Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 10 No. 3 December 2023, 386-394
DOI: 10.20473/jfiki.v10i32023.386-394
Available online at https://e-journal.unair.ac.id/JFIKI/

Green Tea Dregs (*Camellia sinensis* (L.) Extraction Method Effect on *Cutibacterium acnes* and Development of Spot Cream

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Submitted: 31 August 2023
Revised: 12 December 2023
Accepted: 20 December 2023

Abstract

***Background**: Acne is a prevalent skin health problem experienced by teenagers and adults. Green tea is one of the plants that can be used to treat acne. Green tea dregs contain catechins, which have antibacterial activity that causes acne. **Objective**: This study aims to determine the antibacterial activity of green tea dregs extract against Cutibacterium acnes bacteria. **Methods**: This study used two brewing time variations and three green tea dregs with maceration variations. The obtained extract was then analyzed for its catechin content using the total phenolic test. Section, which has a high phenolic content, was then tested for its activity against Cutibacterium acnes bacteria using the microdilution method to obtain the MIC_{50} value. The extract with a brewing time of 2 minutes and the ultrasonic-assisted extraction maceration method had the highest MIC_{50} value of 8.586 mg/mL. The MIC_{50} value references extract concentrations used in acne spot cream formulations. The cream obtained after the stability test is semisolid, brown, and smells like tea. Spot cream is also homogeneous and meets the pH range in cosmetic preparations of 5.5. However, the viscosity of spot cream decreased significantly after storage to 4546 cPoise from 8106 cPoise. The decrease in the viscosity of the cream was caused by the catechin content in green tea dregs extract, which is acidic, thus reducing the effectiveness of the emulator in the form of triethanolamine, which is alkaline. The decrease in viscosity of the cream also caused the spreadability of the cream to increase and the stickiness of the cream to decrease.*

***Keywords**: acne spot cream, brewing time, catechin, Cutibacterium acnes, green tea dregs*

How to cite this article:
Arief, M. O. V., Lieanto, C., Sabani, J. M. & Purwanto. (2023). Green Tea Dregs (*Camellia sinensis* (L.) Green Tea Dregs (*Camellia sinensis* (L.) Extraction Method Effect on *Cutibacterium acnes* and Development of Spot Cream. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 10(3), 386-394. http://doi.org/10.20473/jfiki.v10i32023.386-394

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Acne is a common condition in which there is an overproduction of oil in the skin that leads to clogging the hair follicles. Clogged hair follicles lead to inflammation of skin pores, which is characterized by red discolouration of the skin as well as small pus-filled bumps that are known as pimples (Aslam *et al*., 2015). *Cutibacterium acnes* is present in the fatty acids of sebum secreted by sebaceous glands in skin follicles and produces lipases that facilitate the breakdown of sebum-free short-chain fatty acids that are pro-inflammatory (McLaughlin *et al*., 2019).

Because of this reason, *C. acnes* is a common bacteria that is chosen in the case of antiacne activity analysis of a drug. Clinical and laboratory studies in the last 20 years reported that many natural ingredients are potent antiacne agents.

One of the natural ingredients that is widely used in the treatment of acne is green tea. According to research from Peluso & Serafini (2017), green tea has more substantial antioxidant potential than black tea and oolong tea. These antioxidant properties are attributed to the catechin content in green tea. Catechins are likely and efficacious in treating acne as they have apoptotic, sebo-suppressive, anti-inflammatory, and antibacterial effects against *C. acnes* (Yoon *et al*., 2013).

Indonesians have long used green tea dregs as a facial beauty treatment. According to Soetjipto *et al*. (2012), green tea dregs still have antioxidant and polyphenol activity. Green tea dregs help brighten the skin, overcome acne, remove panda eyes, and soothe the skin. Green tea waste as an antiacne is commonly used as a mask. The green tea dregs mask is left to dry and rinse with water.

Based on this phenomenon, researchers are interested in knowing the potential of green tea dregs as an antiacne agent. Two variables will be tested in this study: tea brewing time and the extraction method of brewed tea dregs. Brewing time will significantly affect the content of dissolved chemicals, colour intensity, and aroma of the tea to be consumed.

