Rosella (*Hibiscuss sabdariffa L*) Extract Quality Test for Phase-1 Clinical Trials

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Abstract. The use of rosella extract administered to humans must pass the quality process test. This study is intended to prepare high quality rosella extract preparations based on Good Manufacturing Practice (GMP) which will be used in phase 1 clinical trials, including the standardization of the extract qualitatively, description of the sensory evaluation test, physicochemical, microbiological. The method used in the extraction process performed by solvent extraction method by maceration using ethanol in acidic conditions with a ratio of 4: 1. Determination of anthocyanin levels using the method of determining pH differentiation, to determine levels of flavonoids by using the aluminum chloride method, visual description sensory evaluation, and physicochemical. Then the microbiological test was conducted using the Total Plate Number (ALT) method. Screening shows rosella extract contains flavonoids and anthocyanins. Roselle extract contains anthocyanin at 10,6 mg/L and total flavonoids equivalent to quercetin 1,3 % w/w. The description sensory evaluation tests have a dark red, homogeneous appearance, smell and taste character like rosella. Physicochemical test results showed particle size 0,82 mm, water solubility 14,2%, alcohol solubility 5,6 %, loss on drying at a temperature of 70 ºC at 9,8 %, density 0.72 g / ml, pH at 25 ºC was 2.62. Rosella Extract free from aflatoxin and metal. The result test of microbiological aerobic plate count is <1,1E+3 cfu/g, Yeast and Mold <1,1E+3 cfu / g, Coliform APM negative, Salmonella sp negative, Shigella negative, Escherichia coli negative, Staphylococcus aureus negative, Pseudomonas aeruginosa negative. Conclusion, the qualitative test results of ethanol extract can be administered as ready-to-use products in phase 1 clinical trials.

1. Introduction

Rosella is a native plant originated from Africa and now widely cultivated in Indonesia, including Malang. The main content of rosella is anthocyanin and flavonoids with a characteristic red to purple [1]. Anthocyanins have more stable characteristics at low pH (acidic conditions) which produce red pigments [2]. Generally plants that contain flavonoids usually produce a yellow color [3]. Rosella has advantages as an antioxidant, currently providing opportunities as an alternative to immunostimulatory supplements [4].

Rosella as an immunostimulant has a good ability as a prophylactic to free radicals. The pharmaceutical process has a very important role in the use of rosella, caused the drug is a foreign substance that enters the body so it needs safety, efficiency both in compounding preparations and contamination free from microorganisms [5].

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Factors that influence the performance of the drug include humidity, solubility and particle size. Unequal particle size especially in solid preparations will cause poor mixing with excipients [6]. In the extraction drying process, the excipient have a considerable role as a filler to keep the active compound are not broken when drying at high temperatures [7].

2. Materials and Methods

2.1 Sample Preparation

Simplisia dried rosella taken from domestic medicinal plant farmers mashed using a blender.

2.1.1. Maceration and Extraction. The powder was extracted using a 70% alcohol solvent with the addition of ethyl acetic acid in a 4: 1 ratio then soaked for 72 hours and filtered using filter paper. The pulp is removed and the filtrate part is taken, then the filtrate is separated with a solvent using a vacuum rotary evaporator.

2.1.2. Drying Extract. The extract drying process using an oven at a temperature of 70 ºC with the addition of maltodextrin in a ratio of 1: 1.5 until a homogeneous mass. Dry extracts are sterilized using UV light to start the packaging process.

2.2. Phytochemical Screening Test

2.2.1. Flavonoid. The process uses 2 drops of NaOH and is shaken vigorously. Samples that are proven to contain flavonoids when the solution changes color prominently from the initial color of light green to yellow, red, brown, or green

2.2.2. Anthocyanin. 1 g dry extract with 2 mL HCl 2 N. The appearance of a pale red color that turns purple after adding ammonia indicates the presence of anthocyanins.

