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Original Article

Anti-Elastase, Anti-Tyrosinase, And Anti-Oxidant of *Rubus Fraxinifolius* Stem Methanolic Extract

Yesi Desmiaty[1,2], Fadlina Chany Saputri[1], Muhammad Hanafi[2,3], Rini Prastiwi[4], Berna Elya[1,*]

ABSTRACT

Introduction: Some Rubus were reported had anti-skin aging activity. *Rubus fraxinifolius* was one of Rubus genus which lives in Indonesian highland. **Objective:** This study was to examine elastase, tyrosinase, and oxidant inhibitory activity of *R. fraxinifolius* stem (RFS) extract. **Methods:** Extraction was done by a Soxhlet apparatus using methanol as solvent. Elastase inhibition activity was determined, which based on the formation of p-nitroaniline. Tyrosinase inhibition activity evaluated based on inhibition of mushroom tyrosinase by the sample with L-DOPA as substrate. The activity of antioxidant was determined using the DPPH radical scavenger method. LC-MS was used for prediction of naturally occurring phytochemicals. **Results:** The RFS extract yield was 9.03 %. The RFS extract revealed inhibition activity against elastase and tyrosinase with IC_{50} 128.85 ppm, and 155.19 ppm, respectively. DPPH radical scavenging activity gave IC_{50} 63.04 ppm. Total phenolic content of the extract was 387.99±3.21 mg GAE/g extract. The LC-MS analysis showed the presence of at least 13 different organic compounds in RFS extract, which might contribute to the bioactivity. **Conclusion:** Therefore, this experiment further proved that RFS extract might be useful as a natural product ingredient of anti-photoaging skincare products because of its ability to inhibit elastase, tyrosinase, and as an antioxidant.

Key words: Anti-elastase, Antioxidant, Anti-tyrosinase, *Rubus fraxinifolius* stem.

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INTRODUCTION

One of the most common dermatologic concern is skin photoaging. There are many synthetic compounds which claimed as cosmetic anti-aging ingredients, but they can produce adverse reactions such as irritant and allergic contact dermatitis, and photoallergic reactions. Hence, it needs to find a new potent compound as skincare products ingredients from natural resources such as herbal extract. Many in vitro research showed that herbal extracts containing phenolic compounds could scavenge free radical and inhibit elastase, hyaluronidase and tyrosinase enzymes.[1-3]

Many plants are growing in tropical mountains environments mostly unexplored. *Rubus fraxinifolius* was one of the plants which live in Indonesia high-elevation and has potential as fresh fruits, beverage raw materials, and medicinal plants.[4] Some Rubus genus were reported had a potential anti-skin aging activity such anti elastase, antioxidant, anti collagenase, anti-tyrosinase, etc.[5-7] Some publication reported the antioxidant activity, nutrition content, and polyphenol content of *R. fraxinifolius* fruit.[8-10] There is no found report about the stem phytochemical content or activity. Therefore, in this research, we examined the activity of *R. fraxinifolius* stem (RFS) extract to inhibit elastase and tyrosinase enzyme, and the capability to reduce free radical level.

MATERIAL AND METHODS

Chemicals and reagents 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methanol, Buffer Trizma base (T1503), Porcine pancreatic elastase (E1250), N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) (S4760), quercetin, Tyrosinase from mushroom (T3824), L-3,4-dihydroxyphenylalanine (L-DOPA), Folin-Ciocalteu's reagent and gallic acid, were purchased from Sigma-Aldrich.

Plant material collection and extract preparation

Rubus fraxinifolius stem was collected from Cianjur, West Java at altitude 1384 m asl. The taxonomic identification of the plant was confirmed a botanist at Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia. Before all analysis, the stem was cut and cleaned to remove any foreign materials and dust, then air-dried and grounded into a fine powder.

The stem powder (50 g) were extracted using a Soxhlet apparatus with methanol (750 mL). The extract was evaporated using rotary evaporator (Buchi) under reduced pressure, and then was dried using vacuum oven yield *Rubus fraxinifolius* stem (RFS) methanolic extract.