The extraction method used in this study is maceration with several modifications, that are maceration at room temperature, maceration at 60ºC (digestion), and maceration with ultrasonic-assisted extraction. Acne patch cream preparation was chosen to treat acne to obtain local effects in drug delivery to the skin layer, especially on problem skin (Rai *et al*., 2019).

MATERIALS AND METHODS

Materials

Green tea (Brand A), technical ethanol (Bratako), distilled water, Na_2CO_3 (Merck), Folin reagent (Merck), Brain Heart Infusion media (Merck), *Cutibacterium acnes* bacteria, 96 well microplates (Iwaki), blue tip (Socorex), yellow tip (Socorex), white tip (Socorex), stearic acid, glycerin, propylparaben, methylparaben, triethanolamine, cetyl alcohol, and pH stick (Merck).

Tools

Analytical scales (Mettler Toledo AL204), flannel cloth, oven (Memmert), hot plate, autoclave (Hirayama Hiclave HVE-50), incubator (Sakura, Japan), laminar airflow (Nuaire Airegard NU-126-400E), Bunsen burner, micropipette (Socorex), microplate reader (AMR-100-Allsheng), vortex (Thermo Scientific), mortar, stamper, overhead stirrer (IKA) and Brookfield viscometer (Lamy Rheology B One Plus).

Method

Determination of green tea leaf

Determination of green tea leaf (*Camellia sinensis* (L.) O. Kuntze) used in this study was carried out at the Cell Biology-Microbiology Laboratory, Department of Pharmaceutical Biology, Gadjah Mada University.

Brewing and preparation of dry dregs

Green tea leaves were weighed as much as 300 g and then brewed using 1000 mL of distilled water at 100˚C for 2 minutes and 5 minutes. The brewed green tea was then filtered by a flannel cloth until the water was drained. The green tea dregs were then dried in an oven at 50˚C for 1x24 hours until completely dry. The dried material was then pulverized by a mortar and stamper.

Extraction

Extraction optimization was carried out on tea grounds in three extraction methods, namely cold maceration for 3x24 at room temperature, maceration with digestion for 6 hours at 60ºC using a water bath, and maceration with ultrasonic-assisted extraction (UAE) for 30 minutes at room temperature by an ultrasonic chamber. The weight of dry tea leaf dregs for one maceration was 75 grams. Ethanol with 70% concentration is used as a solvent because it is polar and can attract polyphenolic compounds in green tea pulp, which are opposite in nature. In addition, ethanol has a low boiling point, which is 79ºC, so it requires less heat for the concentration process. The ratio of the weight of the dry tea leaf dregs to the volume of the liquid was 1:7 *b/v*. Filtration was carried out to separate the filtrates and

residue. The filtrates were then evaporated in a water bath at 60ºC until a slightly thick extract was obtained and then dried by the freeze-drying method.

Total phenolic test with folin-ciocalteau colorimetric method

The Folin-Ciocalteau colourimetric method is based on the chemical reduction of the reagent, a mixture of tungsten and molybdenum oxide based on the method (Singleton *et al.*, 1999). The content of total phenolic compounds in the extract was expressed as gallic acid equivalents (mg/g extract) based on the regression equation of the gallic acid calibration curve. Sample solution (150 uL) and gallic acid standard solution (0, 25, 50, 75, 100, 150, 175, and 200 uL) were pipetted into test tubes. Distilled water was added to 4 mL and 250 uL Folin-Ciocalteau. The mixture was then shaken until homogeneous. After standing for 8 minutes, 750 ul of 20% Na_2CO_3 was added and homogenized. The mixture was then allowed to stand for 2 hours at room temperature. The absorbance was measured at a wavelength of 749 nm. The measurement was repeated in triplicate to obtain the phenol content as gallic acid equivalent (mg GAE/g sample).

