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Original Article

Phytocompounds and *in vitro* antiaging activity of ethanolic extract and fractions of *Rubus fraxinifolius* Poir. leaves

[Fitocompuestos y actividad antienvejecimiento *in vitro* de extracto etanólico y fracciones de hojas de *Rubus fraxinifolius* Poir.]

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Abstract

Context: The leading cause of skin sunburn is exposure to ultraviolet radiation (UVR), which can also cause reactive oxygen species (ROS), which accelerates aging. *Rubus fraxinifolius* Poir. leaf ethanolic extract may have antiaging properties by preventing elastase enzyme activity.

Aims: To compare the antioxidant activity and elastase enzyme inhibition from old and young leaves using Soxhlet extraction and evaluate the antioxidant activity and elastase enzyme inhibition utilizing a variety of extraction techniques, including conventional Soxhlet extraction and unconventional methods like microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UAE).

Methods: Using various extraction methods, samples were extracted with 96% ethanol (Soxhlet, MAE, and UAE). The active extract was then transformed into n-hexane, ethyl acetate, and distilled water fraction. Following the total phenolic and total flavonoid content analysis, the antioxidant activity was evaluated using the radical scavenging tests of 1,2-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power assay (FRAP), and ABTS radicals, or 2,2'-azino-bisphenol S (2,2'-ABPS) (3-ethylbenzothiazoline-6-sulphonic acid). The antiaging potential of skin breakdown enzymes was examined using elastase.

Results: According to the *R. fraxinifolius* leaf's optimization type, extract from older leaves performs better than from younger leaves in terms of antioxidant activity and elastase enzyme inhibitory activity. In terms of optimization extraction, UAE approaches to produce the best outcomes than Soxhlet and MAE. Additionally, using the chosen UAE extraction method, multilayer fractionation was done using n-hexane, ethyl acetate, and distilled water solvents, with distilled water fraction producing the best results.

Conclusions: The distilled water fraction from *R. fraxinifolius* leaf's shows the potential for future research to support its efficacy as an active component in antiaging cosmetics.

Keywords: aging; antioxidant; elastase; *Rubus fraxinifolius*.

Resumen

Contexto: La principal causa de quemaduras solares en la piel es la exposición a la radiación ultravioleta (RUV), que también puede causar especies reactivas del oxígeno (ROS), lo que acelera el envejecimiento. El etanol de extracto de hoja de *Rubus fraxinifolius* Poir. puede tener propiedades antienvejecimiento al prevenir la actividad de la enzima elastasa.

Objetivos: Comparar la actividad antioxidante y la inhibición de la enzima elastasa de hojas viejas y jóvenes utilizando la extracción Soxhlet, y evaluó la actividad antioxidante y la inhibición de la enzima elastasa utilizando una variedad de técnicas de extracción, incluyendo la extracción Soxhlet convencional y métodos no convencionales como la extracción asistida por microondas (MAE) y la extracción asistida por ultrasonidos (UAE).

Métodos: Utilizando varios métodos de extracción, las muestras se extrajeron con etanol al 96% (Soxhlet, MAE y UAE). A continuación, el extracto activo se transformó en fracciones de n-hexano, acetato de etilo y agua destilada. Tras el análisis del contenido total de fenoles y flavonoides, se evaluó la actividad antioxidante mediante las pruebas de eliminación de radicales 1,2-difenil-2-picrilhidrazilo (DPPH), ensayo de poder antioxidante férrico reductor (FRAP) y radicales ABTS o 2,2'-azino-bisfenol S (2,2'-ABPS) (ácido 3-etilbenzotiazolina-6-sulfónico). El potencial antienvejecimiento de las enzimas de degradación cutánea se examinó utilizando elastasa.

Resultados: De acuerdo con el tipo de optimización de la hoja de *R. fraxinifolius*, el extracto de las hojas más viejas se comporta mejor que el de las hojas más jóvenes en términos de actividad antioxidante y actividad inhibidora de la enzima elastasa. En términos de extracción de optimización, UAE se acerca a producir los mejores resultados que Soxhlet y MAE. Además, utilizando el método de extracción UAE elegido, se realizó un fraccionamiento multicapa utilizando disolventes n-hexano, acetato de etilo y agua destilada, siendo la fracción de agua destilada la que produjo los mejores resultados.

Conclusiones: La fracción de agua destilada de las hojas de *R. fraxinifolius* muestra potencial para futuras investigaciones que apoyen su eficacia como componente activo en cosméticos antienvejecimiento.

Palabras Clave: antioxidante; elastasa; envejecimiento; *Rubus fraxinifolius*.

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INTRODUCTION

Indonesia is a country with a tropical climate. Air humidity in Indonesia can reach 80% with relatively high temperatures (35°C) and scorching sunlight. This can harm the skin due to direct exposure to UV rays every day, which include skin damage, brown skin, reddish skin, dry skin, sunburn, wrinkled skin, skin irritation, and premature aging (Masaki, 2010). The biological process of premature aging is hugely complex, and in addition to being influenced by specific genetic variables, extrinsic factors like diet, alcohol consumption, smoking, and environmental conditions can also significantly impact appearance (Getoff, 2007).

Excessive exposure to UV rays is one of the extrinsic factors that contribute the greatest influence on the formation of premature aging. Exposure to UV rays causes the formation of free radicals in cells, damaging cell components and causing dry, wrinkled, dull skin, which can lead to aging (Nur et al., 2023a). Molecularly, UV radiation on the skin will trigger free radicals and reactive oxygen species (ROS) with one or more unpaired electrons due to oxidative metabolic reactions. The skin contains lipids, proteins, carbohydrates, and DNA which are the targets of oxidative stress. ROS can induce various processes that play an essential role in skin aging, including damage to membrane proteins. Increased activity of extracellular matrix (ECM) degrading enzymes such as collagenase, elastase, and hyaluronidase are also triggered by ROS when the skin is exposed to UV light (Nur et al,. 2023b). Therefore, compounds that have antioxidant activity significantly play a role in antiaging therapy (Garg et al., 2017; Masaki, 2010).

In developing compounds with antiaging properties, an approach to natural ingredients is needed, assuming that natural ingredients, especially from plants, contain phytoconstituents that allow them to have strong antiaging and antioxidant properties and minimal side effects (Garg et al., 2017). The search for efficacious compounds from natural ingredients is carried out by an ethnobotanical approach empirically used by the community as an antiaging agent. *Rubus fraxinifolius* Poir. (family *Rosaceae*), also known as the strawberry (Das et al., 2022), is one of the many plants used empirically by people in Indonesia in various traditional medicines, including as an antiaging agent. Ethnomedically, the genus *Rubus* is used for antiaging activity due to polyphenols, saponins, terpenoids, polyphenols, and flavonoids. It was scientifically reported that the leaf extract of *R. fraxinifolius* has great potential to slow signs of aging on the skin based on the results of *in vitro* tests that have been carried out by previous studies (Desmiaty et al., 2018). some of the chemical compounds that have been reported include 2-propenoic acid; 2-propenyl esters; furfural; 1,3-butadiene-1-carboxylic acid; 2,6-dihydroxypyridine; 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; 1,1,2-triacetoxyethane; 3-deoxy-d-mannoic lactone (Bakar et al., 2016) ; 2,3-O-ethyleneglycol, 19-hydroxyurs-12-en-23,28-dioic acid and 2,3-O-propanediol,19-hydroxyurs-12-en-28-oic acid may play a role in preventing premature aging (Desmiaty et al., 2021).

Although it scientifically shows that *R. fraxinifolius* extract has reported antiaging activity, conducting a more in-depth study of *R. fraxinifolius* leaves as antiaging for further development is necessary. In this study, several parameters were considered to assess the bioactivity of *R. faxinifolius* leaves as antiaging, including the type of leaf used and optimization of the extraction method used to extract the phytochemical content of *R. fraxinifolius* leaf extract. This study aims to optimize the extraction of compound content from *R. fraxinifolius* leaves so that it can be developed as an active antiaging ingredient in the future. The optimization results made it possible to continue future research by withdrawing pure compounds with antiaging properties, multiplying compounds through synthesis, and continuing with appropriate antiaging formulas.

MATERIAL AND METHODS

Plant material

Old and young leaves of *Rubus fraxinifolius* were used as plant materials. They were taken from Sindangjaya, Cibodas West Java (lat -6.74238°, long 107.006648°), and their identities were verified at Herbarium Bogoriensis, BRIN Cibinong, Indonesia (B-2454/II.6.2/DI.05.07/7/2022).

Chemical and reagents

Chemicals included Trizma base, PPE (porcine pancreas elastase), SANA (N-succinyl-Ala-Ala-Ala-p-nitroanilide), TPTZ (2,4,6-tris(2-pyridyl)-triazine), and Folin Ciocalteu's phenolic reagent from Sigma Aldrich, Germany; DPPH (2,2-diphenyl-1-picrylhydrazyl) from TCI, Japan; quercetin, ascorbic acid, gallic acid, potassium dihydrogen phosphate, sodium hydroxide, aluminum chloride, sodium acetate, iron (III) chloride hexahydrate, ethanol pa, methanol pa, and iron (II) sulfate heptahydrate from Merck, Germany; water pro injection (Pharmaceutical Laboratories, Indonesia).

