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Isolation and Antioxidant Activity of Flavonoid Compound in Ethanolic Extract of Celery Leaves (*Apium graveolens* L.)

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Abstract

Celery (*Apium graveolens* L.) is a plant that belongs to the *Apiaceae* family and is widely used as a medical plant for low blood pressure, heart tonic, and to prevent cardiovascular disease. This study aims to obtain flavonoid compounds, identify, and test the antioxidant activity of flavonoid compounds and crude extracts from celery leaves. The research procedures consisted of four steps, the first of which was a preliminary test. The second step involved isolating and separating flavonoid components by vacuum liquid chromatography, gravitational column chromatography, and preparative thin layer chromatography. The third step was to identify flavonoid compounds using reagent shift, FTIR, and LCMS/MS. And finally, antioxidant activity was evaluated using the DPPH method. The preliminary test result showed that the ethanolic extract of leaves and stems had a total flavonoid content of 13.99 and 2.46 mg QE/g of dry weight. Both dry leaves and crude extract of celery leaves contained alkaloids, saponin, flavonoid, tannin, quinone, and steroid/triterpenoid, as determined by phytochemical screening. Isolation and separation of flavonoids yielded A2.I and A2.II isolates, with respective weights of 8 mg and 14 mg. Identification of flavonoid compounds using reagent shift showed that two isolates have the basic structure of the flavone group. A2.I isolate had OH groups at 4', 5, and 7, while A2.II isolate had OH groups at 3', 4', 5, and 7. The FTIR analysis revealed that both compounds contain functional groups, including O-H, C=O, C=C aromatic, C-O ether, C-O alcohol, and C-H aromatic ring. According to LCMS/MS analysis, the molecular weights of A2.I and A2.II were 270 g/mol and 286 g/mol, respectively. All of the identification methods for isolates showed that A2.I was apigenin and A2.II was luteolin. Antioxidant activity by DPPH method for a viscous extract of celery leaves, A2.I, and A2.II were 775.41, 288.95, and 184.35 µg/mL, respectively.

1. Introduction

Celery (*Apium graveolens*) is a plant that belongs to the Apiaceae family. This plant grows throughout the tropical and subtropical regions [1]. The micronutrients and secondary metabolites in celery, such as limonene, selinene, procoumarine glycoside, flavonoid, and vitamins A and C, allow the celery to be utilized as traditional medicine [1]. Celery can prevent cardiovascular disease [2], reduce blood glucose levels, and improve heart function [3]. Some studies reported that celery had antifungal [4] and anti-inflammatory properties [5].

In celery, flavonoids, phenolic acid, and phenylpropanoids had antioxidant activity [6]. The leaves and roots of celery can inhibit the mean oxidation of DPPH (2,2-diphenyl-1-picrylhydrazyl) and reduce the intensity of liposomal peroxidation [7]. Many flavonoid compounds are found in celery herbs [8]. Flavon is the predominant flavonoid group found in celery [9].

Major flavonoid content in celery are apigenin, apiin, luteolin, and kaempferol [10]. Li *et al.* [11] reported that flavonoid compounds in celery had an antioxidant activity based on *in vitro* and *in vivo* analysis of the compounds. Apiin (apigenin-7-apisoylglucoside) was

found as a major compound by isolation using LC/ESI/MS/MS from ethanolic celery extracts and proved to have antioxidant activity. The isolation of flavonoid aglicon or glycosides from celery extracts has not been widely reported.

In this study, ethanolic extracts of celery leaves were isolated. The flavonoid compounds were identified using reagent shift, FTIR, and LCMS/MS. Furthermore, the extract and the compounds were tested for antioxidant activity by DPPH assay.

2. Methods

2.1. Materials

Materials used in this study were celery leaves (*Apium graveolens* L.), quercetin standard (purity: ≥97%) and DPPH (Sigma Aldrich), aluminum chloride, ethanol 96%, n-hexane, chloroform, ethyl acetate, methanol, 1-butanol (Merck), thin layer chromatography (Merck), silica gel 60 H and 60 F_{254} (Merck), ammonia, magnesium powder, amyl alcohol, HCl, 10% $FeCl_3$, Dragendorff reagent, Mayer reagent, H_2SO_4, NaOH, acetic acid anhydrate, 5% $AlCl_3$, sodium acetate, and H_3BO_3.

