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SHORT COMMUNICATION

Anti-elastase, Antioxidant, Total phenolic and Total Flavonoid Content of Macassar Kernels (*Rhus javanica* L) from Pananjung Pangandaran Nature Tourism Park- Indonesia

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Abstract

Background: Phytochemicals are present as important substances in natural resources and therefore, have aided in invention of the anti-oxidant, and anti-elastase properties of polyphenol compounds present in Indonesian herbs such as Macassar Kernels (*Rhus javanica* L). **Objective:** This research aimed to investigate anti-elastase, and antioxidant properties. **Methods**: Ethanolic extract of Rhus Leaves (RL), Rhus Stem (RS), Rhus Greenish Fruit (RGF), and Rhus Blackish-grey Fruit (RBF) were prepared individually by solvent extraction method. Anti-elastase activity was carried out with elastase from porcine pancreas. For quantitative phytochemical screening, DPPH radical scavenging assay, Ferric Reducing Antioxidant Power Assay (FRAP), Total Phenolic Content (TPC), Total Flavonoid Content (TFC) of Rhus javanica were estimated. **Result:** RS showed highest anti-elastase activity (45.30%±0.087), compared with RL (12.30%±0.004), and there were no activities in RGF and RBF. In DPPH assay, RS had lowest activity (IC50 561.05 µg/ml), compared with RBF (IC50 239.28 µg /ml), RGF (IC50 189.3I µg/ml), and RL (IC50 157.81 µg/ml). RS also has lowest FRAP activity (% inhibition = 26.60%±0.002), and TPC value (28.50±0.03 mgGAE/g dry weight). **Conclusion:** Test extracts showed anti-aging properties in different mechanisms. RS possessed the highest anti-elastase activity but had the weakest antioxidant activity.

Keywords: Antielastase, Antioxidant, *Rhus javanica* L., Total Flavonoid Content, Total Phenolic Content

Abbreviation

DPPH (2,2 -diphenyl-1-picrylhydrazyl)
FCR (Folin Ciocalteu Reagent)
FRAP (Ferric Reducing Antioxidant Power Assay)
PPE (Porcine Pancreas Elastase)
RBF (Rhus Blackish-grey Fruit)
RGF (Rhus Greenish Fruit)
RL (Rhus Leaves)
ROS (Reactive Oxygen Species)
RS (Rhus Stem)
TFC (Total Flavonoid Content)
TPC (Total Phenolic Content)
TPTZ (2, 4, 6 -tris(2-pyridyl)-striazine)
Q (Quercetin)

1. Introduction

Oxidative stress in skin plays a major role in the aging process. Photoaging as UV-radiation causes a severe

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emanation of bare skin[1]. UV induced ROS (Reactive Oxygen Species) in human skin is responsible for excess proteolytic activity that agitates the skin's three-dimensional integrity[2]. Proteins such as elastin undergo modifications and subsequent conformational changes when certain amino acids were changed to their oxidized forms during oxidative stress[3]. Human macrophage metalloelastase (MMP12) is the most active protease in elastin degradation. Dermal elastin bundles are denatured and consequently results in loss of elasticity, which is directly related to the reduced synthesis of elastin and increased degradation or de-structuring of elastic fibers[4].

Anti-oxidants could be an effective medication to treat skin aging caused by oxidative stress. Phytochemicals are important substances of natural resources and therefore, the investigation of the anti-oxidant, and anti- elastase properties of polyphenolic compounds present in Indonesian herbs such as Macassar Kernels (*Rhus javanica* L) of the family Anacardiaceae needs to be assessed. Macassar kernels have been used traditionally to treat dysentry and diarrhea. Ethanolic extract of Rhus javanica leaf contains high percentage of phenolics, medium percentage of glycosides, and low presence of flavonoids[5].