Phytochemical screening

The leaf extract was examined for alkaloids, tannins, flavonoids, saponins, and steroid-triterpenoid substances using preliminary and conclusive tests. Alkaloids were found using Mayer's and Dragendorff's tests, tannins were found using the ferric chloride test, flavonoids were found using aluminum chloride spotting in UV 366 nm, saponins were found using the formation of frothing, and steroid-triterpenoid was found using the Liebermann-test Burchard's (Cane et al., 2023).

Microscopy

A light microscope (Nikon Eclipse E-200, Nikon, Japan) with magnification objectives at 40× was used to examine the microscopy of *R. fraxinifolius* leaves (both powder and fresh leaves) to see the identifier fragment, a specific part used to identify the plant. Field-Emission Scanning Electron Microscopes (SEM) (FE SEM FEI Inspect F50, FEI, Czech Republic) were used to scan the material's surface, including topography and morphology. The material surface was identified to determine the degree of surface damage caused by different extraction techniques (Mutalib et al., 2017).

Extraction

The extraction process for young and old leaf samples used three methods, i.e., Soxhlet, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE) method. The extraction process aims to optimize the best extraction process and provide the strongest bioactivity. A 96% ethanol was used for Soxhlet extraction with a total solvent of 450 mL from 30 g of leaf powder (the ratio of leaf powder and the solvent used was 1:15 based on the optimization results of previous research conducted by Desmiaty et al. (2020). UAE method was carried out by adding 150 mL of solvent to 15 g of leaf powder (the ratio of leaf powder and the solvent used was 1:10). Then extracted for 30 min using an ultrasonic bath with a frequency of 40 kHz and a temperature of 40°C. While MAE (microwave-assisted extraction) method is carried out by adding 150 mL of solvent to 15 g of leaf powder (the ratio of leaf powder and the solvent used was 1:10). Then extracted using a microwave at power ± 240 Watt for 6 min (Altemimi et al., 2017).

A rotary vacuum evaporator (Buchi R-300, Buchi Labortechnik AG, Switzerland) was used to remove the solvent after each extract was filtered to collect the supernatant (temperature: 50°C, rotational speed: 40 rpm). The viscous extract was then evaporated with a water bath at 40°C until the solvent disappeared, then the yield was calculated and stored at 4°C in the refrigerator until the next test.

Fractionation

A separating funnel was used to extract the active component of *R. fraxinifolius* leaves through fractionation (liquid-liquid partition). The ratio of extract to solvent was 1: 10, and the solvents were non-polar (n-hexane), semipolar (ethyl acetate), and polar (distilled water) solvents. The liquid fractions of n-hexane, ethyl acetate, and water (residue) were evaporated to create the dry fraction and assess its activity (Ibrahim, 2019).

Determination of total phenolic compounds

Determination of the total phenolic content of *R. fraxinifolius* leaf extract was carried out colorimetrically using the Folin-Ciocalteu reagent according to previous studies with slight modifications (Wiliantari et al., 2022). A total of 5 mL of Folin-Ciocalteu LP solution (7.5% in water) was put into a 10 mL volumetric flask containing the sample solution (1 mL each) and then incubated at room temperature for 8 min. 4 mL of 1% NaOH was added to the mixture, then incubated at room temperature for one hour, protected from light. The absorbance of each sample solution was measured at a wavelength of 730 nm (UV/Vis Spectrometer T80+, PG Instrument Ltd, United Kingdom). Gallic acid was used as a standard with serial concentrations to obtain a standard gallic acid curve ($y = 0.0175x - 0.0794$ with $R^2 = 0.998$). The total phenolic content of each sample was calculated based on the gallic acid standard curve equation with replication (n = 3). The total phenolic content of each sample was expressed as gallic acid equivalents per gram of dry extract (mg GAE/g dry extract).

Determination of total flavonoids

The total flavonoid content of each extract was determined based on previous studies with slight modifications (Lukitaningsih et al., 2020; Wiliantari et al., 2022). A total of 1 mL of each sample solution was put into a 10 mL volumetric flask and reacted with 3 mL of ethanol P, 5.6 mL of distilled water, 0.2 mL of 10% aluminum chloride, and 0.2 mL of 1 M sodium acetate. The mixtures were shaken, incubated at room temperature, and protected from light for 30 min. Absorption was measured using a UV-Vis spectrophotometer (UV/Vis Spectrometer T80+, PG Instrument Ltd, UK) at a maximum wavelength of 438 nm. The test was carried out three times. Standard quercetin with serial concentrations was used as a standard curve, and the equation $y = 0.0081x + 0.0606$ with $R^2 = 0.998$ was obtained. The total flavonoid content was calculated using the standard curve equation and

expressed as quercetin equivalents per gram of dry extract (mg QE/g dry extract).

Antioxidant activity

Three separate tests 1,2-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used to measure antioxidant capacity.

DPPH method

Testing the DPPH radical inhibitory activity in each extract solution was carried out based on the procedure of Desmiaty et al. (2018) and Nur et al. (2019). A 0.3 mM DPPH radical solution was prepared by dissolving 2.957 mg of DPPH powder in methanol p.a solvent up to 25 mL. Each extract solution was prepared by weighing 50 mg of extract and dissolved with methanol p.a to 50 mL (1000 µg/mL). The scavenging of DPPH radicals by each sample was carried out by taking a certain serial volume of sample solution and reacting it with 1 mL of DPPH radical solution in a 5 mL volumetric flask. The mixture was made up to 5 mL with methanol p.a. Each sample mixture was incubated for 30 min at room temperature and in the dark. The absorbance of each sample solution was measured at 516 nm with a UV-Vis spectrophotometer (UV/Vis Spectrometer T80+, PG Instrument Ltd, UK). Quercetin was used as a positive control in this study. Both sample and positive control were triplicated (n = 3). The antioxidant strength of each sample solution is expressed as a value of 50% inhibitor concentration (IC_{50}). Each sample's percentage inhibition and IC_{50} values were calculated based on equations 1 and 2.

$$\%\ Inhibition = \frac{(control\ absorbance - sampel\ absorbance)}{control\ absorbance}\ x\ 100\% \quad [1]$$

Then, a linear regression curve is constructed with x representing the concentration (g/mL) and y representing the percentage of inhibition (%) to derive the equation y = a + bx. It was done after collecting the percentage of inhibition from each concentration. Equation [2] provides the formula for calculating the IC_{50} value.

$$IC50 = \frac{(50 - a)}{b} \quad [2]$$

FRAP method

Antioxidant activity in reducing iron from *Rubus fraxinifolius* extract was carried out using the Ferric Reducing Antioxidant Capacity (FRAP) method. A total of 50 µL of each sample solution was reacted with 150 µL of FRAP reagent consisting of acetate buffer pH 3.6, TPTZ (10 mM in hydrochloric acid), and $FeCl_3$ (20 mM in distilled water) with a ratio of 10:1:1 in a 96-well plate. The mixture was incubated for 8 min at 37°C. The mixture was shaken for 30 s, and the absorption was measured at a wavelength of 590 nM using a microplate reader (Biotek 800 TS Absorbance Reader, Germany). The $FeSO_4$ compound was used as a standard, which was prepared using serial concentrations to obtain a standard curve equation (y = 0.0176x – 0.1176, with R^2 = 0.995) with triplicate. The magnitude of the antioxidant capacity in reducing iron from each sample solution was expressed as the FRAP value equivalent to $FeSO_4$ [equations 3 and 4].

$$FRAP\ Value\ (\frac{\mu mol}{g\ sample}) = \frac{C\ x\ V\ x\ Fp}{m} \quad [3]$$

where C is the concentration of standard Fe_2SO_4 $7H_2O$, which is equivalent to the sample (µmol/mL), is obtained by entering the net absorbance value of the sample into the calibration curve equation. V is the sample volume (mL), Fp is the dilution factor, and m is the sample weight (g).

The antioxidant activity of the sample expressed as g of Fe_2SO_4 equivalent per 100 g of extract, could be calculated using equation [4].

$$AA\ (\%) = FRAP\ value \times 10^{-6} \times MW\ Fe_2SO_4.7H_2O \times 100 \quad [4]$$

where AA: antioxidant activity, MW: molecular weight.

ABTS method

Testing the activity of inhibiting ABTS radicals by the extract solution was carried out colorimetrically using a UV-Vis spectrophotometer at a wavelength of 752 nm (Nur et al., 2021a). ABTS radical solution was prepared by reacting 25 mL of ABTS solution (7.0 mM in distilled water) with potassium persulfate solution (2.45 mM in distilled water) in a tightly closed dark container. The mixture was incubated for 16-20 h in a dark place at room temperature. The mixture was diluted to 100 mL with ethanol p.a. For analysis needs, a stock solution of ABTS: ethanol p.a was prepared with a ratio of 1:10. A total of 1 mL of ABTS radical solution was reacted with 1 mL of each sample solution series (Soxhlet extract (2-10 µg/mL), UAE or MAE extract (2–6 µg/mL), n-hexane fraction (15–35 µg/mL), ethyl acetate fraction (10–20 µg/mL), and water distillation fraction (1–6 µg/mL)) and made up to 5 mL with ethanol p.a. The mixture was incubated in a dark place at room temperature for 30 min. After the incubation period, the absorbance of each sample solution was measured with a UV-Visible spectrophotometer (UV/Vis Spectrometer T80+, PG Instrument Ltd, United Kingdom). Quercetin was used as a positive control in this study. Both sample and positive control were triplicated (n = 3). Each sample solu-

tion's percentage inhibition and IC_{50} value were calculated based on the formulas in equations [1] and [2].

