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STABILITY EVALUATION OF ROYAL JELLY GEL

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ABSTRACT

Scars that occur in surgical wounds are still a health problem worldwide. Excessive scarring significantly affects the patient's quality of life, both physically and psychologically, and can also cause pain, pruritus, and contractures and cost a considerable amount of money. Therefore, it is essential to find dressings that improve a patient's prognosis. Topical therapy for treating scars is becoming popular because it is easy to use, convenient, non-invasive, and relatively inexpensive. However, no single therapy provides optimal results to eliminate hypertrophic scar formation. For most people in the world, herbs are easier to obtain because they are widely available at relatively affordable prices, so herbal therapy is still the main choice in wound healing management. Nowadays, the selection of natural ingredients such as Royal Jelly (RJ) has increased in popularity and is in demand by the public for improving the healing of surgical wounds. The study found that the flavonoid content in the sample was equivalent to quercetin and that the total phenol content in the royal jelly extract ranged from 1.41% to 1.66%. The stability test showed that the royal jelly was stable in different conditions for up to 28 days, with no significant changes observed in pH, color, smell, texture, or spreadability.

Keywords: scar, royal jelly, surgical wounds, dressing

INTRODUCTION

Hypertrophic scars and keloids are complications of surgical treatment with high incidence. Both are aesthetically, physiologically, and psychologically disturbing and often re-grow after treatment (Rosyidah *et al*., 2018). These scars also affect the patient's quality of life, causing pain, pruritus, and contractures and costing a considerable amount of money (Ault *et al*., 2018). The incidence of scars ranged from 40% to 70% of surgical procedures and 91% due to burns (Gauglitz *et al*., 2010). Out of 1566 patients, 58 patients had complications in the form of dehiscence, 34 patients required re-stitching, 951 patients with wounds healed well, 615 patients received wound therapy for more than 4–6 days, and five of them underwent scar revision due to hypertrophic scars (Lee *et al*. 2015). The risk factors for scar hypertrophy are deep dermis

wounds and prolonged inflammation (Mohammadi *et al*., 2013). Hypertrophic scars can occur in all body parts, but the predilection is increased in the sternum, shoulders, upper arms, earlobe, and cheek.

Topical therapy for the treatment of scars is becoming popular because it is easy to use, comfortable, non-invasive, and relatively affordable (Chen *et al*., 2012). For most people, herbs and traditional medicine are easier to obtain because they are widely available at relatively affordable prices, consequently resulting in herbal therapy becoming the primary choice in wound healing management. Nowadays, the selection of natural ingredients such as Royal Jelly (RJ) is starting to be in demand by the public to heal wounds. RJ is a thick secretion from the mandibular and hypopharyngeal glands of the worker bee (Apis mellifera) and is known as the food of the queen bee (Pasupuleti *et al*., 2017; Kocot *et al*., 2018; Balan *et al*., 2020; Kunugi and Mohammed, 2019). RJ has also been widely used in commercial products, dietary supplements, and cosmetics (Kamakura, 2011). Active chemical compounds such as flavonoids and phenols in royal jelly have been shown to aid in wound healing through their antioxidant and anti-inflammatory properties (Vazhacharickal, 2021). Antioxidants help to protect against cellular damage caused by free radicals, while anti-inflammatory properties reduce swelling and redness. These actions, combined with the presence of vitamins, minerals, and enzymes, support the skin's natural healing process and improve the tissue's overall health.

RJ cream at a dose of 10% can reduce the inflammatory response in Wistar rats induced by ultraviolet radiation (Fatmawati *et al*., 2019). Topical RJ from Yamada Bee Farm (YBF), Japan, made in the form of an ointment with a concentration of 0.01% and 1%, is known to improve contact dermatitis in mouse models (Yamaura *et al*., 2013). Studies regarding the preparation of RJ gel have not been explored yet, including its stability.

This study aims to look upon the chemical (especially flavonoids and phenol contents) and physical characteristics of the royal jelly sample from Indonesia and connect it to its purported health benefits in humans, especially its effects on inflamations and wound healing.

MATERIALS AND METHODS

Royal Jelly

The royal jelly sample was brought from Griya Madu Salsabila Farm, Sragen, Indonesia. RJ was harvested in December 2021. Royal jelly is placed in the refrigerator at a temperature of -86° after harvesting. RJ is still in frozen form when received.

