Majalah Obat Tradisional (Trad. Med. J.), October-December 2025
Vol. 30(3), p 218-226
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 11-12-2023
Revised : 14-10-2024
Accepted : 13-11-2024

Phytochemical Analysis and Anti-Collagenase Activity of Celery (*Apium graveolens* L.) Extract

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ABSTRACT

Aging is a normal process experienced by humans. One of the causes of aging is the body's intrinsic activity, such as the activity of one of the extracellular matrix-breaking enzymes, collagenase. Demand for skin care to prevent aging is increasing, especially plant-based skin care. This study aims to explore the potency of celery extract as an anti-collagenase. In this study, we used celery extract as a sample obtained from the maceration process by seventy percent ethanol 70% as the solvent. Sample compounds are then detected by LC-HRMS to determine the major compounds contained in a sample and their concentrations. Anti-collagenase activity was observed by enzymatic assay. The LC-HRMS analysis confirmed that apigenin, hispidulin, and catechin were included in the celery extract, with apigenin as the major compound of celery with AUC 73479.21×10^3, the AUC of hispidulin is 17063.21×10^3, and catechin 61.14×10^3. The percentage of collagenase inhibition of celery extract up until 200 µg/mL was 59.22% with an IC_{50} value of 132.48 µg/mL. These data indicated that celery containing apigenin, hispidulin, and catechin has an anti-collagenase activity worth further exploring for a healthy skin supplement.

Keywords: Anti-collagenase; Celery; Enzymatic Assay; LC-HRMS; Skin aging.

INTRODUCTION

The aging process occurs in every organ of humans. Signs of aging, such as wrinkles, are caused by aging in the skin. Exposure to intense internal and external factors can cause oxidative stress and induce collagenase enzyme expression, leading to collagen degradation. When collagen is degraded at a high rate, signs of aging will appear more quickly (Ganceviciene et al., 2012). Collagen degradation is mainly carried out by the collagenase enzyme, one of the enzymes from the matrix metalloproteinase (MMP) family protein (Murphy, 2016). Collagen synthesis and breakdown are in balance under normal circumstances or homeostasis. Reduced collagen synthesis or increased collagenase enzyme output can both impair the balance of the body. The release of the enzyme collagenase can be stimulated externally by events outside of the body or inside by aging and hormones. One of the external factors that can accelerate the aging process through inducing collagenase secretion is exposure to UV radiation (Zhang and Duan, 2018). UV rays can raise the skin's levels of reactive oxygen species (ROS), which disrupt skin homeostasis by activating MMPs and breaking down collagen molecules (de Jager et al, 2017; Martin-Martinez et al., 2022). High levels of ROS also contribute to oxidative stress and can trigger cellular damage (Muchtaridi et al., 2024).

The increasing levels of ROS can be neutralized by antioxidants, including endogenous, such as enzymes, or exogenous, such as flavonoids (Juan et al., 2021). Besides having antioxidant abilities, flavonoids can also be inhibitors of collagenase enzymes with the mechanism of action as chelating agents on the active side of the enzyme by binding Zn (II) atoms and Ca (II) atoms contained in MMP molecules, including collagenase enzymes (Mandrone et al., 2018). Generally, the high flavonoid content in plant extracts is correlated with high antioxidant activity and collagenase enzyme inhibition.

Numerous plants in Indonesia possess diverse phytochemical compositions that hold the potential for anti-collagenase agents. One plant

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that grows well in high altitudes is celery, which is easily found in Indonesia due to the country's vast number of plateaus. Celery is a member of the Apiaceae family that is known for its characteristics of strong and distinct flavors (Turner et al., 2021). Traditionally, celery has been used as a spice, dietary supplement, or medicine to treat wounds and prevent several illnesses (Nuningtyas et al., 2020). This usage has also been supported by research that showed the antimicrobial activity of celery in wounds infected with methicillin-resistant *Staphylococcus aureus* (MRSA) helped skin re-epithelialization, fibroblast proliferation, and cytokeratin 17 (CK-17) expression (Prakoso et al., 2020).

