Formulation of Submicro Particle Extract of Kesum Leaves (*Polygonum minus* Huds.) As Antioxidants

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Abstract. The body needs antioxidants to overcome and prevent oxidative stress. An example of a plant that has the potential to be developed because it allegedly has antioxidant activity is kesum leaves (Polygonum minus Huds.). This study aims to formulate ethanol extract of kesum leaves into submicro particle preparations with antioxidant activity. Making extracts using the maceration method with 70% ethanol solvent. The identification test of compound content was carried out qualitatively using TLC analysis and GC-MS analysis. Preparation of Kesum leaf extracts into submicron particle preparations using chitosan and sodium alginate polymers and calcium chloride (CaCl2) by ionic gelation method. Three formulas (F1-F3) were differentiated by the amount of extract 250, 500, and 750 mg for each formula. Submicro particle preparations were tested for particle size and polydispersity index and evaluated for antioxidant activity by an antioxidant test against DPPH free radical compound with quercetin as a control. Based on the TLC test, kesum leaf extract contains flavonoid compounds. GC-MS results show that kesum leaves contained octadecanoid, hexadecanoic, and cyclolongifolene oxide. The optimal formula analyzed falls into the submicron particle range, and good PDI results were obtained. The results of antioxidant activity submicro particle of kesum leaves have IC50 values of 13.3 μg/mL (F1), 6.6 μg/mL (F2), 2.6 μg/mL (F3) and is a very strong antioxidant category. It can be concluded that the ethanol extract of kesum leaves contains flavonoid and terpenoid compounds, and the preparation of a submicron-particle carrier of ethanol extract of kesum leaves has strong antioxidant activity

Keywords: Submicro particle, Polygonum minus, Antioxidant

1 Introduction

Degenerative diseases are a severe health problem and are responsible for the majority of deaths in Indonesia. Oxidative stress is crucial in the biology of aging and other degenerative diseases, including diabetes mellitus and its consequences, cancer, and atherosclerosis, which leads to heart disease, vascular issues, and stroke. The body has antioxidants, which can neutralize free radicals. Some of these antioxidants include glutathione, catalase, and the enzyme superoxide dismutase (SOD). Foods with phenolic compounds, vitamins C, E, and beta-carotene can also provide antioxidants. Foods that can serve as natural sources of antioxidants are grains, fruits, vegetables, tomatoes, papaya, oranges, pomegranates, spices, chocolate, and so on (1). The body need antioxidants to manage and avert oxidative stress. Numerous natural substances in Indonesia possess antioxidants and other active compounds.

Antioxidants from natural products are needed to improve public health quality at an affordable price; an example of a plant that has the potential to be developed because it allegedly has antioxidant activity is kesum leaf (*Polygonum minus* Huds.). The phytochemical content in this plant includes carotene, retinol, calcium, magnesium, L-ascorbic acid, copper, zinc, and flavonoid-derived secondary metabolite compounds such as catechins and rutin (2).
Previous research on antioxidant effects with in vitro antioxidant activity tests on n-hexane extract of kesum leaves showed that the plant has antioxidant activity. The n-hexane extract of kesum leaves has an antioxidant effect, and the resulting IC50 value is 7.57 ± 0.67 ppm. The total phenol content is 48.7 ± 1.8 mg (GAE/100 g) with a total flavonoid amount of 235.9 ± 17.1 μmol (FE (II) / g) (3). Furthermore, research by winanta (2024) demonstrated that the antioxidant activity of kesum leaf ethanol extract, yielded an IC50 value of 41.46 μg/mL, categorizing it as a very potent antioxidant (4). Flavonoid compounds are generally included in the polyphenol class. These compounds have low bioavailability in human tissue absorption (5).

