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Red pomegranate (*Punica granatum* L.) peel extract mud mask formulation and tyrosinase-inhibition activity test

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ABSTRACT: The peel of red pomegranates (*Punica granatum* L.) contains gallic acid and ellagic acid, which inhibit tyrosinase, an enzyme that aids melanin formation. This study aimed to determine how variations in the concentration of kaolin base affected the evaluation outcomes of mud mask formulations containing *P. granatum* fruit peel extract. *P. granatum* fruit peel extract was used in 3 formulas with variations in the kaolin base concentration of 19.5%, 22.5%, and 26.3%. The three formulations were subsequently evaluated through a series of procedures, which included organoleptic, homogeneity, spreadability, viscosity, pH measurements, and drying time. The enzyme-linked immunosorbent assay (ELISA) method was employed to determine the inhibitory activity of the tyrosinase enzyme. Formula 2 gives the best results with the characteristics of having a distinctive odor and thick brownish-green color, homogeneous preparation, spreadability of 20.5863 cm^2, a viscosity of 16040 cPs; pH of 5.86, and drying time of 16.67 minutes. Inhibition of the tyrosinase enzyme by kojic acid, extracts, and preparations resulted in respective IC_{50} values of 31.64 µg/mL, 155.49 µg/mL, and 276.15 µg/mL. The results of the viscosity test, pH test, and mask drying time were significantly different as the concentration of the kaolin base varied ($p<0.05$).

KEYWORDS: Mud mask, *Punica granatum*, tyrosinase.

INTRODUCTION

Apart from being exposed to cosmetics and pollution, the face is also exposed to direct sunlight, which can cause a darkening facial skin color. The compounds ellagic acid and gallic acid are compounds that can prevent the darkening of skin color which can be found in natural ingredients such as the peel of red pomegranate (Punica granatum L.) from the Punicaceae tribe, which has a phenol content above 1.80%, calculated as gallic acid [1]. The ellagic acid and gallic acid compounds in the peel of *P.granatum* fruit have activity as inhibitors of the tyrosinase enzyme. This enzyme plays a role in the formation of skin pigment so that skin color does not darken. Copper inhibits tyrosinase enzyme activity in the tyrosinase active site, which has an affinity for ellagic acid [2]. As much as 30-40% of the total *P.granatum* fruit is fruit peel [3]. The 70% ethanol extract of *P.granatum* fruit peel has a total phenolic content of 490.12 µg GAE/g and an IC50 value of 0.69 ± 0.98 µg/mL for tyrosinase enzyme inhibition in *P.granatum* Sripanya fruit peel extract [4].

One widely used facial care product is a facial mask. Facial masks use active ingredients to achieve healthy facial skin according to the wishes of using the mask. One type of mask that can remove facial dirt is a clay mask or mud mask. This mask must be made with mineral clay, such as kaolin and bentonite. Kaolin functions to absorb dirt in facial pores, makes facial skin smooth, prevents acne, and improves blood circulation. At the same time, bentonite acts as a softener by absorbing dirt that clogs facial skin pores. Kaolin and bentonite are the two bases used to make mud masks because this combination of ingredients provides excellent benefits for facial skin. The ability of *P.granatum* fruit peel to inhibit the formation of melanin is used as an active ingredient in mud mask preparations. The benefit of a mud mask base, which can absorb pore-clogging dirt, is that it is a good combination choice as a cosmetic preparation because it can achieve healthy skin. The purpose of using mud mask preparations in this research is to overcome skin problems often exposed to outside air, especially pollution and long-term exposure to direct sunlight [5]

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MATERIALS AND METHODS

Materials

Plant material

Red pomegranate peel powder (*Punica granatum* L.) was from BALITTRO, Bogor, Indonesia. *P. pranatum* was determined at the Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia, under the reference number 981/UN2.F3.11/UN2.F3.11/2022.

Chemical

The materials used for this research were tyrosinase enzyme, L-DOPA substrate, and kojic acid from Sigma Aldrich, USA. Kaolin, bentonite, xanthan gum, propylene glycol, phenoxyethanol, purified water, NaOH, and 70% ethanol were obtained from PT. Bronson & Jacobs, Indonesia.

