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Artikel Penelitian

H_2O_2-Scavenging Activity and Hyaluronidase Inhibition Scutellarin and Apigenin in Basil Leaf Extract

Aktivitas Pemerangkapan H_2O_2 dan Inhibisi Hyaluronidase Scutellarin dan Apigenin dalam Ekstrak Daun Kemangi

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ABSTRACT

The potential utilization of flavonoid compounds, especially scutellarin, and apigenin, contained in basil (Ocimum basilicum L.) *leaf extract to manage the aging effects on the skin, that occurs because of over-activated hyaluronidase enzyme and oxidative stress due to hydrogen peroxide (H_2O_2) radicals, is not well known. This study was conducted to assess the H_2O_2 scavenging activity and the inhibition of hyaluronidase from scutellarin and apigenin at various concentrations. The study was conducted by measuring H_2O_2 scavenging inhibition and hyaluronidase inhibition of scutellarin and apigenin in various concentrations using the spectrophotometry method. The various activity was tested using the One-Way ANOVA test followed by Tukey post hoc test. IC_{50} values were calculated based on linear regression equations of H_2O_2 scavenging inhibition and hyaluronidase inhibition. The analysis showed the highest H_2O_2 scavenging activity was found in scutellarin with IC_{50} 158.76 µg/mL. Scutellarin has greater scavenging activity than apigenin. Hyaluronidase inhibition of scutellarin with IC_{50} 35.25 µg/mL, while apigenin was 162.86 µg/mL. Scutellarin has higher hyaluronidase inhibition activity than apigenin. Antioxidant and antiaging effects of basil leaf extract caused by phytochemical compounds contained, especially scutellarin.*

Keywords: *Antiaging, antioxidant, hyaluronidase, hydrogen peroxide, Ocimum basilicum L.*

ABSTRAK

Potensi pemanfaatan senyawa flavonoid, khususnya *scutellarin* dan *apigenin*, yang terkandung dalam ekstrak daun kemangi (*Ocimum basilicum L.*) untuk mengatasi efek penuaan pada kulit, yang dapat disebabkan oleh aktivitas enzim hialuronidase yang berlebihan serta stres oksidatif akibat radikal hidrogen peroksida (H_2O_2), belum diketahui dengan pasti. Penelitian dilakukan untuk menguji aktivitas pemerangkapan radikal H_2O_2 dan inhibisi hialuronidase dari senyawa *scutellarin* dan *apigenin* pada berbagai konsentrasi. Penelitian dilakukan dengan mengukur aktivitas pemerangkapan H_2O_2 dan penghambatan hialuronidase senyawa *scutellarin* dan *apigenin* dalam berbagai konsentrasi menggunakan metode spektrofotometri. Perbedaan aktivitas tersebut diuji dengan uji *One Way ANOVA* dilanjutkan Tukey HSD *post hoc test*. Nilai IC_{50} dihitung berdasarkan persamaan regresi linier pemerangkapan H_2O_2 dan inhibisi hialuronidase. Hasil analisis menunjukan aktivitas pemerangkapan radikal H_2O_2 tertinggi ditemukan pada senyawa *scutellarin* dengan IC_{50} 158,76 µg/mL. *Scutellarin* memiliki inhibisi hialuronidase dengan nilai IC_{50} 35.25 µg/mL, sementara *apigenin* 162,86 µg/mL. *Scutellarin* memiliki aktivitas inhibisi hialuronidase yang lebih tinggi daripada apigenin. Efek antioksidan dan anti penuaan ekstrak daun kemangi kemungkinan diakibatkan oleh senyawa fitokimia yang terkandung, khususnya scutellarin.

Kata Kunci: Antiaging, antioksidan, hidrogen peroksida, hyaluronidase, *Ocimum basilicum L*.

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INTRODUCTION

Skin aging is a complex natural process characterized by the loss of skin ability to retain its physiological functions and structural components (1). The diminishment or reduction of structural components in the skin mainly occurred, especially in the extracellular matrix region located in the dermal layer which is indicated by the formation of wrinkles and sagging. Various factors affecting the aging can be classified as external factors such as genetic and internal factors including lifestyle and exposure to ultraviolet (UV) radiation (2). These factors modulate the skin aging process through multiple pathways and are often characterized by the changes of reactive oxygen species (ROS) concentration.