Our objectives in this work were to examine the phytochemical composition of celery and quantify its anti-collagenase activity. We performed the extraction by maceration and then continued with liquid chromatography combined with high-resolution mass spectrometry (LC-HRMS) to detect and identify the secondary metabolites contained in the extract. The quantitative test of total flavonoid content (TFC) was then carried out to determine the content of flavonoid compounds in the extract. The TFC values in the sample should then give a general idea of the extract's anti-collagenase activity. The test was then followed by a collagenase enzyme inhibitory activity assay by a spectrophotometric assay with a chromogenic substrate, N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA). The collagenase enzyme will break down the FALGPA substrate, reducing the test solution's absorbance over time (Ingale et al., 2018; Rosenblatt et al., 2017).

MATERIALS AND METHODS

Materials

Fresh celery was obtained from Sleman, Yogyakarta. The extraction method used maceration with 70% ethanol as the solvent and VirTis Benchtop Pro 9EG freeze dryer to obtain dry samples. The enzymatic assay was then performed by the Collagenase Activity Colorimetric Assay Kit (MAK293) from Sigma-Aldrich. The kit contains collagenase enzyme from *C. histolyticum* (0.35 U/mL), FALGPA as substrate, 1,10-phenanthroline as positive inhibitor control, and collagenase assay buffer. The sample extract used dimethyl sulfoxide (DMSO) as a solvent from Emsure and aquades from Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. Absorbance values were read with MultiSkan GO (ThermoFisher).

Methods

Preparation and Extraction

The fresh *Apium graveolens* L. was collected from Turi, Sleman, Special Region of Yogyakarta. A determination of samples was conducted at the Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada, with the letter number 32.16.1/UN1/FFA.2/BF/PT/2023. Fresh samples were dried for 24 hours in the oven at 50°C, then grounded to reduce the size of the dried simplicia. The dried plants were then macerated with 70% ethanol for three days and re-macerated for one day (Rahayu et al., 2023). The macerates were evaporated and dried by the freeze-drying method, according to Krakowska-Sieprawska et al. (2022). This process was carried out by VirTis Benchtop Pro 9EG freeze dryer. The condition used was -65 °C and 80 mTorr for 1 day to obtain a dry extract. The yield percentage was calculated by dividing the dry extract by the initial weight of 100 grams of dried *Apium graveolens* L, then multiplied by 100%.

Total Flavonoid Content Assay (TFC)

The TFC was performed by colorimetric method following Farmakope Herbal Indonesia (Kemenkes, 2017). The principle is to mix the sample extract with 10% aluminum chloride P compound and 1 M sodium acetate (Suharyanto and Prima, 2020). The reaction will produce color, which can be detected by Spectrophotometry UV-Vis. We used a double-beam spectrophotometer UH5300 (Hitachi). The absorbance is measured at the maximum wavelength, which has been previously determined. We used quercetin as a standard with the concentration series of 20, 40, 80, 100, and 120 µg/mL. The maximum absorption wavelength, according to Nur et al. (2017), is at a wavelength of 437 nm.

The absorbance results from the concentration series were used to make a regression curve and equation. The linear regression equation can be used to calculate the TFC. The R-value of the equation can be used to check the linearity of the graph. The analytical method is linear if the R^2 value is > 0.995 (de Bievre et al., 1998). The TFC in the samples was calculated by multiplying the concentration of the samples with the volume (5 mL) and dilution factor (df). The multiplication result was then divided by sample weight. TFC was expressed as mgQE/g or milligram quercetin equivalent per gram sample because quercetin was used as the standard.

$$Total\ Flavonoid\ Content\ (mgQE/g) = (C \times V \times df)/W$$

Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS)

The LC-HRMS condition was done according to Artanti *et al.* (2023). The 20 mg sample was extracted with 1 mL of MS-grade methanol and filtered before being injected for LC-HRMS analysis. The sample was analyzed by Liquid chromatography (Thermo Scientific™ Vanquish™ UHPLC Binary Pump) and Orbitrap high-resolution mass spectrometry (Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High-Resolution Mass Spectrometer). An analytical column of Thermo Scientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 µm, and gradient technique of the MS-grade water (Fisher Scientific, USA) containing 0.1% formic acid (Merck, Germany) (A) and MS-grade acetonitrile (Fisher Scientific, USA) containing 0.1% formic acid (B) were used as stationary phase and mobile phases respectively.

