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Reviewers Comments

Reviewer A 2016-09-01 05:29 PM	Subject: Antioxidant and Antiaging assays of Oryza sativa Extracts, Vanillin and Coumaric Acid

For author and editor

(a) *The antioxidative assessment was conducted using chemical assays, no primary cells or cell lines been used to test the toxicity and antioxidative activity.*

This is the preliminary study to observe the antioxidant and antiaging effects of rice extract. Surely, it will be further tested in cell culture to examine the toxicity and its activity as well. However, we conducted preliminary study to evaluate the free radicals scaveging activity. Afterward, the activity of extracts will be tested in oxidative stress-induced cell by measuring its ROS level with flowcytometer.

(b) *Although the authors compare the antioxidative activity of Oryza sativa with its isolated phytochemical entities yet such comparison using, existing results should be included in the discussion to evaluate the potential antioxidative activity of tested bodies. Also, comparative analysis of IC50 value regarding concentration unit should be same for verified data (Oryza sativa extract & phytoconstituents).*

We have mentioned existing results in discussion. We have convert

the antioxidant and antiaging activities compunds (coumaric acid, vanillin) from µM to µg/ml so we can compare among *O. sativa* extract and compounds

(c) Please look into the script of paper which requires grammatical and space correction at some places. Notably, the discussion should explore some interpretation on Ayurvedic lines used in boosting the health benefits.

We have corrected the grammatical and space errors. The corrected sentences are highlighted. We have also added Ayurvedic point of view in the beginning of the discussion.

Antioxidant and Antiaging assays of *Oryza sativa* Extracts, Vanillin and Coumaric Acid *

Running title: *Oryza sativa* as an antioxidant and antiaging

ABSTRACT

Aging is a natural process in human as accumulation of oxygen-derived free radicals which leads to the activation of hyaluronidase, collagenase and elastase, that can further contribute to cellular and tissue damage cell. Bioactive compounds from plants has been used as antioxidant that might inhibit aging processes as well. This study aimed to determine antioxidant and antiaging properties of *Oryza sativa* extract (OSE), and its compounds, vanillin and coumaric acid. The phytochemical analysis of OSE was performed with Farnsworth modified method. Antioxidant activities were performed by measurement of 2,2-diphenyl 1-pichylhydazyl (DPPH) free radical scavenger, ferric reducing antioxidant power (FRAP), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reducing activity, while antiaging assay were observed through inhibitory of elastase, collagenase, and hyaluronidase activities. Phytochemical analysis showed the presence of terpenoids and saponins in high level. OSE showed lowest DPPH activity (IC_{50}=314.51 µg/mL) compared to vanillin (IC_{50}= 283 µg/mL) and coumaric acid (IC_{50}= 255.69 µg/mL). In ABTS assay, OSE resulted lowest activity (IC_{50}= 145.67 µg/mL), compared to vanillin (IC_{50}= 4.96 µg/mL) and coumaric acid (IC_{50}= 1.67 µg/mL). OSE also showed the lowest FRAP-reducing activity (21.26 µM Fe(II)/µg), compared to vanillin (35.05 µM Fe(II)/µg) and coumaric acid (48.52 µM Fe(II)/µg). OSE showed lowest collagenase, elastase, and hyaluronidase inhibitory activity (IC_{50} = 816.78, 107.51, and 203.13 µg/mL), compared to vanillin (IC_{50} = 16.27, 14.46, 45.23 µg/mL respectively) and coumaric acid (IC_{50} = 146.89, 25.38, 8.21 µg/mL respectively). In summary, OSE possess lowest antioxidant and antiaging activities compared to vanillin and coumaric acid.

Keywords: *Oryza sativa*, vanillin, coumaric acid, antioxidant, antiaging.

1. INTRODUCTION

Skin aging is the natural process due to photoaging by environmental factors such as chronic UV radiation. The repetitive exposure to UV radiation cause accelerated physical changes in the skin and connective tissue through the formation of lipid peroxides, the cell contents and Reactive Oxygen Species (ROS)[1]. It leads to loss of skin elasticity implicated in

formation of wrinkling, uneven pigmentation, brown spots, laxity and leathery appearance, solar elastosis, actinic purpura, precancerous lesions, skin cancer, and melanoma[2,3,4].

During aging process, collagen, elastin, and hyaluronic acid decrease, that causes loss of strength and flexibility in the skin, resulting in visible wrinkles. It is also related to increasing enzymes activity including collagenase, elastase and hyaluronidase. Collagenase is known as an enzyme that plays role in the degradation of collagen. Collagen is the main component with percentage of 70-80% of the total skin weight, the increasing degradation of collagen is significant in the photoaging process[5-6]. Hyaluronan or hyaluronic acid is one of important components of the tissue matrix substance and has a role in the development, growth, and repair of damaged tissue[6]. Meanwhile, elastin play a role in the maintenance of skin elasticity, but elastase can degrade it[7]. Degradation of the extracellular matrix (ECM) has been directly linked to skin aging and is correlated with an increase in activity of certain enzymes involved in skin aging[8-9]. Inhibition of these enzymes is crucial in anti-aging prevention[10].

It has been reported that skin aging occurs in the presence of cumulative endogenous damage due to reactive oxygen species (ROS)[11]. ROS are defined as oxygen-containing, highly reactive species. ROS are generated constantly during normal cellular metabolism which is essential for biological functions. Excessive ROS causes oxidative stress and damage to biological molecules[12-13]. Previous studies have investigated that continuous ROS exposure can stimulate skin aging through antioxidant system destruction, wrinkle formation, and melanogenesis[12]. ROS are usually removed from the body through antioxidant defense system[14]. Thus, maintaining antioxidant homeostasis is an appropriate strategy to prevent skin aging.

(**Figure 1**. Chemical structure of vanillin and coumaric acid)

Antioxidant properties derived from natural sources have been proposed for aging prevention[15]. Bioactive compounds contained in plants such as isoflavones, anthocyanins, and catechins may have promising antioxidant activity against ROS[16]. *Oryza sativa* L. is one of the most produced and consumed cereals in the world that contain phenolic compounds, tocopherols, tocotrienols, and g-oryzanol. Phenolic acids were identified in the lignin fraction of rice grain *(O. sativa)* such as caffeic, chlorogenic, p-coumaric, ferulic, gallic acids, p-hydroxybenzoic, protocatechuic, syringic and vanillin[17-19]. Vanillin and coumaric acid have antioxidants activity that can inhibit aging processes[20-21]. In the present study, free radical scavenging activity of *O. sativa* extract (OSE) and its compounds, vanillin and coumaric acid (Figure 1), were evaluated, as well as inhibitory activity of collagenase, elastase, and hyaluronidase.

2. MATERIALS AND METHODS

2.1. Preparation of *O. sativa* extracts

The plant of *O. sativa* were collected from the plantation in Tasikmalaya, West Java. The plant were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The grain of *O. sativa* (600 g) were mashed, extracted using distilled ethanol 70% (2,750 mL) by a maceration method. Every 24 h the ethanol was filtered and the wastes were re-macerated in triplicate. The ethanol filtrate collected was condensed using 50 °C rotavapor to obtainOSE extract. The extract in pasta form (5.64 g) was stored at -20 °C, and used for further assay[22]. Standards compounds used in this study were vanillin with 99% purity [Sigma V1104, USA] and coumaric acid (CA) with 98% purity [Biopurify Phytochemical 14111707, China].

2.2. Qualitative phytochemical screening assay

The phytochemical assay was conducted on *O. sativa* extracts (OSE) using modified Farnsworth method to qualitatively identify presence of phenols, steroid/triterpenoids, saponins, tannins, terpenoids, flavonoids, and alkaloids as listed below[23-25].

2.2.1. Phenol identification

Around 10 mg of sample was placed on a dropping plate, then 1% $FeCl_3$ [Merck 1.03861.0250, USA] was added into the sample. The color formation of green/red/purple/blue/black shows presence of phenol[23-25].