This study aims to formulate ethanol extract of kesum leaves into sub microparticle preparations with antioxidant activity. The advantage of making sub microparticle preparations is that it can increase the extract's stability and reduce side effects due to its small size and dose. Drug delivery with a size of 200-500 nm can penetrate the layers of the skin quickly so that it can facilitate the penetration of drugs into the skin more efficiently (6,7). Research conducted by (8), encapsulation of sub microparticles in beluntas leaves is better in healing abscesses when compared to extracts without formulation (8).

2 Material and Methods

2.1 Materials

Silica gel F254 (Merck®), etanol grade for analysis (Merck®), DPPH (1,1- difenil-2-pikrilhidrazil) (Sigma Aldrich®), Quercetin grade for analysis (Aldrich®), aquadest pH 7 (Shagufta®), citosan grade for analysis (Aldrich®), Methanol grade HPLC (Merck®), Methanol grade for analysis (Merck®), Etil Acetat grade for analysis (Merck®), Amonia 25% (Merck®), $CaCl_2$ grade for analysis (Merck®), Natrium alginat grade for analysis (Merck®), Acetat glacial acid grade for analysis (Merck®), Format acid grade for analysis (Merck®), Etil acetat grade for analysis (Merck®).

2.2 Methods

2.2.1 Material Collection

Kesum leaves obtained from Bengkayang district, West Kalimantan province, were analyzed at the Ahmad Dahlan University Biology Laboratory under reference number. 350/Lab.Bio/B/VII/2023. After sorting, washing, and draining, kesum leaves were dried in an oven at 40ºC for six days until completely dried. After drying, the test was continued to determine the water content of simplisia to <10%; kesum leaves were mashed using a blender until they became fine powder and then filtered using a 20mesh sieve.

2.2.2 Extraction

This research uses the maceration method, which produces ethanol extract from kesum leaves using 70% ethanol solvent. In this study, 400 grams of kesum (*Polygonum minus* Huds.) leaf simplisia powder was macerated with 5 liters of 70% ethanol for five days and then macerated for two days to increase the effectiveness of the extraction, with stirring done every 12 hours. The ratio of material and solvent was 1:10 (Ministry of Health, 2013). The extract was concentrated using a water bath at 50-60 ^{0}C (9).

2.2.3 Flavonoid identification Using TLC

Identification by TLC method using cilia gel F254 stationary phase, with a mobile phase comprising a mixture of toluene, ethyl acetate, and formic acid in a ratio of 5:4:0.2 with ammonia vapor as a stain spotter. The comparator compound used was quercetin. On observation with visible light, a positive reaction was shown by the formation of a yellow-brown stain after ammonia vapor. The blue stain at UV 366 nm indicates quercetin, a standard flavonoid compound (10).

2.2.4 GC-MS Analysis

The ethanolic content of Kesum leaf extract was analysed using the GC-MS technique at LPPT UGM. The Kesum extract component was analysed utilising qualitative methods employing GC-MS. The Thermo Scientific Trace 1310 and Thermoscientific ISQ Singel Quadrupole were used to perform GC-MS analysis. The analysis utilized an HP-5MS UI column with dimensions of 30 m x 0.25 mm x 0.25 µm. Helium UHP (he) is a carrier gas that has a flow rate of 1.0 ml/min. Simultaneously, 0.5 grammes of parity fruit extracts were completely dissolved in 1.5 mL of ethyl alcohol (EtOH). The sample vial was injected with 1 ml using a syringe into the GC column. The acquired mass spectra are compared to the database specific to that instrument.

2.2.5 Formulation Submicro Particle

Kesum leaf ethanol extract was used with different variations of extract concentrations, namely 250 (F1), 500 (F2), and 750 mg (F3) in each variation of the submicro particle formula to obtain the optimal formulation, the difference in concentration in this extract refers to previous research researched by (8)with minor modifications. Table 1 illustrates three formulations including distinct extract changes according to the employed recipe.

