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Research Article

Antioxidant and Antiaging Activity of Ethanolic Extract of Red Beetroot *(Beta vulgaris* L.): An In Vitro Study

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ABSTRACT

Aging is a process of gradual changes in the skin caused by intrinsic and extrinsic factors. Intrinsic and extrinsic aging is the effect of increasing the reactive oxygen species (ROS) produced by cells resulting in cell damage. The use of natural materials has expanded for skin damage therapy, one of which is the utilization of red beetroot (*Beta vulgaris* L.). This study aims to discover the antioxidant and antiaging activity of *Beta vulgaris* L. *in vitro*. The process begins with the extraction of the maceration method using 70% ethanol as a solvent followed by identification of secondary metabolite compounds by phytochemical screening. Antioxidant activity test used the DPPH method with quercetin as a comparison. Antiaging activity testing was conducted *in vitro* with cell viability test using Human Dermal Fibroblast adult (HDF-a) by MTT Assay and the inhibitory effect of skin degradation enzymes (antiaging) was carried out with elastase using Human Neutrophil Elastase (HNE). The result of phytochemical screening tests showed *Beta vulgaris* L. contains alkaloids, saponins, and triterpenoid compounds. Red beetroot extract has weak antioxidant activity with IC_{50} of 3284.42 µg/mL. Antiaging activity testing showed that the extract can maintain cell viability and has the inhibitory activity of the enzyme elastase. This study concludes that red beetroot extract *(Beta vulgaris* L.*)* has potential as a cosmetic active ingredient for antiaging.

INTRODUCTION

Aging is a complex process due to physical changes in the skin by intrinsic aging that occurs with age and extrinsic associated with exposure to external factors causing functional disorders (Girsang *et al*., 2020). Extrinsic aging is often referred to as photoaging because the primary cause is sun exposure that contains ultraviolet (UV) radiation (Ahmad, 2018). UV exposure can trigger the formation of Reactive Oxygen Species (ROS), which is then followed by the expression of genes and proteins that can trigger skin damage and skin cancer (Hart and Norval, 2018).

Intrinsic and extrinsic aging manifests as an increase in ROS in cells that can cause cell damage (Jia *et al*., 2014; Kim *et al*., 2016). Skin cells that decrease in number with age include keratinocytes, fibroblasts, and melanocytes. The decrease in fibroblast cells causes a decrease in skin collagen biosynthesis, leading to wrinkles (Ahmad, 2018). In addition, it also results in the overproduction of several enzymes such as elastase, collagenase, tyrosinase, and hyaluronidase which are involved in the degradation of the protein elastin, collagen, and hyaluronic acid.

In recent decades, modern science has created alternative solutions with few side effects, one of which is natural plants (Juliana *et al*., 2020). The mechanism of plants protecting the skin can be through reducing the reactivity of ROS, inhibiting the oxidation process, absorbing UV rays, suppressing enzyme activity, and reducing the formation of skin wrinkles (Nur *et al*., 2017). Nowadays, scientific research is the source of many cosmetic advancements. Physicians and patients alike frequently have questions about the indications and efficacy of a

wide range of skin-care products since their applications have grown to be so varied and intricate. Many commercially available materials used in the cosmetics industry make claims about the impact they can have on the skin when applied topically. In general, even if the results could be slight, they are important and can enhance the appearance and sensation of the skin with regular use (Ramos-e-Silva et al., 2013).

Plants have traditionally been utilized in the cosmetics business, particularly as sunblock and skin lighteners. In vitro research has demonstrated that plants have the capacity to lower antioxidant levels and block the activity of hyaluronidase, collagenase, elastase, and tyrosinase (Sumantran *et al*., 2007; Dlovu *et al*., 2013). One of the plants that can be used is red beetroot *(Beta vulgaris* L.*)* which has antioxidant activity. According to the test results, red beetroot (*Beta vulgaris* L.) has antioxidant activity with an IC_{50} value of 21.8878 g/mL. Red beetroot is reported to have betacyanin, flavonoid, tannin, triterpenoid, and steroid compounds (Elisabeth, 2020). Red beetroot also contains rich pigments such as betalains, betanin, isobetanin, and vulgaxtanin (Guilani *et al*., 2016). These data support the development of red beetroot extract as an active ingredient in antiaging cosmetics. The lack of information related to the antiaging activity of red beetroot prompted this research to determine the antiaging activity through fibroblast cell viability and elastase inhibition testing.