Preparation of Royal Jelly

Frozen form RJ processed into powder form at the Integrated Laboratory Research Center (ILRC) at the University of Indonesia, Depok. The process for producing RJ extract lyophilized powder consists of the following steps: RJ is placed in the freezer at minus 86 °C for 1 × 24 hours, the next day RJ in a frozen condition was put into the Buchi Lyovapor L-300 chamber for manual drying with a pressure of 5 millibars with a condenser temperature of 103.5 °C until dry, RJ that has dried, comes out in the form of chunks of stone and then ground with a mortar until it becomes a powder (freeze dried).

Royal Jelly Standardization

Royal jelly was sent to the Badan Riset dan Inovasi Nasional (BRIN) for standardization after being powdered. Quantitative examination was carried out to calculate the percent (%) of flavonoids and phenols as well as a qualitative examination of Liquid Chromatography Mass Spectrometry (LCMS), which was to isolate/identify the active substance content. HPLC-MS device and methods used were Waters Acquity UPLC I-Class and XEVO G2-XS Qtof (column: ACQUITY UPLC® BEH C18 1.7 µm 2.1 × 50 mm; injection volume 1 µl; full scan m/z 100 – 1200 ESI mode; mobile phase Solvent A: H2O + 0.1% Formic Acid (FA), Solvent B: ACN + 0.1% FA).

Determination of Total Flavonoid Compound Content

The total flavonoid compound content was calculated based on the aluminum chloride method. The content of total flavonoid compounds was expressed as quercetin equivalent (mg/g extract) based on the regression equation of the calibration curve. The total content of flavonoids was determined by the aluminum chloride colorimetric assay method.

Quercetin and samples were weighed as much as 4 mg each dissolved in 4 ml of methanol (mother liquor 1000 µg/ml). Standard curve measurement: A series of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml standard solutions were prepared by pipetting quercetin standard solutions (50, 100, 150, 200, and 250 µl) into in a test tube. While the sample measurement is done by pipetting as much 250 µL into a test tube. Then treated the same as the standard. In each of the above test tubes, 2 ml of distilled water and 150 µl of 5% NaNO2 were added, after 5 minutes, 150 µl of 10% AlCl3 was added. Six minutes later, 2 ml of 1 M NaOH was added, the volume was measured to 5 ml with the addition of distilled water. The mixture was homogenized, and its absorbance was measured at λ 510 nm with a UV-Vis spectrophotometer. Then a calibration curve is made by connecting the absorption value as the coordinate (Y) and the concentration of the standard solution as the abscissa (X).

Flavonoid content (mg/g extract) = C × FF × V/m (1)

C.....TFC content from the curve equation,
FF .....dilution factor (1 ml/volume (ul) of measured sample),
V.....total volume of mother liquor,
M .....sample weight in mother liquor.

Determination of Total Phenol Compound Content

A total of 10.0 mg of ethanol extract of royal Jelly was dissolved to a volume of 10.0 ml with a mixture of ethanol: distilled water (1:1). Pipette the extract solution obtained 300 µl and added 1.5 ml of Folin-Ciocalteau reagent and shaken. Silent for 3 minutes, added 1.2 ml of Na2CO 37.5% solution and allowed to stand again at range operating time at room temperature. The absorbance of the extract solution was measured by UV-Vis spectrophotometer at maximum absorbance wavelength. Two repetitions of these process where conducted.

Data analysis was first performed using the standard curve, regression method linear ($y = bx + a$) was made based on absorbance data and concentration of the solution standard, then the total phenolic content was calculated. Total phenolic content in the extract royal jelly ethanol was calculated by including the absorbance data in the equation gallic acid standard curve as the *y* value, where the *x* value obtained is milligram equivalent of gallic acid in each gram of extract (GAE).

Qualitative Examination of Liquid Chromatography Mass Spectrometry (LCMS)

The sample to be analyzed by LCMS will go through liquid chromatography to separate the components present in the sample. Furthermore, these components or molecules will be continued to mass spectrometry. The molecule can go through the ionization process, which can be done in various ways. However, one of the most frequently used ionization techniques is electrospray ionization (ESI). The sample in the form of a liquid will be pumped through the capillary and converted into tiny droplets.

Furthermore, heat and nitrogen will convert these droplets into the gas phase. In this process, the electric charge from the drop will be transferred to the molecule that is being detected. The molecule detected can be positively or negatively charged and can be detected by the machine according to the desired settings.