Table 1. Submicro Particle Formula of Kesum leaf extract

Sample	F1	F2	F3
Kesum Extract (mg)	250	500	750
Chitosan (mg)	21	21	21
Natrium Alginat (mg)	3,2	3,2	3,2
$CaCl_2$ 18 mM (µL)	40	40	40

2.2.6 Submicro Particle Preparation

It weighed 1.5 grams of kesum leaf extract and added 20 ml of distilled water. The extract solution was combined with a magnetic stirrer for one hour at 75 rpm at room temperature (23°C) until homogenous. The solution was permitted to sit until the residual extract dissolved. The ethanol extract solution of kesum leaves for the three formulas was pipetted every 3.33 (F1), 6.67 (F2), and 10 mL (F3) with the content of each formula, 250, 500, 750 mg (8).

Chitosan solution was made by weighing as much as 0.42 grams and then put into a glass beaker, then dropping 0.63 ml glacial acetic acid and adding 100 mL aquadest ad. They were then mixed until homogeneous using a magnetic stirrer (IKA-217M) for 24 hours at ambient temperature (23ºC). In a glass beaker, 9.6 mg of sodium alginate was dissolved in 30 mL of distilled water. Then, the sodium alginate solution was mixed until homogeneous using a magnetic stirrer at 75 rpm for 30 minutes at room temperature (23oC). 10 mL of sodium alginate solution was pipetted, with each formula containing 3.2 mg (8).

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Calcium chloride solution 0.018 M was made from dilution of 18 mM concentration by putting 2 grams of CaCl2 powder into 100 mL of API and mixing until homogeneous with a magnetic stirrer at 75 rpm for 30 minutes (23ºC). This solution was diluted by taking 1 mL of 0.18 M CaCl2 solution and then adding 10 mL (8).

2.2.7 Submicro Particle Manufacturing

Kesum leaf extract solutions from FI, II, and III of 3.33, 6.67, and 10 mL were put dropwise into 5 mL of chitosan solution in the vial as mass 1 (using a magnetic stirrer at 750 rpm). Sodium alginate solution was placed on a magnetic stirrer at 750 rpm, and 10 mL of this solution was taken as mass 2. Mass 1 was dripped into mass two dropwise using a 100 µL micropipette on a magnetic stirrer at 750 rpm for 60 minutes at room temperature (23oC). For each formula, 40 µL of calcium chloride solution was added. Then, to reduce the particle size, it was homogenized using a Covaris S220 bath sonicator with a frequency of 42 kHz, and aquabidest was added up to 30 mL on a magnetic stirrer at 750 rpm at room temperature (8).

2.2.8 Characterization of Submicro Particle

Submicro particle suspension of kesum leaf extract from the optimum formula was pipetted as much as 50 µL and diluted using distilled water by a factor of 1: 100. A total of 50 µL was put into the PSA cuvette, then monochromatic light was released by the instrument and captured by the detector to produce diameter and PDI measurements (8).

2.2.9 Antioxidant Activity Assay by DPPH Method

10 mg of DPPH was measured and dissolved in 100 ml of methanol p.a to achieve a concentration of 100 ppm, so creating a DPPH stock solution. Quercetin solution and kesum leaf extract sub-particle preparations were formulated in three variations (F1, F2, F3) at 5 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm concentrations. Each level series is supplemented with 10 ml of methanol p.a. Two milliliters of each series of submicron particle solution were placed into a test tube, followed by the addition of three milliliters of diluted DPPH solution (50 ppm). The mixture was put through a homogenization process and then incubated for thirty minutes at room temperature in a dark setting. The solution's absorbance was measured with a UV-Vis spectrophotometer at a wavelength of 515 nm. Methanol functioned as the control sample, whereas quercetin functioned as the positive control.

3 Result and Discussion

3.1 Flavonoid identification Using TLC

Flavonoids in kesum leaf extracts were identified using the TLC method. The result is shown in Figure 1.

