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IN VITRO ANTIOXIDANT AND ANTI-TYROSINASE ACTIVITY ASSAY OF GARLIC (*ALLIUM SATIVUM L.*) ETHANOL EXTRACT CREAM FOR ANTI AGING

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ABSTRACT

Garlic (Allium sativum L.) is traditionally used for hypertension, cholesterol, anti-cancer and antioxidants. Garlic (Allium sativum L.) has active compounds that function as antioxidants, such as flavonoids, phenols, and organosulfur compounds. This study to determine the antioxidant and tyrosinase activity of the ethanol extract and garlic (Allium sativum L.) ethanol extract cream and its evaluation. The garlic powder from freeze drying is dissolved in ethanol, then sonicated for 20 minutes. The supernatant was evaporated with a rotary evaporator. Non spesific parameter of extract standardization were water content and ash content. The extract was standardized and then formulated in to cream dosage form with 1 and 2% concentration of extract, then cream evaluated for physical properties including organoleptic test, homogeneity, pH, temperature stability, spreadability, type of emulsion, and viscosity. The results showed rendemen of extract 36.547%, Loss on drying of extract 9.93% % and ash content total of extract 1.07% and for the physical properties test of cream preparations obtained homogeneous cream, semisolid form, white bone color, smell of extract garlic, stable at 4ºC, 25ºC and 40 ºC for 24 hours, pH ~ 7, type of emulsion o/w. Garlic extract obtained in this study fulfills the requirements of non spesific parameter standardization from Farmakope Herbal Indonesia and cream formulation of garlic extract has good physical properties. Determination of antioxidant activity was carried out using the modified Ferric Reducing Antioxidant Power (FRAP) method. The antioxidant activity of the ethanol extract yielded 0.2457 Fe^{+2} / 100g, cream 1% 0.0222 and cream 2% 0.0275 Fe^{+2} / 100g. The ethanol extract of garlic was formulated into cream with different

concentrations namely 1% and 2%. The in vitro anti aging test was carried out by determining the activity of tyrosinase inhibitor in ethanol extract and cream. The result of tyrosinase inhibitor test in ethanol extract was 190,907 ppm, meanwhile for cream preparation it was done on a cream that had high antioxidant activity is 2% cream. The results of the 2% cream test had tyrosinase enzyme inhibitor activity, namely 217.566 ppm. Although the ethanol extract and 2% cream have weak tyrosinase enzyme activity, further research is needed with other extraction methods such as maceration and percolation.

KEYWORDS: Garlic, Cream, Tyrosinase, Antioxidant, FRAP, Anti Aging.

INTRODUCTION

The desire of women to look beautiful, they do not realize the safety of a cosmetic product. Skin care is one of example treatments that are widely used, especially skin care that provides anti-aging in the form of serums, creams and lotions.[1,2] Aging is a biological process that cannot be avoided by every human being, but aging faster than his age is undesirable. Many factors can cause skin aging faster than normal, ultraviolet radiation (UV), excess alcohol consumption, tobacco abuse and environmental pollution.[3] Of all the factors and physical signs of aging skin, there are important factors associated with the aging process, namely free radicals ROS (Reactive Oxygen Species). The largest source of free radicals comes from exposure to ultraviolet (UV) rays.[4]

Free radicals can be prevented by antioxidants.[4] Garlic (Allium sativum L.) is a plant that is used by the community as a cooking spice and herbal medicine. Garlic is traditionally used for the prevention and therapy of several diseases, such as antihypertensives, anti-cholesterol, antithrombotic and antioxidant properties.[5,6] The compound in garlic that has an effect as a strong antioxidant is allicin.[5] Allicin is the main compound in garlic which has an antioxidant effect. This compound can be used to reduce premature aging by working to inhibit the leukocyte storefront protein, which is the cause of aging. By inhibiting leukocyte storefronts, the cycle of apoptosis and oxidative stress is disrupted, so it can reduce premature aging which is marked by loss of skin elasticity.[5,6]

The high antioxidant activity in garlic is the reason for making the formula into a cosmetic anti aging cream. Where cream is one of a series of skin care that has benefits as a moisturizer. Creams can reduce fine lines and protect facial skin (American Academy of Dermatology Association, n.d.). The in vitro test for the anti-aging effect of garlic ethanol

extract and cream is a tyrosinase enzyme inhibitor activity test. Tyrosinase enzyme inhibitor is an in vitro anti aging test, which works by inhibiting the tyrosinase enzyme to produce excess melanin. The in vitro test of the anti aging effect of extracts and creams aims to see if there is any change in the anti aging effect before and after the formulation.

