Potential Combination of Pomelo Peel and Stevia Leaves Powder on The Stability of Red Blood Cell Membranes in Vitro

Ni Made Harisna Laksmi Dirtayanti, Dedia Asha Maha Dani, Burhannuddin and Heri Setiyo Bekti

Department of Medical Laboratory Technology, Poltekkes Kemenkes Denpasar, Bali-Indonesia
Corresponding author: harisnalaksmi@gmail.com

Abstract. Inflammation is a local proteactive response caused by damage to tissue because damaging chemicals, physical or microbiological trauma. Chronic excessive inflammatory reactions can also give rise to several diseases such as damage to tissues, resulting in damaging reactive radicals and autoimmune diseases. The research was conducted at Chemistry Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Denpasar. in this study the stability of red blood cells as the dependent variable and the variation of pomelo peel and stevia leaves powder as the independent variable which will affect the results of the stability of red blood cells. Pomelo peel and stevia leaves are dried in an oven at 50°C and then mashed into powder. The combination of pomelo peel and stevia leaves powder was made variation I, variation II and variation III with a different composition of pomelo peel and stevia leaves powder and then the absorbance of the sample was measured using a UV-Vis Spectrophotometer. This study was designed with *a true experimental design method with* complete randomized design, with testing the stability of red blood cell membranes tested in vitro. Anti-inflammatory activity data were analyzed using *ANNOVA's One Way SPSS (Statistical Package for Social Science)* test followed by the *Mann-Whitney test*. In phytochemical tests, the combination of pomelo peel and stevia leaves powder is positive for secondary metabolite compounds, namely flavonoids, alkaloids, tannins and saponins. The results showed that the average percentage of stability of red blood cell membranes was variation I (99.05%), variation II (98.72%), variation III (98.62%) and positive control (99.64%). So, it can be concluded that in vitro test combination of pomelo peel and stevia leaves powder has the potential to maintain red blood cell stability.

Keywords: Pomelo Peel, Stevia Leaves, Anti-inflammatory, Stability of Red Blood Cell Membrane

1 Introduction

Inflammation is a local proteactive response caused by damage to tissue because damaging chemicals, physical or microbiological trauma. Inflammation itself plays a

role in reducing, destroying or localizing both damaging agents and damaged tissues (Agustina, R., 2015). The inflammatory response is usually associated with proinflammatory cytokines and variations in plasma protein changes. In the acute phase of the inflammatory response is usually caused by a systemic reaction, in which some plasma protein concentrations increase or decrease in the inflammatory response. During the inflammation process, pro-inflammatory cytokines IL-1, IL-6 and TNF-α occur as stimulators of acute phase proteins and as a marker of chronic inflammation. Red blood cell stabilization can be used for this anti-inflammatory test because of the structural similarity between red blood cells and lysosomal cells (Tavita, G.E et al, 2022). The inflammatory response is limited by the stability of the lysosomal membrane which prevents the release of lysosomal enzymes from neutrophil granules in the inflammatory process. This enzyme is released during the inflammatory process (due to neutrophil activation) so that it can cause complications of further tissue damage. Therefore, hypotonic solution-induced stabilization of red blood cell membranes can also be used as a measure to determine the stabilization of lysosomal membranes (Kumar, V et al., 2021). Inflammation is the cause of several comorbidities including macrovascular complications which cause the highest mortality in diabetic patients (Melasari, W.P et al., 2021). In addition, excessive chronic inflammatory reactions can also cause several diseases such as damage to tissues, producing damaging reactive radicals and autoimmune diseases.