2.3. Determination of phytochemical levels

2.3.1. Flavonoid. 500 µL of sample combined with 1.50 mL of 95% ethanol, 0.10 mL of 10% aluminum chloride (AlCl3 • 6H2O), 0.10 mL of sodium acetate (NaC2H3O2 • 3H2O) (1 mol L -1) and 2.80 mL distilled water. Tubes stored at room temperature for 40 minutes. Absorbance was measured at 475 nm using a spectrophotometer. Flavonoid concentrations were calculated with a calibration curve (10, 15, 20, 25, 30, 35) ppm using quercetin as standard. Linear regression is calculated from this data, and line equations are obtained for subsequent use in actual sample quantification. The results are expressed in milligrams equivalent to quercetin per gram of extract. Tests were performed in triplicate.

Linear regression equation: $y = a + bx$

2.3.2. Anthocyanin. Total anthocyanin was determined by the differential pH method. Briefly, a buffer pH = 1 was prepared using 0.2 M KCl and 0.2 N hydrochloric acid and a buffer pH = 4.5 was prepared using 0.2 M sodium acetate and 2.0 M acetic acid. 1 gram of the sample is running first to determine dilution factor and wavelength absorption of 510 and 700 nm. Then measuring the absorbance wavelength at each pH with distilled water as a blank.

Determination of Absorbance:

$$A = (pH1 - pH4.5)_{510\ nm} - (pH1 - pH4.5)_{700\ nm}$$

Anthocyanin levels:

$$= \frac{(A \times MW \times Vd \times 1000)}{\varepsilon \times L} \times 100\ \%$$

ε = Sanidin-3-glucoside molar absorptivity
= 26,900 Ll (mol.cm)
L = cuvette width = 1 cm
MW = molecular weight of Cyanidine-3-elucoside (449.2 g/mol)
Vd = final dilution volume

2.4. Description sensory evaluation test
2.4.1. Visual. The extract was visually color observed and then smelled and felt.

2.4.2 Physicochemical. Drying shrinkage is measured by measuring the remaining extract after drying at a temperature of 70 ºC to a constant weight expressed as percent value.

$$\text{Drying Shrinkage} = \frac{\text{Initial Extract - Final Extract}}{\text{Initial Extract}} \times 100\%$$

This measurement is repeated until a constant weight is reached. Density test is determined by weighing the weight of the granules, then put into a measuring cup to see the volume

$$\text{Density Tapped} = \frac{\text{M (mass)}}{\text{V (volume)}}$$

Then the pH is measured.

2.5. Microbiological test
2.5.1. Aerobic Plate Count. Samples of 1.1 g each were added with 10 mL of Peptone Dilution Fluid (PDF) media resulting in dilutions of up to 10^{-1}, then a stratified dilution up to 10^{-7}, by taking a homogeneous sample from a 10^{-1} dilution of a 3 mL, 2 mL for each 1 mL pipetted into a duplo petri dish, each cup containing 1 mL sample in 9 mL of diluent media. Dilution 10^{-1} dilution was diluted gradually with 10^{-2},10^{-3}, 10^{-4}. Then homogeneous with vortex. 500 mL Tryptic Soy Agar (TSA) and 2.5 mL Triphenyl Tetrazolium Chloride (TTC) 0.5% are added to each petri dish ± 20 mL, each petri dish is moved to resemble a figure 8 and the motion is stopped until the media solidifies. Then incubated at 37 ° C for 24 hours in an inverted position.

$$\text{Total Plate Count (TPC)} = \frac{\text{colonies count}}{\text{Dilution factor}}$$

2.5.2. Yeast and Mold. 2 grams of sample is mixed with 18 mL distilled aqua, then shake until homogeneous (10^{-1} dilution), then prepare 3 tubes each containing 9 mL distilled aqua. Pipette 1 mL of 10-1 dilution into the first distilled aqua tube until a 10-2 dilution is obtained and shaken until homogeneous. Subsequent dilutions are made up to 10^{-4}. From each dilution then pipette 0.5 mL, poured on Potato Dextrose Agar, immediately shaken while rotating so that the suspension is spread evenly, the media is made duplo. To determine the sterility of the media and diluent, a blank test is carried out containing media only and allowed to solidify. All petri dishes were incubated at 20-25 ºC for 5-7 days. After 5 days of incubation, recorded the number of fungal colonies, the last observation after 7 days of incubation.