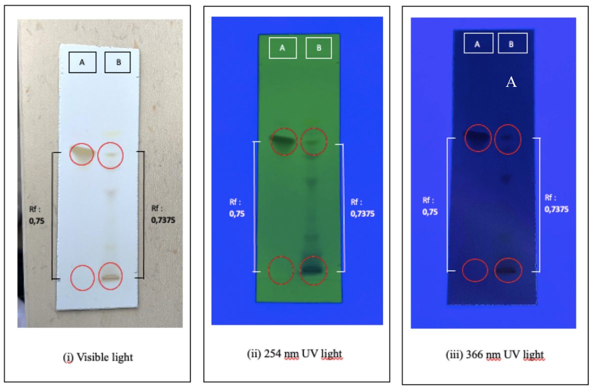

Figure 1. TLC identification under UV light after amonia vapour (NH_3)

Description:

A. Quercetin standard
B. Kesum leaf extract

The observation of the chromatogram pattern in (Figure 1) shows that the sample has the same stain pattern as the quercetin standard based on the elution results on the TLC plate, which has been examined under visible light, UV light at a wavelength of 254 nm and 366 nm. The kesum leaf extract sample produced a spot with Rf 0.7375, while quercetin has Rf 0.75. According to Peter in 2010, the Rf value can be used as evidence to identify a compound; compounds with the same or almost the same Rf value can indicate that the compounds have the same or comparable characteristics.

3.2 GC-MS Analysis of Kesum leaf extract

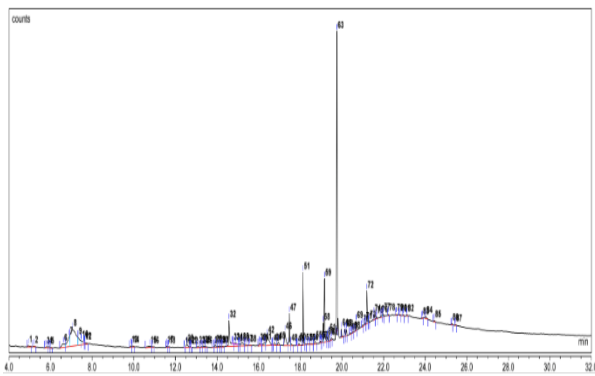

Figure 2. GC-MS chromatogram of kesum leaf extract

Table 2. Chemical compounds of kesum leaf extract with the largest percentage.

Peak	RT	Chemical Coumpounds	Molecular Formula	Molecular Weight	Ret. Area %
32	14.57	Cyclolongifolene oxide	C15H22O	218	5.36
47	17.47	Hexadecadienoic acid, methyl ester	$C_{17}H_{34}O_2$	270	3.11
51	18.13	Hexadecadienoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	5.49
59	19.16	6- Octadecadienoic acid (Z,Z), methyl ester	$C_{19}H_{36}O_2$	296	6.59
63	19.75	(E)-9-Octadecenoic acid ethyl ester	C20H38O_2	310	21.52
72	21.19	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	3.34

Separation of compounds in the Kesum leaf extract produced 6 separate major compounds (figure 1 & table 2). The main compound of the extract is (E)-9-Octadecadienoic acid, ethyl ester which was identified at peak number 63 with a retention time of 19.75 minutes. However, there is one compound that is quite interesting from the results of the GC-MS identification. The compound is Cyclolongifolene oxide, this compound is part of the terpene group. This compound appeared at a retention time of 14.57 as peak number 32. The Cyclolongifolene oxide molecule was observed to have m/z 218.

3.3 Submicro Particle Characterization

Tabel 3. The result of submicro particle characterization

Formula Submikro Ekstrak Daun Kesum	Ukuran Partikel (nm)	Indeks Polidispersitas (%)
F1	186,4	0,358
F2	244,3	0,1884
F3	326	0,1058

The diameter and particle size distribution (PDI) of the submicroparticles that were characterized from F1 to F3 were determined with the help of a particle size analyzer which is 186.4 nm (F1), 244.3 nm (F2), 326 nm (F3) (particle size) and 0.358% (F1), 0.1884% (F2), 0.1058% (F3) (polydispersity index). The diameter measurement findings indicated that the best formula examined was within the submicron particle range. Particle size is an essential factor in producing nano- and micro-sized particles, and one of the parameters that are also important in manufacturing is the homogenization process of the preparation. Mixing polymers using only a magnetic stirrer without sonicating will result in a large preparation particle size due to sufficient kinetic e nergy to shrink the particle size (6). Particle size reflects the stability of the formulation.