Preparation of *P. granatum* fruit peel extract

A total of 500 g of dry simplicia powder was weighed, put into a kinetic maceration vessel, and added with 5 L of 70% ethanol solvent. Then, the ethanol extract was stirred every 6 hours at a speed of 500 rpm and left for 18 hours, and the maceration results were filtered using a Buchner funnel. Then, the filtrate was concentrated using a rotary vacuum evaporator with a vacuum pressure of 175 mmHg at a speed of 40 rpm at a temperature of 50 °C to obtain a thick extract. The concentrated extract was then subjected to organoleptic and water content tests with Karl Fischer, weighed, and the yield of the extract and DER-native was calculated using the following formula:

Yield value = (extract weight)/(powder weight)x 100%

DER-native = (powder weight)/(extract weight)

Phytochemical screening of *P. granatum* fruit peel extract

Phytochemical screening of *P. granatum* fruit peel extract was carried out using the Farnsworth method [6]. Compounds were examined, including alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides.

Mud mask formulation

The composition of the mask is shown in Table 1. The bentonite was put into a mortar, then hot pure water was added gradually until it became wet. The expanded xanthan gum was put into a mortar containing bentonite and then crushed until homogeneous. Some kaolin was put into the mortar little by little, and the other part was dissolved in hot pure water in a glass beaker, then put into the mortar and crushed until homogeneous. The homogenized base was transferred into a glass beaker, and then the extract was dissolved in propylene glycol and added. Phenoxyethanol was put into a glass beaker containing the base and extract, then stirred with a stirrer at a speed of 200 rpm for 10 minutes. The homogenized preparation was then put into a closed container. The evaluation was carried out on the preparation of the mud mask.

Table 1. Mud mask formula for *P. granatum* fruit peel extract

Materials	F1 (%)	F2 (%)	F3 (%)
P. granatum fruit peel extract	0.3	0.3	0.3
Kaolin	19.5	22.5	26.3
Bentonite	2.25	2.25	2.25
Xanthan gum	2	2	2
propylene glycol	5.5	5.5	5.5
Phenoxyethanol	0.5	0.5	0.5
Purified water ad to	100	100	100

Evaluation of mud mask [7]

Organoleptic

The preparation results were visually observed, and then the consistency of the mask preparation was tested by applying it to the finger or palm. The odor that arose from the preparation was smelled.

Homogeneity test

The object glass was coated with 0.3 g of the mask preparation and subsequently held in conjunction with another glass object while being examined for uniformity of surface smoothness and homogeneity of the mask preparation.

Spreadability test

A total of 0.5 g of the mask preparation was placed in a spreading power tool, covered with another spreading power glass tool, given a weight weighing 200 g, and waited for 2 minutes until it formed a circle. Afterward, the vertical diameter and horizontal diameter were measured using a caliper.

Viscosity and rheology test

A Brookfield-type RV viscometer with spindle number 6 was used at 20, 50, and 100 rpm speeds. The mask preparation was placed in a glass cylindrical container or beaker. The spindle was inserted to a predetermined limit and rotated at a certain speed until the viscometer needle rested on a constant scale. The viscosity was then determined using the equation:

Viscosity = Scale x factor (cPs)

Shear stress (F) = Scale x Kv (dyne/cm^2), Kv = 7187,00 (dyne/cm^2)

The flow properties were determined by a curve between the shear rate (rpm) and shear stress (dyne/cm^2).

pH determination

A pH meter (Mettler Toledo) measured as much as 1% of the mask preparation.

Drying time

A total of 0.3 g of the mud mask preparation was applied to the skin of the arm, which had been marked 4 cm long and 1 cm wide. Then, the time needed for the preparation to dry was measured. The preparation was considered dry if it formed a thin film layer, became stiff, and changed color to a more grayish shade.

Tyrosinase enzyme inhibitor activity test

The tyrosinase enzyme solution dissolves the tyrosinase enzyme with 50 mM phosphate buffer pH 6.5 to obtain a concentration of 75 U/mL. The L-DOPA substrate solution was prepared by dissolving the L-DOPA substrate (BM = 197.19) in 50 mM phosphate buffer pH 6.5 with a concentration of 10 mM. The positive control solution of kojic acid was prepared by dissolving kojic acid in 50 mM phosphate buffer pH 6.5 and making a concentration series of 50 ppm, 20 ppm, 10 ppm, 5 ppm, and 2.5 ppm. The extract and mud mask solution was made by dissolving it in 70% ethanol until a concentration of 1,000 ppm was obtained as a stock solution. Then dilutions are made with concentrations of 500 ppm, 400 ppm, 300 ppm, 200 ppm, and 100 ppm. Phosphate buffer solution (50 mM pH 6.5), tyrosinase enzyme solution, test solution, and finally, the L-DOPA substrate solution were added into a 96-well microtiter plate *(*Table 2*)*. Each sample was made into a blank (the blank did not have the tyrosinase enzyme solution added) and then incubated for 20 minutes at 37 °C. Then, the absorbance of the mixture was measured with an ELISA reader at a wavelength of 470 nm.