MATERIALS AND METHODS

Materials

The plant studied was garlic (Allium sativum L.) which was found in the Lembang Kec. Selayo, Solok City, West Sumatra, Indonesia. The chemicals used are ethanol (Merck, Germany), ortho-phenanthroline (Sigma- aldrich, Germany), iron (II) sulfate heptahydrate (Sigma-aldrich, Germany), sodium acetate trihydrate (Sigma- aldrich, Germany), aquadest (Bratacho, Indonesia), iron (III) chloride hexahydrate (Sigma-aldrich, Germany), L-DOPA (Sigma-aldrich, Germany), Aquabidest (Bratacho, Indonesia), dimethyl sulfoxide (DMSO) (Merck, Germany), kojic acid (Sigma aldrich, Germany), enzyme tyrosinase (Sigma aldrich, Germany), NaH2PO4.2H2O (Merck, Germany), Na2HPO4 (Merck, Germany), iron (III) chloride hexahydrate (Sigma- aldrich, Germany), Aquades (Bratacho, Indonesia)), propylene glycol (Bratacho, Indonesia), triethanol amine (Bratacho, Indonesia), cetyl alcohol (Bratacho, Indonesia), stearic acid (Bratacho, Indonesia), ascorbic acid (sigma-aldrich, Germany), nipagin (Bratacho, Indonesia), and glycerin (Bratacho, Indonesia).

Preparation of Garlic (Allium sativum L.) Ethanol Extract and Qualitative Screening Assay

500 g of garlic is cleaned from the skin, pressing out juice from whole garlic without removing the husk, the remaining was freeze drying (powdering). Store the garlic powder in a closed container and protected from light until it will be used.[7,8,9,10] Then 50 g garlic powder was mixed with 100 mL of ethanol, sonicated for 20 minutes, then centrifuged at 3750 rpm for 10 minutes, until a supernatant was obtained. The collected supernatant is evaporated with a rotary evaporator until a thick brown liquid is obtained.[11] The phytochemical assay was conducted on garlic (Allium sativum L.) Extracts using modified Farnsworth method to qualitatively identify presence of flavonoids, phenol, saponins, alkaloids, triterpenoids and steroids.[4]

Flavonoid Identification

The flavonoid test was carried out by using the shinoda test, namely Mg and HCl. Add a few

drops of Mg and HCl to the sample solution and observe the color change. If a reaction is positive it is marked with red or orange color after a few minutes.

Phenol Identification

Solutions containing phenolic compounds were carried out by adding FeCl3 solution and observing the color reaction that occurred, namely blue or purple black.

Saponin Identification

Saponin test uses concentrated H2O or HCl reagent, if the reaction is positive, it is indicated that foam is formed which does not disappear with the addition of concentrated HCl.

Alkaloid Identification

Mayer reagent is a specific reagent for alkaloids. A positive sample solution contains alkaloids if a white precipitate is formed or a cloudy solution is present.

Terpenoid and Steroid Identification

The sample solution was tested with the liebermann-burchard solution reagent. A positive reaction if there is a red or purple ring in the top or bottom solution of the sample for triterpenoids. But if there is a green or green blue ring is steroid positive.

Creams Formulation

Table 1: Formula of Cream.[12]

Material	Formulation (g)		
	0 (Blanko)	F1 (1%)	F2 (2%)
Extract	-	1	2
Propylen Glycol	7	7	7
Triethanolamine	1	1	1
Vaselin	5	5	5
Na_2EDTA	0,05	0,05	0,05
Cetil alcohol	3	3	3
Stearic acid	3	3	3
Glycerin	0,2	0,2	0,2
Methyl paraben	0,2	0,2	0,2
Aquades	Ad 100	Ad 100	Ad 100

Evaluation Organoleptic test

Cream was evaluated for its organoleptic properties like color, odour, and state.[1] Results are listed in Table 4.

Homogeneity test

Homogeneity test was carried out on the glass surface by visual appearance and touch.[3]

Stability test

Creams were divided into four parts and stability test was performed at 4°C in refrigerator and at 25°C, 40°C. The above parameters were observed for 8 weeks at weekly intervals.[3] Physical stability of cream by centrifuge at 3750 rpm with vibration are widely used for the evaluation of the physical stability of cosmetic products. Parameters of stability was phase separation, crystallization or precipitation of ingredients, color changes. Result are listed in Table 5.

pH test

pH test used pH meter was calibrated. About 1 g of the cream was weighed and dissolved in 10 ml of distilled water and its pH was measured.[3]