2.2. Preliminary Study

Preliminary studies were performed to determine the total flavonoid content and phytochemical screening of celery. The total flavonoid content of celery was examined by extracting 50 g of leaves and 50 g of the stem using 96% ethanol. Then, 0.1 g of leaves and stem extracts were dissolved in methanol: water (1:1). One milliliter of the solution was added by 3 mL of methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1M CH_3COONa, and subsequently distilled water up to 10 mL. One milliliter of quercetin standard (0, 1, 2, 4, 6, 8, 10 ppm) was mixed with 3 mL methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1 M CH_3COONa, and added distilled water up to 10 mL. The quercetin, leaves extracts, and stem extracts were left for 30 min at room temperature. The absorbance was measured at the maximum wavelength of quercetin (432.5 nm) using UV-Vis spectrophotometer Genesys 10S.

2.3. Isolation of Flavonoid

One kilogram of dry celery leaves was extracted by maceration method using 96% ethanol until the filtrate was colorless. The filtrate was evaporated, and the crude extracts were weighed. Ethanolic extract was dissolved in ethanol, added to distilled water (1:1), and left for 24 h to precipitate the chlorophyll. The filtrate was extracted using n-hexane, and the polar layer (ethanol layer) was separated and evaporated. The ethanolic-water extract was hydrolyzed using 1.2 M HCl for 90 min at 74°C. The hydrolyzed fraction was fractionated using ethyl acetate, evaporated, and weighed.

Ethyl acetate fraction was isolated by vacuum liquid chromatography using silica gel 60 H as the stationary phase and chloroform → ethyl acetate → methanol by gradient polarity as the mobile phase. The fractions were collected every 35 mL in vial bottles. Each fraction was profiled using thin-layer chromatography under UV light at 254 and 366 nm. The fractions with similar profiles were mixed (A, B, C, D, E, F, G) and weighted. The fractions containing flavonoid spots were isolated by gravitational column chromatography using chloroform: ethyl acetate: 1-butanol (2:4:1) as the mobile phase and silica gel 60 F254 as the stationary phase. The fractions were collected every 10 mL in a vial bottle (A1, A2, A3, etc.). The fraction containing more flavonoid spots was isolated by preparative thin-layer chromatography using chloroform: ethyl acetate: 1-butanol (6:3:0.5) as the mobile phase. Purity tests of the compounds were performed by 1D and 2D thin-layer chromatography. The structure of flavonoid compounds was then identified by reagent shift, FTIR, and LCMS/MS Xevo G2-XS QTof.

2.4. Antioxidant Activity

The antioxidant activities of extracts and flavonoid compounds from celery leaves were conducted by DPPH assay. Quercetin was used as the positive control. Several concentrations of flavonoid compounds were made at 30, 60, 90, 120, 150, 200, 250, and 300 ppm, and the extracts at 10, 50, 100, 150, 200, 300, 400, and 500 ppm. One milliliter of each concentration was reacted with 3 mL of 0.1 mM DPPH. The mixture was homogenized and left for 30 min in a dark room. After 30 min, the absorbance was measured at 516 nm using UV-Vis Spectrophotometer. The same treatment was done to quercetin standard at 2, 4, 6, 8, 10, 12, and 14 ppm. The absorbance value was used to calculate the percentage of DPPH inhibition using Equation (1).

$$\%\text{inhibiton} = \frac{\text{A.blank} - \text{A.sample}}{\text{A.blank}} \text{x } 100\% \quad (1)$$

The IC_{50} value was determined using a calibration curve with the x-axis as concentration and the y-axis as the percentage of DPPH inhibition (%inhibition). The IC_{50} is the sample concentration that can decrease 50% of DPPH radical activity.

3. Results and Discussion

3.1. Preliminary Studies

The total flavonoid content was determined for the leaves and stems of celery. This test aimed to identify which plant part had the highest flavonoid concentration to proceed to the flavonoid separation process. The total flavonoid content was calculated using a standard calibration curve of quercetin. The maximum wavelength for the measurement was 429.0 nm. The results of total flavonoid content are shown in Table 1. Based on the results of the total flavonoid content of the leaves, the isolation process was performed to obtain flavonoid compounds.