Antibacterial activity test by microdilution method

C. acnes from glycerol stock was streaked on sterile Brain Heart Infusion (BHI) medium in a petri dish and incubated at 37ºC for 24 hours under anaerobic conditions in a jar incubator. To prepare the starter, one tip of the *C. acnes* culture was inoculated in 5 mL of Brain Heart Infusion Broth (BHIB) media in a test tube aseptically, then vortexed and incubated at 37ºC for 2 hours. The bacterial suspension was standardized using a spectrophotometer at a wavelength of 600 nm to obtain an OD of 0,08 – 0,13, which is equivalent to 1,5 x 10^8 CFU/mL (CSLI, 2017). The antibacterial activity test was carried out by microdilution method in BHI media with varying concentrations of green tea dregs extract of 3 mg/mL, 5 mg/mL, and 10 mg/mL in 1% DMSO solution. A mixture of 5 µL of *C. acnes* bacteria suspension, 50 µL of extract, and 145 µL of BHI media were pipetted into the wells as treatments and replicated three times. The negative control consisted of 145 µL of BHI media, 5 µL of bacterial suspension, and 50 µL of 1% DMSO. The inhibition calculation used the following formula (CLSI, 2017).

$$\%Inhibtion = \frac{(Negative\ control\ absorbance - Treatment\ absorbance)}{Negative\ control\ absorbance} \times 100\%$$

Acne spot cream formulation

The formulation of green tea dregs extract spot cream preparation refers to the formula optimization results of Karmilah & Musdalipah (2018), with minor modifications, which can be seen in Table 1 (Arief, 2023).

Physical stability test of cream preparation

The stability test conducted in this study was an accelerated stability test by a climatic chamber for one month with a temperature of 40ºC ± 2ºC and RH 75% ± 5% and testing intervals every one week (CSLI, 2012). Tests that are conducted every week include organoleptic, homogeneity, viscosity, pH, spreadability, and adhesion tests, as seen in Table 2.

Table 1. The formula of green tea dregs extract spot cream (Karmilah & Musdalipah (2018)). The amount of extract was performed based on the MIC50 value of microdilution analysis

Composition	Cream Formula (%)		Usability
	Control	Extract	
Stearic acid	10	10	Oil base
Glycerin	10	10	Humectants
Cetyl alcohol	3	3	Oil base
Liquid paraffin	2	2	Emollients
Triethanolamine	1	1	Emulgator
Green tea dregs extract	-	0,9	Active substance
Methylparaben	0,2	0,2	Preservatives
Propylparaben	0,05	0,05	Preservatives
Distilled water	ad 100	ad 100	Solvents

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 2. Test methods are conducted every week in the physical stability test of cream preparation. Each test was performed in triplicate

Tests	Test methods
Organoleptic test	The organoleptic test was carried out by observing the physical appearance of the preparation consisting of shape, colour, and odour (Saryanti *et al*., 2019).
Homogeneity test	The homogeneity test was conducted by weighing 0.1 g of cream and then applying it evenly to the glass object. The cream preparation must be homogeneous, characterized by absence of coarse particles that appear in the preparation (Saryanti *et al*., 2019).
Viscosity test	Viscosity measurements were carried out at 25ºC using a Brookfield viscometer, namely by installing spindle No. 6 on the device and then dipping it into the preparation to a specific limit and setting a speed of 100 rpm for 15 seconds (Saryanti *et al*., 2019).
pH test	The pH measurement was carried out using a Merck brand pH stick. The pH tolerance range of the cream ranges from 4.0-7.5 (Saryanti *et al*., 2019).
Spreadability test	A 0.5 g of cream preparation was placed on a glass on a millimetre block of paper. The glass was then covered with another transparent glass and left for 1 minute to obtain the diameter of the spread. Next, a load of 50 g to 250 g was added to the glass every 1 minute, and the diameter of the spread formed was observed. The cream preparation is expected to spread readily and evenly.
Adhesive test	A 0.5 g of cream preparation is placed on a glass object marked 4 x 2 cm. Another object glass is placed on top of the preparation and given a load of 1 Kg for 5 minutes. The test glass was placed on the test device and given a load of 80 grams. The load that has been installed is dropped, and the time until the two glass objects are released after the load is dropped is recorded.