A mass spectrometer consisting of four metal rods arranged in parallel will select the molecule to be detected based on each molecule's mass-to-charge ratio (m/z). Molecules with unwanted m/z ratios are discarded, while molecules or analytes with desired m/z ratios are passed to the detector. The detector will produce peaks if the desired molecule is present in the sample.

The Steps for Making RJ Gel

RJ powder was also sent to the UI pharmacy laboratory to make RJ gel with a concentration of 10%. Topical preparation in the form of a gel is made by mixing the gelling material, namely carboxymethyl cellulose (CMC), as the basic ingredient of the gel and the active ingredient in the form of royal jelly extract dissolved in aquadest. Before the manufacture of gel containing royal jelly extract, optimization of gel consistency was carried out without the addition of royal jelly extract. Several concentrations were made in the optimization process, 2.5%, 5%, 7.5% and 10% CMC content. The contraceptive gel is created by heating aquadest at a temperature of approximately 80 °C; then, the royal jelly extract is dissolved in water. After that, 7.5% CMC was added according to the concentration determined from the optimization results. After CMC dissolves with distilled water, stir all ingredients until homogeneous, and a gel consistency is formed. Store the gel preparation in a small plastic tube. Then the preparation was stored at room temperature.

RJ Stability Test

Gel pH Test

The gel that has been formed is then tested for dispersion and pH measurements. The pH measurement for the gel preparation was carried out by dissolving the gel in distilled water and then measuring it using litmus paper. Measurement of pH was carried out in several time periods, namely day 1, day 7, day 14, day 24, day 28 at each concentration.

Gel Spreadability Test

The measurement of gel dispersion was carried out using microblock paper, a pair of glass containers, weights (50 and 100 grams), and a ruler. The glass cover container was weighed first and the weight was recorded, as much as 0.5 grams of gel was placed in the center of the glass container and covered with a glass lid. After 1 minute, count the distance formed on 3 different sides. Then, add a load of 50 grams in the middle, let it go for 1 minute and measure it on 3 different sides. Then the load is changed to 100 gram and measured in the same way.

Consistency and Discoloration Gel Test

Physical stability test was carried out by storing the preparation at 4 °C and 27 °C at room temperature. Then, the gel preparation was observed every week to see changes in the color and pH of the preparation. Color and pH recording starts from the first day.

RESULT

As previously mentioned, the total flavonoid contents from the sample were measured using aluminum chloride colorimetric assay method. From the calibration curve, a linear regression equation is obtained, namely y = 0.0073x + 0.0802, with a correlation coefficient (r) = R^2 = 0.955. The levels of flavonoids (C) obtained were equivalent to quercetin (mg/g extract). Tab. I represents absorbance rate based on different concentrations of the samples.

I: *Measurement results of total flavonoid*

Concentration (µ/ml)	Absorbance		
	A1	A2	A average
0	0.0506	0.0522	0.0514
10	0.1805	0.1813	0.1809
20	0.2973	0.3023	0.2998
30	0.3977	0.3958	0.3968
50	0.5050	0.5056	0.5053

The absorbance of the extract solution was measured by UV-Vis spectrophotometer at maximum absorbance wavelength to find the phenol content. Two repetitions of these process were conducted with different volumes of royal jelly (Tab. II). LCMS measurements were also conducted on the samples and components inside the royal jelly (Tab. III).

Stability test for the royal jelly were conducted in 28 days in three different conditions: room temperature, 4 °C, and exposed to the sun to see the versatility and stability of the royal jelly in different situations (Tab. IV).

DISCUSSION

Levels of total flavonoids were 0.21 ± 0.03 mg equivalent of keusetin/g extract. Whilst Flavonoids are an important natural product due to its antioxidants, anti-inflammatory, anti-mutagens, anti-viral, and anti-cancer-causing properties that is combined with the ability to control significant cell enzyme functions (Jo *et al.,* 2020; Frabasile *et al.,* 2017; Zakaryan *et al.,* 2017). The exact amount of flavonoids in royal jelly can vary depending on various factors such as the production process and geographic location. However, it is known to contain various flavonoids such as apigenin, quercetin, and kaempferol. The exact concentration of these flavonoids in royal jelly has rarely been determined.