Enzymatic Assay

Collagenase enzyme activity inhibition was assessed by a colorimetric method, slightly modified from Thring et al. (2009). We used Sigma-Aldrich Collagenase Activity Colorimetric Assay with catalog number MAK293. First, we prepared the sample by dissolving 10 mg of dried celery extract with 500 µL of dimethyl sulfoxide (DMSO) solvent to obtain a 2×10^4 µg/mL solution concentration. A concentration series of 12.5, 25, 50, 100, and 200 µg/mL was prepared by distilled water.

The assay was performed by pipetting the celery extract, buffer, enzyme, and substrate into a microplate according to Table I. A standard inhibitor was used as a positive control. We also prepared a sample control to observe the effect of sample color on the measurement. All prepared samples were incubated for 10 minutes at room temperature. After incubation, 100 µL of FALGPA substrate was added to each treatment group. Each test treatment was replicated three times.

The absorbance values were then observed by a microplate reader with a constant wavelength of 345 nm for 20 minutes with a 30-second interval immediately after adding the substrate. The collagenase activity was calculated by the following formula:

$$Collagenase\ Activity = \frac{(\frac{-\Delta A_{345\ test}}{\Delta T} - \frac{-\Delta A_{345\ blank}}{\Delta T}) \times 0.2 \times df}{0.53 \times V}$$

ΔA_{345} is the difference between A_2 (absorbance at T_2) and A_1 (absorbance at T_1), ΔT is the time difference between T_2 and T_1, 0.2 is the total volume (µL) of the test, df is the dilution factor (100), the value of 0.53 is the millimolar extinction coefficient of FALGPA, and V is the volume of enzyme used which is 0.01 mL. Furthermore, the percentage inhibition value was calculated by the following formula:

$$\%collagenase\ inhibition = 100\% - (\frac{activity_{inhibitory}}{activity_{enzyme}} \times 100\%)$$

Activity inhibitory was obtained from measuring the enzyme activity with inhibitors, either standard inhibitor or celery extract samples. Meanwhile, activity enzyme is the enzyme activity without any inhibitors, it was obtained from the enzyme control group.

Statistical Analysis

Data analysis was carried out on the results of anti-collagenase activity-percentage in different concentrations. The enzymatic assay was replicated three times for every concentration series. The results were then comparatively tested with a One-Way ANOVA by IBM SPSS 26 with 95% levels of confidence. The ANOVA test is a statistical technique for comparing the means between three or more groups. The means indicated significantly different if $P<0.05$ (Mishra *et al.*, 2019).

RESULTS

***Apium graveolens* L. Extraction with Ethanol 70%**

The extraction from 2 kilograms of wet celery plant samples yielded 210.6 grams of dry samples. Furthermore, 100 grams of dry samples were macerated. The extraction results sample gave a sample yield of 23.02%. The dry *Apium graveolens* L. extract has a dark green color and hydrophilic properties.

Total Flavonoid Content (TFC)

We used 436 nm as the maximum wavelength to read absorbance data, as the quercetin 120 µg/mL showed a maximum wavelength of 436 nm with an absorbance of 0.822. The absorbance data results from reading the standard curve series were then used to calculate the total flavonoid content by the regression equation y = 0.0061 x - 0.0045.

Furthermore, the flavonoid concentration results from the regression equation were calculated with the quercetin equivalence formula. The calculation of mg quercetin equivalents (mgQE/g extract) is shown in Table II. Based on these data, it is known that the average flavonoid content equivalent to quercetin in the celery extract is 5.65 ± 0.16 mgQE / g extract.