Data analysis

Data analysis was carried out using SPSS version 20.0. The obtained data were then analyzed for normality. The normality test ensures that the research data comes from a population with a normal distribution. The normality test was conducted by Saphiro-Wilk with a confidence level of 95%. If the significance value of p-value > 0.05, it can be said that the data is normally distributed and vice versa (Rohman, 2014). Normally distributed data were tested for significance with One-Way ANOVA with a 95% confidence level.

RESULTS AND DISCUSSION

Brewing brand A green tea for 2 and 5 minutes at 100°C was chosen because it is the time and temperature commonly used by the public. In addition, 100˚C produces the highest catechin content compared to 70˚C and 85˚C (Chadijah & Qaddafi, 2021). After brewing, the green tea was drained by a flannel cloth, then arranged on a tray, and dried in an oven at 50˚C for 1x24 hours. The temperature of 50˚C was chosen because catechins are not resistant to high heat and will degrade at 98˚C (Vuong *et al*., 2011). The code of variation of brewing time and extraction method can be seen in Table 3.

Table 3. Brewing time variation code and extraction method

Code	Brewing time	Extraction method
2A	2 minutes	Cold maceration
2B	2 minutes	Maceration with digestion
2C	2 minutes	Maceration with ultrasonic-assisted extraction (UAE)
5A	5 minutes	Cold maceration
5B	5 minutes	Maceration with digestion
5C	5 minutes	Maceration with ultrasonic-assisted extraction (UAE)

The drying of green tea dregs macerate was carried out using the vacuum freeze-drying method. This method is drying materials by reducing the ambient temperature pressure to allow sublimation, transferring the solid phase to the gas phase without going through the liquid phase. This method is used because it can dry materials without heating, thereby reducing product damage due to high temperatures, and the dried product has an attractive physical shape.

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E-ISSN: 2580-8303

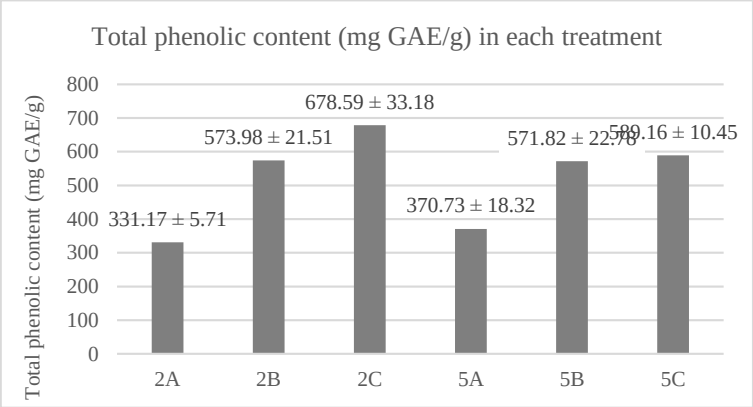

Figure 1. Total phenolic content (mg GAE/g) in each treatment. Each sample was performed in triplicate

Percent extract yield (Table 4) was obtained by calculating the ratio of the tea dregs extract produced's dry weight to the raw material used multiplied by 100%. The extract obtained is brownish green, smells typical of tea, and tastes like chelate. The yield of green tea dregs extract ranges from 12.56 - 20.99%. Green tea dregs extract yield with a brewing time of 2 minutes is greater than 5 minutes, following the theory that the faster the brewing time, the more compounds are left in the dregs.

Table 4. The yield of green tea dregs extracts of each treatment

Brewing time	Extraction method	Mass of extract (gram)	Extract yield (%)
2 minutes	Cold maceration	9.76	13.02
	Maceration with digestion	15.74	20.99
	Maceration with UAE	15.07	20.09
5 minutes	Cold maceration	9.42	12.56
	Maceration with digestion	12.81	17.08
	Maceration with UAE	11.95	15.93

The extract was then tested for total phenolic content by a colourimetric method. The maximum wavelength of gallic acid was determined by measuring a gallic acid solution with a concentration of 150 ppm in the wavelength range of 400 - 800 nm with a UV-Vis spectrophotometer. The maximum wavelength obtained was 749 nm. The maximum wavelength is needed because it will provide optimal absorption of phenolic compounds with high sensitivity. Gallic acid reacts with the Folin-Ciocalteau reagent in an alkaline atmosphere to produce a blue colour, indicating the presence of phenolic compounds. The more intense the blue colour formed, the greater the concentration of phenolic ions formed.