Anti-elastase assay

The RFS extract solution and pancreatic elastase (PPE) were mixed in Trizma®-HCl buffer (pH 8.0), then pre-incubated at 25°C for 5 min. Substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) was added to the mixture and incubated at 25°C for 20 min in 96-well microplate (Nunc). The optical density due to the formation of p-nitroaniline was

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measured at 401 nm with a microplate spectrophotometer (Versamac). The reaction mixture was contained 0.07M Trizma®-HCl buffer, 16 mU elastase, 0.29 mM substrate. The sample was performed in triplicate. The elastase inhibitory activity of each sample was calculated according to the following formula:

Elastase inhibition activity (%) = $(OD_{blank} - OD_{extract}) \times 100/OD_{blank}$

where OD_{blank} and $OD_{extract}$ were the optical densities in the absence and presence of extract, respectively.[11]

Anti-tyrosinase assay

Tyrosinase inhibitory assay was performed according to the method previously described by Moon with modification.[12] The reaction was done with a potassium phosphate buffer (0.1 M, pH 6.7) containing 10 mM L-DOPA and mushroom tyrosinase aqueous solution (50 units/mL) at 37°C in Nunc 96 well microtitre plate. The mixture was incubated for 15 min before adding the substrate. The change of the absorbance of dopachrome was measured at 475 nm using a microplate spectrophotometer (Versamac). The sample was performed in triplicate. The tyrosinase inhibitory activities were calculated as described in the elastase inhibitory activity.

Antioxidant assay

Antioxidant activity of RFS extract was determined using DPPH free radical reagent with the method previously described with slight modification.[13] Briefly, an amount of 20 µL of five serial concentrations of RFS diluted extract (12.5–100 µg/mL), and 180 µL of DPPH (60 µmol/L) in methanol were mixed in each well of the 96-well microplate. The absorbance was measured at 516 nm after 30 min in the dark by a microplate reader (Versamac). Gallic acid was used as positive controls. The experiment was done in triplicate. The DPPH radical scavenging activity was calculated according to the equation: % Inhibition activity = [(Acontrol − Asample)/Acontrol] × 100, where Acontrol was the absorbance of the control and Asample was the absorbance of the sample. The IC_{50} value which was the concentration of the sample that reduction 50% of the DPPH radical.

Total phenolic content assay

The total phenolic assay was assessed using Folin Ciocalteau reagent (FCR) with some modification of microplate method.[13] FCR is a mixture of phosphotungstate and phosphomolydate. This reagent will oxidize phenolic group and reduces the heteropoly acids to yield a blue complex. Diluted RFS extract (20 µL) was mixed with 1:4 diluted FCR (100 µL) and 75 µL of sodium carbonate (100 g/L) in a 96-well plate. After a 120 min at room temperature and light protected, the absorbance of the reaction mixture was measured using a microplate reader (Versamac) at 750 nm. The standard curve was using gallic acid dilutions (10-200 mg/L) as a reference. The analysis was done in triplicate. The result was expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract.

Phytochemical determination using LC-MS

RFS extract was analyzed by Liquid chromatography equipped with mass spectrometry (Waters UPLC-MS XEVO G2-XS QTOF) in positive ionization mode using electrospray ionization and acetonitrile as solvent. The chromatogram of the sample was identified by comparing their mass spectra with the library data.

RESULT AND DISCUSSION

Nowadays, it is a new era in the development of cosmetic products. There are many variations of techniques for producing cosmetic products to improve the appearance of the skin and delaying premature aging. An understanding of skin physiology continues to develop. The skin can be affected by the use of topical preparations, along with the availability of new active ingredients and advanced formulations, useful in developing more effective products. Some scientific research has been carried out to check the anti-aging effects of natural ingredients and cosmetic products from nature. In vitro research has shown that plants contain secondary metabolites which have been widely used in the cosmetics industry world because of their significant impact on skin aging as antioxidants, skin brightening, and sunscreen agents. The new cosmeceuticals manufacturing technology can facilitate skin to improve wrinkles, which leads to a younger-looking, healthier-looking face, radiant skin, and against skin aging. This natural skin care product is quickly absorbed by superficial layers of the skin and is usually hypo-allergenic.[1,14-16]

Tyrosinase and elastase inhibitory effects were performed using in vitro methods and the result shown in Figures 1 and 2. The RFS extracts inhibited tyrosinase, elastase, and DPPH in a dose-dependent manner with IC_{50} 155.19 ppm, 128.85 ppm, and 63.04 ppm, respectively.

