 30 minutes. Afterward, the filtrate was concentrated using a rotary evaporator, yielding the crude extract.

Hydrolysis and partition

The hydrolysis process was carried out by adding 2 N hydrochloric acid (HCl) with a ratio of samples and HCl was 1:2. Then, it continued with the stirring for an hour and adding NaHCO₃ until the pH (degree of acidity) was neutral (pH 7). After that, 25 mL of ethyl acetate was used thrice in the partition step. When the organic fraction was separated and able to collect, it was concentrated using a rotary evaporator, yielding the partitioned fraction.

Column chromatography isolation of flavonoid

Column chromatography was utilized for this study for isolation process. It started with activating silica slurry in a column with a diameter of 1 cm and a

length of 50 cm. A total of 15 g of sample: silica ratio (1:150) was saturated in the column using the best eluent from thin layer chromatography (TLC) analysis, namely n-hexane and ethyl acetate, with a ratio of 7:3 within 24 hours. After that, the elution stage of 0.1 g of sample produces an eluate, collected into a glass bottle with a volume of 2 mL every minute. Afterward, isolated products from the elution were observed using the TLC method, which used an adsorbent as an activated F₂₅₄ silica gel plate with a size of 200 x 100 mm. For better spots in the TLC plate, spraying occurred before and after the observation. The observation was done under the 366 and 254 nm ultraviolet (UV) lights to identify the separation results.

Samples in this research were the 70% ethanol crude extract, partitioned-fraction isolate, and column chromatography isolate. Those were identified using Fourier Transform Infrared (FTIR) Varian 1000 and spectrophotometer UV-Vis Arian Carry 50. According to the prior analysis, only the partitioned-fraction isolates were characterized using liquid chromatography/ LC-Accella 1250 Mass Spectrophotometer (TSQ Quantum Access Max) instruments. The qualitative analysis of secondary metabolite compounds was conducted using specific reagents to locate the bioactive compounds such as alkaloids, flavonoids, tannins, saponins, steroids, and triterpenoids.

Antioxidant activity analysis

An antioxidant activity test was set up using a mixture of 3 mL of 98% ethanol and 1 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH), measured using a spectrophotometer UV-Vis. Followed by 5 mg of sweet flag isolate dissolving in 20 mL of 98% ethanol. Multiple sets of isolate concentrations ranged from of 10-50 ppm. A total of 3 mL of isolate with various concentrations was added with 1 mL of 0.2 mM DPPH. Afterward, the incubation took 30 minutes under 37°C conditions before the absorbance measurement was done. This test was repeated five times for each concentration of isolate

RESULTS AND DISCUSSION

Sample preparation

The first step in the isolation process was sweet flag rhizomes ultrasonication-assisted extraction (UAE) using 70% ethanol. Ultrasonication uses the ultrasonic wave to have a cavitation effect for degrading the cell walls of plants and releasing the subcellular contents. The essential plus point of ultrasonication relies on the nonthermal effects when undertaking the high-volatility flavonoid compounds

extraction process. In addition, this is related to the thermosensitive physicochemical characteristics of main compounds isolated for flavonoids. Afterward, the rotary evaporator removed the solvent from the crude extracts [5]. This step aims for the high-viscosity liquid with dark brown color extracts, of which the quantity is 12%. **Figure 1** shows the results of a 70% ethanol-concentrated extract of sweet flag rhizomes.



Figure 1. Results of 70% ethanol concentrated extract and ethyl acetate fraction of sweet flag rhizomes

The second step was hydrolysis of the crude extract sample before partitioning. This hydrolysis reaction used the 2 N hydrochloric acid (HCl), which is beneficial for degrading the glycoside bonds to obtain the secondary metabolite compounds. The hydrolyzed extract neutralized the acidity level using sodium bicarbonate (NaHCO₃), which showed a good impact in terminating the degradation of the flavonoid target compound [6]. The sweet flag rhizome extract was under the fractionation process with ethyl acetate as the solvent, attaining a 17.09% yield in proportion to the crude extracts from the previous step. Utilizing ethyl acetate as a solvent was the best option since its polarity characteristics are semipolar, which physiochemically can interact with target compounds with the same polarity as the “like dissolved like” theory [7]. In other words, the yield from the fractionation process can also be called an ethyl acetate fraction. Using a phytochemistry screening test, those two extraction process products, crude or partitioned extract, were analyzed to scrutinize secondary metabolites in sweet flag rhizomes.