Reactive oxygen species, one of them is hydrogen peroxide (H_2O_2) radicals, the build-up is an important marker of cell aging (3,4). In skin aging, superoxide dismutase (SOD) is highly expressed in keratinocyte, while catalase is absent. ROS is very easily transferred between cells which causes the surrounding cells such as skin fibroblasts to be easily exposed to radicals (5). Exposure of radicals induced apoptosis in fibroblasts through a series of cascade pathways that activate protein caspase (6). This process does not only result in the loss of cellular integrity but also its extracellular matrix. Furthermore, ROS also affects the protein expression associated with matrix remodeling and pigmentation. Therefore, the inhibition of radical compounds served as an important strategy for treating skin aging. Besides, hyaluronic acid (HA) also plays an important role in human skin. These anionic non-sulfate glycosaminoglycans form the core of proteoglycans, which are responsible for maintaining proper skin volume and flexibility. Under oxidative stress, hyaluronidase (HAase), an enzyme responsible for depolymerization of HA, is over-activated and excessively breaks down HA, leading to the destruction of proteoglycan tissue. This results in skin homeostasis deregulation, worsens inflammation and allergic conditions, also improves the appearance of aging skin (7).

Phytochemical compounds are secondary metabolites derived from plants that have very diverse biological activities (8). This underlies the utilization of plants as herbal medicines, including basil that is often found in Indonesia. The previous studies have shown that basil has biological activities such as anti-inflammatory, anti-diabetic, anti-hypertensive, antimicrobial, and also anti-aging (9,10). The previous study showed that basil leaf contains flavonoid compounds, scutellarin, and apigenin (11,12) which are responsible for various biological activities (13-15). There are indications that flavonoid consumption is associated with antiaging potential (16), and antioxidant activity (15) but the potential utilization of basil extract and its compound scutellarin and apigenin toward aging is not widely understood. Flavonoid compounds found in basil extract, scutellarin and apigenin are subjected to H_2O_2 scavenging and hyaluronidase inhibition. This research was conducted to describe the H_2O_2 scavenging activity and hyaluronidase inhibition potential of scutellarin and apigenin compounds in basil extract.

METHODS

Scutellarin, Apigenin Preparation

The scutellarin [Biopurify Phytochemicals Ltd, BP1277] and apigenin [Biopurify Phytochemicals Ltd, BP0177], that known to be flavonoid compounds in basil leaf extract (11,12), were purchased from Biopurify Phytochemicals (Chengdu, China). Each compound for H_2O_2 scavenging assay was dissolved in dimethyl sulfoxide (DMSO) (Merck, 102952) until it reached the following concentrations: 50, 25, 12.5, 6.25, 3.13, and 1.56 μg/mL, while for hyaluronidase inhibition assay, the compounds were dissolved in DMSO until reaching the following concentrations: 83.33, 41.67, 20.83, 10.42, and 5.21 μg/mL.

Hydrogen Peroxide Scavenging Assay

The H_2O_2 scavenging assay was carried out to measure the antioxidant activity of the intended compound, especially the activity against radical peroxide. The assay was done using a colorimetric assay based on radical reduction and stain formation. Antioxidants play a role through their ability to continue contributing hydrogen atoms, transforming radical compounds into stable forms so that the levels of antioxidant activity of compounds reflect the ability to reduce oxidation (21). The level of H_2O_2 scavenging activity of the tested compounds was measured based on their reduction capacity against peroxide radicals. Neutralized radicals are unable to oxidize iron ions; thus, Fe (II) will react with phenanthroline, a poly organic compound that is capable of forming complexes with metal ions. The O-Phenanthroline-Fe^{2+} complex has a strong orange color which can be quantified by a spectrophotometer (17). The greater the absorbance measured by the spectrophotometer indicated the higher rate of captured peroxide radical.

Hydrogen peroxide scavenging activity was measured using a method found by Mukhopadhyay *et al.* with slight modifications (4,17,18). The prepared mixture was introduced into a 96-well plate and incubated for 5 minutes at room temperature. Then, 75 μL 1,10-phenanthroline (Sigma Aldrich, 131377) was added to the mixture and re-incubated for 10 minutes at room temperature. The absorbance was measured using a spectrophotometer at a wavelength of 510 nm. The results were described as scavenging percentage calculated using the following equation:

$$\%\ \text{scavenging} = \frac{c-s}{c} \times 100$$

C: absorbance of activity without sample

S: absorbance of activity with the addition of samples tested

Hyaluronidase Inhibition Assay

The hyaluronid $\%\ \text{scavenging} = \frac{c-s}{c} \times 100$ measured using a method devel nd Tu & Tawata (2015) with minor modifications (4,19,20). The mixture consisting 25 μL samples (0.78–50 μg/mL), 3 μL enzymes hyaluronidase from bovine testes type IS (0.02 mg/mL) (Sigma Aldrich H3506), and 12 μL phosphate buffer (300 mM, pH 5.35) (Sigma Aldrich, 0751) was incubated at 37°C for 10 minutes. The control mixture containing 3 μL enzymes, 37 μL phosphate buffers, and a blank containing 15 μL phosphate buffers and 25 μL samples was incubated at 37°C for 10 minutes. A mixture of 10 μL hyaluronic acid was added and re-incubated at 37°C for 45 minutes. The stop solution (100 μL albumin) was added into the solution and stored at room temperature for 10 minutes. The absorbance was measured using a spectrophotometer at a wavelength of 600 nm. The results are described as inhibition percentage calculated using the following equation:

% inhibition = (1 - c/s) x 100

C: absorbance of enzyme activity without sample

S: absorbance of enzyme activity with the addition of samples tested

Data Analysis

The data of H_2O_2 scavenging and anti-hyaluronidase activity were analyzed using the One-Way ANOVA test followed by Tukey HSD post hoc test. Furthermore, the IC_{50} value of each activity was calculated based on a linear regression equation.

RESULTS

Hydrogen Peroxide Scavenging Activity

The results (Table 1), showed that contained flavonoid in the basil leaf extract was related to the decreasing peroxide levels in the test solution. The highest activity of scutellarin was found at 50 µg/mL concentration, while the lowest activity was at 1.56 µg/mL concentration. The differences among concentrations of H_2O_2 scavenging activity can be seen in Table 2 for scutellarin and apigenin as the result of one-way ANOVA test. The p-value was showed that among the compound's concentration was significantly different except for scutellarin at 3.13 µg/mL and 1.56 µg/mL. The smaller samples (scutellarin and apigenin) show the smaller H_2O_2scavenging activity.

Table 1. H_2O_2 scavenging activity of scutellarin and apigenin

Concentrations of Scutellarrein and Apigenin (µg/mL)	Average Activity Trapping of H_2O_2 (%)	
	Scutellarin	Apigenin
50.00	98.03 ± 2.32	81.14 ± 0.90
25.00	72.23 ± 6.67	47.24 ± 0.39
12.50	49.30 ± 0.29	35.04 ± 0.40
6.25	34.81 ± 0.65	24.09 ± 0.49
3.13	25.85 ± 1.91	19.82 ± 0.42
1.56	24.66 ± 0.93	16.58 ± 0.48

Note: Data were displayed as mean ± standard deviation. Each sample was done triplicate.

The IC_{50} value in peroxide radical scavenging activity was defined as the concentration of the compound needed to bind 50% of the peroxide radical contained in the solution. It was determined by linear regression analysis in each compound. The IC_{50} value of scutellarin and apigenin was showed in Tabel 3. The concentrations of scutellarin and apigenin greatly influenced its scavenging activity based on the R^2 value that was close to 1. The results of the analysis showed that the highest peroxide radical scavenging activity was found in scutellarin compounds with IC_{50} around 158 µg/mL. Scutellarin compounds tend to have greater scavenging activity than apigenin.

Table 3. IC50 value trapping H_2O_2 by scutellarin and apigenin

Samples	Equation	R^2	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Scutellarin	Y = 0.1519x + 26.699	0.92	153.40	
Scutellarin	Y = 0.1522x + 25.213	0.98	162.86	
Scutellarin	Y = 0.1603x + 24.349	0.96	160.02	158.76 ± 4.8
Scutellarin (Average)	Y = 0.1548x + 25.42	0.96	158.76	
Apigenin	Y = 0.1291x + 16.002	0.99	263.35	
Apigenin	Y = 0.1315x + 16.028	0.99	258.34	
Apigenin	Y = 0.1308x + 15.711	0.99	262.15	261.28 ± 2.6
Apigenin (Average)	Y = 0.1305x + 15.914	0.99	261.28	

Note: The IC_{50} were displayed as mean ± standard deviation. The assay was done triplicate

Hyaluronidase Inhibition Assay

Hyaluronidase inhibition assay was performed to measure the inhibitory capacity of the compound against hyaluronidase. The hyaluronidase inhibition assay was based on the determination of the turbidity level of the solution. Dissolved hyaluronic acid increases the turbidity level, and the degradation degree will reduce the turbidity of the solution. The greater the absorbance value measured by spectrophotometry, the higher the level of hyaluronidase inhibition activity of a compound.