2.2.2. Steroid / triterpenoid identification

Approximately 10 mg of sample was placed on a dropping plate, then soaked with acetate acid until the sample was covered. After 10-15 min, one drop of absolute sulfate acid (H_2SO_4) [Merck 109073, USA] was added to the sample. The formation of green/blue color indicates the presence of steroid while red/orange sediment indicates the presence of triterpenoid[23-25].

2.2.3. Saponin identification

Approximately 10 mg of sample was put into the test tube with some water and boiled for 5 min. It was shaken vigorously and saponin content was indicated by persistence of froth on the surface[23-25].

2.2.4. Tannin identification

Approximately 10 mg of samples was added with 2 mL of HCl 2N [Merck 1003171000] in the test tube, then heated on a the water bath for 30 min. The mixture was cooled down and filtered, the filtrate was added with amyl alcohol [Merck 10979, USA]. Purple colour formation indicates positive reaction for tannins[23-25].

2.2.5. Terpenoid identification

Around 10 mg of sample was added into a dropping plate, then vanillin and H_2SO_4 was added to the sample. Terpenoid presence was indicated by the formation of purple color on the mixture[23-25].

2.2.6. Flavonoid identification

About 10 mg of sample was added into a test tube, then Mg [Merck EM105815, USA] and HCl 2N was added to the sample. The mixture sample was heated for 5 to 10 min, then filtered after it was cooled down. Subsequently, amyl alcohol was added into the filtrate. The positive reaction was shown by the formation of red or orange color[23-25].

2.2.7. Alkaloid identification

The small amount of sample (10 mg) was introduced into a test tube, then 10% ammonia was added into the sample. After chloroform added to the mixture, two layers of liquid was formed and the bottom layer was collected. HCl 1N was added to the liquid, forming two layers.The upper layer collected and added with 1-2 drops of draggendorf solution. The presence of yellow colour indicated positive result[23-25].

2.3. 2,2-Diphenyl-1-picrylhydrazil (DPPH) assay

The DPPH assay was conducted using the method from Widowati *et al.* (2015) study[26]. The method is based on the reduction of alcoholic DPPH solution in the presence of a

hydrogen-donating antioxidant due to the formation of the non-radical from DPPH-H[27]. Briefly, 50 µL of various level of samples (50-400 µg/mL for extract and 50-400 µM for compounds in the DMSO) were added to each well in a 96-well microplate. It was then followed by addition of 200 µl of 2,2-Diphenyl-1-picrylhydrazil (DPPH) [Sigma D9132, USA] solution (0.077 mmol/L in methanol) into the well. The mixture was then incubated in the dark for 30 min at room temperature. Afterward, the absorbance was read using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA) at 517 nm wavelengths. The radical scavenging activity was measured using the following formula:

$$\% \text{ Scavenging} = (Ac - As) / Ac \times 100$$

Ac = negative control absorbance (without sample)
As = sample absorbance

2.4. ABTS-reducing activity assay

The antioxidant capacity of OSE, vanillin, and CA were measured using 2,2'-Azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) [Sigma A1888-2G, USA] diammonium salt-free radical assay[28]. $ABTS^{\bullet+}$ solution was produced by reacting 14 mM ABTS and 4.9 mM potassium persulfate [Merck EM105091, USA] (1:1 volume ratio) for 16 h in dark condition at room temperature, then the mixture was diluted with 5.5 mM PBS (pH 7.4) until the absorbance of the solution was 0.70 ± 0.02 at wavelength 745 nm. In brief, 2 µl of various level of samples were added to each well at 96-well microplate, then to the samples the fresh 198 µl $ABTS^{\bullet+}$ solution were added. The absorbance was measured at 745 nm after the plate incubated for 6 min at 30 °C. The percentage inhibition of ABTS radical (%) was determined by the ratio of reducing of $ABTS^{\bullet+}$ absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control). The median inhibitory concentration (IC_{50}) were also calculated[28-29].

2.5.Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay (FRAP) was estimated using modified method from Mishra *et al.* (2006) and Widowati (2014)[28,30]. The FRAP reagent was prepared freshly by mixing 10 mL of acetate buffer 300 mM, 1 mL of ferric chloride hexahydrate [Merck 1.03943.0250, USA] 20 mM dissolved in distilled water, and 1 mL of 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) [Sigma 3682-35-7, USA] 10 mM dissolved in HCl 40 mM. In 96-well microplate, 7.5 µl of various level of samples (12.5-100 µg/mL for OSE and 12.5-100 µM for compunds) were mixed with 142.5 µL FRAP reagent then incubated for 30 min at 37 °C. The absorbance value was measured at 593 nm with a microplate reader. The standard curve was made using $FeSO_4$,between 0.019 and 95 µg/mL $FeSO_4$. The results of samples were expressed in µM Fe (II)/µg extract[28].

2.6.Collagenase inhibitory activity assay

Collagenase inhibitory activity was measured according to modified method of Sigma-Aldrich dan Thring *et al.* (2009)[7]. Mixed solution included 10 µL *Collagenase from Clostridium histolyticum* [Sigma C8051, USA] (0.01 U/mLin the cool aquades), 60 µL*Tricine* buffer (50 mM, pH 7.5, content of 10mM $CaCl_2$ dan 400 mM NaCl), 30 µL of various level of sample (0-250 µg/mL for OSE and 0-250 µM for compounds in the DMSO), then incubated at 37 °C for 20 min. After incubated added 20 µL N-[3-(2-Furyl)acryloyl]-leu-gly-Pro-Ala

[Sigma F5135, USA] (1mM in the *Tricine* buffer) substrate. Absorbance was measured at 335 nm wavelengths.

$$\% \text{ Collagenase inhibition} = (1-B/A) \times 100\%$$

A= sample absorbance
B= control absorbance

2.7. Elastase inhibitory activity assay

Elastase inhibitory activity was measured by modified method of Sigma Aldrich and Thring *et al.* (2009)[7]. A mixture of 10 µL of various level of samples (0 - 66.67 µg/mL for OSE and 0 - 66.67 µM for compounds), 5 µL *elastase from porcine pancreas* [Sigma 45124, USA] (0.5 mU/mL in the cool aquades) and 125 µL Tris buffer was pre-incubated for 15 min at 25 °C. Mixed solution was added N-Sucanyl-Ala-Ala-Ala-p-Nitroanilide substrate [Sigma 54760, USA] and then incubated for 15 min at 25 °C. Absorbance was measured by 410 nm wavelengths.

$$\%\text{Elastase inhibition} = (1-B/A) \times 100\%$$

A= sample absorbance
B= control absorbance

2.8. Hyaluronidase inhibitory activity assay

Hyaluronidase inhibitory of activity was measured by modified method of Sigma Aldrich and Tu and Tawata (2015)[31]. A mix of 25 µL of various level of samples (0 - 166.67 µg/mL for OSE and 0 - 166.67 µM for compounds) and 3 µL hyaluronidase from bovine testes type I-S [Sigma H3506, USA] was preincubated for 10 min at 37 °C and then added 12 µL phosphate buffer (300 mM, pH 5.35) for 10 min at 37 °C. Afterward 10 µL hyaluronic acid substrate [Sigma H5542, USA] was added and incubated for 45 min at 37 °C. Decomposition reaction of hyaluronic acid was stopped by adding 100 µL acidic albumin acid. Mixed solution incubated at room temperature for 10 min, then absorbance was measured at 600 nm wavelengths.
Quantification of inhibition activity by formula:

$$\% \text{ Hyaluronidase inhibition} = (1-B/A) \times 100\%$$

A= sample absorbance
B= control absorbance

3. RESULTS

3.1. Phytochemical screening of OSE

Phytochemical screening of the plants showed the presence of phenols, flavonoids, terpenoids, saponins, tannins and alkaloids. The result of OSE phytochemical screening can be seen in Table 1.

Table 1: The result of qualitative phytochemical screening of OSE

Phytochemical content	OSE
Phenols	+
Steroids/ Triterpenoids	-/+
Terpenoids	+++
Saponins	+++
Flavonoids	-
Tannins	-
Alkaloids	-

*++++: very high content; +++ : high content; ++ : moderate content; + : low content; - : not detected

Phytochemical screening of OSE aimed to detect presence of phenols, steroids, saponins, flavonoids, and tannins in OSE. Terpenoids and saponins was detected in high content (+++), phenols and triterpenoids were low content (+), while steroids, flavonoids, tannins and alkaloids were not detected (-) (Table 1).