The third step was the highly recommended separation process to attain only the most selective bioactive compounds with high efficacy in pharmacology and non-gradient column chromatography. The basic principle of this column chromatography uses the interaction of stationary and mobile phases physically and chemically. According to this study, silica gel acted as the stationary phase. On the other hand, the mobile phase was the multiple sets concentration of n-hexane and ethyl acetate. While

the elution of this column chromatography was in progress, the eluate was being collected for each minute running the system and gathering about 2 mL eluate in the vial. By the time elution reached the end, all the gathered-eluate was observed under thin layer chromatography (TLC), which used F₂₅₄ silica gel adsorbent as stationary phase and *n*-hexane: ethyl acetate (7:3) solvent as the eluent acting as mobile phase. TLC was running and producing the spots observed under a 366 nm UV lamp. In addition, liquid ammonia spraying was used in the visualization step to stabilize the color of TLC spots for more specific identification results of flavonoid compounds in the sample. The eluates that produce the same TLC spot characters are then gathered in a larger vial for further analysis; this is called column isolate.

According to the TLC results, only 60-118 of 150 vials that produced spots were suspected of flavonoids. The color of the spots was purple with a retention factor (R_f) value of 0.45. It is claimed to be a flavonoid compound in the flavon/flavonol groups [8,9]. A scientific explanation of flavonol and flavon group compounds produce purple spots under 366 nm UV lamp observation and do not change their purple color when reacted to liquid ammonia (NH₃).

The subsequent analysis results were reinforced by phytochemical analysis on column isolate fractions using (1) Wilstater reagent with the addition of magnesium metal and concentrated hydrochloric acid. Column isolate samples containing flavonoids will be reduced to benzopyrylium salts, where the sample undergoes a clear-to-yellow color change, and (2) 10% NaOH test, where flavonoid compounds produce a reddish-yellow color change due to the formation of sodium salts.

Phytochemical screening test of extracts, fractions, and column isolate results

A phytochemical screening test was done due to it is the rapid qualitative analysis to identify many bioactive compounds, such as alkaloids, tannins, flavonoids, steroids, terpenoids, and saponins, which are possibly in the crude extract, ethyl acetate fraction, and column isolate of sweet flag (*Acorus calamus* L) rhizomes. Regarding the test, it only used the color transformation observation for flavonoids, precipitation products for alkaloids and tannins, ring products in the test tubes for steroids or triterpenoids, and foaming in the saponin identification test [10].

The results of the phytochemical screening test of this study are shown in **Table 1** data interpretation, which explains the detrimental effect that has occurred for its phytochemistry compounds. A wide range of bioactive compounds in the crude extract downsized to

a small range of only flavonoid presence in the column isolate. In line with the phytochemical screening test results, the extraction process may take part in several bioactive compound degradation when it has been through several steps, such as hydrolysis and fractionation, before producing column isolate of sweet flag rhizomes [11].

Table 1. Phytochemical analysis of sweet flag rhizomes

Secondary Metabolite Compounds	Crude Extract	Ethyl acetate fraction	Isolate column chromatography
Flavonoid			
- Wilstater	+	+	+
- NaOH 10%	+	+	+
Alkaloid			
- Mayer	+	-	-
- Dragendorf	+	-	-
- Wagner	+	-	-
Tannin	+	+	-
Saponin	+	-	-
Steroid	+	-	-
Terpenoid	+	-	-

Chemical functional groups validation Fourier-Transform Infrared (FTIR) spectrophotometry analysis

Ensuring sweet flag extracts in this study have the correct functional groups within their bioactive compounds, this study carried out Fourier-Transform Infrared (FTIR) spectrophotometry. This instrument is potent in detecting specific functional groups that may represent those compounds' chemical structures [12,13]. It correlates with the fact that each bioactive compound has different absorption values on infrared (IR) spectrums (4000-400 cm⁻¹) according to their peculiar functional group contents.