2.5.3. Coliform APM. Samples of 2.2 g each added 20 mL Peptone Dilution Fluid (PDF) resulting in dilutions of up to 10-^{1}, then dilutions of up to 10^{-3}, from 10^{-1} dilutions pipetted 3 mL each, for each 1 ml pipetted into a screw tube containing 9 ml of MacConkey agar media with triplo. The results of 10^{-1} dilution are then diluted gradually with 10^{-2} and 10^{-3} dilutions. The dilution made is homogeneous with vortex. Homogenisation results in the screw tube were incubated for 24 hours at 37 ° C.

2.5.4. Salmonella sp. Each sample taken 2.2 g added 20 mL Buffered Peptone Water (BPW). Homogenization results were incubated at 27 ° C for 4 days, then on the 5th day they were stored in the freezer for 24 hours. Salmonella sp. test on the 6th day, each homogenisation sample was taken 0.1 mL using a micropipette, then pipetted into a screw tube containing 9 mL Rappaport Vassiliadis Broth (RVB) media, create duplo. The results of the dilution were incubated for 24 hours at 42 ° C.

2.5.5. Staphylococcus aerus. Each sample was taken 2.2 g and added 20 mL Buffered Peptone Water (BPW) resulting in a 10^{-1} dilution. The dilution made is homogeneous with vortex. Homogenisation results from each sample were taken 1 mL, then pipetted into a petri dish containing the media of Baird Parker Agar (BPA) made in duplo. Then flattened using a spreader until evenly distributed and incubated for 24 hours at 37 ° C in not inverted position.

3. Result and Discussion

The results explained that the quality made of rosella extract was in accordance with the raw materials provisions according to Materi Medika guidelines. The choice of solvent is adjusted to the polarity of flavonoids and anthocyanins which are polar compounds so that alcohol is used to increase the withdrawal of flavonoids and anthocyanins. The addition of maltodextrin which works to water draw so that it can increase the drying speed of the extract.

3.1. Screening and phytochemicals determination.

3.1.1. Phytochemicals screening. Rosella extract was proven positive for flavonoids and anthocyanins in phytochemical screening tests. The flavonoids detected are yellow while the anthocyanins are red.

Table 1. Phytochemical test results of rosella extract (*Hibiscuss sabdariffa L*)

Secondary Compounds	Test Result	Color
Flavonoids (NaOH 10%)	+	Yellow
Anthocyanins (HCl)	+	Red

(a) (b)

Figure1. Phytochemical screening color (a) Flavonoids and (b) Anthocyanins

The occurrence of a yellow color in flavonoids is caused by flavonoids having many OH groups with high electronegativity differences, and working as antioxidants so that their polarity forms hydrogen bonds [8]. Anthocyanins are also part of flavonoids with a red color characteristic, with methoxyl or hydroxyl groups which if acid (HCl) added will cause a dark red color [9]. The red pigment from anthocyanin originates from the Flavylium cation [10].

3.1.2. Phytochemical levels determination. Flavonoid content of dry rosella extract with filler 1: 1.5 get a 1.3% w / w equivalent to quercetin (Table 3). While the level of anthocyanin is 10.6% (mg / L) (table 3).