3.2. 2,2-Diphenyl-1-picrylhydrazil (DPPH) assay

DPPH is a reagent for investigating the free radical scavenging activities of compounds. In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow coloured diphenyl picryl hydrazine[32-33]. The percentage of DPPH scavenging activity of OSE, vanillin, and coumaric acid can be seen in Figure 2. and the median inhibitory concentration (IC_{50}) of samples toward free radical scavenging DPPH activity can be seen in Table 2.

(**Figure 2.** DPPH scavenging activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 50.00; 100.00; 200.00; 400.00 (µg/mL; µM).)

Table 2: IC_{50} value DPPH scavenging activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y = 0.13 x + 8.36	0.98	-	314.51
Vanillin	Y = 0.02 x + 16.05	0.93	1865.16	283.76
Coumaric Acid	Y = 0.02 x + 23.36	0.95	1557.78	255.69

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Based on Figure 2, DPPH radical scavenging activity was concentration-dependent manner, in which higher concentration increased DPPH activity. At the highest concentration (400 µg/mL), OSE has the highest DPPH scavenging activity compared to vanillin and coumaric acid (59.62 ± 5.81%, 23.86 ± 0.57%, and 17.39 ± 0.16%, respectively) (Figure 2). However, IC_{50} value of coumaric acid has the lowest value (255.69 µg/mL) compared to OSE (314.51 µg/mL) and vanillin (283 µg/mL) (Table 2). These results indicate OSE has low DPPH-scavenging activity among treatments.

3.3. *ABTS - reducing activity assay*

ABTS-reducing activity assay measures the relative ability of antioxidant to scavenge the ABTS generated. The ABTS is generated by reacting a strong oxidizing agent (potassium permanganate/potassium persulfate) with the ABTS salt. Reduction of blue-green ABTS

radical colored solution by hydrogen-donating antioxidant is measured by the long wave absorption spectrum[34]. The result ABTS-reducing activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 3.

Table 3: IC_{50} value ABTS-reducing activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y = 0.33 x + 2.41	0.99		145.67
Vanillin	Y = 1.39 x + 4.72	0.99	32.63	4.96
Coumaric Acid	Y = 3.17 x+ 17.76	0.95	10.18	1.67

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

The antioxidant activity of OSE, vanillin, and coumaric acid were evaluated by ABTS-reducing activity assay. The IC_{50} of ABTS-reducing activity of OSE, vanillin, and coumaric acid can be seen in Table 3. OSE has the lowest ABTS-reducing activity as indicated by highest IC_{50} (145.67 µg/mL) compared to vanillin (4.96 µg/mL) and coumaric acid (1.67 µg/mL). The result indicated OSE has weak activity antioxidant compared to these compounds.

3.4. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyl triazine $Fe(TPTZ)^{3+}$ to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Absorbance of Fe(II) complex at 593 nm produced by antioxidant reduction of corresponding tripyridyl triazine Fe(III) complex[35]. FRAP activity of OSE, vanillin, and coumaric acid can be seen in Figure 3.

(**Figure 3.** FRAP Activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 12.50; 25.00; 50.00; 100.00 (µg/mL; µM).)

The result of the present study showed FRAP activity in concentration-dependent manner, in which higher concentration increased FRAP activity (Figure 3). At the highest concentration (100 µg/mL) OSE has a lowest ferric reducing with value (21.26 ± 0.21 µM Fe(II)/µg) compared to vanillin (35.05 ± 0.80 µM Fe(II)/µg) and coumaric acid (48.52 ± 0.24 µM Fe(II)/µg). That indicates OSE has the lowest antioxidant activity in the FRAP assay compared to vanillin and coumaric acid.

3.5. Collagenase Inhibitory Activity

A spectrophotometric method was used to collagenase activity assay, and to detect potential collagenase inhibitors[36]. The collagenase inhibitory activity of OSE, vanillin, and coumaric acid can be seen in Figure 4.

(**Figure 4.** Collagenase inhibitory activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 31.25; 62.50; 125.00; 250.00 (µg/mL; µM).)

Collagenase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 4.

Table 4: IC_{50} value collagenase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	y= 0.06x + 0.42	0.99	-	816.78
Vanillin	y= 0.15x + 34.16	0.92	106.99	16.27
Coumaric Acid	y= 0.05x + 3.47	0.93	894.83	146.89

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

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Figure 4 shows collagenase activities of OSE coumaric acid and vanillin in concentration-dependent manner. Collagenase activities of OSE (15.46 ± 1.12%) and coumaric acid (15.68 ± 1.67%) were comparable, but lower than vanillin (68.91 ± 1.56%). However, IC_{50} value of OSE was the highest (816.78 µg/mL) compared to coumaric acid (146.89 µg/mL) and vanillin (16.27 µg/mL) (Table 4). These findings indicate OSE has very low collagenase inhibitory activity, compared to coumaric acid and vanillin.

3.6. Elastase Inhibitory Activity

Elastase inhibitory activity can be used to evaluate the inhibitory activity of OSE. The percentation elastase inhibitoryactivity of OSE, vanillin and coumaric acid can be seen in Figure 5.

(**Figure 5.** Elastase inhibitory activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 8.33; 16.67; 33.33; 66.67 (µg/mL; µM).)

The IC_{50} value an elastase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 5.

Table 5: IC_{50} value elastase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y = 0.29 x + 19.07	0.91	-	107.51
Vanillin	Y = 0.45 x + 7.31	0.98	95.07	14.46
Coumaric Acid	Y = 0.22 x + 16.13	0.94	154.66	25.38

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Elastase inhibitory activity of OSE, vanillin, and coumaric acid showed the highest inhibition percentage at the highest concentration (36.82 ± 0.40%, 36.28 ± 0.20%, 29.86 ± 0.45% respectively) (Figure 5). However, vanillin showed the highest activity in elastase inhibition with IC_{50} value 14.46 µg/mL. OSE and coumaric acid have IC_{50} value 107.62 µg/mL and 25.38 µg/mL, respectively (Table 5). The result showed that OSE posses low elastase inhibition compared to vanillin and coumaric acid.

3.7. Hyaluronidase inhibitory activity

Hyaluronidase was assayed by a highly sensitive spectrophotometric method, based on precipitation of HA with cetylpyridinium chloride, which is used for high throughout screening for hyaluronidase inhibitors[37].

Hyaluronidase inhibitory activity can be used to evaluate inhibitory activity of OSE. The percentation hyaluronidase inhibition activity of OSE, vanillin, and coumaric acid can be seen in Figure 6.

(Figure 6. Hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid. OSE, vanillin and coumaric acid were diluted in DMSO to reach the final concentration of 5.21; 10.42; 20.83; 41.67; 83.33; 166.67 (µg/mL; µM).)

The IC_{50} value for hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 6.

Table 6: IC_{50} value hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	y= 0.24x + 0.34	0.91	-	203.13
Vanillin	y= 0.15x+5.47	0.99	297.33	45.23
Coumaric Acid	y= 0.89x + 5.01	0.94	50.11	8.21

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Based on Figure 6, OSE showed the moderate activity with percentage 36.86 ± 2.28% compared to coumaric acid (48.48 ± 0.40%) and vanillin (20.84 ± 4.78%). Coumaric acid has the lowest IC_{50} value (8.22 µg/mL) compared to OSE (203.13 µg/mL) and vanillin (45.23 µg/mL) (Table 6). These results show OSE has low hyaluronidase inhibitory activity compared to vanillin and coumaric acid.