Antielastase activity

The antiaging activity of *R. fraxinifolius* extract was determined based on the parameters of elastase inhibition by enzymatic colorimetry. The formation of a yellow color marked the reaction between elastase and SANA (N-succinyl-Ala-Ala-Ala-p-nitroanilide), and its absorption was observed at a wavelength of 405 nm using a microplate reader (Glomax GM 3000, Promega, USA). Elastase inhibitory activity by *R. fraxinifolius* extract samples was carried out by reacting 30 µL of extract and fraction solutions (10-1000 µg/mL) with 15 µL of elastase (4 units/mL) in a 96-well plate containing tris-HCl buffer solution pH 8.0 and then incubated for 10 min at 25°C. The mixture was added with 30 µL of substrate solution (1.3 mM SANA) and re-incubated at 25°C for 50 minutes using a Memmert IN55 incubator. After the incubation period, the absorbance of the mixture was measured at 405 nm using a microplate reader. Elastase inhibitory activity by extract and fraction samples was expressed as IC_{50} value (Desmiaty et al., 2020; Oh et al., 2020). Quercetin was used as a positive control in this study. Both sample and positive control were triplicated (n = 3). Each sample solution's percentage inhibition and IC_{50} value were calculated based on the formulas in equations [1] and [2].

LC-MS-MS test

The most active samples were examined for active compounds using a high-performance liquid chromatography (UPLC) system made up of LC (ACQUITY UPLC® H-Class System, Waters, USA) and a mass spectrometer (Xevo G2-S QTof, Waters, USA). The LC analysis was carried out using mobile phases of water + 5 mM ammonium formic (A) and acetonitrile + 0.05% formic acid with a flow rate of 0.2 mL/min (step gradient), running for 23 min (see slide moving phase), and an injection volume of 5 L (initially filtered through a 0.2 m syringe filter) (B). Electrospray ionization (ESI) in positive mode was used to carry out the mass spectrometry (MS) analysis (Ismed et al., 2022). Data collection and analysis were also performed using Masslynx software version 4.1.

Statistical analysis

The results of the bioactivity test for each sample were analyzed using the Microsoft Excel 12 version and were produced in mean ± SD form. The significance of each sample was analyzed through an independent T-test and LSD one-way ANOVA using SPSS 23 Version with significant differences $p<0.05$. Data correlation between TPC and TFC with antioxidant and anti-elastase activity were analyzed using Pearson correlation by Minitab 20 version.

RESULTS

Phytochemical screening

The secondary metabolites of the *R. fraxinifolius* extract were identified through phytochemical screening analysis, and the chemical components can now be employed in a variety of ways. The test results showed on the Table 1, that ethanol extract of *R. fraxinifolius* leaves from the active extraction method (UAE) contained alkaloids, flavonoids, hydrolyzed tannins, saponins, and triterpenoids. These screening's findings are consistent with earlier study by Desmiaty et al. (2020), which demonstrated that *R. fraxinifolius* contains flavonoids and triterpenoid.

Microscopy

The microscopic results of *R. fraxinifolius* leaf powder typically shows thickening of the cuticle, three layers of palisades, and a simple hair covering. Detailed microscopic findings can be seen in Fig. 1.

R. fraxinifolius fresh leaf microscopic also show thickening of the cuticle, three layers of palisades, and a simple hair covering. Fig. 2 displays specific microscopic results.

Table 1. Phytochemical test results in UAE extract.

Parameter	Reagent	Observation	Results
Alkaloid	Dragendorff	Brown orange precipitate is formed	Positive (+)
	Mayer	White precipitate is formed, which is dissolved in methanol	Positive (+)
Flavonoid	$AlCl_3$	Yellow fluorescence is visible at 366 nm UV	Positive (+)
Hydrolyzed tannin	$FeCl_3$	Blue black color is formed	Positive (+)
Saponin	Hot water	1-2 cm foam is formed and persists after adding HCl	Positive (+)
Triterpenoid	Liebermann-Burchard	Blue green color is formed	Positive (+)

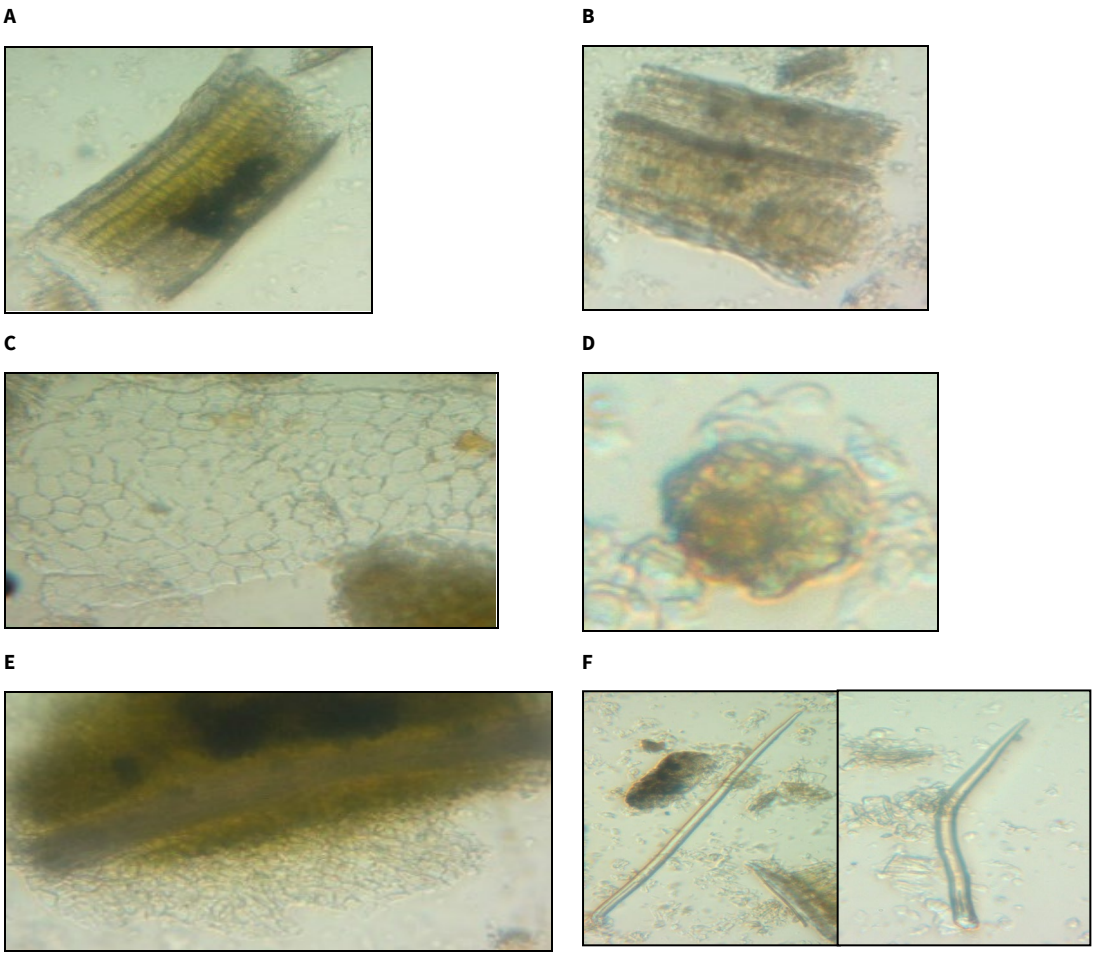


Figure 1. Microscopy of *R. fraxinifolius* leaf powder.
(A) Palisade (3 layers); **(B)** Calcium oxalate in palisade; **(C)** Upper epidermis; **(D)** Glandular hair; **(E)** Epidermis and stomata; **(F)** Hair cover.

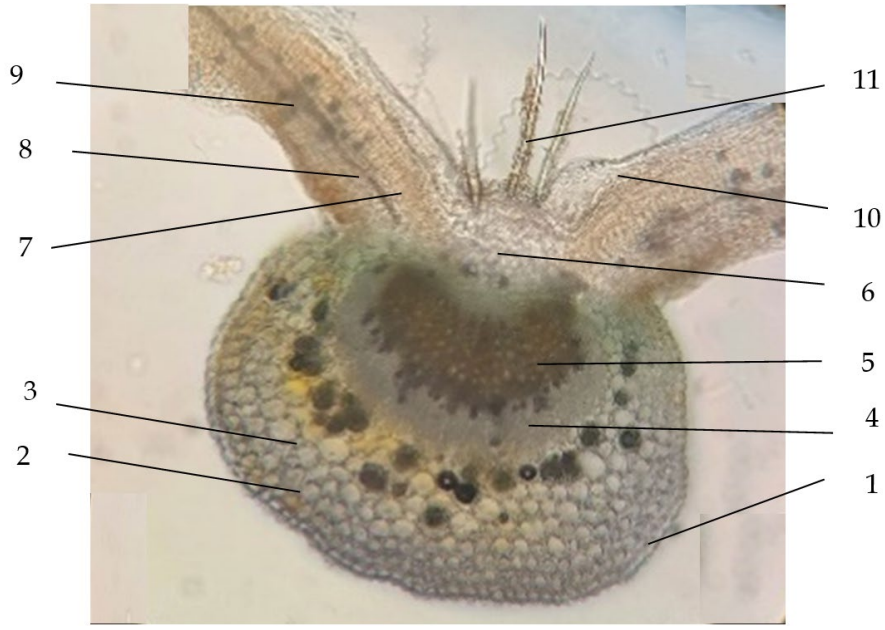


Figure 2. Microscopy of *R. fraxinifolius* fresh leaf.
(1) Lower epidermis; **(2)** Chollenchyma; **(3)** Parenchyma; **(4)** Floem; **(5)** Cylem; **(6)** Sponge parenchyma; **(7)** Palisade (3 ply); **(8)** Vascular tissue; **(9)** Calcium oxalate crystals; **(10)** Cuticle; **(11)** Hair cover.