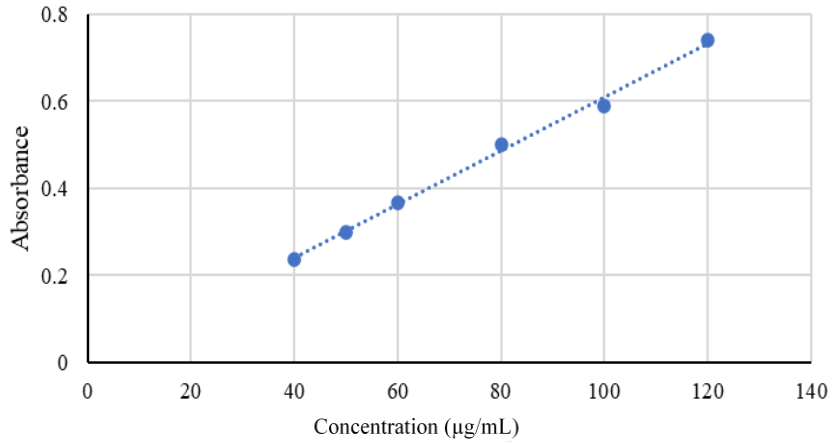

Figure 1. Quercetin standard curve graphs. We performed the quercetin standard curve with a concentration range of 40, 60, 80, 100, and 120 µg/mL. The results showed regression equation for total flavonoid content was y = 0.0061x - 0.0045 with R^2 0.9958.

Table I. Composition for collagenase enzymatic assay

Reagent	Enzyme Control	Standard Inhibitor Control	Sample	Sample Control	Blank
Plant extract	-	-	2 µL	2 µL	-
Standard Inhibitor (1,10-phenanthroline 1M)	-	2 µL	-	-	-
Collagenase	10 µL	10 µL	10 µL	-	-
Buffer	90 µL	88 µL	88 µL	98 µL	100 µL
Incubation (temp./duration)	25˚C/10 minutes				
Substrate FALGPA	100 µL	100 µL	100 µL	100 µL	100 µL
Total Volume	200 µL	200 µL	200 µL	200 µL	200 µL

Table II. Total Flavonoid Content of Celery

	Cons. (µg/mL)	Abs+$AlCl_3$	Abs-$AlCl_3$	Abs.	Flavonoid Cons. (µg/mL)	Quercetin Equiv. (mgQE/g extract)
Celery 1	10120	0.492	0.132	0.36	59.36	5.87
Celery 2	10010	0.479	0.142	0.337	55.62	5.50
Celery 3	10030	0.464	0.121	0.343	56.60	5.59
Average					57.19	5.65
Standard Deviation (SD)					1.94	0.19
Coefficient of Variation (CV)					3.40	3.40

Compounds Analysis of Celery Extract

Compounds present in the celery extract were analyzed by the LC-HRMS technique. According to the LC-HRMS analysis, apigenin, hispidulin, and catechin compounds were detected in celery extract (Figure 1). The apigenin content in celery extract was shown in the abundant level and followed by hispidulin and catechin, respectively. This result is similar to the article by Arsenov et al. (2021), in which catechin levels in *Apium graveolens* L. were significantly lower than apigenin.

Enzymatic Assay

We performed an enzymatic assay to measure the inhibitory activity of the celery extract. The inhibitory activity increased as the concentration of celery extract increased with the highest inhibition being 59.22%. The one-way ANOVA statistical analysis showed no significant difference between the celery extract and the positive control anti-collagenase activity, except for the lowest extract concentration (12.5 µg/mL). The inhibition activity data was then used to calculate the IC_{50} value, and it was found that celery

extract had an IC_{50} value of 132.48 µg/mL. Previous research conducted by Lee *et al.* (2007) reported that apigenin, which is a typical compound of celery plants with an amount of up to 240.2 µg/g extract, shows considerable collagenase enzyme inhibition activity, which reaches 85.3% at a concentration of 500 µM.

DISCUSSION

This study explores the potency of celery extract as an anti-collagenase agent. Celery is already known as an important medicinal plant with rich bioactive compounds. Numerous studies have shown the activity of celery extract, specifically on their flavonoid compounds (Singh *et al.*, 2022; Jia *et al.*, 2020). Farmakope Herbal Indonesia (2017) states that apigenin, a flavonoid compound, is an identity compound of celery.