This FTIR analysis has an essential role in validating the bioactive compounds in sweet flag extracts, which remains a high level of uncertainty. Furthermore, the profiling of phytochemical compounds might have a positive relationship with the FTIR spectra (**Figure 2** and **Table 1**). As shown in **Table 1**, some bioactive compounds such as alkaloids, tannins, saponins, steroids, and terpenoids had been through the degradation phase, with only flavonoids left unbreakable in the column isolate. This fact stands for the same point as in FTIR spectra that interpret all those extracts as having flavonoid compounds (**Figure 2** and **Table 2**).

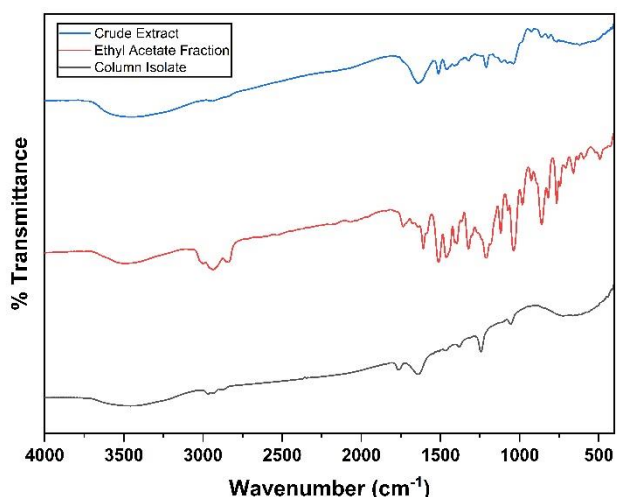


Figure 2. FTIR spectra results of crude extract, ethyl acetate fraction, and column isolate of sweet flag rhizomes

Table 2. Interpretation of FTIR spectra for the crude extract, ethyl acetate fraction, and column isolate of sweet flag rhizomes

Wavelength (cm ⁻¹)			Functional Group
Crude extract	Ethyl acetate fraction	Column Isolate	
3452	3503	3458	O-H stretching
2938	2935	2937	Csp ³ -H asym
1639	1608	1642	C=O stretching
1511	1511	1525	C=C aromatic
1211	1210	1244	C-O-C stretching
1114	1118	1055	C-O stretching alcohol
766	765	725	C-H aromatic
528	595	615	C-H

All sweet flag extracts underwent the ultraviolet-visible (UV-Vis) screening test for a wavelength absorption range of 200 to 800 nm. This study utilized the quercetin standard to quantify wavelength absorption areas that work for the sample since it is helpful to validate the presence of flavonoids as the primary secondary metabolite compounds [14]. Amidst the wavelength absorption areas of all sweet flag rhizome extracts, column isolate has the highest wavelength number, which is 342 nm in band I and 280 nm in band II, in comparison to the other two extracts, which are in similar positions, 256 nm in band II and around 310 in band I [Figure 3]. The band I absorption

area of 300-550 nm is typical of a conjugated C=C double bond with a $\pi \rightarrow \pi^*$ electron transition [15]. Conversely, band II, with an absorption area of 210-285 nm, relates to the absorption of a single chromophore group C=O bond with an electron transition $n \rightarrow \pi^*$. According to the absorption results in this study, column isolate is suspected to have a crucial chemical structure of conjugated C=C and the C=O groups, which are the typical functional groups representing flavonoids.