4. DISCUSSION

Ayurveda, which means science of long life, is at least a 5,000-year-old system of Indian medicine (1500–1000 BC) designed to promote good health and longevity rather than to fight disease and was practiced by physicians and surgeons. Ayurveda usually recommends the use of several plant extracts in combination. Toxic effects of modern medicine are insufficient due to a lack of other components derived from the plants. Each herbal formulation contains multiple active compounds that may operate synergistically, producing therapeutic benefits and lowering the risks on adverse effects[38]. Rice (*O. sativa)*, a herb belongs to Poaceae, has been known as a potential source of antioxidants in food, pharmaceutical and cosmetic industries. It has been utilized in the treatment of cancer and aging[39]. *O. sativa* has bioactive compounds, including many phenolic compounds as an antioxidant[17]. Five phenyl compounds isolated from the roots of *O. sativa* L. are vanillin, methyl trans-ferulate, trans-p-coumaric acid methyl ester, N-benzoyltryptamine, and N-(trans-cinnamoyl)tryptamine[40]. Phenols can be divided into two subgroups according to their structure, p-hydroxybenzoic acid derivatives such as gallic, protocatechuic and syringic acids, and hydroxycinnamic derivatives such as caffeic, ferulic, p-coumaric and chlorogenic acids[19]. The present study showed that OSE contained low phenols and triterpenoids, high terpenoids, and saponins. Phytochemical analysis of *O. sativa* was conducted in the previous study that showed 11–13% protein, approximately 11% fiber and 20% of its weight in oil, as well as functional compounds and antioxidants[41]. Phenolic compounds, such as benzoic or cinnamic acid derivatives, have been documented in Poaceae plants such as *Sorghum* sp.[42-43].

UV irradiation generates free radicals that can induce expression of certain members of the matrix metalloproteinase (MMP) family, which degrades collagen and other extracellular matrix (ECM) proteins that consist of the dermal connective tissue[44]. In this study, OSE had the lowest scavenging free radical activity as indicated by reduced ABTS and FRAP activities and DPPH scavenging activity compared to vanillin and coumaric acid. A recent study on *O. sativa* in different solvent showed that *O. sativa* ethanol extract have the lowest antioxidants (IC_{50}= 4.78 µg/mL) compared to *O. sativa* methanol extract (IC_{50}= 2.6 µg/mL) and *O. sativa* isopropanol extract (IC_{50}= 2.4 µg/mL)[45]. *O. sativa* methanol and isopropanol extracts exhibited better free radical scavenging activity, which is associated with increasing phenolic compounds that increases antioxidant activity[46]. Coumaric acid has been reported to protect against oxidative stress and play a key role as an antioxidant and anti-inflammatory[47-48]. *O. sativa* oils such as coumaric acid and vanillin exhibited very good oxidative state with range oxidative stability 5.99-7.40[49].

OSE has the lowest DPPH scavenging activity compared to vanillin and coumaric acid, this data was not validated with previous study showed that *O. sativa* extract has rich secondary metabolites, especially alkaloids and phenolic acids that have strong antioxidant activity[50]. Phenolic and flavonoid contents are associated with strong antioxidant activity and especially in terms of DPPH and nitric oxide free radical scavenging[51]. The phenolic extracts from fermented *O. sativa* showed slow inhibition kinetics of the DPPH radical, as IC_{50} value obtained was 250 mg/g compared to unfermented *O. sativa* with value 213 mg/g, while feluric acid 235.00 mg/g[52]. In another study, Poaceae plants such as *Z. mays* ethanolic extract has low antioxidant activity with IC_{50} value of DPPH 163.45 ± 6.34 µg/mL,while *T. aestivum* bran ethanol extract (0.5 mg/kg) has DPPH value 58.00 ± 1.1%[53-54]. *T. aestivum* methanol extract showed good radical scavengers with the inhibition of 12% at 1 mL/mL concentration[55]. *T. aestivum* has shown potential anti inflammatory, antioxidant, and antiaging properties[56].

Collagen, elastin, and hyaluronic acid are the skin main components and have an important role in maintaining its structure and hydration[57]. Enzymes causes repetitive collagen fibers breakdown and responsible for structural defect in dermis and wrinkle development[58]. Collagenase and elastase contribute in production and degradation of these fibers, which is also induced by free radical oxidative stress[59]. In the present study, *O. sativa* had lowest activity in collagenase inhibition compared to coumaric acid and vanillin. *O. sativa* has been studied in anti-aging treatment. Water-soluble enzymatic extract from *O. sativa* served as a protective screen against UV-B radiation on cultivated keratinocytes and in vitro reconstructed skin[60]. In the other study, the extract of *O. sativa* plants callus improve the human-skin barrier function[61]. Mechanisms of antiaging are via up-regulation of collagen sythesis in normal human dermal fibroblast and down-regulation of matrix metalloproteinase[62]. Vanillin content was also reported to inhibit matrix metalloproteinase-9 expression through down-regulation of nuclear factor-kB signaling pathway in human hepatocellular carcinoma cells[63].

Elastin is a fibrose protein that compose 2–4% of the ECM and involved in the hydration of the skin[64]. Elastase is responsible for increased tissue permeability, inflammation progress and delayed wound healing[65]. Elastase is also the key enzyme that attacks all the major connective tissue matrix protein[66]. In elastase assay, OSE showed lowest activity compared to coumaric acid and vanillin. Low activity of OSE in the present study might be due to low content of phenol and absence of flavonoids in phytochemical screening. Several studies showed that phenol and flavonoid content of *O. sativa* possess anti-elastase activity[51,67-68], OSE had lowest antielastase might be correlated with an absence of phenol and flavonoid. However, vanillin has the highest elastase inhibitory activity.

Our present study showed that OSE had the lowest hyaluronidase inhibitory activity compared to CA and vanillin. These results might be correlated with an absence of tannin in OSE. Tannin-rich plants have been reported to inhibit hyaluronidase and elastase release from

stimulated neutrophils in vitro[69]. Hyaluronidase (Haases) selectively degrade hyaluronic acid (HA), which is a megadalton acidic structural polysaccharide found exclusively in the extracellular matrix (ECM). Haase inhibitors are thus potent regulators that maintain HA homeostasis and they might serve as antiinflammatory, antiaging, antimicrobial, anticancer and antitoxin and contraceptive agents[70]. Hyaluronidase degrades hyaluronic acid by lowering its viscosity and increasing the permeability[71].

CONCLUSION

Vanillin and Coumaric acid have antioxidant activities through DPPH scavenging, ABTS-reducing activities, FRAP and antiaging through inhibitory activity of collagenase, elastase and hyaluronidase higher than *O. sativa* extract.

ACKNOWLEDGMENT

This research was supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for a research grant, laboratory facilities, and research methodology. We are thankful to Neng Sri Mulkiyatul M.A, Kartiko Adi Poerbo and Muhammad Bihar Jafarian as the interns at Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for their valuable assistance.

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Journal of Natural Remedies
October 06, 2016

Acceptance Letter

Manuscript No. : 7220

Authors: Wahyu Widowati, Nurul Fauziah, Merry Afni, Ervi Afifah, Hanna Sari W. Kusuma, Hayatun Nufus, Seila Arumwardana, Peni Astrini, Dwi Davidson Rihibiha

Title: Antioxidant and Antiaging assays of *Oryza sativa* Extracts, Vanillin and Coumaric Acid.

Dear Dr. Wahyu Widowati:

The Editorial Team of Journal of Natural Remedies (JNR) is pleased to inform you that your manuscript "Antioxidant and Antiaging assays of *Oryza sativa* Extracts, Vanillin and Coumaric Acid" has been accepted. Thank you for making the payment for the same. The article is scheduled to be published in Volume 16, Issue 3, 2016.

Thank you for your submission.