Anti-inflammatory drugs of the steroid class (AIS) and nonsteroidal class (AINS) are drugs commonly used for anti-inflammatory. The mechanism of action of steroid-class anti-inflammatory drugs is to inhibit the release of prostagladin from the source cells. Pharmacological therapy using these drugs gives side effects to patients such as kidney damage, headaches, gastrointestinal irritation, diarrhea, pancreatitis, depression and this therapy is sometimes aggressive and less effective in some cases. Steroid and nonsteroidal anti-inflammatory drugs have many side effects so it is necessary to develop anti-inflammatory drugs derived from natural ingredients, especially in planting (Ramadhani,N et al., 2017). One of the natural ingredients that can be used because of its content and easy to found is pomelo peel and stevia leaves. The phytochemical content of pomelo peel, including flavonoids, alkaloids, saponins and terpenoids. Flavonoids have antiinflammatory effects by lowering the expression pressure of TNF-α as the main proinflammatory cytokine. In addition, flavonoids can also inhibit cyclooxygenase or liposigenase and inhibit the accumulation of leukocytes in the blood so that it can be anti-inflammatory (Narande, J.M et al., 2017). The largest phytochemical content of stevia leaves is glycosides, steroids and tannins (Sevani Pongoh, G, et al., 2020). Stevia leaves as a natural sweetener have two main components, namely stevioside (3-10% of the dry weight of the leaf) and rebaudioside (1-3% of the dry weight of the leaf) which gives a sweet taste sensation. The antioxidant activity of tannins can inhibit the production of oxidants by macrophages, neutrophils and monocytes that act as anti-inflammatory (Hardani, R., 2015). Several studies related to anti-inflammatory in vitro with the red blood cell membrane stability test method were carried out because they have the same principle in influencing the inflammatory process. Based on research (Armadany, F.I et al, 2020) using the red blood cell membrane stability test method, the leaf content of bamboo-bamboo plants (Polygonum pulchrum blume) shows a very high level of anti-inflammatory activity. So, in this study, using the same method and

the application of different ingredients was applied in testing anti-inflammatory activity in vitro by combining pomelo peel and stevia leaves powder.

Based on the background and the potential of natural ingredients pomelo peel and stevia leaves, in this study did a combination of powder from both natural ingredients to be developed as an anti-inflammatory drug made from natural ingredients. In this study, a combination of pomelo peel and stevia leaves powder was carried out anti-inflammatory tests in vitro with the method of stability of red blood cell membranes.

2 Materials and Method

2.1 Research Location

The research was conducted at Chemistry Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Denpasar which included preparation of samples for making pomelo peel and stevia leaves powder, phytochemical tests, making red blood cell suspensions and anti-inflammatory tests.

2.2 Research Time

The research was carried out during 22 until 28 May 2023

2.3 Research Variable

The dependent variable is a variable that is influenced by the independent variable. Meanwhile, independent variables is variables that cause changes in the dependent variable. So, in this study the stability of red blood cells as the dependent variable and the variation of pomelo peel and stevia leaves powder as the independent variable which will affect the results of the stability of red blood cells.

2.4 Research Methods

This study used a pure experimental research design (True Experimental Design) with complete randomized design in vitro which was analyzed using SPSS (Statistical Package for Social Science) ANOVA (Analysis of Variance) one way with a follow-up test, namely the Mann-Whitney test. This method was used to determine the difference in the percentage of red blood cell stability in the combination of samples of pomelo peel and stevia leaves powder with diclofenac sodium as positive control, in determining anti-inflammatation activity in vitro through red blood cell stability tests [2]. The data obtained consists of primary data when testing the stability of red blood cells on samples. Secondary data is obtained based on a review of library sources from journals or related research references.

2.5 The Making of Pomelo Peel and Stevia Leaves Powder

Pomelo peel is obtained from fruit sellers and traders who sell pomelo and stevia leaves obtained from local farmers. Pomelo peel and stevia leaves that have been collected are cleaned using running water until clean. Then, the pomelo peel is cut thinly to facilitate the drying process. The drying process of pomelo peel and stevia leaves is carried out using an oven temperature of 50°C until the moisture content is below 10%. Next, the ingredients are blended until smooth and stored in a waterproof and sealed container. After the material has finished drying, it is made with three variation Table 1, as follows:

Table 1. Composition of Variations of Pomelo Peel and Stevia Leaves

No	Variations of Pomelo Peel and Stevia Leaves	Composition
1	Variation I	0,5gram pomelo peel powder + 1,5gram stevia leaves powder
2	Variation II	1gram pomelo peel powder + 1gram stevia leaves powder
3	Variation III	1,5gram pomelo peel powder + 0,5gram stevia leaves powder