Table 1. Total flavonoid content of celery leaves and stems

Samples	Absorbance	Total Flavonoid Content (mg QE/g dw)
Leaves	0.3528	13.99
Stems	0.6345	2.46

Phytochemical screening was conducted to identify the secondary metabolite content in celery leaves and

extracts. Table 2 shows the results of the phytochemical screening.

Table 2. Phytochemical screening of dry leaves and crude extracts of celery

Test	Results	
	Dry leaves	Crude extract
Alkaloid	+	+
Flavonoid	+	+
Saponin	+	+
Quinon	+	+
Tannin	+	+
Steroid	+	+
Triterpenoid	+	+

3.2. Flavonoid Isolation

1025 g of celery leaves dry powder were macerated using 96% ethanol, filtrated, and evaporated. The yield of crude extract obtained after evaporation was 482.68 g (48.27%). The chlorophyll from crude extracts was precipitated using ethanol: water (1:1), then the filtrate was fractionated using n-hexane. The ethanol-water fraction was hydrolyzed to break the O-glycoside bond in the flavonoid, resulting in flavonoid aglycon and glucose. Flavonoid aglycones were fractionated using ethyl acetate, yielding a 20.85 g fraction from 119.68 g ethanol-water fraction.

Table 3. Fractions from isolation by vacuum liquid chromatography

Code	Number of vial	Color
A	9-17	Yellow-greenish
B	18-26	Yellow-greenish
C	27-32	Brown
D	33-38	Brown
E	39-41	Brown
F	42-45	Brown
G	46-49	Brown

Ethyl acetate fraction (10 g) was isolated using gradient polarity mobile phase starting from chloroform, chloroform/ethyl acetate, ethyl acetate, ethyl acetate/methanol, ending with methanol, resulting in 73 fractions being collected. The fractions with similar spots were mixed (shown in Table 3).

A-fraction was analyzed by thin-layer chromatography using ammonia and 5% $AlCl_3$ as a reagent spray. A-fraction was further isolated by gravitational column chromatography using chloroform: ethylacetate: 1-butanol (1:2:0.5) as the mobile phase. Each fraction was analyzed by thin-layer chromatography (TLC). The results of TLC profiling are shown in Figure 1.

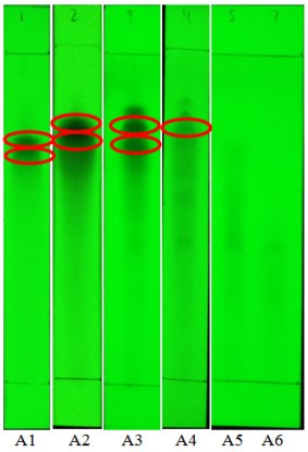

Figure 1. TLC profiles of A1-A6 fractions (left to right) under UV light at 254 and 366 nm

According to the TLC profile, A1-A3 fractions exhibit two identical spots, which are flavonoid spots (Figure 1); hence, preparative TLC was used to separate the two spots. The A1, A2, and A3 were obtained as much as 7.3 mg, 51.4 mg, and 26.8 mg, respectively. A2-fraction was chosen for further isolation. The mobile phase was chloroform: ethyl acetate: 1-butanol (6:3:0.5). Figure 2 shows the result of the separation.

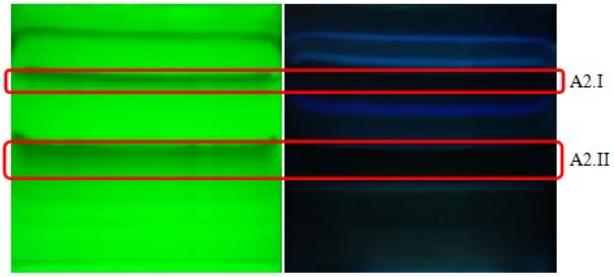

Figure 2. TLC profile of A2-fraction under UV light at 254 nm (left) and 366 nm (right)

A2.I (Rf = 0.75) dan A2.II (Rf = 0.41) bands were cut, dissolved, and filtrated to obtain A2.I and A2.II isolates. Each isolate was tested for its purity using 1D and 2D TLC. The profile is shown in Figures 3 and 4.