According to our LC-HRMS result (Figure 2), at least three flavonoid compounds were present in abundant levels in the celery extract. They were apigenin, hispidulin, and catechin. The mechanism of apigenin in inhibiting collagenase enzymes differs for each type of MMP. For MMP-1 molecules, apigenin and glycoside derivatives of apigenin inhibit MMP-1 activity by reducing the expression of MMP-1 molecules by interfering with Ca^{2+}-dependent MAPKs and AP-1 signaling (Lim and Kim, 2007; Hwang et al., 2011). However, for MMP-8 molecules, apigenin and glycoside derivatives of apigenin inhibit MMP-8 activity by downregulating the MMP-8 expression (Juurikka et al., 2019).

Hispidulin is one of the flavonoid compounds contained in celery extract, and it has a derivative glycoside form as well as apigenin and apigenin-7-O-glucoside. The mechanism of hispidulin in inhibiting collagenase is by forming hydrogen bonds with the enzymes and assisted by electrostatic interactions with side chains and zinc ions in the collagenase enzyme (Crascì et al., 2017). Other research conducted by Akram et al. (2015) demonstrated that homoplantaginin or hispidulin-7-O-glucoside, the glycosides derivative of hispidulin, inhibited NO and PGE2 production and COX-2 through heme oxygenase-1 (HO-1) induction via activation of nuclear factor erythroid 2-related factor 2 (Nrf2). This process may affect MMP-1 activity declines (Chaiprasongsuk et al., 2017). Furthermore, hispidulin inhibits the production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which in turn lessens the collagen degradation brought on by their actions (Qu et al., 2009).

Catechin is a flavonoid group compound with several forms of derivative compounds. According to Lee et al. (2020), catechin effectively inhibits collagenase enzymes by reducing their activity and halting the breakdown of collagenase by triggering TNF-α. Furthermore, the catechin-collagen complex may block the active regions in collagen chains, inhibiting the interaction between collagenase and collagen. Thus, the catechin and collagenase competitively interact with collagen. The glycoside derivative of catechin, epigallocatechin gallate (EGCG), also denotes a higher collagenase inhibition than catechin. It is caused by the ability of EGCG to make a stronger hydrogen bond and hydrophobic interactions with collagenase (Madhan et al., 2007; Jackson et al., 2010).

Our results on the collagenase inhibition assay showed that the celery extract inhibition activity has a comparable potency with the standard inhibitor (Fig. 3). It also has a dose-dependent manner effect with the optimum inhibition at 200 µg/mL. Although the lowest extract concentration showed low collagenase inhibition, the higher concentration showed no significant difference from the standard inhibitor. The flavonoid compounds in celery extract have the potential to be anti-aging agents. Flavonoids are known to have antioxidant activity by inhibiting enzymes responsible for producing superoxide anions, such as cyclamine oxidase and protein kinase C. In addition, flavonoids have also been shown to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, and NADH oxidase, which is responsible for forming reactive oxygen in the body (Pietta, 2000). In addition to their antioxidant properties, flavonoids have the potential to inhibit collagenase enzymes with the general mechanism as chelating agents on the active side of the enzyme by binding Zn (II) atoms and Ca (II) atoms contained in MMP molecules, including collagenase enzymes (Mandrone et al., 2018). Generally, the high flavonoid content in plant extracts will align with the high antioxidant activity and collagenase enzyme inhibition.