Table 2. Absorbance of quercetin standard solution λ 475 nm

Concentration (ppm)	Absorbance	Regression Equation
10	0,653	
15	0,656	
20	0,656	y =33,818x + 4,712
25	0,659	
30	1,101	
35	1,103	

Table 3. Rosella extract flavonoid levels (*Hibiscus sabdariffa L*)

Replication	Absorbance	Value	Value Average
I	0,215	1,33 %	
II	0,215	1,33 %	1,33 %
III	0,216	1,33 %	

According to Desmiaty and Alatas [11] the optimal level of optimal absorption of quersetin at hydrolysis temperature is 80 ºC. In this study, the resulting level is smaller because the hydrolysis of flavonoid glycosides temperature is 70 ºC. Quercetin as a bioflavonoid which belongs to the class of flavonol has a high level of solubility in alcohol [12]. The addition of acid as a catalyst and the temperature used during drying are still within the stability tolerable range, causing an increase in the quantity of quercetin in rosella extract production.

Table 4. Wavelength data and anthocyanin levels of rosella extract (*Hibiscus sabdariffa L*)

Replication	pH				Absorbance	Value %	Average Value
	pH 1 (A)		pH 2 (A)				
	λ 510	λ 700	λ 510	λ 700			
I	0,897	0,256	0,889	0,262	0,014	21,2±	
II	0,830	0,216	0,825	0,214	0,003	4,55±	10,6 %
III	0,738	0,155	0,736	0,517	0,004	6,1 ±	

Anthocyanin pigment contained in this rosella extract can be delphinidin-3 sambubiosid, cyanidin-3 sambubioside, delphinidin-3 glucosid4 and cyanidin-3-glucoside which shows red color at low pH. According Wahyuningsih [2] anthocyanin spectrum can be seen at absorbance of 400-600 nm which will be stable at low pH and will be brownish at high temperatures. Based on Table 4 is generated that the wavelength of 510 nm have a higher absorbance than the wavelength of 700 nm. Anthocyanin determination in this study using the pH determining method. At pH 1 anthocyanin is bright red because it is a strong acid that causes anthocyanins to form oxonium (flavilum) cations, while at pH 4.5 anthocyanin will turn into chalcone in the form of hemiketal (figure 1) [13].

pH 1 pH 4.5

Figure 2. Color change of rosella extract (*Hibiscus sabdariffa L*) at pH 1 and pH 4.5

3.2. Description sensory evaluation and physicochemical

Observation of chemical and physical characteristics of a drug substance is a very important stage in the development of solid dosage forms. Identification of the chemical properties can be metal content (impurities), allergen, then physical properties such as solubility, polymorphism, hygroscopicity, particle size, density, etc. [14]. Particle shape measurement is a way to see the solubility of the extract, the smaller size of the particles, the easier it is to dissolve [6]. In this research the dried ethanol extract of rosella which had been added with maltodextrin filler had a red color with a distinctive rosella aroma, sour taste and easily dissolved in aquadest with acidic pH. The uniqueness in the sour taste causes rosella is often used as a dietary supplement by women [1].

Table 5. Sensory evaluation and physicochemical test of rosella extract (*Hibiscus sabdariffa L*)

Test	Result
Sensory Evaluation	
- Appearance	Powder
- Odor	Characteristic odor of Hibiscuss sabdariffa
- Color	Red-purplish red
- Taste	Sour
Physicochemical	
- Extract Ratio (native extract: Filler Ratio)	1:1.5
- pH (10%)	2,62
- pH (15%)	2,67
- Particle size	0,82 mm
- Tapped density	0,27 g/ml
- Water solubility	14,2%
- Alcohol solubility	5,6 %
- Loss on drying	9,8 %
- Ash Content	5,6%
- Water content	<10%
- Allergen Aflatoxin (B1, B2, G1 dan G2)	Negative
- Pesticide residues	Negative
- Metal (Pb, Cd, As, Hg)	Negative

The resulting extract is classified as weak acid (2.5-3) so that it allows a little ionization because the extract pH is lower than gastric acid. So that it has a lower degree of ionization, it can increase the active substances presentation in absorbed rosella extract [15].