Viscosity and Rheology

Viscosity measurement was determined by Brookfield Viscometer. The viscosity measurements using LV-6 spindle. The developed formulation was poured into the adaptor of the viscometer and the angular velocity increased gradually from 2, 4, 10 and 20 rpm, then 20, 10, 4, and 2 rpm.[13]

Spreadability test

The sample is placed in a round glass of a certain diameter and has been coated with graph paper and then the prepared sample is loaded with 0,5 g for 15 seconds, so that the sample is distributed. Then give the load on it, with a dosage weight of 1, 3.5 and 7 g, then let stand for 60 seconds and calculate the increase in area that occurs on the basis.[14]

Emultion test

A blue dye from metylen blue was mixed with the cream. A drop of the cream was placed on a microscopic slide, if the disperse globules appear blue the continuous phase colourless, the cream is water-in- oil (w/o) type. The reverse condition is occurs in oil-in-water (o/w) type cream i.e. the disperse globules appear colourless and the continuous phase blue.[15]

Irritation test

An area (1sq.cm) was marked on the left hand dorsal surface of volunteers. Checked up to 24 hours if any irritancy, erythematic, edema.

Antioxidant activity

Preparation of Extract solutions

Accurately weighed 10 g of the extracts and dissolved in 10 ml of ethanol to obtain solutions of 200 mg/ml concentration. Furthermore, 6 mL (200 mg/mL) pippete into volumetric flask 10 mL, add aquadest to obtain solution of 120 mg/mL.

Preparation of Cream solutions

Accurately weighed 5 g of cream and dissolved in 10 ml of methanol to obtain solutions of 500 mg/ml concentration.

Ferric Reducing Antioxidant Assay (FRAP)

The Ferric Reducing Antioxidant Power Assay (FRAP) was estimated using modified method from Vitchipan.[16] The FRAP reagent was prepared freshly in volumetric flask 100 mL by mixing 10 mL acetate buffer 0.3 M, 1 mL of 1,10 ortho-phenantroline 30 mmol/L and 1 mL of ferric chloride hexahydrate 20 mM. In a reaction tube 0.1 mL of sample solution of and 0.3 mL of deionized water were added into 3 ml of FRAP reagent. Absorbance was measured at 510 nm after 30 min. A standard curve was prepared using different concentrations of FeSO4.7H2O (0.1 - 0.5 mM). The antioxidant efficiency of the sample solution was calculated with reference to the standard curve given by a Fe^{2+} solution of known concentration. Ascorbic acid 100 µg/mL was used as a positif control. Ferric reducing power of the sample was expressed in mol Fe^{2+}/100g.

Tyrosinase activity

The tyrosinase inhibition activity of the garlic extract and cream was assayed by the modified dopachrome method using L-DOPA as a substrate.[17] Briefly, 50 µl of five serial concentrations of the extracts (250-15.625µg/ ml) dissolved 10mL in DMSO, 20 µl of 250 units mushroom tyrosinase solution in 50 mM phosphate buffer, 100 µl of L-DOPA solution in 50 mM phosphate buffer, and 30 µl of 50 mM phosphate buffer were added into each well of a 96-well plate. The mixture was incubated at 37±2 °C for 60 min and the absorbance at 450 nm was measured. The absorbance was measured before and after incubation. Kojic acid (1000-62,5µg/mL) was used as a positive control. All experiments were performed in triplicate.

Table 2: Tyrosinase activity assay.

Material	Volume (µL)			
	KS	S	KB	B
Sampel (Extract, Creams, and kojic acid)	50	50	-	-
Phosphate buffer	150	30	200	80
Mushroom tyrosinase	-	20	-	20
L-DOPA	-	100	-	100

Note :

KS : control sampel (Sampel + Buffer phosphate) S : sampel (extract/kojid acid/cream).

KB : control blanko (buffer phosphate).

B : blanko (buffer phosphate, L-DOPA, Mushroom tyrosinase).

RESULT AND DISCUSSION

Preparation of Garlic (Allium sativum L.) Ethanol Extract and Qualitative Screening Assay