Best Regards,

Editorial Team

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DOI: 10.18311/jnr/2016/7220

Antioxidant and Anti Aging Assays of *Oryza Sativa* Extracts, Vanillin and Coumaric Acid

Wahyu Widowati[1*], Nurul Fauziah[1], Heddy Herdiman[1], Merry Afni[2], Ervi Afifah[2], Hanna Sari W. Kusuma[2], Hayatun Nufus[2], Seila Arumwardana[2] and Dwi Davidson Rihibiha[2]

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Abstract

Aging is a natural process in human as accumulation of oxygen-derived free radicals which leads to the activation of hyaluronidase, collagenase and elastase, that can further contribute to cellular and tissue damage cell. Bioactive compounds from plants has been used as antioxidant that might inhibit aging processes as well. This study aimed to determine antioxidant and anti aging properties of *Oryza sativa* Extract (OSE), and its compounds, vanillin and coumaric acid. The phytochemical analysis of OSE was performed with Farnsworth modified method. Antioxidant activities were performed by measurement of 2,2-diphenyl 1-pichylhydazyl (DPPH) free radical scavenger, Ferric Reducing Antioxidant Power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reducing activity, while anti aging assay were observed through inhibitory of elastase, collagenase, and hyaluronidase activities. Phytochemical analysis showed the presence of terpenoids and saponins in high level. OSE showed lowest DPPH activity (IC_{50} = 314.51 µg/mL) compared to vanillin (IC_{50} = 283 µg/mL) and coumaric acid (IC_{50} = 255.69 µg/mL). In ABTS assay, OSE resulted lowest activity(IC_{50} = 145.67 µg/mL), compared to vanillin (IC_{50} = 4.96 µg/mL) and coumaric acid (IC_{50} = 1.67 µg/mL). OSE also showed the lowest FRAP-reducing activity (21.26 µM Fe(II)/µg), compared to vanillin (35.05 µM Fe(II)/µg) and coumaric acid (48.52µM Fe(II)/µg). OSE showed the lowest collagenase, elastase, and hyaluronidase inhibitory activity (IC_{50} = 816.78,107.51, and 203.13 µg/mL), compared to vanillin (IC_{50} = 16.27, 14.46, 45.23 µg/mL respectively) and coumaric acid (IC_{50} = 146.89, 25.38, 8.21 µg/mL respectively). In summary, OSE possess the lowest antioxidant and anti aging activities compared to vanillin and coumaric acid.

Keywords: Antioxidant, Anti aging, Coumaric Acid, Oryza sativa, Vanillin

1. Introduction

Skin aging is the natural process due to photo aging by environmental factors such as chronic UV radiation. The repetitive exposure to UV radiation cause accelerated physical changes in the skin and connective tissue through the formation of lipid peroxides, the cell contents and Reactive Oxygen Species (ROS)[1]. It leads to loss of skin elasticity implicated in formation of wrinkling, uneven pigmentation, brown spots, laxity and leathery appearance, solar elastosis, actinic purpura, precancerous lesions, skin cancer, and melanoma[2–4].

During aging process, collagen, elastin, and hyaluronic acid decrease, that causes loss of strength and flexibility in the skin, resulting in visible wrinkles. It is also related to increasing enzymes activity including

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collagenase, elastase and hyaluronidase. Collagenase is known as an enzyme that plays role in the degradation of collagen. Collagen is the main component with percentage of 70-80 % of the total skin weight, the increasing degradation of collagen is significant in the photo aging process[5,6]. Hyaluronan or hyaluronic acid is one of important components of the tissue matrix substance and has a role in the development, growth, and repair of damaged tissue[6]. Meanwhile, elastin play a role in the maintenance of skin elasticity, but elastase can degrade it[7]. Degradation of the Extracellular Matrix (ECM) has been directly linked to skin aging and is correlated with an increase in activity of certain enzymes involved in skin aging[8,9]. Inhibition of these enzymes is crucial in anti aging prevention[10].

It has been reported that skin aging occurs in the presence of cumulative endogenous damage due to Reactive Oxygen Species (ROS)[11]. ROS are defined as oxygen-containing, highly reactive species. ROS are generated constantly during normal cellular metabolism which is essential for biological functions. Excessive ROS causes oxidative stress and damage to biological molecules[12,13]. Previous studies have investigated that continuous ROS exposure can stimulate skin aging through antioxidant system destruction, wrinkle formation, and melanogenesis[12]. ROS are usually removed from the body through antioxidant defense system[14]. Thus, maintaining antioxidant homeostasis is an appropriate strategy to prevent skin aging.

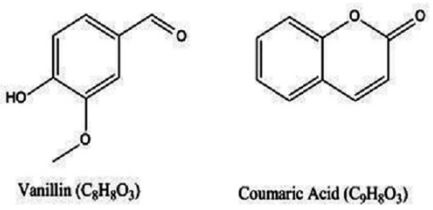

Fig. 1. Chemical structure of vanillin and coumaric acid.

Antioxidant properties derived from natural sources have been proposed for aging prevention[15]. Bioactive compounds contained in plants such as isoflavones, anthocyanins, and catechins may have promising antioxidant activity against ROS[16]. *Oryza sativa* L. is one of the most produced and consumed cereals in the world that contain phenolic compounds, tocopherols, tocotrienols, and g-oryzanol. Phenolic acids were identified in the lignin fraction of rice bran *(O. sativa)* such as caffeic, chlorogenic, p-coumaric, ferulic, gallicacids, p-hydroxybenzoic, protocatechuic, syringic and vanillin[17–19]. Vanillin and coumaric acid have antioxidants activity that can inhibit aging processes[20,21]. In the present study, free radical scavenging activity of *O. Sativa* Extract (OSE) and its compounds, vanillin and coumaric acid (Figure 1), were evaluated, as well as inhibitory activity of collagenase, elastase, and hyaluronidase.

2. Materials and Methods

2.1 Preparation of *O. sativa* Extracts

The plant of *O. sativa* were collected from the plantation in Tasikmalaya, West Java. The plant were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The grain of *O. Sativa* (600 g) were mashed, extracted using distilled ethanol 70% (2,750 mL) by a maceration method. Every 24 h the ethanol was filtered and the wastes were re-macerated in triplicate. The ethanol filtrate collected was condensed using 50°C rota vapor to obtain OSE extract. The extract in pasta form (5.64 g) was stored at -20 °C, and used for further assay[22]. Standards compounds used in this study were vanillin with 99% purity [Sigma V1104, USA] and coumaric acid (CA) with 98% purity [Biopurify Phytochemical 14111707, China].

2.2 Qualitative Phytochemical Screening Assay

The phytochemical assay was conducted on *O. Sativa* Extracts (OSE) using modified Farnsworth method to qualitatively identify presence of phenols, steroid/triterpenoids, saponins, tannins, terpenoids, flavonoids, and alkaloids as listed below[23–25].

2.2.1 Phenol Identification

Around 10 mg of sample was placed on a dropping plate, then 1% $FeCl_3$ [Merck 1.03861.0250, USA] was added into the sample. The color formation of green/red/purple/blue/black shows presence of phenol[23–25].

2.2.2 Steroid /Triterpenoid Identification

Approximately 10 mg of sample was placed on a dropping plate, then soaked with acetate acid until the sample was covered. After 10-15 min, one drop of absolute sulfate acid (H_2SO_4) [Merck 109073, USA] was added to the sample. The formation of green/blue color indicates the presence of steroid while red/orange sediment indicates the presence of triterpenoid[23–25].

2.2.3 Saponin Identification

Approximately 10 mg of sample was put into the test tube with some water and boiled for 5 min. It was shaken vigorously and saponin content was indicated by persistence of froth on the surface[23–25].

2.2.4 Tannin Identification

Approximately 10 mg of samples was added with 2 mL of HCl 2N [Merck 1003171000] in the test tube, then heated on a the water bath for 30 min. The mixture was cooled down and filtered, the filtrate was added with amyl alcohol [Merck 10979, USA]. Purple colour formation indicates positive reaction for tannins[23–25].

2.2.5 Terpenoid Identification

Around 10 mg of sample was added into a dropping plate, then vanillin and H_2SO_4 was added to the sample. Terpenoid presence was indicated by the formation of purple color on the mixture[23–25].

2.2.6 Flavonoid Identification

About 10 mg of sample was added into a test tube, then Mg [Merck EM105815, USA] and HCl 2N was added to the sample. The mixture sample was heated for 5 to 10 min, then filtered after it was cooled down. Subsequently, amyl alcohol was added into the filtrate. The positive reaction was shown by the formation of red or orange color[23–25].