METHODS

Instrumentations and materials

The instrumentations used in this research were analytical balance (Santorius®), LAF cabinet (Labconco®, Kansas), centrifuge (Sorvall®, USA), ultraviolet-visible (UV-Vis) spectrophotometer (Shimadzu®, Jepang), ELISA Reader (Bio-rad Benchmark®, Jepang and Corona electric type SH-1000), rotary evaporator (IKA®, Jerman), micropipette (Socorex®, Swiss), vortex (Shimadzu®, Jepang), Oven (Memmert®, Jerman), incubator (Heraeus®, Jerman), and 96 well plate (Pyrex®). The materials used in this research were ethanol 70% (General Labora®), acetic acid (Bratachem®), Methanol (Bratachem®), Aquadest (General Labora®), DMEM, FBS, antibiotics, trypsin-EDTA, DMSO (Gibco®, New York, USA), H_2O_2, SDS 10%, HCl p.a, and assay buffer (Sigma®), Enzim Neutrophil Elastase (Sigma®), inhibitor a1–antitrypsin (Sigma®), MeO-Suc-Ala-Ala-Pro-Val-MCA substrate (Sigma®), DPPH (Sigma®), Sel HDFa (Gibco®, New York, USA), MTT (Merck®, Darmstadt, Germany), PBS (Sigma®), and SDS HCl 0.01 N (Merck®, Darmstadt, Germany).

Sample Preparation

Red beetroot *(Beta vulgaris* L*)* from the city of Malang, Lowakwaru, East Java, has been determined with No. 220/Lab.Bio/B/IX/2020 and made into simplicia and powder. Then, the maceration method was used for the extraction with 70% ethanol in a ratio of 1:5 in a closed vessel for 2-3 days and stirred every day. Afterward, the result was filtered to produce filtrate and residue. Then, the residue was macerated again (remaceration) for 2 days, while occasionally stirring and thickened with a rotary evaporator until a thick extract was obtained.

Qualitative Phytochemical Screening

Test for Alkaloids: about 50 mg of extract was added to 2 drops of Dragendorff reagent solution. The formation of orange lumpy deposits showed positive samples containing alkaloids (Alasa *et al*., 2017).

Test for Tannin: The extract was diluted with 10 mL aquadest then filtered and added 5 mL of $FeCl_3$ 1 %. A positive reaction to tannins was claimed if there was a black-blue color appearance (Harborne, 1996).

Test for Steroid/Triterpenoid: The extract in chloroform was added to 2 mL of anhydrous acetic acid and H_2SO_4 (Elisabeth, 2020).

Test for Saponin: The extract was diluted with boiled water and added to 1 drop of HCl 2 N. The formation of a stable foam showed positive samples containing saponin (Harborne, 1996).

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Scavenging Activity

Preparation of 0.4 mM DPPH solution and red beetroot extract test solutions with series levels of 1000, 2000, 4000, 6000, and 8000 µg/mL and 1, 2, 3, 4, and 5 µg/mL for quercetin. The blank solution was prepared with methanol to determine the maximum wavelength and operating time. Testing the antioxidant activity by reading the absorbance at the maximum wavelength of DPPH, then calculating the IC_{50} value (Patria and Soegihardjo, 2013).

Cell Viability Assay

The *in vitro* study of cell viability assay has ethical clearance approval with No. 077/EC-EXEM-KEPK FKIK UMY/VIII/2021. Red beetroot extract test solution with concentration series 31.25, 62.5, 125, 250, 500, 1000 µg/mL and 1000 M H_2O_2. HDFa cells 2 x 10^4 cells/well 100 µM, distributed to 96-well plate, incubated 24 hours.