The freeze drying method was chosen because instability of garlic compounds, according to the research of Phadatare et al., (2014)[19], the powder produced from crushed garlic will make allicin inactive until it is used with the appropriate solvent.[19] In this study, 70% ethanol solvent was chosen, because it is a universal solvent in dissolving compounds non-polar, semi-polar to polar compounds.[20] The ethanol extract was obtained by dissolving garlic powder with 70% ethanol then sonication for 20 minutes then centrifuged at 3750 rpm for 10 minutes. The results of centrifugation were evaporated with a rotary evaporator until a thick liquid was obtained with an extract yield of 36.54%. In other literature, using the same solvent, the yield was 20.98% and 8.90%, the difference in the amount of yield obtained was due to the influence of the location of the garlic, powder and extraction time.[10,21] Non-specific characteristics which include determination of water content, and total ash content . Measurement of water content in a material is needed in various fields, especially in an extract derived from the plant. High levels of water can cause mould growth. The loss on draying of extract 9.93%. The purpose of determining loss on drying is to determine the amount of compounds lost during the drying process contained in the extract, because it will affect the active substance content. Total ash content in samples is 1.07 %. The nature and yields of the extracts were given in Table 3. The qualitative phytochemical analysis showed the presence of flavonoid, phenol, saponin, triterpenoid, steroid and absence alkaloid.

Table 3: Qualitative Phytochemical Analysis of The Extracts.

No.	Secondary Metabolites	Reagents	Result
1	Flavonoid	Shinoda Test (Mg powder/ HCl)	+
2	Phenol	Ferric (III) chloride	+
3	Saponin	Water/ HCl	+
4	Triterpenoid	Liebermann-Burchard	+
5	Steroid	Liebermann-Burchard	+
6	Alkaloid	Mayer	-

While the alkaloid test with mayer reagent was not detected. The qualitative test results of these secondary metabolites are in accordance with Priska et al., (2019)[20], the positive ethanol extract contains flavonoids, phenols, and terpenoids, while alkaloids with dragendrophic and mayer reagents are negative. Based on the test results, the ethanol extract of garlic has activity as an antioxidant to ward off free radicals that cause premature aging. This is in contrast to the research of Arify et al., (2018)[22] the results of testing for secondary metabolites do not contain phenol, tannin, and alkaloid compounds, but contain saponins, flavonoids, and terpenoids. The difference in secondary metabolite yields can be due to the difference in the source of garlic obtained, because it can be influenced by soil type, temperature, and the plant environment obtained.

Organoleptic and homogeneity test.

Table 4: Organoleptic test and Homogeneity.

parameters	Formulation		
	Blanko	**F1**	**F2**
Smell	None	Aromatic	Aromatic
Color	White	Pale	Broken white
Texture	Smooth	Smooth	Smooth
homogeneity	Homogenous	Homogenous	Homogenous

Stability test

The stability of the cream was tested in two ways, namely mechanically and by the influence of extreme temperatures, namely 4 ℃ and 40 ℃. The cream was tested mechanically by means of a centrifuge at 3750 rpm, the results showed that the cream was stable and did not experience separation, while stability testing which was influenced by temperature showed that the cream was stable without any separation and physical changes at both extreme temperatures (40 ℃) and cold temperatures (4℃). The stability of the cream is in accordance

with the research of Sanad & Mabrouk (2016)[23], cream from garlic oil with storage at 25 ℃ for 12 months is stable without any separation of phase, odor, color and homogeneity. This is also supported by the research of Nalla & Chinnala (2017)[24], which reported that topical preparations, namely ointments from garlic, were stable at 2, 25 and 35 ℃ for 4 weeks of storage.

Table 5: Stability testing.

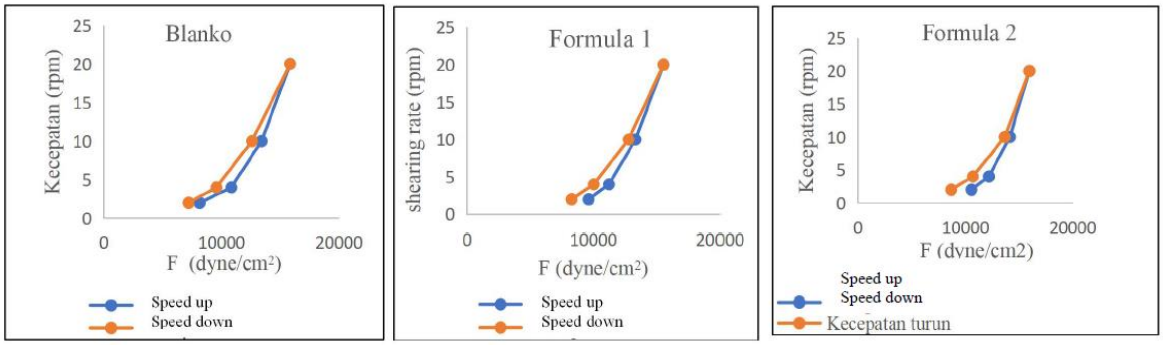
Formulation/ temperature	day to-0	color	Homogeneity	Phase separation
Blanko 25℃ 40℃ 4℃	white pale broken white	None changes	Homogenous	- - -
F1 25℃ 40℃ 4℃	white pale broken white	None changes	Homogenous	- - -
F2 25℃ 40℃ 4℃	white Pale Broken white	None changes	Homogenous	- - -

pH test

The cream's pH has met and requirement of SNI 16 - 4399 – 1996. i.e. having pH corresponding to skin's physiological pH of about 4.5-8.0. The pH value is an important parameter, because the pH is too acidic, it will irritate the skin, if it is too alkaline it causes scaly skin.