2.6 Phytochemical Tests

To determine the content of secondary metabolite compounds, qualitative phytochemical tests were carried out. In the 3 ml alkaloid test, 3 ml samples were added 3 drops of HCl 2N, then divided into 2, tube 1 plus 10 drops of dragendorff reagent and tube 2 plus 2 drops of mayer reagent. Test flavonoids by means of 1 ml sample plus 0.1 gr of Mg powder and 10 drops of concentrated HCl. In the tannin test, 1 ml of sample is added with 3 ml of aquades and 3 drops of 10% FeCl. Then, test saponins by means of 1 ml sample plus 10 ml of hot water then shake for 10 seconds and add 1 drop of HCl 2N. The positive test results of alkaloids using dragendroff reagents are characterized by the formation of reddish-brown deposits, alkaloid tests using mayer reagents form white/yellow deposits, flavonoid tests form pink to dark red solutions, tannin tests form greenish-blue solutions and in saponin tests are marked positive with foam formation and in addition HCL 2N does not disappear (Shaikh, J.R et al, 2020.

2.7 The Making of Red Blood Cell Suspension

A blood sample of 10 mL, poured into a centrifugation tube that has been filled with sterile alsever solution, is centrifuge at a speed of 3,000 rpm for 10 minutes. The supernatant is separated and the precipitate is added with an isosaline solution, centrifuge several times until the supernatant is clear. The final stage is to make a 10% red blood cell suspension, by mixing 2 mL of red blood cells with 18 mL of isosaline solution (Tavita, G.E et al., 2022).

2.8 Red Blood Cell Membrane Stability Test

The sample to be tested for the satibility of red blood cell membrane is made with each variation carried out 3 repetitions so that there are 9 samples as a test solution. In each variation, repetition “a” is carried out, namely brewing a variation bag in the first 5 minutes, then repetition “b” in the next 5 minutes and repetition “c” in the next 5 minutes with the same bag variation. The solutions used for the stability test of red blood cell membranes are presented in Table 2, as follows :

Table 2. Red Blood Cell Membrane Stability Test Solution

No	Test Solutions	Composition
1	Test solutions	1 mL of phosphate (0.15 M), 0.5 red blood cell suspension, 1 mL of sample solution and 2 mL of hyposaline.
2	Diclofenac sodium solution (0.1%) (positive control)	1 mL of phosphate (0.15 M), 0.5 red blood cell suspension, 1 mL of diclofenac sodium solution and 2 mL of hyposaline.
3	Test Control Solution	· Test control 1: 1 mL of phosphate buffer (0.15 M), 0.5 mL of red blood suspension replacement isosaline solution, 1 mL of sample solution and 2 mL of hyposaline.
		· Test control 2: 1 mL of phosphate buffer (0.15 M), 0.5 mL of red blood suspension replacement isosaline solution, 1 mL of isosaline solution and 2 mL of hyposaline.
5	Negative control solution	1 mL of phosphate (0.15 M), 0.5 mL of red blood cell suspension, 1 mL of isosaline solution instead of sample solution and 2 mL of hyposaline.

Diclofenac sodium is an NSAID (nonsteroidal anti-inflammatory) drug that has properties as an analgesic and anti-inflammatory. The mechanism of action of diclofenac sodium is by inhibiting cyclooxygenase (COX) enzymes so that prostaglandins do not form thereby reducing the formation of pain mediators in the peripheral nervous system. The solution that has been made is then incubated 30 minutes at 37°C. Before being tested on a UV-Vis spectrophotometer, the solution is centrifuged first at a speed of 5000 rpm for 10 minutes. The supernatant fluid obtained was taken and the hemoglobin content was calculated using a UV-Vis spectrophotometer with a wavelength of 577 nm. The percentage of stability of red blood cell membranes and diclofenac sodium is calculated by the formula below (Armadany, F.I., 2020):

%Stability of Red Blood Cell

$$= 100 - \left(\frac{Abs.\ test\ solution - Abs.test\ control\ solution\ 1}{Abs.negative\ control\ solution}\right) \times 100\%$$

%Stability of Diclofenac Sodium

$$= 100 - \left(\frac{Abs.diclofenac\ sodium - Abs.test\ control\ solution\ 2}{Abs.negative\ control\ solution}\right) \times 100\%$$

3 Result

3.1 Phytochemical Test

Table 3. Result of Phytochemical Test

Sampel	Alkaloid	Flavonoid	Tanin	Saponin
Variation I	+	+	+	+
Variation II	+	+	+	+
Variation III	+	+	+	+