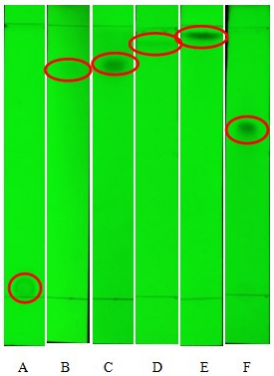

Figure 3. TLC profiles from purity test of A2.I isolate under UV light at 254 nm

Mobile phase: A = chloroform, B = ethyl acetate, C = methanol, D = chloroform:methanol (1:1), E = hexane:chloroform:ethanol (1:2:2), F= chloroform:methanol (20:1).

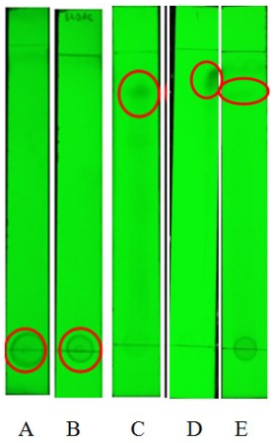

Figure 4. TLC profiles from purity test of A2.II isolate under UV light at 254 nm

Mobile phase: A = chloroform, B = ethyl acetate, C = ethanol, D = ethyl acetate:ethanol (1:1), E = ethyl acetate:ethanol (9:1).

The first and second mobile phases for the 2D TLC profile of A2.I isolate were chloroform: methanol (15:1) and hexane:chloroform: ethanol (2:1:1). Meanwhile, for A2.II isolate were ethyl acetate: ethanol (1:1) and ethyl acetate: ethanol (1.5:1). The result of 2D TLC profiling is shown in Figure 5.

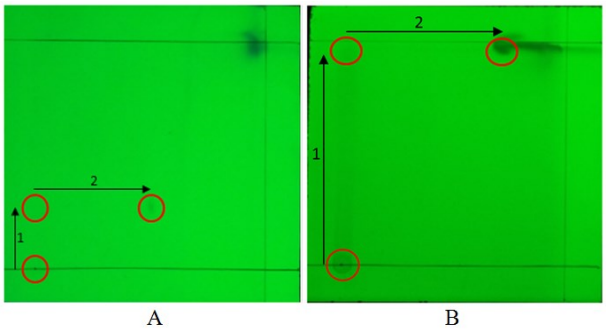

Figure 5. 2D TLC profiling of (A) A2.I and (B) A2.II under UV light at 254 nm

Figure 5 shows the single point in each of the 1D and 2D TLC profiles of the isolates. The purity of both isolates may be confirmed by these results. Both isolates were evaporated, and obtained 8 mg of A2.I isolate and 14 mg of A2.II isolate.

3.3. Structure Identification of A2.I Isolate

The A2.I isolate was identified using reagent shift to determine the parent skeleton and branch of the compound. This study used NaOH, $AlCl_3$, HCl, NaOAc, and H_3BO_3 as the reagent shift and the spectra measurement by UV-Vis spectrophotometer. The result of the reagent shift test is shown in Table 4.

Based on structure identification using reagent shift, A2.I isolate is a flavone group with hydroxyl attached with carbon numbers at 4', 7, and 5. Further identification was performed using FTIR to prove the identical absorbance of each functional group. Figure 6 shows the FTIR spectra of A2.I isolate.

Table 4. The wavelength shift of bands I dan II of A2.I isolate

Reagent	λ of each band (nm)			OH
	I	II	new	
-	338	269	-	-
NaOH	394	275	327	4', 7
NaOH (5 min)	393	275	327	
$AlCl_3$	350, 384	275, 302	-	5
$AlCl_3$/HCl	344, 381	278, 301	-	
NaOAc	387	275	-	7
NaOAc (5 min)	387	275	-	
$NaOAc/H_3BO_3$	342	269	-	-