Based on previous research, 299 plant species in Jeju island were investigated for their potential ability to inhibit elastase activity. Leaf extract *Rhus javanica* showed strong antiaging ability by inhibiting 80. 8 ±0. 5 of elastase activity at 500ppm from extraction using 80% ethanol[6]. The five major phenolic compounds were successfully identified using LC-MS (Liquid chromatography-mass spectrometry) as gallic acid, methyl gallate, syringic acid, pentagalloylglucose, and protocatechuic acid. Those chemical substances might have strong protective effects against oxidative damage which might cause a serious disease and aging process[7]. In an other report, there were seven phytochemicals which were isolated from nutgall stem bark of *Rhus javanica*. These substances were identified as gallic acid (1), 5-methylresorcinol (2), methylgallate (3), 3,4,5-trihydroxyacetophenone 4-O-β-Dglucop yranoside (4), 3-hydroxy-5-methylphenol 1-O-β-D-(6'- galloyl) glucopyranoside (5), scopoletin (6), and phlorizin (7)[8].

Several scientific publications have reported about total polyphenol, flavonoid and antioxidant activity of *Rhus javanica* but the research on its inhibition of elastase activity and the correlation with antioxidant activity has not been carried out yet. Therefore, the present experiment was performed to determine the ability of *Rhus javanica* and its antiaging by inhibiting elastase activity.

2. Materials and Methods

2.1 Plant Materials

Rhus javanica leaf, stem, and fruits (greenish and blackish-grey) were collected from Pananjung Pangandaran Tourism Park (108°39'18" - 108°39'52" E and 7°42'16" - 7°42'35" S) and authenticated (No. 3912/11.CO2.2/PL/2019) at School of Life Science and Technology, Institut Teknologi Bandung, Indonesia. The leaf, stem, greenish fruit, and blakish-grey fruit samples have been indicated as *Rhus* Leaf (RL), *Rhus* Stem (RS), *Rhus* greenish fruit (RGF) and *Rhus* Blackish-grey fruit (RBF).

2.2 Chemicals and Reagents

Trizma base (Sigma Aldrich No.T1503), SANA (N-Succinyl-Ala-Ala-Ala-p-nitroanilide) from Sigma Aldrich SLBR 7591V , PPE (porcine pancreas elastase) from Sigma Aldrich SLBV 9311, quercetin (Sigma Aldrich, India), gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl) ;TPTZ (2,4,6-tris(2-pyridyl)-striazine); Folin and Ciocalteu's phenolic reagent from Sigma Aldrich (Germany), acetic acid; aluminum chloride; DMSO (dimethyl sulfoxide); ethanol 96%; methanol; sodium acetate; hydrochloric acid; ferric chloride; sodium carbonate; FCR (Folin Ciocalteu Reagent) from Merck (Germany).

2.3 Preparation of Extracts

Fresh samples of *Rhus javanica* were separately collected into 4 samples (The leaf, stem, greenish fruit, and blackish-grey fruit). The samples were oven dried at 50°C until a stable weight was attained. The dried samples then were individually ground into fine particles using a grinding machine. A total of 50 g powder was macerated with 500 ml of 96% ethanol. It

was collected in a beaker, covered and protected from light, and stirred often.

Every 24 hours, the powder – solvent mixtures were filtered using Whatman paper. The process was repeated over five cycles and evaporated at 40°C using water bath to attain dryness. The concentrated extracts were then stored at -4°C for further usage.

2.4 DPPH Free Radical Scavenging Activity

Free radical scavenging activity was quantified by DPPH. The measurement was carried out using microplate reader with an established method adapted by Bobo Garcia and Zhang Chengting *et al.* with slight modification[9,10]. The previous publication reported that there was no significant difference between standard method (spectrophotometer) and microplate method[9]. Briefly, 20 µl sample in absolute methanol with various concentrations (100 – 850ppm) and 180 µl DPPH 100 µg/ml were diluted in 96-well microplate. The mixture was combined until it turned homogenous and was incubated in a protected dark room at room temperature for 30 minutes. Quercetin was used as a reference antioxidant and methanol was used as a control. The absorbance was measured at 517 nm in triplicate. The percentage inhibition of the DPPH radical was quantified by using the following formula:

$$\%DPPH = \frac{CA - SA}{CA} \times 100\%$$

CA = Control Absorbance

SA = Sample absorbance

2.5 FRAP Free Radical Scavenging Activity

FRAP (Ferric reducing antioxidant power assay) was a very useful technique to quantify antioxidant capacity on food and plants. FRAP test was carried out by microplate reader with an established method conducted and adapted by Tomasina and Fernandes protocols[11,12]. The FRAP reagent was freshly made by combination of 300 mM acetate buffer pH 3.6, 10mM TPTZ dissolved in 40 mM HCl, and 20 mM ferric chloride hexahydrate in distilled water (10:1:1). Quercetin was used as standard control. The reagent (280 µL) and sample in methanol solution (20 µL) were added to each well in triplicate precisely and incubated in protected dark room for 30 minutes at 37°C. The absorbance was recorded at 593 nm. The percentage inhibition of FRAP was quantified by using the following formula:

% Inhibition = (sample absorbance – control absorbance) x 100%

3. Elastase Inhibitory Activity Assay

Extract from different parts was prepared and diluted into various concentrations. Elastase Inhibitory activity assay was performed using an established protocol from Sigma Aldrich with slight modifications according to the previous research (Wittenauer *et al.* and Popoola *et al.*)[13,14]. 20 µl of plant extract in DMSO solution, 130 µl Tris HCl buffer pH 8.0 and 0.022 unit PPE were incubated within 15 minutes at 25°C. 0.29 mM SANA substrate was then added and mixed immediately and the mixture was then reincubated within 15 minutes with light protection at room temperature. Quercetin was used as standard control. The absorbance was measured at 401 nm using microplate reader Versamax. Anti- elastase activity was calculated using the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{C - S}{C} \times 100\%$$

where, C = Control absorbance; S = Sample absorbance

3.1 Total Phenolic Content

Determination of total phenolic content was conducted according some modified protocols (Shannon *et al.*) and Farasat Method[15,16]. 20 µl extract in absolute methanol, and 100 µl of FCR were mixed homogenously in a 96-well of microplate. 80µl sodium carbonate solution was added into the mixture after 5 minutes' incubation at room temperature. The absorbance was measured at 720 nm after incubated for 120 minutes. Calculation of total phenolic content resulted in gallic acid equivalents per gram of extract (GAE/g) which was extrapolated from a calibration curve of gallic acid (5–25 ppm).

3.2 Total Flavonoid Content

Content of total flavonoid was carried out using a method conducted by Farasat with microplate reader Versamax[16]. Briefly, 20 µl extracts in absolute

methanol, 20µl aluminum chloride solution, 20µl of sodium acetate, and 140 µl distilled water were added immediately in a 96 –well of microplate. Absorbance was measured at 415 nm after 30 minutes' incubation at room temperature. The total flavonoid contents were estimated from quercetin equivalents per gram of extract (QE/g) which was extrapolated from a calibration curve of quercetin (5–15ppm).

3.2 Data Analysis

All statistical analyses were performed using SPSS 24.0 for Windows. The data was analyzed to gain a Pearson correlation. A technique for investigating the correlation of two quantitative data, and finding association of two variables.

4. Result

4.1 DPPH and FRAP Free Radical Scavenging Activity

The antioxidant quantity of the test samples was concluded by DPPH and FRAP techniques generally used in plant and food research for screening antioxidant activity[17]. RL showed the best potency to scavenge DPPH free radicals compared with other samples. The half maximal inhibitory concentration (IC50) of samples toward DPPH free radical scavenging activity can be seen in Table 1. IC50 value with respect to quercetin as standard. Lower IC50 value showed more antioxidant potential. The DPPH values were also remarkably good for RGF in contrast, RS revealed a lack value of antioxidant activity.