Flavonoids are naturally occurring compounds in fruits, vegetables, and other plants; in this study keusetin were used as representation of flavonoids. They have been shown to promote wound healing by stimulating cell proliferation, reducing oxidative stress, and reducing inflammation. Flavonoids are thought to enhance the activity of certain enzymes in wound healing, such as collagen synthesis, which is essential for forming new tissue. Additionally, flavonoids have been shown to increase the production of growth factors that promote wound healing, such as transforming growth factor-beta

II: *The average total phenol content in the extract*

Sample	Test	Volume (µL)	Absorbance	TFC in extract (%)	Average TFC in extract (%)
Royal Jelly	1	250	0.1103	1.53	1.52 ± 0.10
	2	250	0.1135	1.66	
	1	500	0.1436	1.41	
	2	500	0.1482	1.50	

III: *The examination of LCMS levels*

	Component Name	Observed (m/z)	Neutral Mass (Da)	Observed RT (min)	Detector Counts	Response	Adducts	Formula	Mass Error (mDa)
1	Adenosine	268.1039	267.09675	0.64	153513	132444	+H	C10H13N5O4	-0.2
2	Mahuannin G	543.1331	542.12130	0.50	239684	174737	+H	C30H22O10	4.5
3	trans-4-Hydroxy-3-methyl-6-(1-methylethyl)-2-cyclohexen-I-one	169.1221	168.11503	3.28	23478	20928	+H	C10H16O2	-0.2
4	trans-Dihydrocarvone	153.1274	152.12012	5.53	34126	30114	+H	C10H16O	0.0
5	Vitamin B5	220.1179	219.11067	1.38	49289	42451	+H, +Na	C9H17NO5	0.0
6	Candidate mass C10H15N3O5	258.1103	257.10117	0.50	719365	-	+H	C10H15N3O5	1.8

IV: *Royal Jelly stability test*

Day 1						
Parameters	Room Temperature		4 °C		Exposed to the sun	
	5%	10%	5%	10%	5%	10%
pH	5.17	5.19	-	-	-	-
Color	Brownish yellow (normal)	Slightly darker (normal)	-	-	-	-
Smell	Characteristic odor (normal)	Characteristic odor, slightly more pungent (normal)	-	-	-	-
Texture/appearance	typical normal gel	typical normal gel	-	-	-	-
Spreadability:						
1. No Load	2.50	2.45	-	-	-	-
2. 50 gram load	3.10	2,95	-	-	-	-
3. 100 gram load	3.35	3.20	-	-	-	-
4. 150 gram load	3.65	3.40	-	-	-	-
Day 7						
Parameters	Room Temperature		4 °C		Exposed to the sun	
	5%	10%	5%	10%	5%	10%
pH	5.19	5.21	5.18	5.20	5.22	5.24
Color	Brownish yellow (normal)	Slightly darker (normal)	normal	normal	normal	normal
Smell	Characteristic odor (normal)	Characteristic odor, slightly more pungent (normal)	normal	normal	sweat on the wall	sweat on the wall
Texture/appearance	typical normal gel	typical normal gel	normal	normal	nomal	normal
Spreadability:						
1. No Load	2.50	2.45	2.40	2.43	2.65	2.50
2. 50 gram load	3.15	2.95	3.10	2.95	3.20	3.00
3. 100 gram load	3.38	3.20	3.35	3.16	3.40	3.24
4. 150 gram load	3.65	3.40	3.60	3.37	3.68	3.45
Day 14						
Parameters	Room Temperature		4 °C		Exposed to the sun	
	5%	10%	5%	10%	5%	10%
pH	5.20	5.21	5.19	5.20	5.19	5.18
Color	Brownish yellow (normal)	Slightly darker (normal)	normal	normal	normal	normal
Smell	Characteristic odor (normal)	Characteristic odor, slightly more pungent (normal)	normal	normal	a bit rancid	a bit rancid
Texture/appearance	typical normal gel	normal	normal	nomal	Sweat on the wall	sweat on the wall
Spreadability:						
1. No Load	2.45	2.50	2.50	2.45	2.65	2.55
2. 50 gram load	3.15	2.95	3,15	3.00	3.25	3.00
3. 100 gram load	3.40	3.20	3.40	3,25	3.45	3.25
4. 150 gram load	3.60	3.45	3.75	3.40	3.65	3.50

Day 21						
Parameters	Room Temperature		4 °C		Exposed to the sun	
	5%	10%	5%	10%	5%	10%
pH	5.20	5.22	5.20	5.21	5.18	5.18
Color	Brownish yellow (normal)	Slightly darker (normal)	normal	normal	normal	normal
Smell	Characteristic odor (normal)	characteristic odor, slightly more pungent (normal)	normal	normal	a bit rancid	a bit rancid
Texture/appearance	typical normal gel	typical normal gel	normal	normal	Sweat on the wall	Sweat on the wall
Spreadability:						
1. No Load	2.40	2.55	2.70	2.55	2.55	2.60
2. 50 gram load	3.10	2.90	3.15	2.95	3.20	3.00
3. 100 gram load	3.45	3.15	3.35	3.25	3.40	3.30
4. 150 gram load	3.50	3.40	3.80	3.45	3.70	3.50