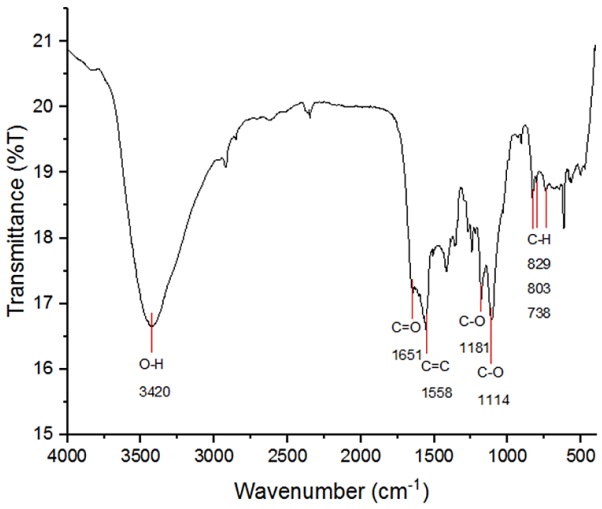

Figure 6. FTIR spectra of A2.I isolate from celery leaves

FTIR spectra of A2.I isolate shows the absence of a specific band at 1100-1000 cm^{-1} due to glycosidic bond [12], indicating that the A2.I isolate is not a flavonoid-glycoside—furthermore, the band at 3420.42 cm^{-1} shows stretching vibration of the O-H group [13]. A band at 1651 cm^{-1} indicates the C=O stretching vibration [14]. The band at 1558.8 cm^{-1} shows the C=C aromatic group (substitution of aromatic compounds) [15]. The C-O ether group is shown by stretching vibration at 1181.54 cm^{-1} [16] and C-O alcohol at 1114 cm^{-1} [17]. The C-H aromatic ring bending vibration is apparent at 829.25, 805, and 738 cm^{-1} (substitution of aromatic compounds).

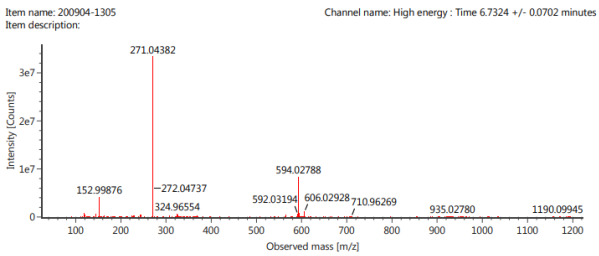

Figure 7. MS result of A2.I isolate from celery leaves

LCMS/MS analysis (Figure 7) shows that A2.I compound has a retention time of 6.74 min with an m/z value of 271.04. The LCMS/MS using ESI positive [M+H] so the molecular weight of A2.I was 270 g/mol. The fragmentation showed that A2.I isolate is apigenin (Figure 8).

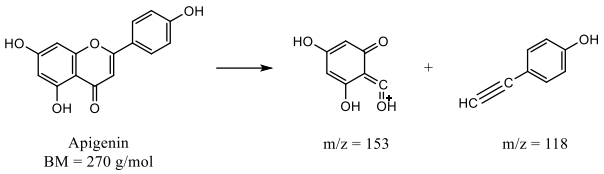

Figure 8. Fragmentation of A2.I compound from celery leaves

3.4. Structure Identification of A2.II Isolate

The pure A2.II isolate was identified by reagent shift to determine the parent skeleton and branch of the compound using NaOH, $AlCl_3$, HCl, NaOAc, and H_3BO_3, then observed the UV-Vis spectrum. The A2.II identification result using reagent shift is shown in Table 5.

Table 5. The wavelength shift of bands I and II of A2.II isolate

Reagent	λ of each band (nm)			OH
	I	II	new	
-	350	255, 268	-	-
NaOH	404	272	336	4', 7
NaOH (5 min)	403	271	336	
$AlCl_3$	326, 425	273, 300	-	5
$AlCl_3$/HCl	360, 386	266, 275	297	
NaOAc	401	271	-	7
NaOAc (5 min)	403	270	-	
$NaOAc/H_3BO_3$	372	261	-	3', 4'

From Table 5, the A2.II isolate is a flavon group with hydroxyl on carbon numbers at 3',4', 7, and 5. FTIR was employed for future analysis to prove and determine the specific absorbance for each functional group. The FTIR spectrum is shown in Figure 9.