The collagenase inhibitory activity of the extract occurred because of flavonoid glycosides present in the extract (Xie et al., 2022). Glycoside flavonoid compounds generally have a larger structure than the flavonoid compound alone. Thus, their bioavailability is lower compared to an aglycone structure (Wang and Khalil, 2018). However, the activity of flavonoids with glycoside forms, such as rutinoside glycone, tends to be more active and more resistant to hydrolysis in inhibiting the collagenase enzymes including, MMP-1, MMP-8, and MMP-13 molecules. The activity of flavonoids that have a glycoside chain can form a strong additional hydrogen bond with the collagenase enzyme. It could be involved in

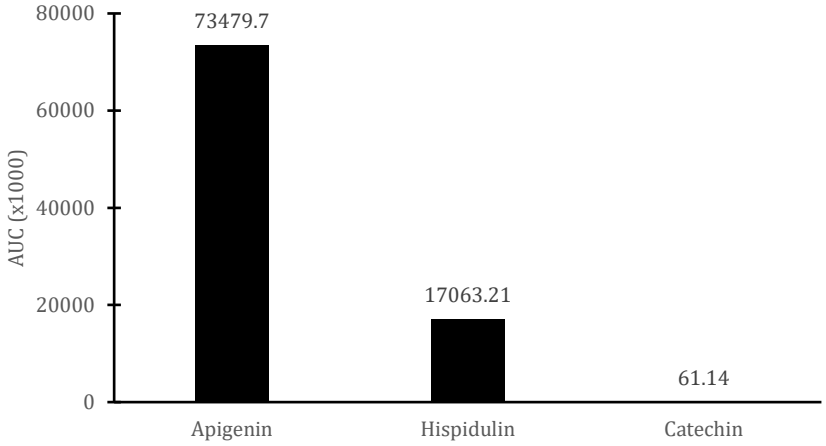

Figure 2. **LC-HRMS results of celery extract.** We performed LC-HRMS to identify the compounds in the celery extract as described in the Methods. We found the presence of apigenin, hispidulin, and catechin compounds in celery extract, as shown in the graph.

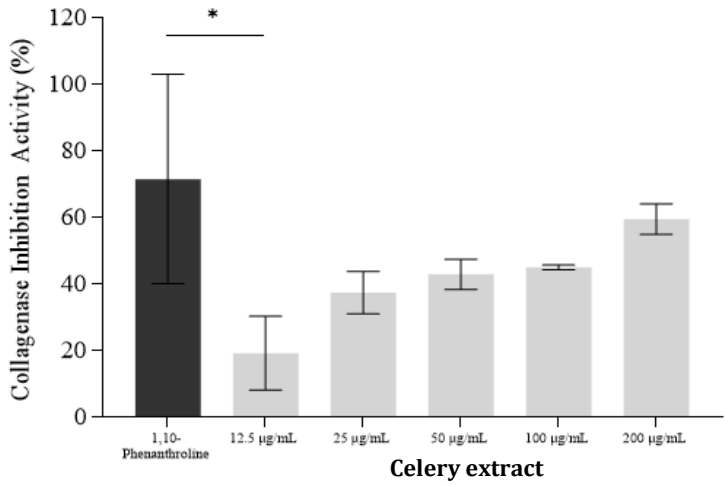

Figure 3. Anti-collagenase activity of celery extract. Collagenase inhibition activity graph of standard inhibitor control (1,10-phenanthroline) and celery extract in a series of concentrations. The data was means of 3 replications. The asterisk (*) indicated that $P< 0.05$.

forming electrostatic interactions with side chains and zinc ions so that they can hold the structure of the compound with the enzyme more strongly (Crascì et al., 2017).

CONCLUSION

The LC-HRMS results showed that apigenin, hispidulin, and catechin can be found in *Apium graveolens* L. The highest compounds are apigenin, followed by hispidulin and catechin. The 200 µg/mL *Apium graveolens* L. extracts can inhibit collagenase enzyme activity until 59.22%, similar to the standard inhibitor, 1,10-phenanthroline. These results indicated that celery extracts have an anti-collagenase activity, and their flavonoids, such as apigenin, hispidulin, and catechin, may contribute to this activity. Therefore, exploring celery activity as a skin anti-aging agent is noteworthy.

ACKNOWLEDGEMENT

We thank Hibah Penelitian Penunjang Skripsi 2023 from the Faculty of Pharmacy, Universitas Gadjah Mada (UGM), for funding this research. Some of the data presented in this study are part of Fahrizal Amanda Permana and Meitha Khansa Khairunnisa's final thesis.

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