2.2.7 Alkaloid Identification

The small amount of sample (10 mg) was introduced into a test tube, then 10% ammonia was added into the sample. After chloroform added to the mixture, two layers of liquid was formed and the bottom layer was collected. HCl 1N was added to the liquid, forming two layers. The upper layer collected and added with 1-2 drops of Draggendorf solution. The presence of yellow colour indicated positive result[23–25].

2.3 2,2-Diphenyl-1-picrylhydrazil (DPPH) Assay

The DPPH assay was conducted using the method from Widowati et al., study[26]. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical from DPPH-H[27]. Briefly, 50 µL of various level of samples (50-400 µg/mL for extract and 50-400 µM for compounds in the DMSO) were added to each well in a 96-well micro plate. It was then followed by addition of 200 µl of 2,2-Diphenyl-1-picrylhydrazil (DPPH) [Sigma D9132, USA] solution (0.077 mmol/L in methanol) into the well. The mixture was then incubated in the dark for 30 min at room temperature. Afterward, the absorbance was read using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA) at 517 nm wavelengths. The radical scavenging activity was measured using the following formula:

$$\% \text{ Scavenging} = (Ac - As) / Ac \times 100$$

Ac = negative control absorbance (without sample).

As = sample absorbance.

2.4 ABTS-Reducing Activity Assay

The antioxidant capacity of OSE, vanillin, and CA were measured using 2,2'-Azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) [Sigma A1888-2G, USA] diammonium salt-free radical assay[28]. $ABTS^{\bullet+}$ solution was produced by reacting 14 mM ABTS and 4.9 mM potassium persulfate [Merck EM105091, USA] (1:1 volume ratio) for 16 h in dark condition at room temperature, then the mixture was diluted with 5.5 mM PBS (pH 7.4) until the absorbance of the solution was 0.70 ± 0.02 at wavelength 745 nm. In brief, 2 µl of various level of samples were added to each well at 96-well microplate, then to the samples the fresh 198 µl $ABTS^{\bullet+}$ solution were added. The absorbance was measured at 745 nm after the plate incubated for 6 min at 30°C. The percentage inhibition of ABTS radical (%) was determined by the ratio of reducing of $ABTS^{\bullet+}$

absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control). The median Inhibitory Concentration (IC_{50}) were also calculated[28,29].

2.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric Reducing Antioxidant Power Assay (FRAP) was estimated using modified method from Mishra et al., and Widowati[28,30]. The FRAP reagent was prepared freshly by mixing 10 mL of acetate buffer 300 mM, 1 mL of ferric chloride hexahydrate [Merck 1.03943.0250, USA] 20 mM dissolved in distilled water, and 1 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) [Sigma 3682-35-7, USA] 10 mM dissolved in HCl 40 mM. In 96-well micro plate, 7.5 µl of various level of samples (12.5-100 µg/mL for OSE and 12.5-100 µM for compounds) were mixed with 142.5 µL FRAP reagent then incubated for 30 min at 37°C. The absorbance value was measured at 593 nm with a micro plate reader. The standard curve was made using $FeSO_4$, between 0.019 and 95 µg/mL $FeSO_4$. The results of samples were expressed in µM Fe (II)/µg extract[28].

2.6 Collagenase Inhibitory Activity Assay

Collagenase inhibitory activity was measured according to modified method of Sigma-Aldrich dan Thring et al[7]. Mixed solution included 10 µL *Collagenase from Clostridium histolyticum* [Sigma C8051, USA] (0.01 U/mLin the cool aquades), 60 µL *Tricine* buffer (50mM, pH 7.5, content of 10mM $CaCl_2$ dan 400mM NaCl), 30 µL of various level of sample (0-250 µg/mL for OSE and 0-250 µM for compounds in the DMSO), then incubated at 37 °C for 20 min. After incubated added 20 µL N-[3-(2-Furyl)acryloyl]-leu-gly-Pro-Ala [Sigma F5135, USA] (1mM in the*Tricine* buffer) substrate. Absorbance was measured at 335 nm wavelengths.

% Collagenase inhibition = (1-B/A) x 100%

A = sample absorbance

B = control absorbance

2.7 Elastase Inhibitory Activity Assay

Elastase inhibitory activity was measured by modified method of Sigma Aldrich and Thring et al[7]. A mixture of 10 µLof various level of samples (0-66.67 µg/mL for OSE and 0 - 66.67 µM for compounds), 5 µL elastase from porcine pancreas [Sigma 45124, USA] (0.5 mU/mL in the cool aquades) and 125 µL Tris buffer was pre-incubated for 15 min at 25°C. Mixed solution was added N-Sucanyl-Ala-Ala-Ala-p-Nitroanilide substrate [Sigma 54760, USA] and then incubated for 15 min at 25°C. Absorbance was measured by 410 nm wavelengths.

% Elastase inhibition = (1-B/A) x 100%

A = sample absorbance

B = control absorbance

2.8 Hyaluronidase Inhibitory Activity Assay

Hyaluronidase inhibitory of activity was measured by modified method of Sigma Aldrich and Tu and Tawata (2015)[31]. A mix of 25 µL of various level of samples (0-166.67 µg/mL for OSE and 0 - 166.67 µM for compounds) and 3 µL hyaluronidase from bovine testes type I-S [Sigma H3506, USA] was preincubated for 10 min at 37°C and then added 12 µL phosphate buffer (300mM, pH 5.35) for 10 min at 37°C. Afterward 10 µL hyaluronic acid substrate [Sigma H5542, USA] was added and incubated for 45 min at 37°C. Decomposition reaction of hyaluronic acid was stopped by adding 100 µL acidic albumin acid. Mixed solution incubated at room temperature for 10 min, then absorbance was measured at 600 nm wavelengths.

Quantification of inhibition activity by formula:

%Hyaluronidase inhibition= (1-B/A) x 100%

A = sample absorbance

B = control absorbance

3.Results

3.1 Phytochemical Screening of OSE

Phytochemical screening of the plants showed the presence of phenols, flavonoids, terpenoids, saponins, tannins and alkaloids. The result of OSE phytochemical screening can be seen in Table 1.

Table 1: The result of qualitative phytochemical screening of OSE

Phytochemical content	OSE
Phenols	+
Steroids/Triterpenoids	-/+
Terpenoids	+++
Saponins	+++
Flavonoids	-
Tannins	-
Alkaloids	-

*++++: very high content; +++ : high content; ++ : moderate content; + : low content; - : not detected

Phytochemical screening of OSE aimed to detect presence of phenols, steroids, saponins, flavonoids, and tannins in OSE. Terpenoids and saponins was detected in high content (+++), phenols and triterpenoids werelowcontent (+), while steroids, flavonoids, tannins and alkaloids were not detected (-) (Table 1).

3.2 2,2-Diphenyl-1-picrylhydrazil (DPPH) Assay

DPPH is a reagent for investigating the free radical scavenging activities of compounds. In the DPPH test, the extracts were able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine[32,33]. The percentage of DPPH scavenging activity of OSE, vanillin, and coumaric acid can be seen in Figure 2 and the median Inhibitory Concentration (IC_{50}) of samples toward free radical scavenging DPPH activity can be seen in Table 2.

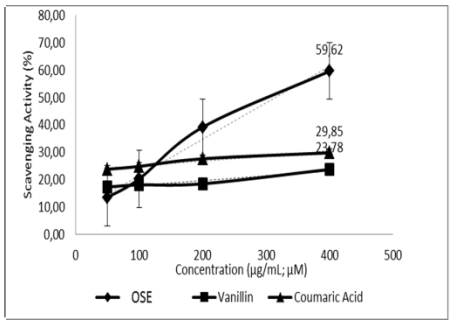

Fig. 2. DPPH scavenging activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 50.00; 100.00; 200.00; 400.00 (µg/mL; µM).