The absorbance of the gallic acid solution obtained is made in the form of a standard curve and the equation $y = 0.0041x + 0.003$ and R^2 0.998, which can be concluded that the data obtained is linear. The eligibility requirements for acceptable analytical methods for the correlation coefficient (R) of the range 0.996 - 1 will be used to determine the total phenolic content of green tea dregs extract. Measurement of total phenolic compounds was made as many as three replicates for precision purposes with phenol content obtained as gallic acid equivalent (mg GAE/g sample) as attached in Figure 1.

Based on Figure 1., the average total phenolic content of green tea dregs extract was 331,17 mg GAE/g in treatment 2A; 370,73 in treatment 5A; 573,98 in treatment 2B; 571,82 in treatment 5B; 678,59 in treatment 2C; and 589,16 in treatment 5C. The highest total phenolic test results were obtained in the 2C extract of 0,679 mg GAE/g, which means that every 1 gram of sample contained 0,679 mg gallic acid equivalent.

Furthermore, data analysis carried out by SPSS indicated that the data is customarily distributed and homogeneous. However, the significance of 0,00 was obtained after the One-Way ANOVA test so it can be concluded that there is a significant difference between the average total phenolic content in each treatment code. The homogeneous subset posthoc test found that green tea dregs' average total phenolic content was grouped into three subsets. In subset 1, there are treatment codes 2A and 5A; in subset 2, there are treatment codes 2B, 5B, and 5C. In subset 3, there is only treatment code 2C.

So, the highest average total phenolic is the sample with treatment code 2C of 0,679 mg GAE/g. The

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E-ISSN: 2580-8303

average total phenolics of samples with treatment codes 2B, 5B, and 5C were not significantly different. The insignificant difference between the average total phenolic content of samples brewed for 2 and 5 minutes in each maceration treatment was due to the narrow difference between the brewing times. The significant differences between the mean total phenolic content of cold macerated green tea dregs, maceration with digestion, and UAE maceration were due to temperature and ultrasonication. Maceration with digestion and UAE did not significantly differ in the average total phenolic content because both maceration processes can increase the solubility of the extracted active substances, whereas in the ultrasonication methods, there is an agitation that helps the diffusion process of active ingredients.

Only the two subsets with the highest phenolic content were then tested for antibacterial activity using the microdilution method because they were considered to have better antibacterial activity against *C. acnes* bacteria. Therefore, the antibacterial activity test was conducted on samples with treatment codes 2B, 2C, 5B, and 5C.

The bacterial suspension was standardized by a spectrophotometer at a wavelength of 600 nm to obtain an OD of 0,08 – 0,13. This OD was chosen because the bacteria are in the exponential phase in this range (Martins *et al*., 2011). The incubation time of 18 hours was determined because it follows CLSI (2012) guidelines, which are 18-24 hours. The incubation temperature of 37ºC was chosen because it corresponds to the optimum temperature for bacterial growth (Tellu *et al*., 2019). Meanwhile, concentrations of 3 mg/mL, 5 mg/mL, and 10 mg/mL were selected based on preliminary tests that fall into the range required to obtain the MIC_{50} value.

The amount of bacterial inhibition was obtained from turbidity readings using a microplate reader at OD 600 nm. The wavelength of 600 nm was chosen because it only measures the level of light scattering caused by bacteria in a culture and does not absorb bacterial growth media, which impacts increasing absorbance (Martins *et al*., 2011). The absorbance obtained was then compared with the absorbance of the negative control to determine the inhibitory value of the extract against *C. acnes* bacteria, and the data attached in Table 5 was obtained.

Based on Table 5, it is known that the highest antibacterial potential is 2C code with a MIC_{50} value of 8,958 mg/mL. This value means that the extract concentration needed to inhibit 50% bacterial growth is 8,958 mg/mL. The order of antibacterial activity in the four samples from the highest is 2C with an MIC_{50} value of 8,958 mg/mL; then 2B at 9,060 mg/mL; 5B at 9,091 mg/mL, and the lowest 5C at 9,189 mg/mL.