Table 1. DPPH Assay

Sample	Equation	R^2	IC_{50}
Quercetin	y=9.1305x- 4.388	0.9908	5.957
RL	y=0.2739x+6.7183	0.9996	157.699
RS	y=0.0612x+15.664	0.9997	561.046
RGF	y=0.2696x-1.0391	0.9992	189.31
RBF	y=0.1809x+6.715	0.9994	239.276

The FRAP was a simple technique which showed high reproducibility. This method calculated the capability of antioxidants to reduce ferric iron[17]. Figure 1 exhibited FRAP value from 4 samples of the extract. Quercetin assumed to have baseline antioxidant potency. Various samples at the same concentration had showed RL to have the highest FRAP value (89.70±0.027%), compared to RGF (72.60±0.007%), RBF (66.40±0.018%), and RS (26.60±0.002%).

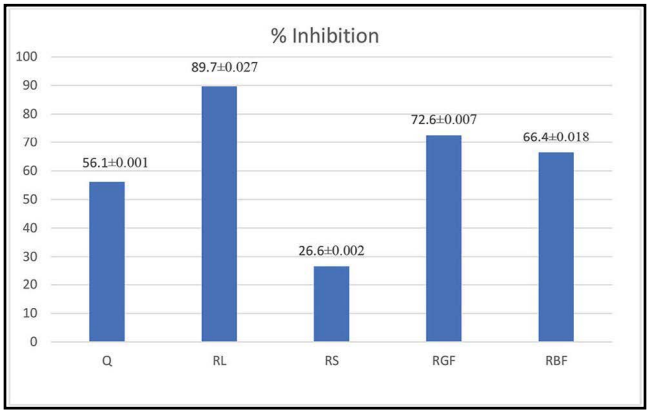

Figure 1. FRAP Activity of Quercetin, RL, RS, RGF, and RBF. There were diluted in methanol to reach the final concentration of 0.67; 66.67; 66.67; 66.67; 66.67 (µg/mL).

4.2 Elastase Inhibitory Activity Assay

Each extract at a concentration of 100 µg/ml was used as a sample test. The detailed percentage of inhibition is given in Table 2. The best anti-elastase potency was given by Quercetin (64.92±16.20%) compared to RS (45.30%), RL (12.30%), while there were no elastase inhibition activities from RGF and RBF.

Table 2. Elastase inhibition activity of *Rhus javanica* extracts from different part

Sample	Elastase inhibition (%)
Quercetin	64.92±16.20
RL	12.30±0.004
RS	45.30±0.087
RGF	NA
RBF	NA

Note: NA= no activity

4.3 Total Phenolic and Flavonoid Content

Based on Figure 2, the highest value of total phenolic content (TPC) was obtained in RGF (53.66 mg/1 g of

extract), compared to RL (51.91 mg/ 1 g of extract), EBF (32.74 mg/ 1 g of extract) and ES (28.50 mg/1 g of extract). As a calibration curve, Gallic acid was used as standard (Figure 3). RL exhibited the highest total flavonoid content (17.78±0.002 mg/ 1g of extract), whereas RBF contained a lower amount of flavonoid (11.3±0.02 mg/ 1 g of extract), followed by RGF (9.4±0.012 mg/1g of extract), and RS (5.06±0.004mg/ 1 g of extract). As a calibration curve, Quercetin was used as a standard (Figure 4).

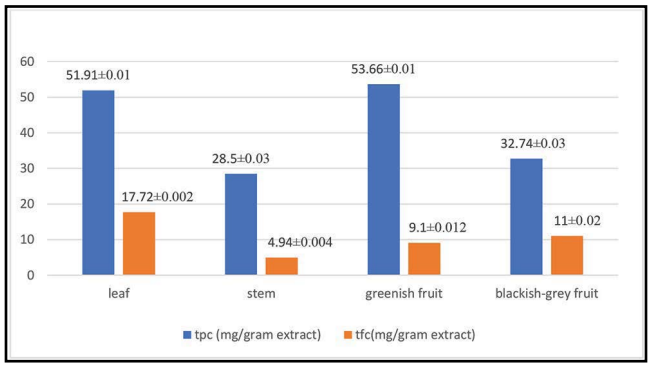

Figure 2. Total Phenolic and flavonoid contents of Rhus javanica extract from different parts.