Table 2: IC_{50} value DPPH scavenging activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y= 0.13x+8.36	0.98	-	314.51
Vanillin	Y=0.02x+16.05	0.93	1865.16	283.76
Coumaric Acid	Y= 0.02x+23.36	0.95	1557.78	255.69

*Linear equations, coefficient of regression (R^2) and IC50 of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Based on Figure 2, DPPH radical scavenging activity was concentration-dependent manner, in which higher concentration increased DPPH activity. At the highest concentration (400 µg/mL), OSE has the highest DPPH scavenging activity compared to vanillin and coumaric acid (59.62 ± 5.81%, 23.86 ± 0.57%, and 17.39 ± 0.16%, respectively) (Figure 2). However, IC_{50} value of coumaric acid has the lowest value (255.69 µg/mL) compared to OSE (314.51 µg/mL) and vanillin (283 µg/mL) (Table 2). These results indicate OSE has low DPPH-scavenging activity among treatments.

3.3 ABTS - Reducing Activity Assay

ABTS-reducing activity assay measures the relative ability of antioxidant to scavenge the ABTS generated. The ABTS is generated by reacting a strong oxidizing agent (potassium permanganate/potassium persulfate) with the ABTS salt. Reduction of blue-green ABTS radical colored solution by hydrogen-donating antioxidant is measured by the long wave absorption spectrum[34]. The result ABTS-reducing activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 3.

Table 3: IC_{50}value ABTS-reducing activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y= 0.33x + 2.41	0.99		145.67
Vanillin	Y= 1.39x + 4.72	0.99	32.63	4.96
Coumaric Acid	Y= 3.17x+ 17.76	0.95	10.18	1.67

*Linear equations, coefficient of regression (R^2) and IC50 of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

The antioxidant activity of OSE, vanillin, and coumaric acid were evaluated by ABTS-reducing activity assay. The IC_{50} of ABTS-reducing activity of OSE, vanillin, and coumaric acid can be seen in Table 3. OSE has the lowest ABTS-reducing activity as indicated by highest IC_{50}(145.67µg/mL) compared to vanillin (4.96µg/mL) and coumaric acid (1.67µg/mL). The result indicated OSE has weak activity antioxidant compared to these compounds.

3.4 Ferric Reducing Antioxidant Power (FRAP) Assay

The Ferric Reducing Antioxidant Power (FRAP) method is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the intensely blue coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Absorbance of Fe(II) complex at 593 nm produced by antioxidant reduction of corresponding tripyridyltriazine Fe(III) complex[35]. FRAP activity of OSE, vanillin, and coumaric acid can be seen in Figure 3.

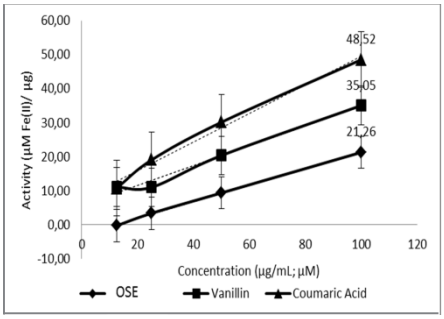

Fig. 3. FRAP Activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 12.50; 25.00; 50.00; 100.00 (µg/mL; µM).

The result of the present study showed FRAP activity in concentration-dependent manner, in which higher concentration increased FRAP activity (Figure 3). At the highest concentration (100 µg/mL) OSE has a lowest ferric reducing with value (21.26±0.21 µM Fe(II)/µg) compared to vanillin (35.05±0.80µM Fe(II)/µg) and coumaric acid (48.52±0.24 µM Fe(II)/µg). That indicates OSE has the lowest antioxidant activity in the FRAP assay compared to vanillin and coumaric acid.

3.5 Collagenase Inhibitory Activity

A spectrophotometric method was used to collagenase activity assay, and to detect potential collagenase inhibitors[36]. The collagenase inhibitory activity of OSE, vanillin, and coumaric acid can be seen in Figure 4.

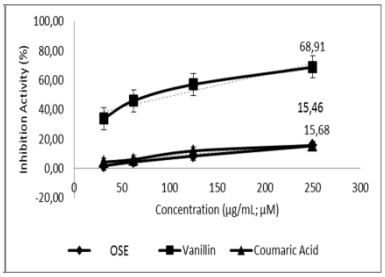

Fig. 4. Collagenase inhibitory activity of OSE, vanillin and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 31.25; 62.50; 125.00; 250.00 (µg/mL; µM).

Collagenase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 4.

Table 4: IC_{50} value collagenase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	y= 0.06x + 0.42	0.99	-	816.78
Vanillin	y= 0.15x + 34.16	0.92	106.99	16.27
Coumaric Acid	y= 0.05x + 3.47	0.93	894.83	146.89

*Linear equations, coefficient of regression (R^2) and IC50 of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Figure 4 shows collagenase activities of OSE coumaric acid and vanillin in concentration-dependent manner. Collagenase activities of OSE (15.46±1.12%) and coumaric acid (15.68±1.67%) were comparable, but lower than vanillin (68.91 ±1.56%). However, IC_{50} value of OSE was the highest (816.78 µg/mL) compared tocoumaric acid (146.89 µg/mL) andvanillin (16.27 µg/mL) (Table 4). These findings indicate OSE has very low collagenase inhibitory activity, compared to coumaric acid and vanillin.

3.4 Elastase Inhibitory Activity

Elastase inhibitory activity can be used to evaluate the inhibitory activity of OSE. The percentation elastase

inhibitory activity of OSE, vanillin and coumaric acid can be seen in Figure 5.

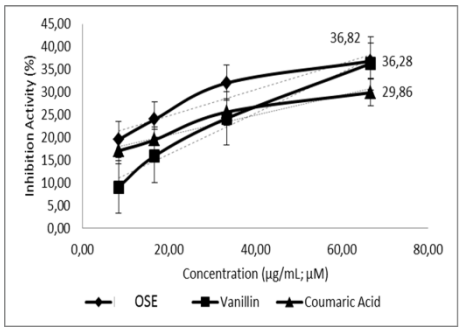

Fig. 5. Elastase inhibitory activity of OSE, vanillin, and coumaric acid. OSE, vanillin, and coumaric acid were diluted in DMSO to reach the final concentration of 8.33; 16.67; 33.33; 66.67 (µg/mL; µM).

The IC_{50} value an elastase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 5.

Table 5: IC_{50} value elastase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	Y= 0.29x+19.07	0.91	-	107.51
Vanillin	Y= 0.45x+7.31	0.98	95.07	14.46
Coumaric Acid	Y=0.22x+16.13	0.94	154.66	25.38

*Linear equations, coefficient of regression (R^2) and IC^{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Elastase inhibitory activity of OSE, vanillin, and coumaric acid showed the highest inhibition percentage at the highest concentration (36.82 ± 0.40%, 36.28 ± 0.20%, 29.86 ± 0.45% respectively) (Figure 5). However, vanillin showed the highest activity in elastase inhibition with IC_{50} value 14.46 µg/mL. OSE and coumaric acid have IC_{50} value 107.62 µg/mL and 25.38µg/mL, respectively (Table 5). The result showed that OSE posses low elastase inhibition compared to vanillin and coumaric acid.

3.7 Hyaluronidase Inhibitory Activity

Hyaluronidase was assayed by a highly sensitive spectrophotometric method, based on precipitation of HA with cetylpyridinium chloride, which is used for high throughout screening for hyaluronidase inhibitors[37].

Hyaluronidase inhibitory activity can be used to evaluate inhibitory activity of OSE. The percentation hyaluronidase inhibition activity of OSE, vanillin, and coumaric acid can be seen in Figure 6.

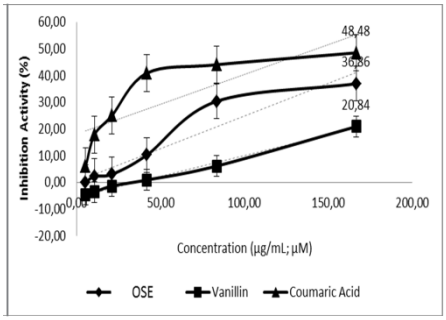

Fig. 6. Hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid. OSE, vanillin and coumaric acid were diluted in DMSO to reach the final concentration of 5.21; 10.42; 20.83; 41.67; 83.33; 166.67 (µg/mL; µM).

