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Original Article

UNRIPE FRUIT OF *RUBUS FRAXINIFOLIUS* AS A POTENTIAL SOURCE OF ANTIOXIDANT AND ANTIELASTASE AGENT

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ABSTRACT

Objective: This research aimed to examine the anti-oxidant activity, antielastase activity, and the content of total phenolic and total flavonoid of *R. fraxinifolius* unripe fruit.

Methods: The dried unripe fruit was extracted using Soxhlet apparatus with sequence solvent: n-hexane, ethyl acetate, and methanol. Each extract was determined the anti-oxidant and antielastase activity, total phenolic, and total flavonoid content.

Result: The result showed the extracts (n-hexane, ethyl acetate, and methanol) gave anti-oxidant IC_{50}>200; 186.84; and 19.74 ppm, and the ability of elastase inhibition was 6.84+0.9%; 52.23+7.1%; and 57.81+5.5% at 100 ppm, respectively. The methanolic extract contained phenolic 202.2 mg GAE/g extract and flavonoid 43.89 mg QE/g extract.

Conclusion: *R. fraxinifolius* unripe fruit has shown potential as a DPPH (α, α-diphenyl-β-picrylhydrazyl) radical scavenger and anti-elastase. This study provides an excellent effect to underline the importance of *R. fraxinifolius* unripe fruit, and it can be developed as nutricosmetics, nutraceuticals, or herbal anti-wrinkle cosmetics.

Keywords: Raspberry, Nutricosmetics, Anti-wrinkle, DPPH, Soxhlet extraction

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INTRODUCTION

Premature aging of the skin is known to be caused by two mechanisms: intrinsic and extrinsic mechanisms. Enzymes in the skin such as tyrosinase, elastase, collagenase, and hyaluronidase are examples of intrinsic factors and reactive oxygen species (ROS) as extrinsic factors are the cause of premature skin damage. The use of nutricosmetics is one of the latest trends in skincare. Nutricosmetics is a food and nutritional supplement, which is useful for improving the health and visual appearance of the skin. Currently, the cosmetics industry is trying to continue to look for ingredients from natural sources of nutricosmetics because of its competitive effectiveness and lower toxicity effects [1].

Elastase is a member of the chymotrypsin protease enzymes, and it is responsible for the decomposition of elastin (a protein fundamental for elastic properties of the skin extracellular matrix). Excessive degradation of elastin skin fiber tissue can cause a loss of skin elasticity. This enzyme dysfunction can cause premature wrinkles and skin aging process. Substances possessing inhibitory actions on elastase and anti-oxidant might play significant roles as anti-wrinkle and, in the deceleration of the skin aging process, also could potentially be developed for cosmetic purposes [2–4].

Rubus fruit is a soft and small berry, colorful, have essential nutritional, edible, has been consumed for a long time throughout the world because they taste good and known contained anthocyanins, phenolic monomers, and phenolic polymers [5, 6]. Some *Rubus sp.* is reported to have activities like anti-oxidants, anti-elastase, anti-collagenase, antithrombotic, anti-bacterial, and has the potency to treat disorders due to the radical, mainly cancer and other inflammatory diseases. The plants also have a large content of polyphenols and flavonoids [7–15].

Fruit of *Rubus fraxinifolius,* locally known as "arben/arbei" (West Java) and had mercantile value due to its ability to harvest the fruit throughout the year [16]. This raspberry has a high content of sugar, vitamin C, and iron [17]. The leaves and fruit have an excellent radical scavenger activity (FRAP method and DPPH method) and high polyphenol content [10, 18, 19]. There are no prior data available found *R. fraxinifolius* as antielastase in the literature. Bravo and colleagues (2016) have tested the *in vitro* anti-aging activity of several maturity levels of *R. glaucus* and *R. robustus* fruit. The test results showed that unripe fruit provides a higher inhibitory enzyme activity [8]. This statement is also supported by several other studies in some Rubus fruit [7, 20, 21], therefore in this study were selected the unripe fruit.