Anti-elastase assay

Figure 1 summarizes the result of elastase inhibition for RFS extract at concentrations of 50-250 ppm. RFS extract (IC_{50} 128.85 ppm) has lower elastase inhibition activity in comparison with control positive (quercetin IC_{50} 78.70 ppm), but this study demonstrated that RFS extract has an activity to inhibit elastase enzyme. Quercetin was reported to have an inhibitory effect on elastase enzyme.[17] 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.[18] 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.

Anti-tyrosinase assay

The study revealed that RFS extract inhibited tyrosinase with IC_{50} 155.19 ppm. Figure 2 shows the linear regression of the activity of RFS

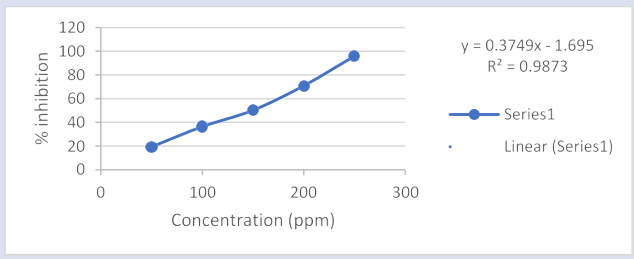

Figure 1: Anti-elastase assay of RFS

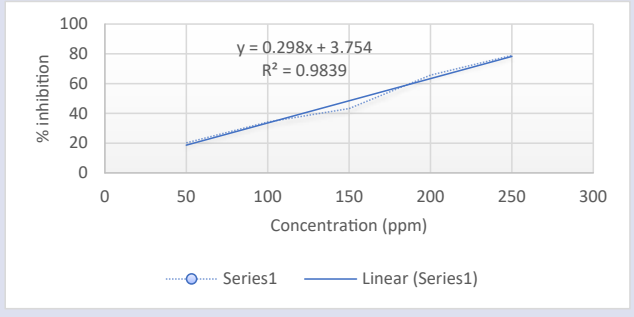

Figure 2: Anti tyrosinase assay of RFS

extract in inhibiting tyrosinase. Tyrosinase is the main enzyme involved in skin pigmentation because it can activate the melanin pigment, so hyperpigmentation is formed in two reactions: (1) the hydroxylation of L-tyrosine become L-DOPA (L-3,4- dihydroxyphenylalanine), and (2) the oxidation of L-DOPA become dopaquinone. This dopaquinone is very reactive and could be polymerized spontaneously to form melanin.[19]

Antioxidant assay

The scavenging activity of RFS extract was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals. The study revealed that RFS extract has an intense antioxidant activity with IC_{50} 63.04 ppm. The linear regression of activity was showed in Figure 3. The result from previous studies in leaves and fruit of *R. fraxinifolius* have excellent antioxidant activity.[20,10]

Total phenolic content assay

In this research showed that RFS extract had high phenol content (387.99 + 3.21 mg GAE/g extract). As informed in other publications, that *R. fraxinifolius* fruit and leaves also contain a high polyphenolic compound.[10,21] Phenolic and flavonoid compounds have been reported to present significant antioxidant properties. Phenolic compounds can act as antioxidants because of their ability as reducing agents. The hydroxyl groups in polyphenolic compounds will donate their hydrogen so it can reduce DPPH radicals. Besides, its low molecular weight also contributes to the high scavenging activity to DPPH. Furthermore, polyphenolics can scavenge and deactivate reactive oxygen intermediates to avert oxidative reactions.[22]

Phytochemical determination using LC-MS

RFS extract was analyzed by UPLC-MS XEVO G2-XS QTOF, the spectra was shown in Figure 4. We identified each compound based on the Waters databases, and the prediction was listed in Table 1. There is no information found about *R. fraxinifolius* stem phytochemical content, and from this research known that RFS contained triterpenoid and its derivatives which might also contribute to the activity. Some report shows that Rubus containing triterpenoid and have significant bioactivity.[23-25]

CONCLUSION

Our results demonstrate that RFS extract has potential activity as anti-tyrosinase, anti-elastase, and antioxidant. Further studies are necessary to investigate the active components and safety of these extracts.