Added 100 µl of the sample which was incubated for 24 hours at a 5% CO_2 incubator, temperature 37^{0}C. Then, the media was discarded, and the PBS cell was removed 100µl. Added H_2O_2 100 µl incubated for 2 hours. Added 100 µl MTT reagent 5 mg/ml in PBS and incubated for 4 hours. The reaction was stopped with a reagent stopper (10% SDS in 0.01 N HCl), incubated for 24 hours at room temperature covered in aluminum foil. The absorbance was read at a wavelength of 595 nm. Tests without H_2O_2 were also done in the same way (Nur, 2017).

Elastase assay

Inhibitory activity of elastase was measured according to the modified method of Sigma Aldrich and (Nur *et al*., 2017), 25µL of the sample (50, 100, 200 µg/mL) in 48 L assay buffer and inhibitor control (100µM). Added 2 µL of elastase enzyme, 48 µL of Tris-HCl buffer for enzyme control, inhibitor control, and 50 µL added to the blank sample. Added 2 µL substrate and incubated for 30 minutes at 37°C. The absorbance was read at a wavelength of 405nm for 10 minutes.

RESULTS AND DISCUSSION

The Qualitative Phytochemical Screening

The yield of beetroot's ethanolic extract was 35.6% w/w. One of the steps before testing an extract is to do a phytochemical test to discover what compounds play a role in the bioactivity of the red beetroot extract. Phytochemical screening of red beetroot extract showed positive compounds containing alkaloids, steroids/triterpenoids, and saponins. The test results can be seen in Table 1.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Scavenging Activity

The antioxidant test was conducted using the DPPH method based on the sample's ability to scavenge DPPH free radicals. Quercetin was used as a comparison because one of the flavonol compounds derived from flavonoids is rich in hydroxyl groups in its compound structure, so it has strong antioxidant activity (Cahyono *et al.,* 2021).

Table 1. Qualitative phytochemical screening result

Chemical Compounds	Test	Result	Observation
Alkaloid	Dragendorff test		(+) Orange lumpy deposits
Tannin	$FeCl_3$ test		(-) No change is observed
Steroid/ Triterpenoid	anhydrous acetic acid + H_2SO_4		(+) (triterpenoid) A reddish-brown coloration
Saponin	Foam test		(+) Foam

Table 2. DPPH Radical Scavenging Activity of Red Beetroot

Test Compounds	Linear Regression Equation	IC_{50} value	Information
Red Beetroot Extract	y = 0.0104x + 15.842 R^2 = 0.9448	3284.42	Weak
Quercetin	y = 4.098x + 23.882 R^2 = 0.945	6.37	Very Strong

Table 3. HDFA Cell Viability Data

Sample	Cell Viability Average (%) on Concentration (µg/mL)					
	1000	500	250	125	62.5	31.25
Cell Control	100±0.01					
H_2O_2 Control	61.42±3.94					
Red beetroot extract	62.52± 1.12	89.55± 1.85	92.41± 2.22	96.81± 3.19	95.73 ±1.42	96.71± 0.97
Red beetroot extract + H_2O_2	85.05± 5.03	86.61± 3.60	80.26± 2.67	82.00± 1.16	81.33± 2.25	84.01± 11.5

According to Table 2, the classification of the antioxidant of red beetroot extract is weak with IC_{50} is 3284.42 µg/mL, while quercetin is classified as very strong with IC_{50} is 6.37 (Blois, 1958). It is suspected that the quercetin used is pure quercetin powder, while the red beetroot extract is not a pure flavonoid compound, so there are compounds containing glycosides. According to (Fukumoto and Mazza, 2000) antioxidant activity increased in the presence of hydroxyl groups and decreased due to glycoside groups. These results are consistent with previous research (Pratiwi *et al*., 2019), which reported that the ethanolic extract of red beetroot has weak antioxidant activity with an IC_{50} value of 1614.72 µg/mL. The difference in IC_{50} value is influenced by the ability of the compound to transfer hydrogen atoms, the chemical structure of free radical scavenger, and the number of hydroxyl groups (Widyawati *et al*., 2010).