In the process of drug formulation, particle size has a very important factor especially in the bioavailability of the drug where the size of the granule can affect drug dissolution [14]. Sieving method is used to determine the particle size distribution, the results of the rosella extract test range from 0.82 mm with tapped density 0.27 g / ml, indicates the flow properties of rosella extract (value <1 indicates good flow properties). The results of the solubility test, rosella extract water soluble with a level of 14.2% can be interpreted that the rosella extract is included in the category of soluble (10-30 parts of the active substance dissolved in the solvent), the bioavailability of the active substance can be increased if the drug solubility in gastrointestinal fluid is high. Essentially each drug is easily absorbed when dissolved in water so that it can reach sufficient concentration in the systemic circulation [16]. Roselle has a low solubility in alcohol around 5.6%. Based on the theory, alcohol can increase the availability of certain drugs, and based on research, acidic compounds have a good solubility in alcohol, especially lipophilic compounds. The presence of alcohol in the small intestine causes an increase in dissolution and availability in the systemic circulation [17]. Ash content shows the characteristics of non-organic compounds. Ash content of Rosella extract included in the limit set <10.2% [18]. A good extract must also be free of aflatoxins and pesticides as well as heavy metal contents such as Pb, Cd, As, Hg. Heavy metal testing uses the Atomic Absorption Spectroscopy (AAS) method. The results showed that rosella extract was free of aflatoxins, pesticides and metals.

The testing of roselle extract drying milk is intended to measure the amount of moisture loss so as to achieve a specific extract granulation [14]. Humidity is identical to water content, the requirements for a good extract to be used in capsule form must contain no less than 10% water, this value is associated with preparation susceptibility to microorganism contamination [19]. The results showed that the drying loss of rosella extract was 9.8%. The drying process of rosella extract is carried out at a temperature of 50-70 ºC, considering that the filler used is maltodextrin which is easily damaged at high temperatures.

3.3. Microbiological test

Microbiological testing is an important component of the quality of preparations that shows safety from microorganisms contamination to avoid the cause of infection. The regulation on the amount of contamination allowed on traditional medicine preparations is regulated in BPOM (Badan Pengawas Obat dan Makanan/Food and Drug Supervisory Agency) regulation on quality requirements of traditional medicine no. 12 of 2014 [19].

Table 6. Test results of microorganism contamination of roselle ethanol extract (*Hibiscus sabdariffa L*) with triplo

Microorganisms	Test results (average) cfu / colony	BPPOM Standard
Total Plate Count	$< 1{,}1 \times 10^3$	$\leq 10^6$ coloni/g
Yeast and Mould	$< 1{,}1 \times 10^3$	$\leq 10^4$ coloni/g
Escherichia coli	Negative	Negative/g
Salmonella spp	Negative	Negative/g
Pseudomonas aeruginosa	Negative	Negative/g
Staphylococcus aureus	Negative	Negative/g
Coliform APM	Negative	Negative/g

Rosella extract which has been through the drying process after the addition of filler is sterilized using UV light for 30 minutes. Mixing extracts and fillers are processed in the LAF (Laminar Air Flow) area to prevent microorganisms contamination process. Microbiological contamination test results of rosella ethanol extract showed that the preparations produced in accordance with BPPOM regulations were free from microorganisms.

4. Conclusion

Rosella extract (Hibiscus sabdariffa Linn) dried with the addition of maltodextrin filler showed a red granule shape with a sour taste. The results of screening tests and phytochemical levels indicate the content of flavonoids and anthocyanins. Extract requirements according to BPPOM that the water content is less than 10%, free from aflatoxins, microorganisms and metals.

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References

[1] N. Harun, E. Darmawan, and L. H. Nurani, "Effect of calyx capsule-ethanol extract Hibiscus sabdariffa L. on renal function of healthy volunteers," *IOP Conf. Ser. Mater. Sci. Eng.*, vol. 259, no. 1, p. 012019, Nov. 2017.

[2] S. Wahyuningsih, L. Wulandari, M. W. Wartono, H. Munawaroh, and A. H. Ramelan, "The Effect of pH and Color Stability of Anthocyanin on Food Colorant," *IOP Conf. Ser. Mater. Sci. Eng.*, vol. 193, no. 1, 2017.