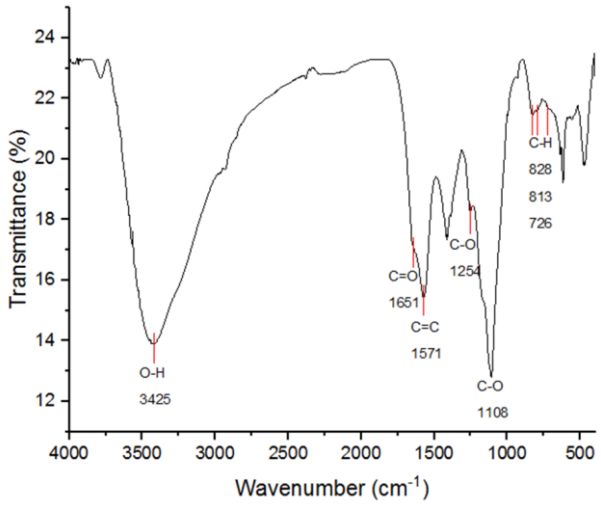

Figure 9. FTIR spectrum of A2.II compound from celery leaves

FTIR spectra of A2.II compound does not exhibit a distinct band at 1100-1000 cm^{-1} due to the glycosidic bond, indicating that it is a flavonoid-aglycone rather than a flavonoid-glycoside. Meanwhile, the O-H group stretching vibration is indicated at 3425.66 cm^{-1}. At 1651 cm^{-1}, there is a band from the stretching vibration of the C=O group. A band at 1571.01 cm^{-1} shows a C=C aromatic vibration. The C-O ether group and C-O alcohol are identified by stretching vibration at 1254 cm^{-1} and 1108 cm^{-1}, respectively. The bands at 828.01, 813, and 792 cm^{-1} are caused by the C-H aromatic ring's bending vibration.

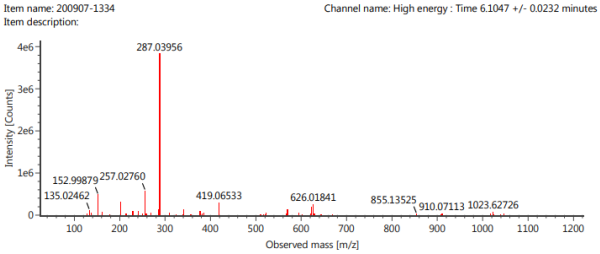

Figure 10. MS result of A2.II compound from celery leaves

LCMS/MS analysis (Figure 10) shows that A2.II compound has a retention time of 6.11 min with an m/z value of 287.04. The ESI-positive [M+H] LCMS/MS resulted in a molecular weight of A2.I of 286 g/mol. The fragmentation shows that A2.II compound is luteolin (Figure 11).

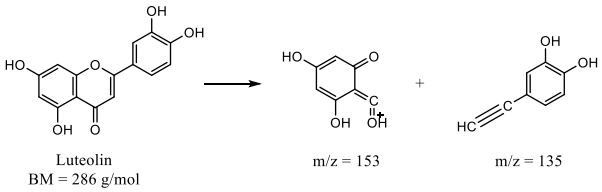

Figure 11. Fragmentation of A2.II compound from celery leaves

3.5. Antioxidant Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method was applied to the crude extracts, flavonoid compound, and quercetin standard to determine their antioxidant activity. Flavonoids reacted with DPPH by reduction reaction resulting in nonradical DPPH. This study was done to determine the Inhibition Concentration (IC_{50}), which is the antioxidant concentration that can inhibit 50% of DPPH radical properties. The result of the IC_{50} value is shown in Table 6.

Table 6. The IC_{50} of crude extracts, flavonoid isolates, and quercetin standard

Sample	IC_{50} (µg/mL)
Extract	775.41
A2.I compound	288.95
A2.II compound	184.25
Quercetin standard	10.08

Taormina *et al.* [18] reported that antioxidant activity is classified into 4 levels, including very strong (IC_{50} <50 µg/mL), strong (IC_{50} 50-100 µg/mL), moderate (IC_{50} 100-250 µg/mL), and weak (IC_{50} 250-500 µg/mL). Hence, it can be concluded that A2.I compound has weak antioxidant activity, whereas A2.II compound has moderate antioxidant activity.

4. Conclusion

Isolation of flavonoid compound from celery leaves (*Apium graveolens* L.) resulted in two isolates, A2.I compound (8 mg) and A2.II compound (14 mg). Structure identification by reagent shift, FTIR, and LCMS/MS showed that A2.I and A2.II compounds were apigenin and luteolin, respectively. The crude extracts and A2.I and A2.II compounds from celery leaves have IC_{50} values of 288.95 µg/mL and 184.25µg/mL, respectively.

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