In Table 4, the anti-hyaluronidase activity of scutellarin and apigenin at various concentrations was showed. The results showed that the addition of scutellarin associated with the dose-dependent manner with the highest hyaluronidase inhibitory activity was found at a concentration of 83.35 µg/mL while the lowest was at a concentration of 5.21 µg/mL. Similar results were also found in apigenin.

Further analysis that can be seen in Table 5 showed that the hyaluronidase inhibitory activity of scutellarin among concentrations of 5.21, 10.42, and 20.83 µg/mL did not have significant differences. This shows that a decrease in the scutellarin concentration decreases the inhibitory activity to a concentration of 20.83 µg/ml, a further decrease in concentration does not make a significant

Table 2. Post hoc test result of the H_2O_2 activity of scutellarin and apigenin

50.00 µg/mL		25.00 µg/mL		12.50 µg/mL		6.25 µg/mL		3.13 µg/mL		1.56 µg/mL	
Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin
-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.000	-	-	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	-	-	0.001	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.001	0.000	-	-	0.032	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.032	0.000	-	-	0.014	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.000	0.996*	0.000	-	-

Note: The symbol (*) indicates that the data was no significant (P>0.05). The p-value was based on One Way ANOVA followed by the Tukey HSD post hoc test.

Table 5. The P-value for the anti-hyaluronidase activity of scutellarin and apigenin

83.35 µg/mL		83.33 µg/mL		41.67 µg/mL		20.83 µg/mL		10.42 µg/mL		5.21 µg/mL	
Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin	Scutellarin	Apigenin
-	-	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.002	-	-	0.005	0.695*	0.000	0.093*	0.000	0.002	0.000	0.001
0.000	0.000	0.005	0.695*	-	-	0.023	0.655*	0.000	0.021	0.000	0.008
0.000	0.000	0.000	0.093*	0.023	0.655*	-	-	0.217*	0.247*	0.186*	0.104*
0.000	0.000	0.000	0.002	0.000	0.021	0.217*	0.247*	-	-	0.100*	0.992*
0.000	0.000	0.000	0.001	0.000	0.008	0.186*	0.104*	0.100*	0.992*	-	-

Note: The symbol (*) indicates that the data was no significant (P >0.05). The p-value was based on One Way ANOVA followed by the Tukey HSD post hoc test

difference. Furthermore, the inhibitory activity of apigenin showed the significant difference was found in concentrations of 83.35 µg/mL.

Ic_{50} was defined in the hyaluronidase inhibition test as the compound concentration needed to inhibit 50% hyaluronidase in the test solution. Linear regression analysis was performed to find out the IC_{50} owned by each compound. The concentrations of scutellarin and apigenin greatly influenced its scavenging activity based on the R^2 value that was close to 1. Scutellarin had an IC_{50} value of 35.25 ± 5.43 µg/mL, while apigenin was 162.86 ± 3.73 µg/mL. Scutellarin compounds tend to have more inhibitory activity than apigenin (Table 6).

Table 6. IC_{50} values of anti-hyaluronidase by scutellarin and apigenin

Samples	Equation	R^2	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Scutellarin	Y = 0.2716x + 42.114	0.95	29.11	35.25 ± 5.43
Scutellarin	Y = 0.2731x + 39.242	0.97	39.41	
Scutellarin	Y = 0.2716x + 39.917	0.93	37.23	
Scutellarin (Average)	Y = 0.2721x + 40.424	0.97	35.25	
Apigenin	Y = 0.193x + 10.701	0.86	203.63	162.86 ± 37.3
Apigenin	Y = 0.3165 x + 8.9477	0.95	130.33	
Apigenin	Y = 0.2651x + 9.0277	0.95	154.62	
Apigenin (Average)	Y = 0.2579x + 9.5589	0.96	162.86	

Note: The IC_{50} were displayed as mean ± standard deviation. The assay was done triplicate

DISCUSSION

Factors that lead to skin aging were ROS and hyaluronidase enzyme activity. ROS, one of them is H_2O_2 radicals, affects the protein expression associated with matrix remodeling and pigmentation while hyaluronidase enzyme could break down hyaluronic acid excessively and leads to the destruction of proteoglycan tissue when it was over-activated (7). Basil leaf was contained flavonoid compounds like scutellarin and apigenin (11,12). In this study, scutellarin and apigenin were used to determine its H_2O_2 scavenging and hyaluronidase activity potential. Result of this study identify that scutellarin and apigenin in basil leaf extract have the ability to bind H_2O_2 radicals and inhibit hyaluronidase enzyme, specifically scutellarin

The hydrogen peroxide scavenging activity assay showed that both of the compounds have antioxidant potential, especially scutellarin. Based on its structure, scutellarin has more hydroxyl groups than apigenin, which affects the ability to scavenge scutellarin because the biological activity of flavonoids is related to its structure (14). Flavonoids are polyphenolic compounds characterized by one or more phenol groups in their structure, thereby reducing radicals to their neutral form (22).