Scanning Electron Microscopy (SEM)

The extraction results showed that the yield of the UAE method was higher than other methods (UAE 27.36%, Soxhlet 21.83%, MAE 19.94%). This is correlated with the SEM results in Fig. 3. UAE extraction method shows a more hollow cell wall surface than other extraction methods' cell walls (Soxhlet and MAE). These results indicate that the UAE extraction process has an impact on increasing the material's surface area, accelerating the solvent's penetration into the material to be extracted.

Yield result

The leaf types were selected to determine the best type of leaf with the strongest antioxidant and anti-elastase activity, whether from old or young leaves. Extraction was performed using the selected Soxhlet method based on previous research by Desmiaty et al. (2020) with same conditions. Old and young leaf yield extraction were obtained at 21.83% and 22.95%, respectively.

The most active leaf species were then varied in their extraction methods to determine which extraction method was most effective in providing the strongest antioxidant and elastase activity values. The extraction method used is the Soxhlet, UAE, and MAE methods. The extraction method can affect the type and number of chemical compounds extracted from a sample. The Soxhlet extraction method was chosen because it was based on previous research by Desmiaty (2020), which compares conventional extraction using Soxhlet, maceration, and reflux. The Soxhlet method reported giving the best results in antioxidant activity and elastase inhibition on Soxhlet methods. This study also uses a few extraction methods, including UAE and MAE. UAE was selected because of less time and less energy requirement. The principle of UAE extraction uses ultrasonic waves to induce physical forces created by acoustic cavitation to rupture plant tissue. It speeds up the release of extractable components into the solvent by improving mass transport (Kumar et al., 2021). MAE was chosen because it has many advantages, such as shorter extraction time, less solvent, higher extraction rates, and lower costs than conventional methods. Microwave radiation speeds up the process of residual water evaporation in raw materials and eventually weakens plant cell walls to facilitate extraction by internal diffusion (Delazar et al., 2012).

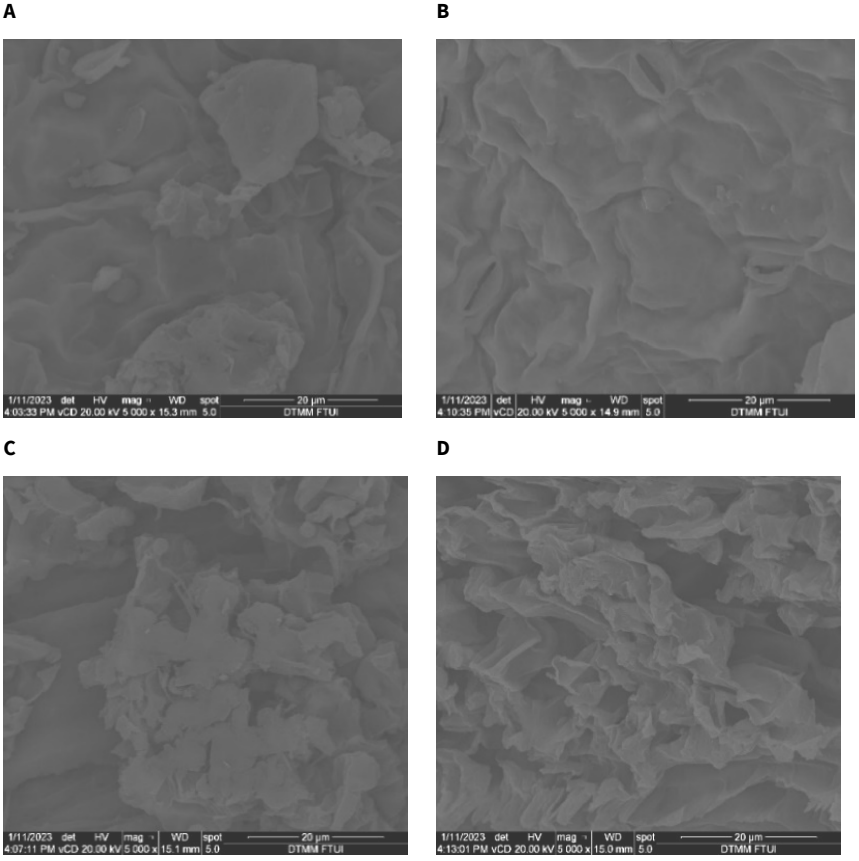


Figure 3. SEM of *R. fraxinifolius* leaf powder from various methods extraction.
(A) Powder; **(B)** Microwave-assisted extraction (MAE); **(C)** Soxhlet; **(D)** Ultrasound-assisted extraction (UAE).

The extraction results showed that the UAE yield was higher (27.36%) than other methods (Soxhlet 21.83%, MAE 19.94%). The extracted content obtained from the UAE method is higher because the cavitation effect on UAE will break down the cell wall mechanically and increase material transfer to extract more target compounds (Zhang et al., 2018).

Selected extracts with the strongest antioxidant and elastase inhibitory properties were then fractionated using n-hexane, ethyl acetate, and water. The fractionation results showed that the water fraction had the highest yield (18.79%) compared to n-hexane (10.01%) and ethyl acetate (13.83%). This shows that the concentration of dissolved chemicals is relatively high in the water (polar) portion and relatively low in n-hexane (non-polar compound) and ethyl acetate (semi-polar compound).

Total phenolic and total flavonoid compounds

Determination of the total phenol content was carried out using the colorimetric method with the Folin-Ciocalteu reagent, and the results are expressed as gallic acid equivalents per gram of dry extract (mg GAE/g dry extract. Polyphenols from plant extracts react specifically with the Folin-Ciocalteu reagent to produce a blue-colored complex detected by UV-VIS spectrophotometry. At the same time, the total content of flavonoids using the $AlCl_3$ colorimetric technique with quercetin as a standard and expressed quercetin equivalents per gram of dry extract (mg QE/g dry extract). As one of the best flavonoid compounds for scavenging free radicals (hydroxyl radicals, superoxide, and peroxyl radicals), quercetin is used as a standard (comparison) solution because it can produce phenolic radicals, which are stabilized by the action of aromatic ring resonance. This compound also inhibits various oxidation reactions (Rocabado et al., 2008; Blainski et al., 2013).

Phenolic compounds and flavonoids have substantial antioxidant effects. Phenolics function as chain bond breakers and free radical acceptors. Phenolics interfere with the rapid oxidation of lipids and other molecules by donating hydrogen atoms to radicals. Meanwhile, flavonoids can capture or bind free radicals in the environment because they contain hydroxyl groups which can become hydrogen donors for free radicals (Nur et al., 2021b).

Based on the results of leaf type optimization tests, old leaves had the highest total polyphenol content significantly (230.623 ± 0.002 mg GAE/g extract) at the same extract concentration of 100 ppm. This follows the research of Pratama et al. (2021), which reported that the phenolic content increased with growing maturity. Plants produce secondary and bioactive metabolites in varying amounts during the growth period, which is controlled by leaf morphology and age (Pratama et al., 2021). Progressive leaf morphology and age will affect the secondary and bioactive metabolites produced, thus affecting the total flavonoid content in the leaves, similar to the effect of total phenol levels (Bait et al., 2022). In this study, the results showed that young leaves had a higher flavonoid content than old leaves. Young leaf extract had the highest amount of flavonoids (9.452 ± 0.001 mg/g extract) for the total flavonoid content at the same extract concentration of 3000 ppm. Table 2 shows the results of total phenol and total flavonoid data.

Table 2. Total phenol and total flavonoid content from leaf type optimization.

Sample	Total phenols (mgGAE/g dry extract)	Total flavonoids (mg QE/g dry extract)
Old leaves	230.623 ± 0.002^{b}	7.736 ± 0.002^{b}
Young leaves	220.733 ± 0.002^{a}	9.452 ± 0.001^{a}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b}indicates significant difference between young leaves versus old leaves with t-test analysis ($p<0.05$, $n = 3$).

In optimizing the extraction method, based on the test results at the same extract concentration of 100 ppm, it is known that the MAE extraction method has the lowest total polyphenol content significantly (180.090 ± 0.001 mg GAE/g dry extract). In contrast, the UAE extraction has the highest total polyphenol content (277.326 ± 0.001 mg GAE/g dry extract). The highest levels of flavonoids were significantly obtained using the MAE extraction method (17.728 ± 0.040 mg QE/g dry extract), and the lowest levels were obtained using the Soxhlet extraction method (7.736 ± 0.084 QE/g dry extract) for the total flavonoid content at the same extract concentration, i.e., 3000 ppm. Table 3 shows the results of total phenol and total flavonoid data.

Table 3. Total phenol and total flavonoid content from extraction methods optimization.

Sample	Total phenol (mg GAE/ g dry extract)	Total flavonoid (mg QE/g dry extract)
Soxhlet	230.623 ± 0.002^{bc}	7.736 ± 0.084^{bc}
UAE	277.326 ± 0.001^{ac}	12.590 ± 0.093^{ac}
MAE	180.090 ± 0.001^{ab}	17.728 ± 0.040^{ab}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c}indicates significant difference between extraction methods with Tukey analysis ($p<0.05$, $n=3$).