3.2 Red Blood Cell Membrane Stability Test

Table 4. Result of Red Blood Cell Membrane Stability Test

No	Formulation	Average percentage (%) of red blood cell membrane stability
1	Variation I (Combination of Pomelo Peel and Stevia Leaves Powder)	99,05 %
2	Variation II (Combination of Pomelo Peel and Stevia Leaves Powder)	98,72 %

3	Variation III (Combination of Pomelo Peel and Stevia Leaves Powder)	98,62 %
4	Positive Control	99,64 %
5	Negative Control	0,03 %

4 Discussion

4.1 Phytochemical Test

In this research, phytochemical tests were carried out on samples by measuring qualitatively using appropriate reagents. Based on table 3 the results of the examination of the content of secondary metabolites of the combination of pomelo peel and stevia leaves powder in variations I, II and III ishowed positive results containing alkaloids, flavonoids, tannins and saponins. The mechanism of alkaloids as anti-inflammatory is by suppressing the release of histamine by mast cells. Alkaloids have also been shown to inhibit prostaglandins and leukotrienes. Leukotrienes and prostagladins are products of arachidonic acid which will later cause leukocyte chemottaxisis. So that, by inhibition of prostaglandins and leumotrients will be able to reduce the number of monocyte cells (Fachri, H.O et al., 2020). Flavonoids are one of the largest natural phenol group compounds where flavonoids function as anti-inflammatory agents by inhibiting cyclooxygenase and lipooxygenase enzymes so that they can provide hope for treating symptoms of inflammation and allergies (Pramitaningastuti A.S et al., 2017). Tannins act as anti-inflammatories in various ways, namely inhibiting the production of oxidants by neutrophils, monocytes, and macrophages (Hardani, R., 2015). The mechanism of saponins as anti-inflammatory is by inhibiting vascular permeability and inhibiting the formation of exudate (Yuni Astika R, et al., 2022).

4.2 Red Blood Cell Stability Test

In anti-inflammatory tests, in vitro research methods are carried out using the method of stabilizing red blood cell membranes or called HRBC (Human Red Blood Cell). In this test, the stability of red blood cell membranes was measured in variations of pomelo peel and stevia leaves powder containing anti-inflammatory compounds. Red blood cells that are in hypotonic conditions and induced by analog heat so as to stimulate the inflammatory process by administering a solution of pomelo peel and stevia leaves powder as anti-inflammatory can be known for their activity in maintaining the stability of red blood cell membranes. Based on the table 4, the average percent stability of red blood cell membranes is highest in variation I. Variation I has the highest concentration compared to the other two variations. However, the results of red blood cell membrane

stability in variations II and III are similar with positive controls. This shows that variations II and III also have the same effectiveness as positive control in maintaining red blood cell stability. The stability of human blood cells is seen from the induction of hypotonic solutions. Damage to the red blood cell membrane is caused by the transfer of low concentration solvents to high concentration solvents in semipermiable membranes so that it can cause significant hemolysis. This hemolysis can occur when red blood cells are induced by a hypotonic solution. The process of measuring hemolysis that occurs can be used as a determinant of inflammatory activity in red blood cells (Kanias, T et al, 2017).

The release of phospholipase A2 is usually triggered by damage to the lysosomal membrane causing hydrolysis of phospholipids to produce inflammatory mediators. Membrane stabilization in red blood cells inhibits lysis and release of contents from the cytoplasm which in this case is analogous to lysosomes which can limit tissue damage and reduce inflammatory response (Yesmin, S et al, 2020). The discharge of hemoglobin from red blood cells is caused by hemolysis so that the solution is red. In UV-Vis spectrophotometry measurements, this color will be checked. The high intensity of the color produced indicates the number of red blood cells that undergo lysis so that the greater the absorbance read on the UV-Vis spectrophotometer (Himbert, S et al, 2020). The combination of pomelo peel and stevia leaves powder with secondary metabolite compounds owned is able to stabilize the red blood cell membrane or stabilize lysosomes by disrupting the initial process of the inflammatory reaction phase, namely the release of phospholipase A2 enzymes. Changes in phospholipids in cell membranes into arachidonic acid are highly reactive and rapidly metabolized in the process of prostaglandin synthesis (cyclooxygenase) by phospholipase A2 (Serhan, C.N and Haeggstrom, J.Z., 2010). The higher percentage of stability of red blood cell membranes is directly proportional to the anti-inflammatory activity of the combination of pomelo peel and stevia leaves powder (Zhao YL et al, 2019). If the percentage of stability of the red blood cell membrane drops it indicates that red blood cells undergo lysis and release hemoglobin into the solution so that there is no red blood cell membrane defense process in the inflammatory process (Mendonca, R et al., 2016). The combination of pomelo peel and stevia leaves powder with secondary metabolite compounds contained in it works optimally by diffusing into the red blood cell membrane and working to maintain the stability of the red blood cell membrane so that no red blood cells undergo lysis shows that the combination of pomelo peel and stevia leaves powder tested in vitro can work as an anti-inflammatory (Myint, K.Z et al., 2020).