Table 1. 96-well microtiter plate loading table

Solution	Control (µL)	Blank control (µL)	Test sample (µL)	Blank sample (µL)
Phosphate buffer pH 6.5	120	150	110	120
L-DOPA substrate	40	50	30	40
Test (kojic acid, extract, or mask)	-	-	30	40
Tyrosinase enzyme	40	-	30	-
Total	200	200	200	200

The enzyme inhibition test was obtained from absorbance measurements with an ELISA reader at the optimum wavelength. The measured absorbance was the absorbance of dopachrome formation. From the absorbance measurements, the % inhibition of the tyrosinase enzyme was calculated using the formula:

$$\%\text{inhibiton} = ((B-S))/B \times 100\%$$

B = control absorbance minus control blank (B1-B0)

S = sample absorbance minus sample blank (S1-S0)

IC_{50} was determined using the absorbance linear regression equation, with sample concentration in logarithms as the x-axis and percent inhibition or %inhibition as the y-axis. These values were then entered into the equation y = a + bx to calculate the IC_{50} value, where it was known that y = 50.

▪ RESULT AND DISCUSSION

Preparation of *P. granatum* fruit peel extract

The resulting extract had a characteristic dark brown color and was thick with a distinctive aroma. The DER-native value obtained was 2.57 g, yielding 38.82%. The extract had good solubility in propylene glycol but was rather difficult to dissolve in water. The resulting pH value was 3.27 ± 0.02. The resulting water content value was 13.07 ± 0.89%.

Phytochemical screening

The screening results showed that the compounds in *P. granatum* peel extract were alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, and glycosides. Compounds that play a role in enzyme inhibitor activity are phenolic compounds, flavonoids, and tannins. One of the phenolic members, namely ellagic acid, plays a role in binding the active site of enzymes, which can become inhibitors. Ellagic acid and tannin are complex compounds that are members of phenolic compounds, and ellagic acid is obtained from the hydrolysis of tannin, namely ellagic acid as a structural component in cell walls and cell membranes, ellagic acid is a glucose ester which, when hydrolyzed produces ellagic acid [8].

Evaluation of mud mask preparations

The research results in Table 3 showed that FI and FII are brownish green while formula III is green. The resulting color is formed by mixing the base color with gray kaolin and bentonite with a dark brown extract. The mask preparations in all formulas are thick due to the presence of a binding ingredient, namely xanthan gum. Meanwhile, the preparation's odor is the base's typical odor, namely bentonite and kaolin. The requirements for viscosity, which is range from 2,000 – 50,000 cPs [9]. In the research, viscosity results met the requirements, with the largest viscosity result being formula III and the smallest viscosity result being formula I. This result shows that formula III is thicker than the other formulas because formula III uses a larger concentration of kaolin base. Formula III has poor homogeneity and the highest viscosity; this causes the spreadability value to be low and not meet the requirements [10] .

The research results show that the pH of the mask preparation falls within the pH range of facial skin, namely between 4.5-6,5 [7]. Measuring the drying time is influenced by the homogeneity of the preparation, where a homogeneous preparation will be difficult to dry because of the uneven drying pattern. Formula II is the composition that produces the best homogeneity so that the drying time is the best. The results of the physical and chemical evaluation of the *P. Granatum* mud mask can be seen in Table 3.