The results of extraction method were confirmed to have a significant impact on the efficiency of bound phenolic release. The amount of bound phenolic released by the UEA extraction method gives a higher

Table 4. Antioxidant capacity from leaf type optimization.

Sample	IC_{50} value (µg/mL)		FRAP assay (Fe_2SO_4/100 g)
	DPPH assay	ABTS assay	
Old Leaves	14.02 ± 0.148^{bc}	5.02 ± 0.217^{bc}	101.159 ± 0.018^{c}
Young Leaves	14.17 ± 0.048^{ac}	5.11 ± 0.090^{ac}	99.875 ± 0.067^{c}
Reference	2.29 ± 0.148^{ab}	0.89 ± 1.064^{ab}	377.91 ± 6.190^{ab}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c}indicates significant difference with Tukey analysis ($p<0.05$, n=3). For the DPPH test ascorbic acid used as reference, FRAP assay used ascorbic acid as reference, while the ABTS test used ascorbic acid used as reference

phenol content. This is in line with a study by Jovanovic et al. (2017), who assessed the effectiveness of polyphenol extraction using several extraction techniques and found that the UEA approach produced the largest total phenolic concentration.

Antioxidant activity

Three different antioxidant assay methods were used to measure the antioxidant capacity of the samples, namely 1,2-diphenyl-2-picrylhydrazyl (DPPH), iron-reducing antioxidant power (FRAP), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

DPPH free radical staining is caused by electron donation from antioxidants, which can neutralize free radical molecules. Unstable radicals (because they lack electron pairs) will turn purple and light yellow because they receive electron donations from antioxidants (Xiao et al., 2020). A linear regression curve between the ratio of sample concentration and DPPH radical scavenging activity (%) was plotted to calculate the inhibitory concentration value (IC_{50}).

FRAP is a method that shows good reproducibility. The ability of antioxidants to reduce ferric iron was calculated using this method. The principle of the FRAP method is a reduction reaction in an acidic medium to form a yellow Fe^{3+} complex compound to a greenish-blue Fe^{2+} complex due to the presence of electron donors from antioxidants. The amount of reduced iron can be determined by measuring the absorbance at 593 nm, which is also associated with antioxidants (Xiao et al., 2020).

In the ABTS method, the test is based on the ability of antioxidants to inhibit the $ABTS^{\bullet+}$ radical, which has a blue-green chromophore and changes to ABTS to produce decolorization. To optimize leaf types, the antioxidant activity of *Rubus* leaf extract resulted in very high IC_{50} values for both old and young leaf extracts. Table 4 shows the information on the results of antioxidant activity, where the older leaf extracts showed stronger antioxidant activity than the younger leaves. Old leaf extract was significantly different from young leaves (IC_{50} 14.02 ± 0.148 µg/mL) using the DPPH and using ABTS methods (IC_{50} 5.02 ± 0.217 µg/mL), and old leaf extract had the highest FRAP value (101.159 ± 0.018 µg/mL). This happens because the level of maturity strongly influences the antioxidant activity of leaves. The more developed the leaves, the higher antioxidant activity resulting from modifications in biochemistry, physiology, and structural elements that occur during leaf growth (Bait et al., 2022). This study shows that leaf age can affect antioxidant activity.

The DPPH method was used to assess antioxidant levels to optimize the extraction process. The results of UAE extraction method providing the significantly antioxidant activity compared to the Soxhlet and MAE extraction methods, with IC_{50} values in the very strong category (7.54 ± 0.115 µg/mL). Results of ABTS method provided the significantly antioxidant activity for UAE extraction compared to the Soxhlet and MAE extraction methods, with IC_{50} values very strong category (2.89 ± 0.131 µg/mL). FRAP method results in UAE providing significantly antioxidant activity compared to the Soxhlet and MAE extraction methods at the same extract concentration of 6 ppm (IC_{50} 192.877 ± 0.003 µg/mL). The antioxidant activity of *R. fraxiniofolius* leaf extract by UAE extraction gives a higher IC_{50} value than MAE and Soxhlet using the DPPH, ABTS, and FRAP methods. Table 5 shows antioxidant activity results on extraction methods optimization. This suggests that cell injury can increase a compound's solubility in solvents and boost extraction yields. The SEM results of UAE extraction, which exhibits more cell damage than MAE and Soxhlet, demonstrate these findings.

The strongest extract was then fractionated to separate the active ingredients in the extract depending on their polarity. As a result of fractionation, active chemicals are produced that are more specific or less complex than in the form of crude extracts. Based on the antioxidant activity test results, the water fraction provided the highest antioxidant activity significantly compared to the other fractions, with an IC_{50} value in

Table 5. Antioxidant capacity from extraction methods optimization.

Sample	IC_{50} value (µg/mL)		FRAP assay (Fe_2SO_4/100 g)
	DPPH assay	ABTS assay	
Soxhlet	14.02 ± 0.148^{bcd}	5.02 ± 0.217^{bcd}	101.159 ± 0.018^{bcd}
UAE	7.54 ± 0.115^{acd}	2.89 ± 0.131^{acd}	192.877 ± 0.003^{acd}
MAE	8.67 ± 0.118^{abd}	3.51 ± 0.142^{abd}	162.911 ± 0.006^{abd}
Reference	2.29 ± 0.148^{abc}	0.89 ± 1.064^{abc}	377.91 ± 6.190^{abc}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c}indicates significant difference with Tukey analysis ($p<0.05$, n=3). For the DPPH test ascorbic acid used as reference, FRAP assay used ascorbic acid as reference, while the ABTS test used ascorbic acid used as reference

Table 6. Antioxidant capacity of fraction.

Sample	IC_{50} value (µg/mL)		FRAP assay (Fe_2SO_4/100 g)
	DPPH assay	ABTS assay	
n-Hexane	65.59 ± 0.221^{bcd}	24.94 ± 0.181^{bcd}	23.020 ± 0.003^{bcd}
Ethyl acetate	9.29 ± 0.188^{acd}	15.98 ± 0.124^{acd}	88.755 ± 0.002^{acd}
Distilled water	7.80 ± 0.204^{abd}	3.99 ± 0.132^{abd}	103.908 ± 0.026^{abd}
Reference	2.29 ± 0.148^{abc}	0.89 ± 1.064^{abc}	377.91 ± 6.190^{abc}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c,d}indicates significant difference with Tukey analysis ($p<0.05$, n = 3). For the DPPH test, used concentration n-hexane fraction (30–90 µg/mL); ethyl acetate fraction (2–10 µg/mL); water distillation fraction (2–10 µg/mL); and ascorbic acid used as reference (0.5–2.5 µg/mL). FRAP assay used a concentration of 12.5 µg/mL for each fraction and ascorbic acid used as reference (3 µg/mL). While the ABTS test used concentration n-hexane fraction (15–35 µg/mL); ethyl acetate fraction (10–20 µg/mL); water distillation fraction (1–6 µg/mL), and ascorbic acid used as reference (0.5–2.5 µg/mL).

Table 7. Inhibition activities (%) of extract *R. fraxinifolius* against elastase (leaf type optimization).

Sample	Extract concentration (µg/mL)				IC_{50} value (µg/mL)
	150	75	37.5	18.75	
Old leaves	67.73 ± 0.79	52.67 ± 1.24	38.83 ± 2.31	38.56 ± 4.16	71.34 ± 3.14^{bc}
Young leaves	30.65 ± 3.67	21.08 ± 1.57	7.99 ± 2.60	7.52 ± 2.82	249.95 ± 3.32^{ac}
Quercetin	-	-	83.69 ± 1.61	72.31 ± 5.42	6.18 ± 4.6^{ab}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c}indicates significant difference with Tukey analysis ($p<0.05$, n = 3). Quercetin was used as a positive control with concentration 1.17, 2.34, 4.69, 9.38, 18.75, and 37.5 µg/ mL.

the very strong category (7.80 ± 0.204 µg/mL). It is possible that polar active substances with antioxidant capabilities, such as flavonoids, terpenoids, phenolics, flavonoid glycosides, and saponins, are present in the water fraction.

Antioxidant levels in fraction were evaluated using the DPPH, ABTS, and FRAP method with detailed data can be seen in Table 6.

Following the antioxidant test results of the ABTS method, the water fraction has an IC_{50} value very strong category (3.99 ± 0.132 µg/mL) antioxidant activity when compared to the other fractions. As with the FRAP method, it showed that water fraction had the highest antioxidant activity when compared to the other fractions (103.908 ± 0.026 µg/mL).

Antielastase activity

Elastase activity can be inhibited in the presence of polyphenols in plants, according to previous research from Brás et al. (2010). Kinetic parameters were also calculated, confirming that the inhibition was reversible and competitive. To denature enzymes and eliminate their catalytic activity, polyphenols can bind to enzymes through hydrogen interactions between the hydroxy groups and their amino acid side chains (Brás et al., 2010). Triterpenoids and polyphenols in *R. fraxiniofolius*, important substances in preventing elastase activity, cause elastase inhibitory activity (Desmiaty et al., 2020).

According to the study's leaf type optimization on the Table 7, an extract of *R. fraxiniofolius* older leaves had a significantly higher level of elastase inhibition

Table 8. Inhibition activities (%) of extract *Rubus fraxinifolius* against elastase (extraction method optimization).