Table 5. Percent inhibition and MIC_{50} value of each treatment. Only samples with maceration treatment B and C were tested for their MIC because of high phenolic content

Sample	Concentration	Percent inhibition	MIC_{50} value
2B	3 mg/mL	30.304	9.060
	5 mg/mL	39.746	
	10 mg/mL	52.294	
2C	3 mg/mL	32.086	8.958
	5 mg/mL	39.834	
	10 mg/mL	52.683	
5B	3 mg/mL	28.221	9.091
	5 mg/mL	39.552	
	10 mg/mL	51.518	
5C	3 mg/mL	29.827	9.189
	5 mg/mL	38.281	
	10 mg/mL	52.171	

Furthermore, data analysis carried out by SPSS indicated that the data is homogeneous. However, the significance of 0.960 was obtained after the One-Way ANOVA analysis, so it can be concluded that there is no significant difference between the MIC_{50} values in each treatment code.

The insignificant difference between the MIC_{50} values in each treatment is associated with the average total phenolic content, which is also not significantly different. Not all phenolic compounds contained in the extract have activity against *C. acnes*. Green tea contains phenolic compounds in the form of catechins, epicatechins, epicatechin gallate, epigallocatechin gallate, gallic acid, teaflavin, and Gallocatechin (Khan & Mukhtar, 2019). In addition, the MIC_{50} value of green tea dregs extract against *C. acnes* obtained was not potent because it was more than 100 µg/mL. The low potency of the material in this investigation could be attributed to a brief maceration treatment lasting approximately 2-5 minutes.Another possibility is the usage of waste. Tea dreg means there is a previous process (maceration for drinking purposes) before maceration to get a sample for antibacterial purposes. In addition, low-quality green tea is used. According to research by Koch *et al*. (2018), the quality of green tea affects the catechin content contained in the extract, where the highest catechin content was obtained in green tea samples from Japan.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

The stability of pharmaceutical preparations is defined as the ability of a preparation in a specific packaging system to maintain physical, chemical, microbiological, and pharmacological specifications during storage and application. The stability test used in this study was an accelerated stability test for one month in a climatic chamber with a temperature of 40ºC ± 2ºC and RH 75% ± 5% and a testing interval of one week. The cream preparation in each replication has a distinctive smell of tea and a brown colour derived from green tea dregs extract. It is homogeneous, which is characterized by the absence of coarse particles and even color visible in the preparation. The cream preparation in each replication also has the same pH of 5.5. This value shows that the preparation is stable both before and after storage with an accelerated stability test.

However, the cream preparation viscosity decreased after storage with accelerated stability (Figure 2). This decrease in value probably can be caused by increasing storage temperature and absorption of water from the surrounding environment by moisture-absorbing ingredients in the formula, such as glycerin. Furthermore, the acidic green tea dregs extract lowers the efficiency of the alkaline triethanolamine, making the preparation thinner.

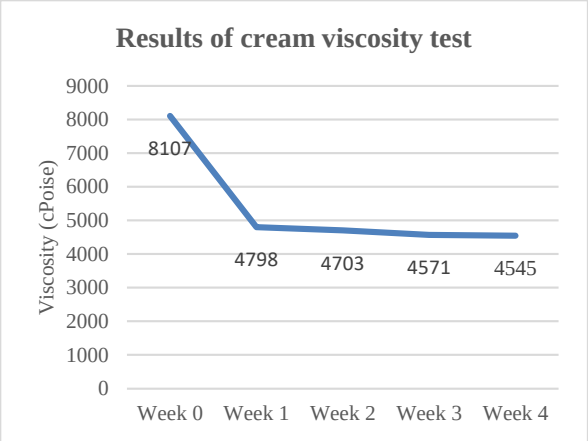

Figure 2. Results of cream viscosity test

The significance of 0,00 was obtained after the homogeneity test so that the One-Way ANOVA analysis could not be carried out and replaced with the non-parametric Kruskal-Wallis test and obtained the results of cream viscosity in week four was not significantly different from weeks 2 and 3, but significantly different from weeks 0 and 1. The cream viscosity of week 3 was not significantly different from week two but significantly different from weeks 0 and 1. On the other parameter, spreadability testing on cream preparations increased after storage with accelerated stability (Figure 3). The increase in spreadability was due to the decreased viscosity of the cream after stability testing.