Day 28						
Parameters	Room Temperature		4 °C		Exposed to the sun	
	5%	10%	5%	10%	5%	10%
pH	5.20	5.23	5.20	5.20	5.18	5.18
Color	Brownish yellow (normal)	Slightly darker (normal)	normal	normal	normal	normal
Smell	Characteristic odor (normal)	characteristic odor, slightly more pungent (normal)	normal	normal	a bit rancid	a bit rancid
Texture/appearance	typical normal gel	typical normal gel	Sweat on the wall	Sweat on the wall	Sweat on the wall	Sweat on the wall
Spreadability:						
1. No Load	2.45	2.55	2.73	2.60	2.65	2.65
2. 50 gram load	3.20	2.95	3.20	3.00	3.35	3.15
3. 100 gram load	3.55	3.33	3.37	3.35	3.55	3.45
4. 150 gram load	3.65	3.50	3.85	3.50	3.85	3.65

(TGF-beta) and epidermal growth factor (EGF). Furthermore, flavonoids have been shown to have anti-inflammatory effects, which are essential in the healing process since chronic inflammation can delay wound healing.

The average total phenol content in the extract (%) was 1.52 ± 0.10. Pure phenol is used in specific medical procedures and as an ingredient in numerous treatments and laboratory applications. Phenol compounds present in royal jelly have been shown to have antibacterial and anti-inflammatory properties. These properties make it a potential natural remedy for promoting wound healing. Royal jelly contains multiple phenols, such as p-hydroxybenzoic acid and p-coumaric acid, which have been found to stimulate the production of growth factors and cytokines that aid in tissue repair. Additionally, phenols in royal jelly have been shown to increase blood flow to the wound site, promoting the delivery of oxygen and nutrients necessary for healing.

From the examination of LCMS levels, it was found that RJ contained Adenosine, vitamin B5, Mahuannin G, Candidate Mass C10H15N3O5, trans-4-Hydroxy-3-methyl-6-(1-methylethyl)-2-cyclohexenl-one, trans-Dihydrocarvone. Adenosine increased cell proliferation, or reproduction, at the wound site and accelerated endothelial cell wound healing. Endothelial cells line blood vessels, such as arteries and capillaries. Topical adenosine may also promote the healing of wounds in individuals with impaired wound healing. Moreover, it can help treat minor injuries, cuts, and burns (Bonyanian *et al.,* 2019). Topical adenosine are also known to increases thickness of hair (Iwabuchi, 2016).

The usage of vitamin B5 is prevalent within the field of dermatology. There is a growing interest in the various effects of nonsteroidal anti-inflammatory drugs (NSAIDs) (Gunaydin *et al.,* 2018). This interest has led to a study that compares the effectiveness of dexpanthenol (an alcoholic analog of D-pantothenic acid) as an alternative treatment

to atopic dermatitis against a standard treatment of hydrocortisone. Overall, the study found that dexpanthenol can potentially treat mild to moderate childhood atopic dermatitis therapy (Udompataikul *et al.,* 2012) Candidate Mass C10H15N3O5, trans-4-Hydroxy-3-methyl-6-(1-methylethyl)-2-cyclohexenl-one, trans-Dihydrocarvone are some of the active ingredients found in royal jelly that can reinforce each other to provide effects such as antibacterial, immunomodulatory, antiviral, wound healing, growth promoter, antioxidant, nephroprotective, and anti-inflammatory (Kunugi *et al.,* 2019). Results obtained from the stability tests showed that the RJ was relatively stable, but the smell changes when the royal jelly is exposed to sunlight; in order to keep it stable, the royal jelly is best placed in the refrigerator.

CONCLUSION

The study revealed that the sample had flavonoid content equivalent to quercetin, the total phenol content in the royal jelly ranged from 1.41% to 1.66%, and the stability test showed no significant changes in pH, color, smell, texture, or spreadability for up to 28 days in different conditions. RJ has active ingredients that provide many benefits for human health. The preparation and stability study of royal jelly gel has been conducted successfully. Our findings indicate that RJ gel establishes good physical characteristics and stabilizes the active ingredient.

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