Sample	Extract concentration (µg/mL)				IC_{50} value (µg/mL)
	67.5	90	112.5	135	
Soxhlet	49.12 ± 4.16	54.25 ± 2.31	59.38 ± 1.24	64.51 ± 0.79	71.34 ± 3.14^{bd}
MAE	31.84 ± 4.26	47.02 ± 4.21	61.14 ± 1.21	76.93 ± 1.94	95.15 ± 4.02^{ad}
UAE	48.38 ± 3.99	57.72 ± 2.19	70.67 ± 1.73	85.44 ± 4.07	75.82 ± 4.19^{d}
Quercetin	-	-	-	-	6.18 ± 4.63^{abc}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c,d}indicates significant difference with Tukey analysis ($p<0.05$, n = 3). Quercetin was used as a positive control with concentrations 1.17, 2.34, 4.69, 9.38, 18.75, and 37.5 µg/ mL.

Table 9. Inhibition activities (%) of extract *Rubus fraxinifolius* against elastase (fractionation).

Sample	Extract concentration (µg/mL)				IC_{50} value (µg/mL)
	67.5	90	112.5	135	
n-Hexane	2.55 ± 1.16	4.78 ± 1.96	7.09 ± 1.34	8.07 ± 0.94	642.82 ± 1.23^{bcd}
Ethyl acetate	30.06 ± 1.00	33.34 ± 1.85	35.46 ± 0.43	38.71± 1.01	191.74 ± 1.14^{a}
Distilled water	42.63 ± 0.17	47.81 ± 1.81	54.44 ± 0.27	58.86 ± 0.18	100.85 ± 1.07^{a}
Quercetin	-	-	-	-	6.18 ± 4.63^{a}

All data was shown as the mean ± SD in which each treatment was repeated for three times. ^{a,b,c,d}indicates significant difference with Tukey analysis ($p<0.05$, n = 3). Quercetin was used as a positive control with concentrations 1.17, 2.34, 4.69, 9.38, 18.75, and 37.5 µg/ mL.

than its younger leaves, with an IC_{50} value of 71.34 ± 3.14 µg/mL.

Optimization of the extraction method gave the enzyme test results as listed in Table 8. The UAE extract from *R. fraxinifolius* had the highest level of elastase inhibition compared to other extraction techniques. The activity of inhibiting elastase enzyme was higher in the UAE method, because the polyphenol and terpenoid content that was successfully extracted was increasing due to ultrasonic vibrations on the cell wall.

At the stages of the fractionation process, the results of enzyme activity as listed in Table 9, the water fraction of *R. fraxinifolius* had the highest level of elastase inhibition compared to the other fractions, but there was no significant difference. The same results were found in a previous study conducted by Desmiaty (2018), who found that the polar methanol fraction had higher antioxidant activity and inhibition of elastase enzymes against the n-hexane and ethyl acetate fractions. (Desmiaty et al., 2018).

Correlation between antioxidant activity

A correlation analysis adapted from the Pearson method was carried out to ensure a correlation between the research findings. The Pearson correlation aims to quantify the intensity and direction of the linear relationship between TPC and TFC's antioxidant and anti-elastase activities. Each extract's concentrations of phenols and flavonoids (n = 3) and its IC_{50} value (n = 3) for its antioxidant and anti-elastase activities were associated (Fig. 4). Different correlations were displayed in the correlation coefficient plot matrix.

Strong relationships were seen in the matrix plot between TFC and antioxidant activity. TFC/DPPH was strongly correlated, reaching 76.3% (r = -0.763) due to the influence of flavonoid compounds. The antioxidant activity measured by the ABTS and FRAP methods showed a strong correlation with flavonoid compounds, reaching 67.8% for TFC/ABTS (r = 0.678) and 64.7% for TFC/FRAP (r = 0.647).

In contrast, a weak correlation between TPC and antioxidant activity was found in the FRAP methods, which reached 29.8% (r = 0.298). The correlation coefficient of TPC with an antioxidant activity using the DPPH and ABTS methods also was categorized as weak, which reached 13.9% for TPC/DPPH (r = 0.139) and 26.0% for TPC/ABTS (r = 0.260).

These findings demonstrate that while phenols do not correlate with DPPH antiradical action, flavonoids strongly do. The antioxidant activity of the FRAP and ABTS methods showed the same correlation activity, indicating a strong correlation between total flavonoid content and antioxidant activity.

The presence of flavonoid compounds in each extract resulted in a strong correlation which reached 90.2% (r = 0.902) for its anti-elastase activity. The anti-elastase activity of each extract also strongly correlated with the phenolic compound content in

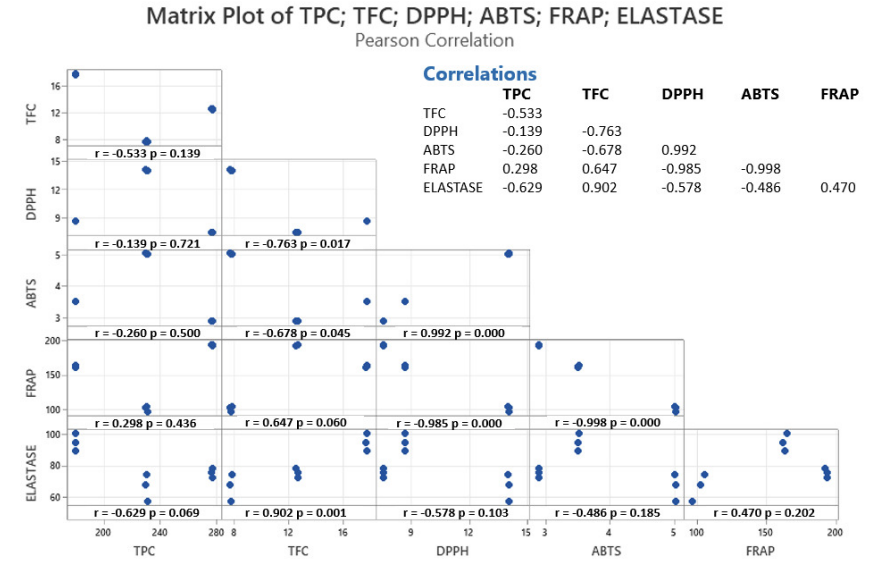


Figure 4. Pearson correlation coefficient analysis between total phenolic content (TPC) and total flavonoid content (TFC) with antioxidant and anti-elastase activity of three extracts with triplicate.

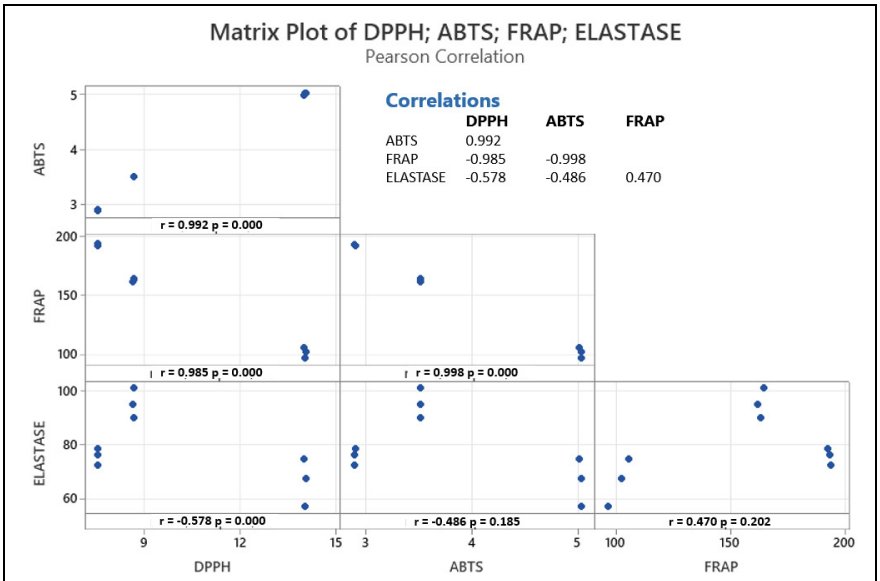


Figure 5. Pearson correlation coefficient analysis between antioxidant and anti-elastase activity of three fraction with triplicate.

each sample, with a correlation coefficient of r = 0.629. However, this study shows that the major compounds in flavonoids were predicted to contribute to their bioactivity as antioxidants and anti-elastase.

Analysis data in the fractionation process using Pearson gives results described in Fig. 5. Elastase enzyme inhibitory activity had a strong correlation with antioxidant activity. This indicates that antioxidant activity has a role in elastase inhibitory activity. Inhibition of elastase has a Pearson correlation value of 0.973 for activity in decreasing DPPH radicals, which suggests that the correlation reached 97.3%. The same with FRAP and ABTS methods gives strong activity, which reached 88.3% for the ABTS method (r = 0.883) and 97.8% for the FRAP method (r = 0.978).

LC-MS-MS

Metabolite profile analysis in this study used UPLC-MS. The UPLC-MS used in this study uses an MS detector with an ESI (+) ion source and an MS analyzer in the form of Q-ToF.