This research was intended to determine the anti-oxidant and antielastase activity of *R. fraxinifolius* fruit. The Soxhlet extraction method was chosen to obtain the bioactive component present in the fruit using n-hexane, ethyl acetate, and methanol solvents. The anti-oxidant activity of extracts was determined using the radical DPPH radical scavenger activity (DRSA) method. *In vitro* anti-elastase activity was determined using porcine pancreatic elastase (PPE), which is based on the formation of p-nitroaniline.

MATERIALS AND METHODS

Chemical and reagents

α, α-diphenyl-β-picrylhydrazyl (DPPH), Folin–Ciocalteu's reagent, Buffer Trizma base (T1503), Porcine pancreatic elastase/PPE (E1250), N-Succinyl-Ala-Ala-Ala-p-nitroanilide/SANA (S4760), gallic acid (G7384), aluminum chloride, quercetin, potassium acetate, methanol, all materials were purchased from Sigma Aldrich (St. Louis, MO).

Plant material

Rubus fraxinifolius fruit was collected from Cianjur, West Java, Indonesia at 1384 MASL, and sorted the unripe fruit. The Biology Research Center, Indonesian Institute of Sciences, determined the plant with specimen number 033/If.07/1/2018. Fresh unripe fruit was cleaned, air-dried, and grounded.

Phytochemical screening

Dried fruit powder was analyzed for the presence of alkaloid, tannin, polyphenol, flavonoid, saponin, and steroid-triterpenoid compounds using preliminary and confirmatory tests. The alkaloid was detected using Mayer's and Dragendorff's tests, tannin, and polyphenol using gelatin and ferric chloride test, flavonoid using the Shinoda's test, saponin using the formation of frothing, and the steroid-triterpenoid was using Liebermann-Burchard's test.

Soxhlet extraction (SE)

The extract was prepared using the Soxhlet apparatus in sequential extraction. Dried fruit (30 g) was extracted, respectively, with each 500 ml of n-hexane, ethyl acetate, and then methanol (each 24 h). Each extract was evaporated under reduced pressure using a rotary evaporator, followed by drying using a vacuum oven and stored in the refrigerator.

Determination of anti-oxidant

The DPPH radical scavenging activity (DRSA) was examined using the modified microplate method [22, 23]. DPPH was dissolved in methanol (6 mg/100 ml), and 180 µl was added to extracts 20 µl (final conc. 50 ppm in methanol) in a 96-Nunc microplate well. The mixture was shaken 5 min and allowed to stand at room temperature in the dark (30 min). The absorbance distinction of the resulting solution was monitored in the VersaMac microplate spectrophotometer at 517 nm. All tests were made triplicate, and gallic acid was used as a positive control [24]. The anti-oxidant activity was calculated by the following equation 1 [10]:

Elastase inhibitory assay

The anti-elastase was determined by a previously described method with slight modification [25]. The test was performed in 100 mmol buffer Trizma®-HCl (pH 8.0). PPE was dissolved in cold buffer (0.2 mg/ml stock solution). The substrate SANA was dissolved in buffer at 2.9 mmol. The extracts (20 µl) were incubated with 20 µl enzyme for 15 min, and the reaction was started by adding 20 µl substrate. The total volume reaction mixture was 200 µl containing 0.29 mmol SANA, 20 mU PPE, buffer, and 100 ppm extract. Gallic acid (100 ppm) was used as a standard reference [24]. Negative controls containing all components without enzymes. The absorbance of p-nitroaniline then measured at 401 nm using a Versamac microplate spectrophotometer. The tests were done in triplicate.