Cell Viability Assay

The cell viability test using the MTT assay method was conducted with two treatments, cell viability without H_2O_2 and cell viability with H_2O_2. H_2O_2 is a precursor of ROS chosen as an oxidant that can cause damage to cells (Craciunescu *et al*., 2012). Excessive ROS production will activate collagenase and elastase which damage elastin fibers in fibroblast cells (Sutjiatmo *et al*., 2020).

Cell viability without H_2O_2 showed that the extract did not affect normal cells, growth at a concentration of 31.25 - 500 µg/mL, while a concentration of 1000 µg/would suppress cells up to 62.55±1.12% (Table 3). Cell viability with H_2O_2 showed that the extract can protect and maintain cell viability from exposure to H_2O_2 to the lowest concentration with weak antioxidant activity. In contrast (Nur *et al*., 2017) reported that the ethanol extract of langsat fruit (*Lansium domesticum* Corr) could protect fibroblast cells with H_2O_2 through the antioxidant activity with percent viability from 77.4% to 106.86% at a concentration of 7.8 - 500 µg/mL.

The cytoprotective effect of red beetroot extract estimated to be due to the inhibition of enzymes due to free radicals (ROS) that are formed in cells like elastase and collagenase

enzymes that can degrade elastin and collagen, the components that makeup fibroblast cells. This is influenced by the flavonoid and triterpenoid compounds in the red beetroot extract, which prevents the activation of elastase and collagenase enzymes. The impact prevents wrinkling, sagging, and cell weakness (Pham *et al.,* 2017; Sahasrabudhe and Deodhar, 2010).

Elastase Assay

Testing with the modified method of sigma Aldrich and (Nur *et al.*, 2017) using the Neutrophil Elastase Inhibitor Screening Kit. The solution used was red beetroot extract (50, 100, and 200 µg/mL), control inhibitor, neutrophil elastase enzyme, and substrate. The inhibitory activity was observed by measuring the absorbance every minute for 10 minutes with an ELISA reader at 405 nm. The results are plotted against time to obtain the slope value. The antiaging activity was expressed in the percentage of enzyme inhibition compared to control.

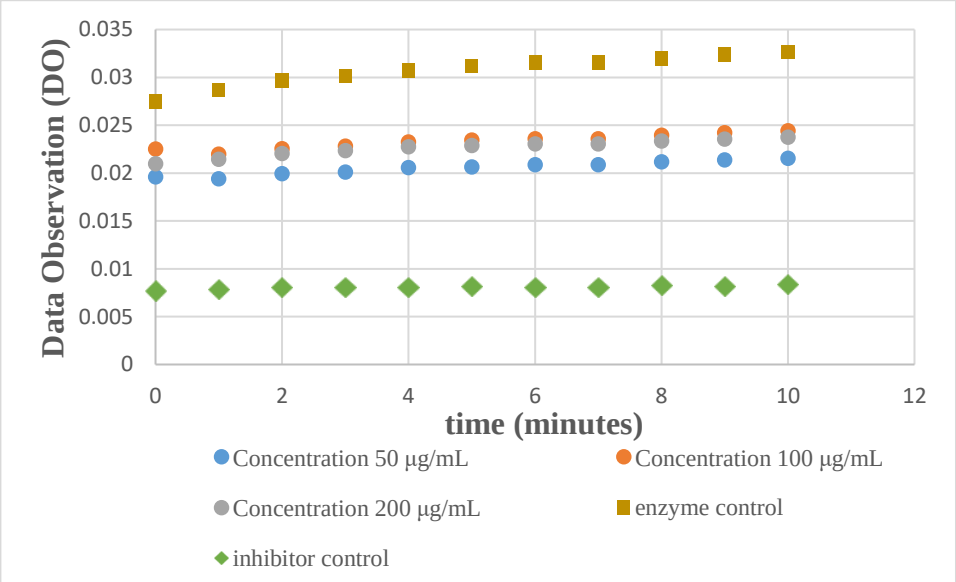


Figure 1. Kinetic Profile of Enzyme Inhibition in Control and Samples.