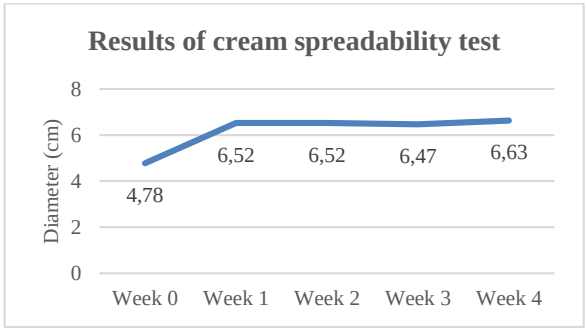

Figure 3. Results of cream spreadability test

A significance of 0,00 was obtained after the one-way ANOVA analysis, so it can be concluded that there is a significant difference between the averages of each week. In the homogeneous subset posthoc test, it was found that the spreadability of the total cream was grouped into three subsets. In subset 1, there is only week 0. In subset 2, there are weeks 1, 2, and 3 (not significantly different). So, the spreadability of green tea dregs extract cream in week 0 experienced a static increase in weeks 1, 2, and 3. Then, increase again in week 4.

Testing the adhesion of the cream preparation showed a decrease after storage with accelerated stability (Figure 4) due to the viscosity of the cream, which decreased after stability testing. A significance of 0,00 was obtained after po analysis, so it can be concluded that there is a significant difference between the means of each week. In the homogeneous subset post-hoc test, it was found that the stickiness of the patch cream was grouped into three subsets. In subset 1, there is only week 4. In subset 2, there are weeks 1, 2, and 3 (not significantly different). So, the adhesion of green tea pulp extract cream in week 0 experienced a static decrease in weeks 1, 2, and 3. Then, it decreased again in week 4.

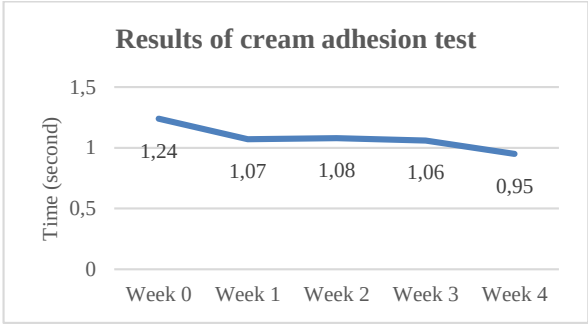

Figure 4. Results of cream adhesion test

P-ISSN: 2406-9388
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CONCLUSION

Green tea dregs have activity against acne-causing *Cutibacterium acnes* bacteria. However, there was no significant difference between green tea brewing time for 2 and 5 minutes in obtaining catechin content in green tea dregs due to the narrow time difference. In addition, there is no significant difference between maceration with digestion and UAE in obtaining catechin content in green tea dregs. In the development of the spot cream dosage form, the cream had a stable characteristic during the accelerated stability test.

ACKNOWLEDGMENT

The authors are grateful to the Department of Pharmaceutical Biology and Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University and staff who assisted in providing places and facilities during the research.

AUTHOR CONTRIBUTIONS

Conceptualization, M. A., P.; Methodology, M. A., P.; Validation, M. A., P.; Formal Analysis, M. A., C. L., J. M., P.; Investigation, M. A., C. L., J. M., P.; Resources, M. A., C. L., J. M., P.; Data Curation, M. A., P.; Writing - Original Draft, M. A., P.; Writing - Review & Editing, M. A., C. L., J. M., P.; Visualization, P.; Supervision, M. A., P.; Project Administration, P.; Funding Acquisition, M. A., P.

FUNDING STATEMENT

This research did not receive any specific grant from funding agencies in the public, commercial, or not for profit sectors.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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