4.3 Data Analysis

The difference in the percentage of stability of red blood cell membranes in the addition of the three variations in the combination of pomelo peel and stevia leaves powder with positive controls was then analyzed statistically. The normality test shows a significance value of 0.000 ($p<0.05$) indicating H0 is accepted and H1 is rejected, thus the data is abnormally distributed. So, the test continued with the Mann-Whitney test to determine the difference in the percentage of red blood cell stability between samples

with the addition of diclofenac sodium (positive control) and samples with the addition of various variations. A significance value of 0.100 (p>0.05) was obtained on positive control tests with all variations, indicating H0 rejected and H1 accepted, so there was no significant difference between the percentage of red blood cell stability of samples added diclofenac sodium (positive control) and samples with variation I, II and III combination of pomelo peel and stevia leaves powder

5 Conclusion

From the research that has been done, it can be concluded that the combination of pomelo peel and stevia leaves powder has anti-inflammatory abilities as evidenced by the results of in vitro tests using the red blood cell membrane stability method showing that the combination of pomelo peel and stevia leaves powder has the potential to maintain red blood cell stability. This can be seen from the average results of the stability of the red blood cell membrane variation I (99.05%), variation II (98.72%), variation III (98.62%) which is not much different from positive control (99.64%) and can also be clrarified with SPSS analysis on the Mann-Whitney test. Further research is needed with in vivo tests using experimental animals to determine its effectiveness in the body of living things and product development is needed so that it can be consumed by the public safely.

6 References

Agustina, R. et al.: Aktivitas Ekstrak Daun Salam (Eugenia Polyantha) Sebagai Antiinflamasi Pada Tikus Putih (Rattus Norvegicus). J. Trop. Pharm. Chem. 2015. 3, 2

Armadany, F.I. et al.: Uji Potensi Antiinflamasi Ekstrak Etanol Daun Bambu-Bambu (Polygonum pulchrum Blume) Dengan Metode Stabilisasi Membran Sel Darah Merah Secara In Vitro. Majalah Farmasetika. 4, (2020). https://doi.org/10.24198/mfarmasetika.v4i0.25873.

Fachri, H.O. et al.: Khasiat Ekstrak Buah Markisa Kuning (P. Edulis Sims) sebagai Antiinflamasi Dilihat dari Jumlah Monosit pada Tikus Wistar Jantan (Rattus norvegicus) (Effect of Yellow Markisa Fruit Extract (P. Edulis Sims) as Antiinflamation Seen from The Number of Monocyte on Male Wistar Rat (Rattus norvegicus)).

Hardani, R.: Uji Aktivitas Antiinflamasi Ekstrak Etanol Daun Pisang Ambon (Musa Paradisiaca L.) Terhadap Tikus Putih (Rattus Norvegicus L.) Yang Diinduksi Karagenan Anti-Inflammatory Activity Test Of Ethanolic Extract Of Banana Leaf (Musa Paradisiaca L.) On Carrageenan-Induced Paw Edema In White Rats (Rattus norvegicus L.). Galenika Journal of Pharmacy 126 Journal of Pharmacy. 1, 2, 126–132 (2015).

Kumar, V. et al.: Evaluation of anti-inflammatory potential of leaf extracts of Skimmia anquetilia. Asian Pac J Trop Biomed. 2, 8, 627–630 (2012). https://doi.org/10.1016/S2221-1691(12)60109-9.