Table 2. Evaluation of mud mask preparations

Parameter	FI	FII	FIII
Organoleptic	Brownish Green Characteristic odor Thick	Brownish Green Characteristic odor Thick	Green Characteristic odor Thick
Homogeneity	homogeneous	homogeneous	inhomogeneous
Viscosity (cPs)	13120	16040	18960
Flow Properties	plastic thixotropic	plastic thixotropic	plastic thixotropic
Spreadability (cm)	5.217 ± 0,23	5.121 ± 0,12	4.813 ± 0,24
pH	5.71 ± 0,02	5.86 ± 0,08	6.13 ± 0,11
Drying time (min)	19.36 ± 0,13	16.67 ± 1,36	22.06 ± 0,08

Tyrosinase enzyme inhibitor activity test

The tyrosinase enzyme is a glycoprotein that works in melanin synthesis in melanocytes. Tyrosinase plays a role in melanogenesis, namely the formation of skin pigment, where tyrosinase regulates melanin biosynthesis by hydroxylating tyrosine to L-DOPA and then oxidizing L-DOPA to dopaquinone. Dopaquinone will form pheomelanin from cysteinyl DOPA's oxidation and polymerization process, namely dopaquinone with excess cysteine. In contrast, dopaquinone, which lacks cysteine, will form dopachrome, which will react by autooxidizing to dihydroxy-indole (DHI) and dihydroxy-indole-carboxyclic-acid (DHICA), both of which will form eumelanin [11]. Inhibition of the tyrosinase enzyme will result in the tyrosinase enzyme not playing a role in melanogenesis, so melanin is not formed.

Tyrosinase-inhibition activity test was carried out on the positive control kojic acid, *P. granatum* fruit peel extract solution, and mud mask preparation solution to compare the IC_{50} values obtained. Tyrosinase has two activities in the catalytic cycle, namely monophenolase activity, where hydroxylation of monophenols (L-tyrosine) becomes O-diphenols (L-DOPA) and diphenolase activity, where tyrosine oxidation of O-diphenols becomes O-quinones (O-dopaquinone) [12],[13]. The working principle of measuring the inhibition of the tyrosinase enzyme is the reduction of dopachrome, a compound resulting from the oxidation of L-DOPA formed from dopaquinone that lacks cysteine. The dopachrome formed will be dark orange to red; if there is inhibitory activity of the tyrosinase enzyme, the dopachrome color will fade so that absorption can be measured at the maximum wavelength in a microplate using an ELISA reader [13].

Kojic acid is a metabolite of chelator fungi that shows inhibitory activity against the tyrosinase enzyme. Kojic acid plays a good role as a copper chelator in the enzyme's active site. Kojic acid can act as a competitive inhibitor in the monophenolase reaction and a mixed inhibitor in the diphenolase reaction. Kojic acid was chosen as a positive control because it is a strong comparison in inhibiting the tyrosinase enzyme. However, its use as a cosmetic skin whitening ingredient is limited due to its side effects. However, kojic acid is an ingredient that is recommended as a positive control for tyrosinase enzyme inhibitors [13], [14]. The results of testing variations in concentration show that the greater the concentration, the greater the inhibition percentage, and a small IC_{50} value is obtained, namely 31.6359 µg/mL. A smaller IC_{50} value indicates that the inhibitor activity will be more potent.

P.granatum fruit peel extract contains compounds that play a role in inhibiting the activity of the tyrosinase enzyme, namely phenolic compounds and flavonoids, which will interact with free radicals produced by the active site of the enzyme. Phenolic compounds such as flavonoids act as alternative substrates for the tyrosinase enzyme. If the enzyme's active site is occupied by phenolic compounds replacing the L-DOPA substrate, the tyrosinase enzyme will not form dopachrome, so melanin will not form

[15]. From the test results, the IC_{50} value was 155.4899 µg/mL. The *P. granatum* fruit peel mask preparation was designed to have tyrosinase enzyme inhibitor activity and produce a preparation that can inhibit the formation of melanin to prevent hyperpigmentation on the skin. From the test results, the IC_{50} value of the preparation was 276.1526 µg/mL. The highest IC_{50} value of the preparation was compared with the IC_{50} value of the control kojic acid and red pomegranate peel extract. The highest IC_{50} result indicated the weakest tyrosinase enzyme inhibitor activity because the mask preparation had been mixed with other ingredients.

CONCLUSION

Punica granatum fruit peel extract has tyrosinase enzyme inhibitory activity with an IC_{50} value of 155.4899 µg/mL. The *P. granatum* fruit peel extract mud mask obtained meets the physical and chemical characteristics. The inhibitory activity of the tyrosinase enzyme in the mud mask preparation of *P. granatum* fruit peel extract has a very weak intensity because the IC_{50} value is 276.1526 µg/mL.

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