Viscosity and rheology

The research result showed that viscosity value is around 29000-35000 cps. This indicates that it qualifies a good cream (2000-50000 cps) of SNI 16 - 4399 – 1996 . Viscosity value in this research is influenced by concentration of extract and storage time. Creams has thixotropic flow are ideal for semisolid preparations.[18] Based on the torque (%) value multiplied by the tool constant, the F value and rotating speed (rpm) are obtained. Make a curve between the x-axis (velocity) and the y-axis (F), the formula flow properties are obtained, namely thixotropic, in making cream according to the flow properties expected in the cream making reference. Sanad & Mabrouk (2016) reported that garlic cream has thixotropic flow properties.



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Gambar 1-3: Rheology cream.

Spreadability test

Spreadability test was conducted to determine the ability of cream's diffusion on the skin. The easier the cream is flattened on the skin, the greater the absorption of its active substances. Formula blanko, FI, and FII have not met the requirement a good dispersing power (5.6 - 6.4 cm). Extract can decrease the disperse of cream.

Irritation test

The formulation blanko, F1 and F2 shows no redness, edema, inflammation and irritation during irritancy studies. These formulations are safe to use for skin.

Antioxidant activity

Determination of antioxidant activity using a modified FRAP method, namely using 1,10 phenantrolin to replace TPTZ. The use of 1,10 phenantrolin has advantages over TPTZ, namely ortho phenantrolin is more accurate, accurate and more correlated with FRAP than TPTZ (Yefrida et al., 2018).[24] The principle of the FRAP method is to reduce Fe^{+3} to Fe^{+2} in order to obtain ethanol extract, and F1 and F2 creams with activity. weak antioxidants, namely 0.2457 Fe^{+2}/100 g; 0.0222 Fe^{+2} / 100g; and 0.0275 Fe^{+2} / 100g. Meanwhile, ascorbic acid as a positive control had a strong antioxidant activity, namely 116,379. The difference in antioxidant activity is because ascorbic acid is already in the form of a pure compound, while garlic is still in the form of an extract with various compound components still present in it. Jang et al., (2017)[9] reported that ethanol extract had the highest antioxidant activity compared to chloroform solvents and distilled water. The low antioxidant activity of cream compared to extracts is because garlic has a compound that is less stable at room temperature, so that it can turn into other compounds that have less activity or have no activity at all (Ganatrika et al., Nd).

The difference in antioxidant activity in black garlic and fresh garlic is five times greater than fresh white garlic. The difference in activity was influenced by differences in the treatment, the type of garlic, the environment where the samples were taken, namely temperature and weather and the difference in the instruments used and the extraction method.

Table 6: Extract ethanol, cream and ascorbic acid has lower activity.

Sampel	regresi	Antioxidant activity (Fe^{+2}/100g)
Extract ethanol	y = 0.696x + 0.161 R = 0.999	0.2457
F1		0.0222
F2		0.0275
Ascorbic acid		116.379

Tyrosinase activity

In the results of tyrosinase activity, ethanol extract, FII cream and kojic acid as positive controls had weak activity in inhibiting the tyrosinase enzyme that causes premature aging, namely black spots. According to Limtrakul (2016)[26] garlic has the ability to reduce premature aging by inhibiting the leukocyte elastase protein which causes fine wrinkles on the face. This contrasts with the research of Somman & Siwarungson, (2015)[27], fresh white bawnag has a high tyrosinase activity compared to processed garlic.

Table 7: Tyrosinase activity from sampel.

Sampel	y = a+bx	IC50 (µg/mL)
Extract ethanol	y = 0.1642x + 13.894	190.907
Cream	y = 0.1675x + 18.023	217.566
Kojic acid	y = 0.027x + 45.926	150.889

CONCLUSION

The ethanol extract of garlic, cream F1 and cream F2 had very weak antioxidant activity and weak tyrosinase activity. The cause of low antioxidant and tyrosinase activity can be influenced by the source of the garlic produced, the instrument used, and the extraction method as maceration, percolation etc.

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