The IC_{50} value for hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid based on IC_{50} value can be seen in Table 6.

Table 6: IC_{50} value hyaluronidase inhibitory activity of OSE, vanillin, and coumaric acid

Samples	Equation	R^2	IC_{50} (µM)	IC_{50} (µg/mL)
OSE	y= 0.24x + 0.34	0.91	-	203.13
Vanillin	y= 0.15x+5.47	0.99	297.33	45.23
Coumaric Acid	y= 0.89x+5.01	0.94	50.11	8.21

*Linear equations, coefficient of regression (R^2) and IC_{50} of each sample were calculated. IC_{50} of OSE was presented in µg/mL, while vanillin and coumaric acid were presented in µM and µg/mL.

Based on Figure 6, OSE showed the moderate activity with percentage 36.86±2.28% compared to coumaric acid (48.48±0.40%) and vanillin (20.84±4.78%). Coumaric acid has the lowest IC_{50} value (8.22 µg/mL) compared to OSE (203.13µg/mL) and vanillin (45.23µg/mL) (Table 6). These results show OSE has low hyaluronidase inhibitory activity compared to vanillin and coumaric acid.

4. Discussion

Ayurveda, which means science of long life, is at least a 5,000-year-old system of Indian medicine (1500–1000 BC) designed to promote good health and longevity rather than to fight disease and was practiced by

physicians and surgeons. Ayurveda usually recommends the use of several plant extracts in combination. Toxic effects of modern medicine are insufficient due to a lack of other components derived from the plants. Each herbal formulation contains multiple active compounds that may operate synergistically, producing therapeutic benefits and lowering the risks on adverse effects[38]. Rice (*O. sativa)*, a herb belongs to Poaceae, has been known as a potential source of antioxidants in food, pharmaceutical and cosmetic industries. It has been utilized in the treatment of cancer and aging[39]. *O. sativa* has bioactive compounds, including many phenolic compounds as an antioxidant[17]. Five phenyl compounds isolated from the roots of *O. sativa* L. are vanillin, methyl trans-ferulate, trans-p-coumaric acid methyl ester, N-benzoyltryptamine, and N-(trans-cinnamoyl) tryptamine[40]. Phenols can be divided into two subgroups according to their structure, p-hydroxybenzoic acid derivatives such as gallic, protocatechuic and syringic acids, and hydroxycinnamic derivatives such as caffeic, ferulic, p-coumaric and chlorogenic acids[19]. The present study showed that OSE contained low phenols and triterpenoids, high terpenoids, and saponins. Phytochemical analysis of *O. sativa* was conducted in the previous study that showed 11-13 % protein, approximately 11% fiber and 20% of its weight in oil, as well as functional compounds and antioxidants[41]. Phenolic compounds, such as benzoic or cinnamic acid derivatives, have been documented in Poaceae plants such as *Sorghum* sp[42,43].

UV irradiation generates free radicals that can induce expression of certain members of the Matrix Metalloproteinase (MMP) family, which degrades collagen and other Extracellular Matrix (ECM) proteins that consist of the dermal connective tissue[44]. In this study, OSE had the lowest scavenging free radical activity as indicated by reduced ABTS and FRAP activities and DPPH scavenging activity compared to vanillin and coumaric acid. A recent study on *O. sativa* in different solvent showed that *O. sativa* ethanol extract have the lowest antioxidants (IC_{50}= 4.78 µg/mL) compared to *O. sativa* methanol extract (IC_{50}= 2.6 µg/mL) and *O. sativa* isopropanol extract (IC_{50}= 2.4 µg/mL)[45]. *O. sativa* methanol and isopropanol extracts exhibited better free radical scavenging activity, which is associated with increasing phenolic compounds that increases antioxidant activity[46]. Coumaric acid has been reported to protect against oxidative stress and play a key role as an antioxidant and anti-inflammatory[47,48]. *O. sativa* oils such as coumaric acid and vanillin exhibited very good oxidative state with range oxidative stability 5.99-7.40[49].

OSE has the lowest DPPH scavenging activity compared to vanillin and coumaric acid, this data was not validated with previous study showed that *O. sativa* extract has rich secondary metabolites, especially alkaloids and phenolic acids that have strong antioxidant activity[50]. Phenolic and flavonoid contents are associated with strong antioxidant activity and especially in terms of DPPH and nitric oxide free radical scavenging[51]. The phenolic extracts from fermented *O. sativa* showed slow inhibition kinetics of the DPPH radical, as IC_{50} value obtained was 250 mg/g compared to unfermented *O. sativa* with value 213 mg/g, while feluric acid 235.00 mg/g[52]. In another study, Poaceae plants such as *Z. mays* ethanolic extract has low antioxidant activity with IC_{50} value of DPPH 163.45 ± 6.34 µg/mL, while *T. aestivum* bran ethanol extract (0.5 mg/kg) has DPPH value 58.00 ± 1.1%[53,54]. *T. aestivum* methanol extract showed good radical scavengers with the inhibition of 12% at 1 mL/mL concentration[55]. *T. aestivum* has shown potential anti inflammatory, antioxidant, and anti aging properties[56].

Collagen, elastin, and hyaluronic acid are the skin main components and have an important role in maintaining its structure and hydration[57]. Enzymes causes repetitive collagen fibers breakdown and responsible for structural defect in dermis and wrinkle development[58]. Collagenase and elastase contribute in production and degradation of these fibers, which is also induced by free radical oxidative stress[59]. In the present study, *O. sativa* had the lowest activity in collagenase inhibition compared to coumaric acid and vanillin. *O. sativa* has been studied in anti aging treatment. Water-soluble enzymatic extract from *O. sativa* served as a protective screen against UV-B radiation on cultivated keratinocytes and in vitro reconstructed skin[60]. In the other study, the extract of *O. sativa* plants callus improve the human-skin barrier function[61]. Mechanisms of anti aging are via up-regulation of collagen sythesis in normal human dermal fibroblast and down-regulation of matrix metalloproteinase[62]. Vanillin content was also reported to inhibit matrix metalloproteinase-9 expression through

down-regulation of nuclear factor-kB signaling pathway in human hepatocellular carcinoma cells[63].

Elastin is a fibrose protein that compose 2–4% of the ECM and involved in the hydration of the skin[64]. Elastase is responsible for increased tissue permeability, inflammation progress and delayed wound healing[65]. Elastase is also the key enzyme that attacks all the major connective tissue matrix protein[66]. In elastase assay, OSE showed the lowest activity compared to coumaric acid and vanillin. Low activity of OSE in the present study might be due to low content of phenol and absence of flavonoids in phytochemical screening. Several studies showed that phenol and flavonoid content of *O. sativa* possess anti-elastase activity[51,67,68], OSE had lowest antielastase might be correlated with an absence of phenol and flavonoid. However, vanillin has the highest elastase inhibitory activity.

Our present study showed that OSE had the lowest hyaluronidase inhibitory activity compared to CA and vanillin. These results might be correlated with an absence of tannin in OSE. Tannin-rich plants have been reported to inhibit hyaluronidase and elastase release from stimulated neutrophils in vitro[69]. Hyaluronidase (Haases) selectively degrade Hyaluronic Acid (HA), which is a megadalton acidic structural polysaccharide found exclusively in the Extracellular Matrix (ECM). Haase inhibitors are thus potent regulators that maintain HA homeostasis and they might serve as antiinflammatory, anti aging, antimicrobial, anticancer and antitoxin and contraceptive agents[70]. Hyaluronidase degrades hyaluronic acid by lowering its viscosity and increasing the permeability[71].

5. Conclusion

Vanillin and Coumaric acid have antioxidant activities through DPPH scavenging, ABTS-reducing activities, FRAP and anti aging through inhibitory activity of collagenase, elastase and hyaluronidase higher than *O. sativa* extract.

6. Acknowledgment

This research was supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for a research grant, laboratory facilities, and research methodology. We are thankful to Neng Sri Mulkiyatul M. A, Kartiko Adi Poerbo and Muhammad BiharJafarian as the interns at Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for their valuable assistance.

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