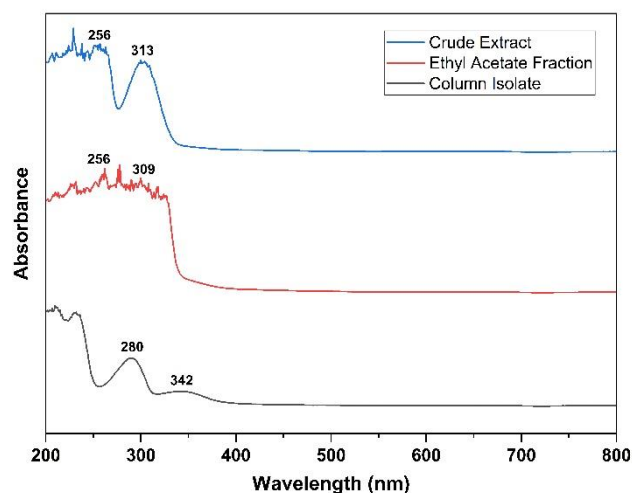


Figure 3. Ultraviolet-visible (UV-Vis) spectra for the crude extract, ethyl acetate fraction, and column isolate of sweet flag rhizomes

Liquid Chromatography Mass Spectrometry (LC-MS) analysis

Paralleling the phytochemical screening test and functional groups profiling using FTIR analysis, this study underwent liquid chromatography-mass spectrometry (LC-MS). This analysis was expected to provide more data on the flavonoid molecules, structures, quantity, and identity of specific sample components [16].

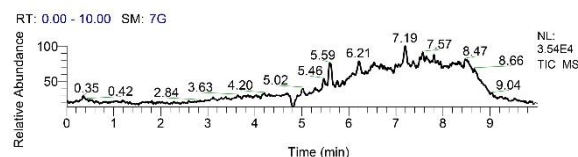


Figure 4. The Total Ionic Chromatogram (TIC) from LC-MS/MS for the ethyl acetate fraction of sweet flag rhizomes

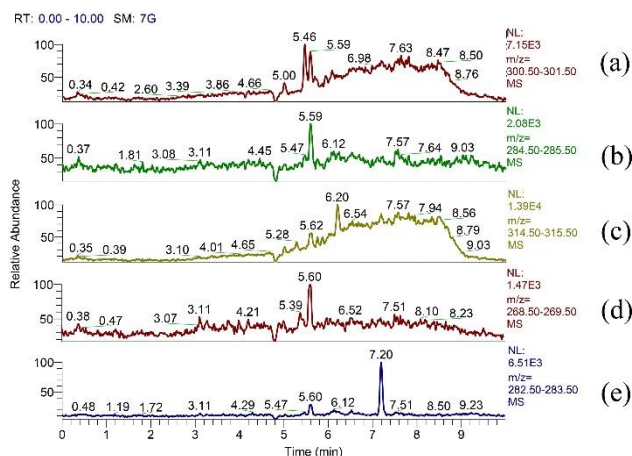


Figure 5. The Extracted Ionic Chromatogram (EIC) for the ethyl acetate fraction of sweet flag rhizomes

Figure 4 shows the chromatogram of TIC with several peaks indicating the presence of several compounds. **Figure 5(a)** is the EIC chromatogram from the ethyl acetate fraction of sweet flag rhizomes showing a peak at a retention time of 5.46 minutes with M-H (M-1) = 301, so the compound has a molecular weight of 302 g/mol, which corresponds to the quercetin compound. **Figure 5 (b-e)** shows a peak at retention time 5.59, 6.20, 5.60, and 7.20 minutes with M-H (M-1) = 285, 315, 269 and 283 so that these compounds have molecular weights of 286, 316, 270 and 284 g/mol which correspond to compounds (b) kaempferol, (c) isorhamnetin, (d) genistein, and (e) glycitein. Regarding the flavonoid functional group classification, the former three compounds, isorhamnetin, quercetin, and kaempferol, are classified in the flavonol group, while the latter two, glycitein and genistein, are in the isoflavone group [17].

Supporting data from the LC-MS analysis results in the Neutral Loss (NL) value of 7.15×10^3 stands for quercetin, 2.08×10^3 for kaempferol, 1.39×10^4 for isorhamnetin, 1.47×10^3 for genistein, and 6.51×10^3 for glycitein [**Figure 5**]. So, the abundance of flavonoid compounds in Jeringau extract is isorhamnetin, quercetin, glycitein, kaempferol, and genistein, respectively. These data sets indicate that flavonol has more present functional groups than other types of flavonoids on the ethyl acetate fraction of sweet flag rhizomes.