[3] N. Harun, M. Indriastuti, A. Fatimah, D. Ega, Y. E. Safitri, and M. Rosmayanti, "Effect of Ethanol Extract Sonchus arvensis Linn Leaf on {BUN}, {SCr}, {ALT}, {AST} in Healthy Male Albino mice (Rattus norvegicus).," *J. Phys. Conf. Ser.*, vol. 1179, p. 12188, Jul. 2019.

[4] N. A. Syahrana, A. Akrom, and E. Darmawan, "Efek Serbuk Bunga Rosella Merah (Hibiscus sabdariffa L.) terhadap Ekspresi IL-10 pada Sukarelawan Sehat," *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 2018.

[5] G. Alderborn and M. E. Aulton, "Pharmaceutics: The Science of Dosage form Design," *Pharm. Sci. Dos. form Des.*, 2002.

[6] D. J. Burgess *et al.*, "Particle Size Analysis : AAPS Workshop Report , Cosponsored by the Food and Drug Administration and the United States Pharmacopeia *," vol. 6, no. 3, 2004.

[7] R. Frida and D. Natalia, "Pengaruh Penambahan Filler dan Suhu Pengeringan terhadap Kandungan Antioksidan pada Daun Physalis angulata yang Diperoleh dengan Ekstraksi Menggunakan Air Subkritik," pp. 1–7, 2016.

[8] S. Kumar and A. K. Pandey, "Chemistry and Biological Activities of Flavonoids: An Overview," vol. 2013, 2013.

[9] H. E. Khoo, A. Azlan, S. T. Tang, and S. M. Lim, "Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits," *Food Nutr. Res.*, vol. 61, no. 1, p. 1361779, 2017.

[10] A. Bakowska-Barczak, "Acylated anthocyanins as stable, natural food colorants : A review," *Polish J. food Nutr. Sci.*, vol. 14, no. 2, pp. 107–116, 2005.

[11] Y. Desmiaty and F. Alatas, "Determination of quercetin in Hibiscus sabdariffa L . calyces by High-Performance Liquid Chromatography (HPLC)," vol. 2008, no. October, pp. 385–388, 2008.

[12] A. V. A. David, R. Arulmoli, and S. Parasuraman, "Overviews of Biological Importance of Quercetin: A Bioactive Flavonoid," *Pharmacogn Rev.*, vol. 10, no. 20, pp. 84–89., 2016.

[13] M. Suzery, S. Lestari, and Bambang Cahyon, "Penentuan Total Antosiaiiin (Hibiscus Sabdariffa L) Dengan Metode Maserasi Dan Sokshletasi," vol. l, 2010.

[14] S. Niazi, "Handbook of Pharmaceutical Manufacturing Formulations Compressed Solid Products," in *Handbook of Pharmaceutical Manufacturing Formulations Compressed Solid Products*, Volume one., Deerfield, Illinois, USA: Pharmaceutical Scientist, Inc, 2009.

[15] S. S. Jambhekar and P. J. Breen, *Basic Pharmacokinetics*. London, UK: The Pharmaceutical Press, 2009.

[16] K. T. Savjani, A. K. Gajjar, and J. K. Savjani, "Drug Solubility : Importance and Enhancement Techniques," vol. 2012, no. 100 mL, 2012.
[17] J. H. Fagerberg, Y. Al-Tikriti, G. Ragnarsson, and C. A. S. Bergström, "Ethanol Effects on Apparent Solubility of Poorly Soluble Drugs in Simulated Intestinal Fluid," *Mol. Pharm.*, 2012.
[18] Anonim, "Keputusan Menteri Kesehatan Republik Indonesia Nomor: 661/MENKES/SK/VII/1994 Tentang Persyaratan Obat Tradisional." Menteri Kesehatan Republik Indonesia, Jakarta, 2009.
[19] Anonim, "Peraturan Kepala BPOM Tentang Persaratan Mutu Obat Tradisional." Badan Pengawas Obat dan Makanan Republik Indonesia, 2014.