The identification of compounds is made using web applications like Chem Spider and Pubchem and m/z of compound literature. The following step is an examines the component profile using LC-MSMS based on antioxidants and elastase inhibition results,

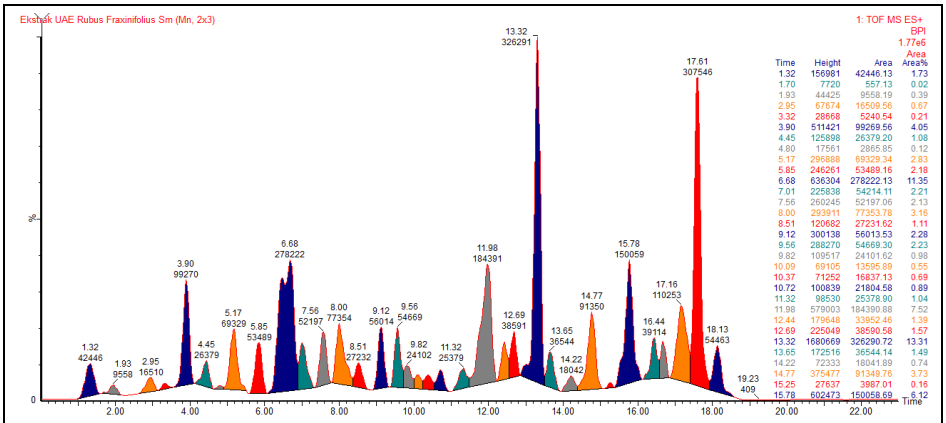


Figure 6. Chromatogram analysis of UAE extract *R. fraxinifolius* leaves.

Table 10. Phytochemical compounds identified in UAE extract of *R. fraxinifolius* leaves.

No.	RT	Area	Chemical	INCI name	Mol weight (Da)
1	17.61	307546	$C_{47}H_{83}N_7O_{13}$	18-[2-[2-[2-[2-[2-[2-[[(5S)-5-Carboxy-5-[[3-[3-(1H-imidazol-5-yl)propanoylamino]-3-methyl-2-oxobutyl]amino]pentyl]amino]-2-oxoethoxy]ethoxy]ethylamino]-2-oxoethoxy]ethoxy]ethylamino]-18-oxooctadecanoic acid	954.200
2	15.78	150058.69	$C_{31}H_{32}N_{10}O_3$	4-[({4-[3-Ethyl-7-(4-morpholinyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-5-yl]phenyl}carbamoyl)amino]-N-[2-(2-pyridinyl)ethyl]benzamide	592.651
3	16.67	29143.449	$C_{35}H_{54}N_2O_3$	(3α,5β,7β)-24-(4-Benzyl-1-piperazinyl)-3,7-dihydroxycholan-24-one	550.815
4	11.98	184390.88	$C_{18}H_{28}O_2$	Phenethyl decanoate	276.400
5	3.90	99269.56	$C_9H_6O_3$	Umbelliferone	162.142
6	9.56	54669.30	$C_{18}H_{26}O_3$	Octinoxate	290.397
7	5.17	69329.34	$C_{11}H_6N_6O_4$	4-Nitro-1-(3-nitro-2-pyridinyl)-1H-benzotriazole	286.203
8	4.45	26379.20	$C_4H_6N_2O_5$	Nitrosoiminodiacetic acid	162.101
9	16.44	39113.746	$C_{39}H_{62}O_5$	[1-(2-{[(3β)-3-Hydroxylup-20(29)-en-28-yl]oxy}-2-oxoethyl)cyclopentyl]acetic acid	610.907
10	14.22	18041.89	$C_{13}H_{28}N_{12}$	1,3-Dimethylimidazolium 1-(2-(5-tetrazolidyl)ethyl)-3-(5-tetrazolidyl)methylimidazolium	352.441
11	8.51	27231.621	$C_{23}H_{48}O_{10}$	7-(2,4-Dihydroxypentyl)-15-(ethoxymethyl)-2,5,8,11,14-pentaoxaicosane-17,19-diol	484.621
12	11.32	25378.90	$C_{21}H_{34}O_4$	10-Gingerol	350.492

which showed that UAE extract gave an excellent activity profile. This study predicted 38 compounds in UAE extracts according to the chromatogram results read in Fig. 6, with details predicted compound in the Table 10.

According to Table 10 results, compounds 5, 8, 11, and 12 are phenolic derivatives with Rt 3.90, 4.45, 8.51, and 11.32, respectively, according to the LC-MS/MS study. Compounds **3** and **9** were identified as steroid terpenoid compounds with Rt values of 16.67 and 16.44, respectively. Significant chemical content of phenolic and steroid terpenoid compounds is predicted by the outcomes of the chemical content screening using the LC-MS technique.

Besides analyzing the content by LCMS in the extract, analysis was also carried out on the most active fraction, namely the distilled water fraction. This study predicted 29 compounds in the distilled water *Rubus* fraction leaves according to the chromatogram results read in Fig. 7, which were determined using UPLC MS with details predicted compound in Table 11.

According to the fractionation LC-MS/MS result in Table 11, compounds **10**, **11**, and **12** are phenolic

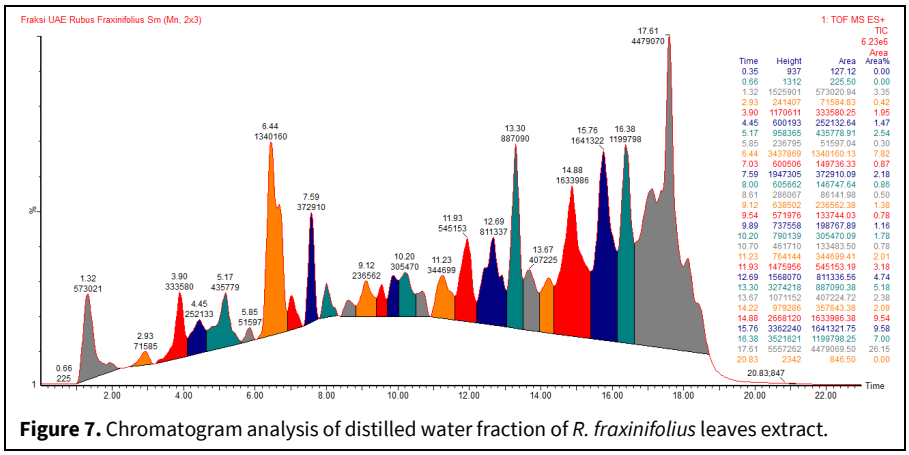


Figure 7. Chromatogram analysis of distilled water fraction of *R. fraxinifolius* leaves extract.

Table 11. Phytochemical compounds identified in the distilled water fraction of *R. fraxinifolius* leaves extract.

No.	RT	Chemical	Compound prediction	Mol weight (Da)
1	17.61	$C_{47}H_{83}N_7O_{13}$	18-[2-[2-[2-[2-[2-[2-[[(5S)-5-Carboxy-5-[[3-[3-(1H-imidazol-5-yl)propanoylamino]-3-methyl-2-oxobutyl]amino]pentyl]amino]-2-oxoethoxy]ethoxy]ethylamino]-2-oxoethoxy]ethoxy]ethylamino]-18-oxooctadecanoic acid	954.200
2	13.30	$C_{22}H_{41}N_9O_4$	N5-(Diaminomethylene)-L-ornithyl-L-prolyl-L-lysyl-L-prolinamide	495.619
3	6.44	$C_{32}H_{50}N_6O_8$	N-{3-[2-({[(3E)-3-Cycloocten-1-yloxy]carbonyl}amino) ethoxy]propanoyl}-D-valyl-N5-carbamoyl-N-[4-(hydroxymethyl)phenyl]-L-ornithinamide	646.775
4	16.38	$C_{39}H_{62}O_5$	[1-(2-{[(3β)-3-Hydroxylup-20(29)-en-28-yl]oxy}-2-oxoethyl)cyclopentyl]acetic acid	610.907
5	15.76	$C_{31}H_{32}N_{10}O_3$	4-[({4-[3-Ethyl-7-(4-morpholinyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-5-yl]phenyl}carbamoyl)amino]-N-[2-(2-pyridinyl)ethyl]benzamide	592.651
6	14.88	$C_{40}H_{54}O_3$	(3R,3'S,5'R,6'S)-7,8-Didehydro-5',6'-dihydro-5',6'-epoxy-β,β-carotene-3,3'-diol	582.855
7	7.59	$C_{32}H_{57}N_7O_9$	L-Leucyl-L-valyl-L-leucyl-L-α-aspartyl-L-prolyl-L-lysine	683.836
8	12.69	$C_{12}H_{31}N_4OP$	N-[diethylamino-(2,2-diethylhydrazinyl)phosphoryl]-N-ethylethanamine	278.380
9	11.93	$C_{18}H_{28}O_2$	Phenethyl decanoate	276.400
10	9.12	$C_{11}H_{16}O_2$	5-Pentylresorcinol	180.243
11	4.45	$C_4H_6N_2O_5$	Nitrosoiminodiacetic acid	162.101
12	2.95	$C_9H_{11}NO_2$	DL-Phenylalanine	165.189

derivatives with Rt 9.12, 4.45, and 2.95, respectively, according to the LC-MS/MS study. Compounds **4** and **6** were identified as steroid terpenoid compounds with Rt values of 16.38 and 14.88, respectively. A significant chemical content of phenolic and steroid terpenoid compounds is predicted by LC-MS/MS method of *R. fraxinifolius* distilled water fraction.

The LC-MSMS test findings for extract and *R. fraxinifolius* fraction indicate that the leaves contain terpenoids and phenolics, which have antioxidant and elastase enzyme-inhibiting properties.

This is consistent with earlier studies conducted by Desmiaty (2021), who discovered that the active components in *R. fraxinifolius* leaves are triterpenoids.