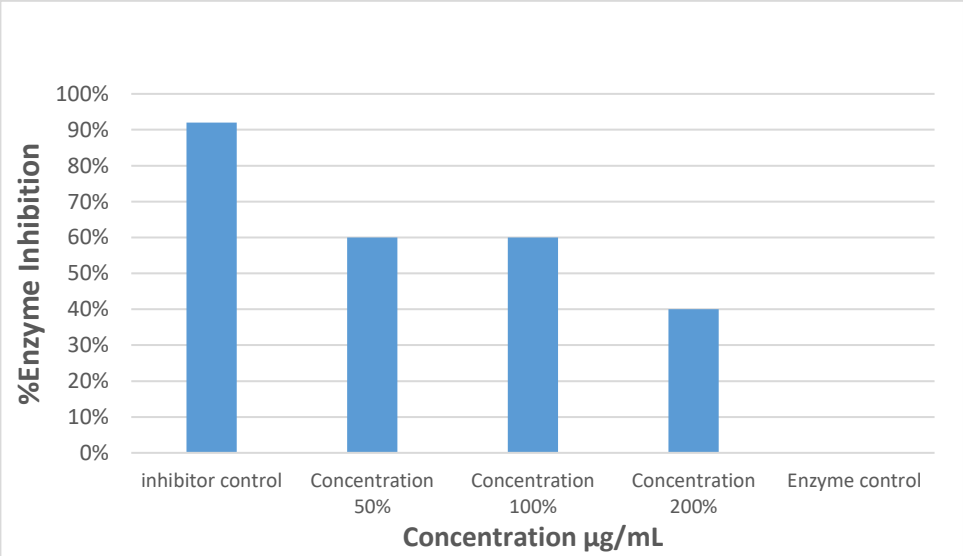


Figure 2. Elastase Inhibitory Activity of Red Beetroot.

Based on the graph above, the data show that the red beetroot extract gave a percentage of enzyme inhibition of 60% and 40% at a concentration of 50 - 200 µg/mL. concentrations of 50 µg/mL, 100 µg/mL, and 200 µg/mL were 37.71%, 33.51%, and 27.94%, respectively. This activity is estimated to be due to the flavonoid and triterpenoid compounds in the red beetroot extract. Flavonoids and phenolic compounds have a phenyl ring with hydroxyl substituents capable of inhibiting ROS, reducing metal ions, modulating protein phosphorylation in enzyme inhibition, and inhibiting lipid peroxidation (Karim *et al*., 2014; Pouillot *et al*., 2011). Based on research (Feng *et al*., 2013) related to the inhibition of human neutrophil elastase by triterpene-pentacyclic, this occurs with the formation of a competitive reversible bond between the pentacyclic triterpene compound and HNE. Based on the test results, the red beetroot extract has the potential as an active ingredient in anti-aging cosmetics that protects skin cells by suppressing the activity of the elastase enzyme and can degrade the components of the skin to prevent wrinkles on the skin.

CONCLUSIONS

Red beetroot contains alkaloids, saponins, triterpenoids, and flavonoids. Red beetroot has weak antioxidant activity by DPPH test based on IC_{50} value. However, red beetroot has good activity in the assay of elastase inhibition. The extract can protect and maintain cell viability from exposure to H_2O_2 to the lowest concentration with weak antioxidant activity This activity is estimated to be due to the flavonoid and triterpenoid compounds in the red beetroot extract. Based on these data, the red beetroot extract sample has the potential as an active anti-aging ingredient.

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CONFLICT OF INTEREST

No conflict of interest between all authors

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