Melasari, W.P. et al.: Effect of Atorvastatin in Lipid Profile Changes and Inflammation Marker TNF-alpha on Diabetes Patient with Dyslipidemia. Folia Medica Indonesiana. 57, 1, 6 (2021). https://doi.org/10.20473/fmi.v57i1.26326.

Narande, J.M. et al.: Uji Efek Antiinflamasi Ekstrak Etanol Daun Suji (Dracaena Angustifolia Roxb) Terhadap Edema Kaki Tikus Putih Jantan Galur Wistar.

Pramitaningastuti, A.S. et al.: Uji Efektivitas Antiinflamasi Ekstrak Etanol Daun Srikaya (Annona Squamosa. L) Terhadap Udema Kaki Tikus Putih Jantan Galur Wistar. Jurnal Ilmiah Farmasi. 13, 1, 8–13 (2017).

9. Ramadhani, N. et al.: Farmaka Artikel Review Aktivitas Antiinflamasi Berbagai Tanaman Diduga Berasal Dari Flavonoid.

10.Sevani Pongoh, G. Et Al.: Uji Efektivitas Ekstrak Kulit Buah Jeruk Bali Citrus Maxima Sebagai Antidiare Pada Tikus Putih Jantan Rattus Norvegitus.

11.Shaikh, J.R., Patil, M.: Qualitative Tests For Preliminary Phytochemical Screening: An Overview. Int J Chem Stud. 8, 2, 603–608 (2020). Https://Doi.Org/10.22271/Chemi.2020.V8.I2i.8834.

12.Tavita, G.E. Et Al.: Phytochemical Testing And In Vitro Anti-Inflammatory Activity On Ethanol Extract Of Akar Kuning (Arcangelisia Flava L) Stems From West Kalimantan. Jurnal Biologi Tropis. 22, 4, 1334–1339 (2022). Https://Doi.Org/10.29303/Jbt.V22i4.4431.

13.Yuni Astika, R. Et Al.: Uji Aktivitas Antiinflamasi Ekstrak Etanol Daun Kayu Manis (Cinnamomum Burmanni) Pada Mencit Putih Jantan. 8, 1, 2022.

Kanias, T., Lanteri, M. C., Page, G. P., Guo, Y., Endres, S. M., Stone, M., & Gladwin, M. T. (2017). Ethnicity, sex, and age are determinants of red blood cell storage and stress hemolysis: results of the REDS-III RBC-Omics study. Blood advances, 1(15), 1132-1141.

Yesmin, S., Paul, A., Naz, T., Rahman, A. A., Akhter, S. F., Wahed, M. I. I., & Siddiqui, S. A. (2020). Membrane stabilization as a mechanism of the anti-inflammatory activity of ethanolic root extract of Choi (Piper chaba). Clinical phytoscience, 6, 1-10

Himbert, S., Blacker, M. J., Kihm, A., Pauli, Q., Khondker, A., Yang, K., ... & Rheinstädter, M. C. (2020). Hybrid erythrocyte liposomes: Functionalized red blood cell membranes for molecule encapsulation. Advanced Biosystems, 4(3), 1900185.

Serhan, C. N., & Haeggstrom, J. Z. (2010). Lipid mediators in acute inflammation and resolution: Eicosanoids, PAF, resolvins, and protectins. fundamentals of Inflammation, 153-174.

Zhao YL, Yang XW, Wu BF, Shang JH, Liu YP, Zhi-Dai, Luo XD. Anti-inflammatory Effect of Pomelo Peel and Its Bioactive Coumarins. J Agric Food Chem. 2019 Aug 14;67(32):8810-8818. doi: 10.1021/acs.jafc.9b02511. Epub 2019 Aug 1. PMID: 31318199

Mendonça, R., Silveira, A.A.A. & Conran, N. Red cell DAMPs and inflammation. Inflamm. Res. 65, 665–678 (2016). https://doi.org/10.1007/s00011-016-0955-9

Myint, K.z., Wu, K., Xia, Y., Fan, Y., Shen, J., Zhang, P. and Gu, J. (2020), Polyphenols from Stevia rebaudiana (Bertoni) leaves and their functional properties. Journal of Food Science, 85: 240-248