CONCLUSION

The results of this study can provide scientific evidence supporting the use of *R. fraxinifolius* leaves as an antiaging agent. Based on the *in vitro* test results, the highest antioxidant activity and elastase inhibition were found in old leaves compared to young leaves. UAE is a method of extracting compounds from *R. fraxiniofolius*, which gives the best results of antioxidant activity and elastase inhibition compared to the Soxhlet and MAE extraction methods. In the fractionation stage, the results showed that the water fraction provided strong antioxidant activity and elastase enzyme inhibition than the other fractions. Based on the results of the studies that have been reported, it can be concluded that the leaves of *Rubus fraxinifolius*

can be developed as an active ingredient for antiaging cosmetic prospects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA (2017) Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6(4): 42. https://doi.org/10.3390/plants6040042

Bait Y, Marsono Y, Santoso U, Marseno WD (2022) Effect of maturity stage on phenolic, flavonoid content, and antioxidant activity of cherry (*Muntingia calabura*) leaves extract. J Agercolere 4(2): 68–77. https://doi.org/10.37195/jac.v4i2.159

Bakar AMF, Ismail NA, Isha A, Mei Ling AL (2016) Phytochemical composition and biological activities of selected wild berries (*Rubus moluccanus* L., *R. fraxinifolius* Poir., and *R. alpestris* Blume). Evid Based Complement Alternat Med 2016: 2482930. https://doi.org/10.1155/2016/2482930

Blainski A, Lopes GC, De Mello JCP (2013) Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L. Molecules 18(6): 6852–6865. https://doi.org/10.3390/molecules18066852

Brás NF, Gonçalves R, Mateus N, Fernandes PA, Ramos MJ, De Freitas V (2010) Inhibition of pancreatic elastase by polyphenolic compounds. J Agric Food Chem 58(19): 10668–10676. https://doi.org/10.1021/jf1017934

Cane HPCA, Musman M, Yahya M, Saidi N, Darusman D, Nanda M, Rizki DR, Puspita K (2023) Phytochemical screening and antibacterial activity of ethnomedicinal plants from Gayo Lues Highland, Indonesia. J Pharm Pharmacogn Res 11(1): 117–128. https://doi.org/10.56499/jppres22.1526_11.1.117

Das S, Mazumder A, Sahu N, Das MK, Chand S, Gupta H, Kumar A (2022) Ethnopharmacology of genus Rubus: Modern science from ancient science. J Nat Remedies 22(4): 529–538. https://doi.org/10.18311/jnr/2022/29886

Delazar A, Nahar L, Hamedeyazdan S, Sarker SD (2012) Microwave-assisted extraction in natural products isolation. Method Mol Biol 864: 89–115. https://doi.org/10.1007/978-1-61779-624-1_5

Desmiaty Y, Elya B, Saputri FC, Hanafi M, Prastiwi R (2018) Antioxidant activity of *Rubus fraxinifolius* Poir. and *Rubus rosifolius* J. Sm. leaves. J Young Pharm 10(2): s93–s96. https://doi.org/10.5530/jyp.2018.2s.18

Desmiaty Y, Hanafi M, Saputri FC, Elya B, Rifai EA, Syahdi RR (2021) Two triterpenoids from *Rubus fraxinifolius* leaves and their tyrosinase and elastase inhibitory activities. Sci Rep 11: 20452. https://doi.org/10.1038/s41598-021-99970-x

Desmiaty Y, Saputri FC, Hanafi M, Prastiwi R, Elya B (2020) Anti-elastase, anti-tyrosinase, and antioxidant of Rubus fraxinifolius stem methanolic extract. Pharmacogn J 12(2): 271–275. https://doi.org/10.5530/pj.2020.12.42

Garg C, Khurana P, Garg M (2017) Molecular mechanisms of skin photoaging and plant inhibitors. Int J Green Pharm 11(2): S217–S232.

Getoff N (2007) Antiaging and aging factors in life. The role of free radicals. Radiat Phys Chem 76(10): 1577–1586. https://doi.org/10.1016/j.radphyschem.2007.01.002

Ibrahim HA (2019) Introductory Chapter: Fractionation. Fractionation. https://doi.org/10.5772/intechopen.78050

Ismed F, Desti WN, Arifa N, Rustini R, Putra DP (2022) TLC-Bioautographic and LC-MS/MS detection of antimicrobial compounds from four semipolar extracts of *Cladonia* species. Proc. 2nd Int. Conf. Contemp. Sci. Clin. Pharm. 2021 (ICCSCP 2021) 40: 49–59. https://doi.org/10.2991/ahsr.k.211105.008

Jovanović A, Đorđević V, Zdunić G, Pljevljakušić D, Šavikin K, Gođevac D, Bugarski B (2017) Optimization of the extraction process of polyphenols from *Thymus serpyllum* L. herb using maceration, heat- and ultrasound-assisted techniques. Sep Purif Technol 179: 369–380. https://doi.org/10.1016/j.seppur.2017.01.055

Kumar K, Srivastav S, Sharanagat VS (2021) Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. Ultrason Sonochem 70: 105325. https://doi.org/10.1016/j.ultsonch.2020.105325

Lukitaningsih E, Nur S, Qonithah F, Zulbayu A, Kuswahyuning R, Rumiyati R (2020) *In vitro* anti-wrinkle and tyrosinase inhibitory activities of grapefruit peel and strawberry extracts. Maj Obat Tradis 25(3): 182–189. https://doi.org/10.22146/mot.59194

Masaki H (2010) Role of antioxidants in the skin: Antiaging effects. J Dermatol Sci 58(2): 85–90. https://doi.org/10.1016/j.jdermsci.2010.03.003

Mutalib, MA, Rahman, MA, Othman, MHD, Ismail, AF, Jaafar J (2017) Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray (EDX) Spectroscopy. In Membrane Characterization. Elsevier B.V. https://doi.org/10.1016/B978-0-444-63776-5.00009-7

Nur S, Aswad M, Yulianty R, Burhan A, Khairi N, Sami FJ, Nursamsiar (2023a) The antioxidant and anti-ageing activity of lyophilisate kersen (*Muntingia calabura* L) fruit *in vitro*. Food Res 7(2): 23–30 https://doi.org/10.26656/fr.2017.7(2).749

Nur S, Mubarak F, Jannah C, Winarni DA, Rahman DA, Hamdayani LA, Sami FJ (2019) Total phenolic and flavonoid compounds, antioxidant and toxicity profile of extract and fractions of paku atai tuber (*Angiopteris ferox* Copel). Food Res 3(6): 734–740. https://doi.org/10.26656/fr.2017.3(6).135

Nur S, Aisyah AN, Lukitaningsih E, Rumiyati, Juhardi RI, Andirah R, Hajar AS (2021a) Evaluation of antioxidant and cytotoxic effect against cancer cells line of *Angiopteris ferox* Copel tuber and its compounds by LC-MS analysis. J Appl Pharm Sci 11(8): 54–61. https://doi.org/10.7324/JAPS.2021.110808

Nur S, Angelina AA, Aswad M, Yulianty R, Burhan A, Nursamsiar N (2021b) *In vitro* antiaging activity of *Muntingia calabura* L. fruit extract and its fractions. J Pharm Pharmacogn Res 9(4): 409–421. https://doi.org/10.56499/jppres20.979_9.4.409

Nur S, Hanafi M, Setiawan H, Nursamsiar, Elya B (2023b) *In silico* evaluation of the dermal antiaging activity of *Molineria latifolia* (Dryand. ex W.T.Aiton) Herb. Ex Kurz compounds. J Pharm Pharmacogn Res 11(2): 325–345. https://doi.org/10.56499/jppres23.1606_11.2.325

Oh YS, Shin SY, Kim S, Lee KH, Shin JC, Park KM (2020) Comparison of antiaging, anti-melanogenesis effects, and active components of raspberry (*Rubus occidentalis* L.) extracts according to maturity. J Food Biochem 44(11): e13464. https://doi.org/10.1111/jfbc.13464

Pratama BP, Supriyadi, Swasono RT, Pranoto Y (2021) Different leaf maturities and withering durations affect the antioxidant potential and aroma compound of Indonesian bay leaf [*Syzygium polyanthum* (Wight) Walp.]. Int Food Res J 28(6): 1196–1203. https://doi.org/10.47836/ifrj.28.6.11

Rocabado GO, Bedoya LM, Abad MJ, Bermejo P (2008) *Rubus* - A review of its phytochemical and pharmacological profile. Nat Prod Commun 3(3): 423–436. https://doi.org/10.1177/1934578x0800300319

Wiliantari S, Iswandana R, Elya B (2022) Total polyphenols, total flavonoids, antioxidant activity and inhibition of tyrosinase enzymes from extract and fraction of *Passiflora ligularis* Juss. Pharmacog J 14(3): 660–671. https://doi.org/10.5530/pj.2022.14.86

Xiao F, Xu T, Lu B, Liu R (2020) Guidelines for antioxidant assays for food components. Food Front 1(1): 60–69. https://doi.org/10.1002/fft2.10

Zhang QW, Lin LG, Ye WC (2018) Techniques for extraction and isolation of natural products: A comprehensive review. Chinese Medicine (United Kingdom) 13: 20. https://doi.org/10.1186/s13020-018-0177-x

AUTHOR CONTRIBUTION:

Contribution	Sulistyowati	Elya B	Iswandana R	Nur S
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x		
Experimental studies	x	x	x	
Data acquisition	x	x	x	
Data analysis	x		x	
Statistical analysis	x		x	
Manuscript preparation	x	x	x	
Manuscript editing	x		x	
Manuscript review	x	x	x	x

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