The anti-elastase was calculated as Equation 2:

$$\text{Anti-elastase (\%)} = (\text{Absorbance}_{control} - \text{Absorbance}_{sample}) \times 100 \; .... \; (2)$$

$(\text{Absorbance}_{control})$

Total phenolic content (TPC) assay

Total phenolic content was determined using a modified Folin Ciocalteau microplate method [22]. Using 96-Nunc well plate, 20 µl extract in methanol (final conc. 100 ppm) were mixed 100 µl FCR (1:4 in aquadest), then add 80 µl of sodium carbonate (100 g/l). Incubation for 120-minute measurements, the absorbance was recorded at 750 nm in a microplate reader (VersaMac). The extract was plotted on a linear regression standard curve of gallic acid (10-200 mg/l). All samples were performed in triplicate. The TPC was expressed as mg of gallic acid equivalence (GAE)/g extract.

Total flavonoid content (TFC) assay

Total flavonoid content determination using Farasat *et al.* method [26] with some modification. Briefly, the 20 µl extract ethyl acetate and methanol in methanol (2000 µg/ml) each added with 20 µl aluminum chloride (10% w/v), 20 µl sodium acetate (1M), and 180 µl aquadest. This mixture was incubated for 30 min at room temperature. The absorbance of the solution was measured on a 415 nm in a microplate reader (VersaMac). Flavonoid quantification was carried out based on a standard curve of quercetin prepared in methanol (25-175 µg/ml), and the results were expressed in mg of quercetin equivalence (QE)/g extract.

RESULTS

In this study were selected unripe fruit. Description of unripe fruit variously ovoid to ellipsoid 0.5-1 cm, green (but exocarp will red when ripe), and have five green sepals (fig. 1).

Fig. 1: Unripe fruit of *Rubus fraxinifolius*

Table 1: Phytochemical screening of unripe fruit *R fraxinifolius*

Phytochemical	Result
Alkaloid	-
Flavonoid	+
Saponin	+
Polyphenol	+
Tannin	+
Quinone	-
Steroid/triterpenoid	+

Note.+: detected,-: No detected

Table 1 showed the phytochemical investigation of dried powder unripe fruit of *R fraxinifolius.* It revealed the presence of tannin, flavonoid, polyphenol, saponin, and steroid-triterpenoid. The anti-oxidant activities were investigated using the free radical scavenging (DRSA) method developed by Blois, which offers the approach to evaluate anti-oxidant activities of the sample, such a compound, extract, or other biological sources [27]. The method is simple, just mixed the compound or extract with DPPH solution and then read the absorbance after the incubation period in the spectrophotometer [28]. Elastase inhibitory assay was using PPE with the substrate SANA (N-Succ-(Ala)3-p-nitroanilide)**.** Table 2 demonstrates the activity of *R. fraxinifolius* fruit extract to scavenge DPPH free radicals and inhibit the elastase enzyme.

Table 2: The antioxidant and anti-elastase activity of *R. fraxinifolius* fruit extract

Extract	Yields (%)	DRSA IC_{50} (μg)	% Inhibition elastase (in 100 μg/ml)		
Hexane	1.14	>200	6.84	+	0.91
Ethyl acetate	3.37	186.84	52.23	+	7.1
Methanol	13.29	19.74	57.81	+	5.53
Gallic acid	-	5.54	64.92	+	16.2

*Value is expressed as for the experiment (mean±SD, n=3)

The determination of total phenolic content and total flavonoid content among ethyl acetate and methanol extract are presented in table 3. The methanolic extract of *R. fraxinifolius* fruit has high phenolic contents.

Table 3: Total phenolic and flavonoid content of *R. fraxinifolius* fruit extract

Extraction methods	TPC (mg GAE/g extract)			TFC (mg QE/g extract)		
n-hexane	-			-		
Ethyl acetate	8.25	+	4.36	58.71	+	2.65
Methanol	202.21	+	4.37	43.89	+	4.22

Value data are given in mean±SD, n=3

DISCUSSION

Phytochemical investigation to dried powder unripe fruit of *R fraxinifolius* revealed the presence of tannin, flavonoid, polyphenol, saponin, and steroid-triterpenoid (table 1). This result was the same in the previous study [10, 29].

The anti-oxidant capacity of extracts on DPPH free radical scavenging was due to its hydrogen-donating process. In this test, the purple color of the reagent solution will turn to yellow in the presence of an anti-oxidant. Table 2 showed that methanol extract had strong activity in scavenging free radical DPPH. Nevertheless, the n-hexane extract had no activity. All values were expressed as the mean±SD of triple experiments.