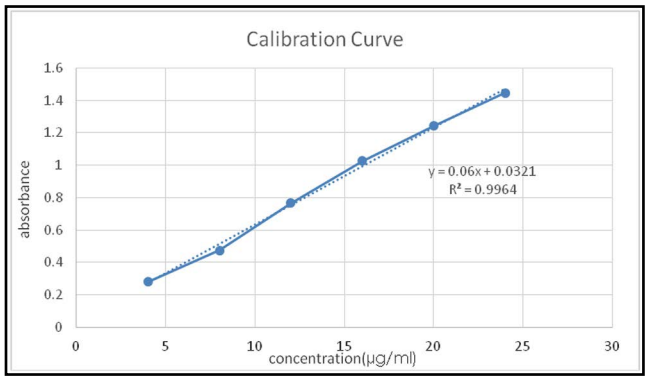

Figure 3. Calibration curve of Gallic acid for TPC value.

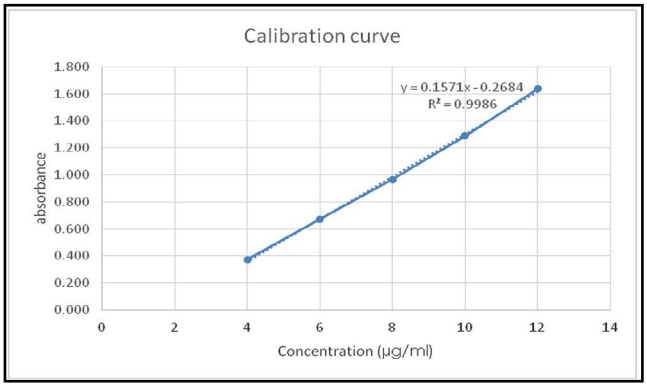

Figure 4. Calibration curve of Quercetin for TFC.

5. Discussion

Based on the principle, each species of nature has its own characteristics. The major phenolic compounds of Rhus Javanica have been reported as were Gallic acid, methyl gallate, syringic acid, pentagalloylglucose, and protocatechuic acid[7]. Due to its chemical compound, polyphenols had potent antioxidants to scavenge Reactive Oxygen Species (ROS). Polyphenols may have inhibited activity of proteolytic enzyme such as elastase[13]. Based on previous research, species of Rhus javanica in Jeju Island had strong anti-aging ability by inhibiting elastase 80. 8 ±0. 5 at 80% ethanol leaf extract 500 µg/ ml (Moon J et al, 2010). In present study, RS showed potent anti-elastase activity that was approximately four-fold greater than RL and slightly weak compared to Quercetin. In numerous studies reported, Quercetin had the ability to deactivate proteinase activity which was the most potent inhibitor of elastase release[18].

Based on Table 3, total phenolic and flavonoid content showed strong correlation with DPPH and FRAP assay. DPPH and FRAP were simple techniques of antioxidant capacity assessment generally used in plant and food research[17]. With regards to DPPH, RL and RGF have high value of TPC and TFC and may have contributed to express potent DPPH scavenging ability. It may be correlated with phenol chemical structure's ability of donating the Hydrogen atom. The result seen in FRAP scavenging ability also ranks similar to DPPH assessment. RL and RFG also showed greater ability to reduce ferric ion compared to RS and RBF which contained a small amount of phenolic and flavonoid.

Aging process impacted many complex pathways. Based on Table 3, it was suggested that value of phenolic and flavonoid content might refer to antioxidant properties to some assessment unless they were not well correlated with anti-elastase activity. RS showed the poorest antioxidant activity but it was the best source of elastase inhibitor.

Table 3. Pearson Correlation's value in some extracts

	TPC	TFC
DPPH	-0.787	-0.789
FRAP	0.808	0.897
Elastase inhibition	-0.569	-0.509

6. Conclusions

All the samples obtained from *Rhus javanica* showed phenolics, flavonoids and antioxidant activity, despite having different competences. The leaves and greenish fruit extracts of *Rhus javanica* had high antioxidant properties but showed poor elastase inhibition activity. Different method of antioxidant and anti protease activity may bring out a different result. Purification of these extracts and further investigations on the different mechanisms shall be studied further.

7. Acknowledgement

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