According to the data of the phytochemical screening test in this study, the presence of the flavonol functional group was affirmed by the fact that the yellow color appears under alkali conditions (NaOH) on the phytochemical test [18]. As for the isoflavones, the functional group can be confirmed using colorless results of the same phytochemical screening tests [19].

Antioxidant activity analysis

Sweet flag rhizome extraction has the primary outcome of producing rich antioxidant agents within the extracts. This study required quantitative assessment using 2,2-diphenyl-1-picrylhydrazyl (DPPH) to obtain inhibition concentration (IC_{50}). Antioxidant activity analysis has a positive response for each level of the extraction process. Furthermore, starting from crude extract, partition, and column isolate, the IC_{50} value has gradually declined as the process comes to the last step of column isolate. After getting the IC_{50} value, the antioxidant activity index (AAI) was also determined.

Table 3. IC_{50} value of antioxidant activity analysis

Sample	IC_{50} (ppm)	AAI (ppm)
Column isolate	28.891	3.461
Ethyl acetate fraction	63.178	0.207
Crude extract	136.068	0.734
Vitamin C	8.346	11.891

This antioxidant activity analysis was used to quantify the bioactive compounds to scavenge the free radical molecules, which has a good health benefit [20]. This study used DPPH as a standard solution in the 10 to 50 ppm. Afterward, the sample solutions, crude extract, partitioned fraction, and column isolate were analyzed for their optimum wavelength absorption, namely 517 nm, and the absorbance results obtained were used to calculate IC_{50} . The optimum wavelength can be used to measure of the antioxidant activity index (AAI).

This study described that crude extract of sweet flag rhizomes has moderate antioxidant activity by aiming for the highest IC_{50} value and the least AAI. Interestingly, on the other hand, the column isolate has a very strong level of antioxidant activity, correlating the lowest IC_{50} value and the highest AAI [**Table 3**]. In addition, this trend data was also supported by FTIR analysis, resulting in the presence of C=O and C-C groups at wavelength absorption of 230 nm in band II and 342 nm in band I [**Figure 3**]. Additionally, the molecules of hydroxyl -OH, aromatic C=C, carbonyl C=O, and aromatic C-H were present as the flavonoid functional groups that are rich in antioxidant agents.

The successful rate of rich antioxidant agent production in the study using a multistep isolation process depends on the flavonoid purity within the column isolate. The higher the purity level of flavonoid and, of course, the fewer unwanted molecules, it correlates to the more potent antioxidant activity in the column isolate sweet flag rhizomes. This trend data may be supported by the absence of antagonist

compounds potentially interfering with the antioxidant activity.

CONCLUSION

In conclusion, sweet flag rhizomes were extracted into a crude extract, ethyl acetate fraction, and column isolate to attain the highly concentrated flavonoid as the main bioactive compound for rich antioxidant products. The decreasing range of phytochemical compounds remained only flavonoid in the column isolate. This isolate was predicted to possess the highest purity flavonoid among other extracts, confirmed by thin layer chromatography (TLC) with the plate purple spots at the retention factor (Rf) value of 0.45 representing the flavonoid compounds. The wavelength absorption area in column isolate that works for flavonoid identification was 342 nm for band I and 280 nm for band II. Fourier-transform infrared (FTIR) spectroscopy analysis successfully identified the representative flavonoid functional groups such as O-H, C=C aromatic, C-H aromatic, C=O carbonyl stretch, C-H aliphatic, C=O alkyl ketone, C-O alcohol secondary, and C-O-C stretching. Liquid chromatography Mass Spectrometry (LC-MS) in ethyl acetate fraction of sweet flag rhizomes identified isorhamnetin, quercetin, kaempferol, glycitein, and genistein. Additionally, this study aims to show that column isolate has the highest purity level with the least inhibitory concentration, 50% (IC₅₀), and the strongest antioxidant activity.

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