The anti-elastase determination was performed to test the ability of the extract to degrade elastase. In terms of premature skin aging, to find inhibitors of elastase can be useful to overcome the loss of skin elasticity and skin sagging [33]. Elastase enzyme, a serine protease that can degrade elastin and hydrolyze almost all extracellular matrix proteins in connective tissue, such as collagen and fibronectin. If the activity of elastase inhibited, it can be a target to protect elastin protein, overcome the ROS, photoaging, and prevent damage to the structure of the extracellular matrix. Table 2 also present that the methanolic and ethyl acetate extract has more than 50% elastase inhibitory activity. This study proved that ethyl acetate and methanol extracts significantly inhibited elastase activity. Methanol extract gave the best activities in both tests and exhibited that it has potent anti-oxidant and anti-elastase activity. This is also consistent with other studies that show that methanol is an efficient extraction medium for a broad spectrum of compounds [1].

All tests were measured with a microplate reader. *In vitro* testing and assay using microplates can save time, cost, and resources, whereas the conventional methods are time-consuming, using a large amount of reagent, and labor-intensive [22].

It has been reported some Rubus species containing phenolic and flavonoid compounds such as chlorogenic acid, ellagic acid, gallic acid, flavonol, and caffeic acid [18, 30]. There were positive correlations between inhibition for radical scavenging activities and antielastase activities with polyphenolics and flavonoids content [20, 31, 32]. Polyphenol compounds are known to react strongly with free radicals by donating hydrogen atoms or chelating electrons to metal ions. The Folin-Ciocalteu method is the most commonly used method for assessing the equivalent amount of pure phenolic compounds (TPC). Various modifications have been made to increase the specificity of this assay, which was developed initially by Singleton and Rossi (1965). Some reports showed that many polyphenols, such as gallic acid, can inhibit elastase activity. The inhibitory activity to proteolytic enzymes maybe by acting as a precipitating or complexing agent. There was a positive relationship between the polyphenol polymerization degree with the activity to inhibit the elastase enzyme [24, 32–35].

To measure total flavonoid content, usually use the aluminum chloride colorimetric method. In this method, flavonoids will perform a complexation reaction with aluminum. Flavonol or flavone complex with $AlCl_3$ will give a bathochromic shift with sodium hydroxide. Among the researched natural compounds, flavonoids play an essential role. In a structure-dependent manner, flavonoids can act as a radical scavenger and inhibit the activity of many enzymes, including elastase. Structure-activity relationship analysis of flavonoid in skin aging prevention mechanisms correlated with a free hydroxyl group in structure. For example, catechol groups in ring B were responsible for the elastase inhibitory activity, so the flavonols have stronger inhibitor activity than flavones or isoflavones [36, 37].

In table 3 has shown that the methanolic extract of *R. fraxinifolius* fruit contains many polyphenols, and this is according to the previous report [10, 18]. Methanol extract contains polar compounds such as polyphenolic, which are known to have anti-oxidant activity, especially phenolic acids and flavonoids [38]. Phenolic acid and flavonoid widely found in plants, especially in fruits and vegetables. Some reports showed the benefit of these compounds could be used for a potent anti-wrinkle agent of skincare or nutraceutical formula.

CONCLUSION

Soxhlet extraction could collect bioactive content in *R. fraxinifolius* fruit, and methanol extract of *R. fraxinifolius* fruit had the best trapping capability of DPPH and also had potential activity as anti-elastase. This investigation provides the vastness promising effect with the importance of *R. fraxinifolius* unripe fruit, and it can be potentially used as a nutraceutical or nutricosmetics ingredient.

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AUTHORS CONTRIBUTIONS

Berna Elya (BE) provided the design for research and edited the paper. Yesi Desmiaty (YD) conducted the experimental work and drafted the manuscript. Both authors discussed and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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