Particle size distribution (PDI) values are used to determine the uniformity and diameter distribution of the particles. The PDI value ranges from 0.0, indicating a perfectly uniform particle size distribution, to 1.0, signifying a highly polydispersed sample with various particle size populations. A PDI close to zero indicates a narrower or smaller particle distribution, which implies a more homogeneous particle size. A more extensive particle distribution can result in particles that are not uniform in size (homogeneous), which can increase agglomeration between particles because small particles will collide with large particles. Particle size and particle size distribution are critical for evaluating sub microparticle performance.

Table 4. Antioxidant activity value from Submicro Particle and Quercetin

Sample	Equation	R^2	IC_{50}	Description
Quercetin	y = 1,4549x + 39,726	R^2 = 0,9976	7,08	Very strong
F1	y = 1,8601x + 25,269	R^2 = 0,9848	13,3	Very strong
F2	y = 1,542x + 39,843	R^2 = 0,9252	6,6	Very strong
F3	y = 1,2894x + 46,654	R^2 = 0,8562	2,6	Very strong

The results of the antioxidant activity test on the sub-particle sample of kesum leaf ethanol extract have IC50 values of 13.3 μg/mL (formula 1), 6.5 μg/mL (formula 2), 2.6 μg/mL (formula 3), and for the IC50 value of the positive control, namely quercetin of 7.08 μg/mL which is included in the category of very strong antioxidants, because it is in the range <50 μg/mL (11). The outcomes of this test are based on research conducted by (12), which states that the test of kesum leaf methanol extract has a very strong antioxidant with an IC50 of 20 ppm.

The high antioxidant activity in submicro particle of ethanol extract from kesum leaves is due to the content of compounds present in the extract and the manufacture and characterization of submicro particle preparations. In ethanol extracts, several compounds, such as flavonoids, terpenoid, phenols and alkaloids, have the potential as antioxidants (13). The content of terpene group such as longifoline (table 2) has contribute of antioxidant activity (14,15).

Flavonoids are intrinsic antioxidants characterized by phenolic groups, demonstrating efficacy in mitigating cellular damage caused by oxidative stress. It is possible for flavonoids to demonstrate their antioxidant properties through either a direct or indirect method. As antioxidants, flavonoids directly donate hydrogen ions to stabilize reactive free radicals. Flavonoids also have protective properties. The antioxidant properties of flavonoids are achieved through the activation of nuclear factor erythroid 2 related factor 2 (Nrf2), which is one of the several ways that flavonoids use to indirectly boost the production of endogenous antioxidant genes. This activation leads to an upregulation of genes responsible for the creation of natural antioxidant enzymes, including superoxide dismutase (SOD) (16). Submicro preparations combined with antioxidant metabolites can enhance antioxidant properties by binding reactive nitrogen and oxygen elements and mimicking antioxidant molecules (17). Microparticle of herbal extract with encapsulation protertise can mantain the stability of antioxidant content that have good corelation with therapeutic effect (18).

4 Conclusion

The ethanol extract of Kesum leaf contains six major compounds, with the most significant percentage of compounds being (E)-9-Octadecenoic acid ethyl ester, which is 21.52%, and Cyclolongifolene oxid with 5.36%. Kesum leaf extract has been identified with flavonoid content with the TLC method. The administration of submicro particle of kesum leaf extract has increased the antioxidant activity effect with the highest effect in Formula III with IC50 2,6 µg/mL. The limitation of this study is that it only looks at antioxidant activity using the DPPH method. So that further research can look at several effects of submicro particles of Kesum leaf extract.

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6 Conflict of Interest

The authors assert that they possess no conflicts of interest.

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