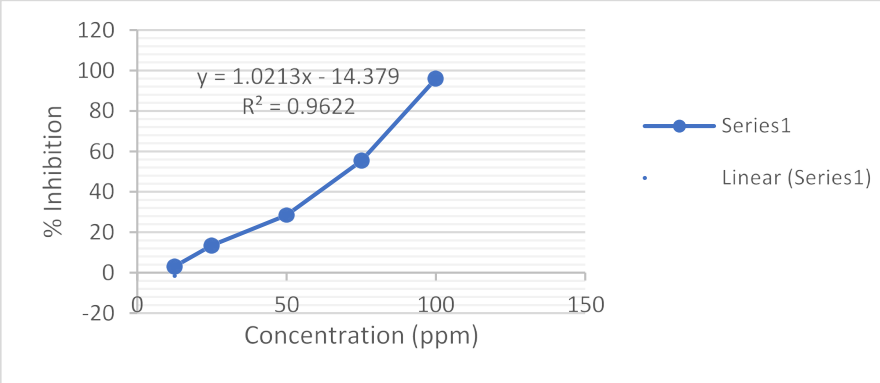

Figure 3: Anti oxidant assay of RFS

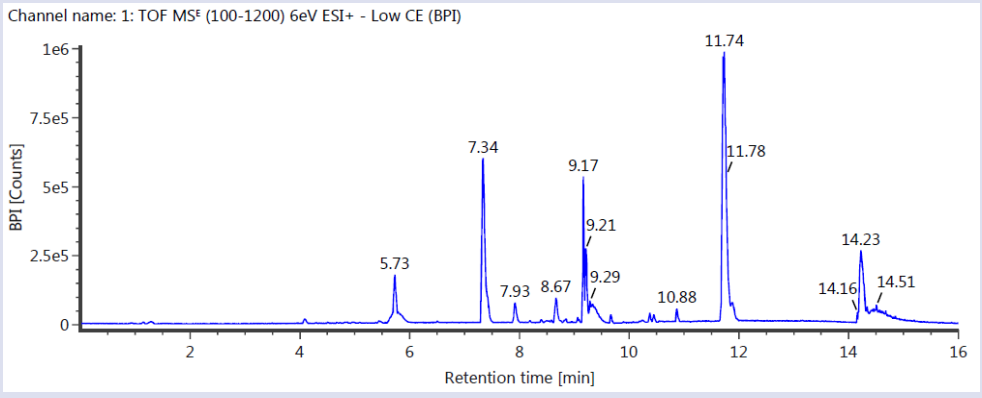

Figure 4: A Liquid chromatogram of the chemical constituents of RFS extract

Table 1: Chemical Composition of RFS extract.

No	Component name	Observed m/z	Retention time (min)	Formula
1	Poricoic acid B	485.3259	7.35	$C_{30}H_{44}O_5$
2	Abrusoside A	647.3789	5.73	$C_{36}H_{54}O_{10}$
3	Epianhydrobelachinal	469.3307	8.67	$C_{30}H_{44}O_4$
4	23-Acetate alisol K	527.3341	7.92	$C_{32}H_{46}O_6$

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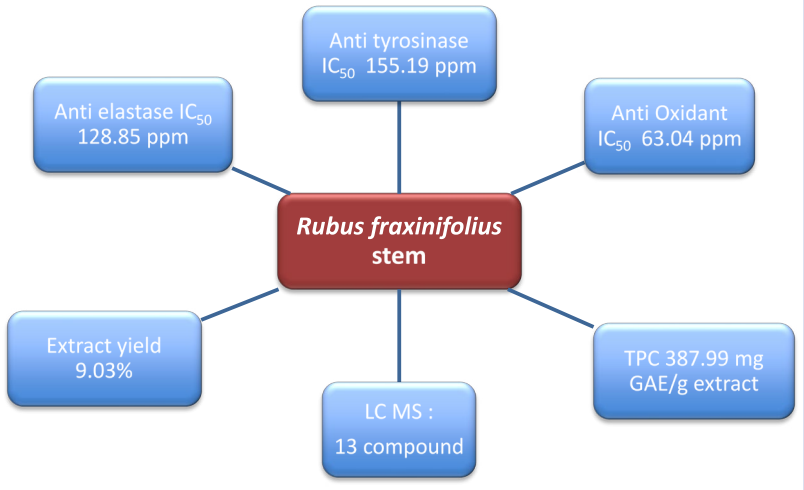
GRAPHICAL ABSTRACT



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