Increased levels of radical compounds found in tissue are the main characteristic of cell aging. This condition is caused by the loss of homeostasis in the production and elimination of free radicals (3). Increased ROS, one of them is H_2O_2 radicals, the concentration may increase further to a condition known as oxidative stress, which is closely related to aging, especially skin aging. This condition can cause various health problems due to the nature of the radicals that are reactive to various biomolecules contained in the body (23). Hydrogen peroxide (H_2O_2) is known to induce apoptosis in skin fibroblast cells through a series of signaling pathways that lead to caspase 3 protein activation (6). This process may further cause structural change on the skin since fibroblasts are involved in the formation of the extracellular matrix. This mechanism is based on the ability of antioxidants to trigger cell proliferation which plays a role in the regeneration of damaged tissue (24).

The ROS plays an important role in signaling the normal function pathways of radical compounds, but only at low concentrations (25). Radical compounds have an important role in modulating the expression of matrix metalloproteinase (MMPs), an important enzyme that plays a role in the degradation of collagen type 1, 2, and 3 found in many extracellular matrices. Oxidative stress caused by UV irradiation in test animals is known to trigger excess MMP1 synthesis (26). Previous in vivo studies found topical N-acetylcysteine (NAC), a glutathione precursor compound (GSH), that are known to inhibit MMP1 protein expression (26). Another study found that catalase can reduce MMP1 levels in the animal model (27). Because of its ability to scavenge peroxide radicals, scutellarin and apigenin have great potential in indirectly inhibit MMP1.

Chronic UVA and UVB irradiations in test animals are strongly related to the loss of elastic tissue of the skin (28), which occurs due to a dramatic increase in elastase activity. Not only inducing MMP1 as described previously, but oxidative stress is also thought to contribute to elastase activation strongly. The administration of plant extracts is known to inhibit the degradation of elastase activity and maintain skin elastase (29,30). These findings support scutellarin and apigenin potentials to play a role in maintaining skin elasticity due to their antioxidant activity.

In addition to the structural changes, hyperpigmentation is another characteristic of skin aging. Pigmentation in human skin is caused by the formation of pigment molecules such as melanin, which are controlled by rate-limiting of the enzyme tyrosinase in melanocyte cells (30). Radical compounds can affect melanin production in melanocytes through modulation of the expression of melanogenic tyrosinase and tyrosinase-related protein 1 factors (31,32). Synthesis of alpha melanocytes and melanogenesis hormones can be prevented by administering GSH antioxidant compounds. Based on this, the antioxidant activity of scutellarin and apigenin has the potential to protect ROS-induced hyperpigmentation skin, especially hydrogen peroxide.

Adjacent to collagen and elastase, there are glycosaminoglycans (GAGs) that contribute to the development of extracellular matrix in skin tissue. Glycosaminoglycans are strong hydrophilic compounds that can hold water as much as 1000 times the volume they have (33). Because of its nature, GAGs, especially hyaluronic acid (HA) has an important role in maintaining the moisture possessed by the skin. In addition to its function in maintaining skin water content, HA has also been found to have a role as a periphery protein that connects elastin and collagen. The loss of HA is thought to contribute to a decrease in the integrity of the extracellular matrix of the skin tissue, which results in the formation of wrinkles (34,35), so the addition of hyaluronidase enzyme also plays an important step in skin aging. Hyaluronidase (HAase) enzyme responsible for depolymerization of HA and under oxidative stress it could over-activated and excessively break down HA (7).

This study shows that both flavonoid compounds have hyaluronidase inhibitory activity, and scutellarin was found to have activities that tend to be better than apigenin. Hyaluronidase inhibitory activity in flavonoid compounds is related to the number of hydroxyl groups it has (36). Flavonoid compounds could spontaneously bind hyaluronidase mainly through electrostatic forces, as well as hydrophobic interactions and hydrogen bonding. The presence of flavonoids was changed microenvironment and conformation of hyaluronidase. Since the binding of flavonoid affected the microenvironment of the hyaluronidase activity site, flavonoid caused the inhibition of hyaluronidase activity (37).

It can be concluded that the two phytochemical compounds, scutellarin and apigenin, in basil leaf extract have the ability to bind H_2O_2 radicals and inhibit hyaluronidase enzyme that support the potential for antioxidant and antiaging effects, specifically scutellarin.

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