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STUDY ON THE OPTIMIZATION OF MULBERRY LEAF EXTRACT BY MACERATING ETHANOL AND MICROWAVE ASSISTED EXTRACTION METHOD (MAE) WITH NATURAL DEEP EUTECTIC SOLVENTS (NADES)

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ABSTRACT
Ethanol extract from the leaves of Morus alba, an herb, has been widely used for medicine, including commercial formulations. This study aims to compare the characteristics of NADES extract using the MAE method and the maceration method. Mulberry leaf simplicia (Morus alba. L) ethanol extract was made with various ethanol concentrations of 50%, 60%, 70%, 80%, and 90%, and NADES (Natural Deep Eutectic Solvents) extract with a solvent ratio of 1:20 and 1: 30 on the MAE (Microwave Assisted Extraction) method with a time of 5 and 10 minutes. Measurement of total flavonoid content and total phenolic content was carried out by UV-vis spectrophotometry with quercetin and gallic acid as reference standards. The results showed that the ethanol extract with ethanol concentrations of 50%, 60%, 70%, 80%, and 90% had an average total flavonoid content; and total phenolic: 0.73%, 0.9%, 1.32%, 1.18%, 0.77%; total phenolic 96.789 mg/g, 71.262 mg/g, 126.465 mg/g, 116.643 mg/g, 93.366 mg/g. NADES extract with a solvent ratio of 1:20 at MAE 5" and 10" minutes obtained flavonoid levels of 0.36%, 0.4%, and total phenolic levels of 36.099 mg/g; 75.621 mg/g. While a ratio of 1:30 with an MAE time of 5" and 10" minutes, the flavonoid content was 0.44%; 0.48%, and the total phenolic content of 61.884 mg/g; 121.237 mg/g. Results of water-soluble essence of ethanol extract; NADES is 26%–31%; 21–24%. Result of ethanol soluble extract content of ethanol extract; NADES is in the range of 36%–42%; 85%–86%. Result of the specific gravity of ethanol extract; NADES is in the range of 0.82 g/mL–0.95 g/mL; 1.08 g/mL–1.18 g/mL. The ethanol extract pH test results were; NADES in the range 6.90–7.01; 7.59–7.89.

Keywords: Total Flavonoid; Total Phenolic; Mulberry Leaf; NADES

1. INTRODUCTION

The ethanol extract of mulberry leaves (*Morus alba L*) is a plant extract that has been widely used in medicine, including as a commercial formulation (De Oliveira, A. *et al.*, 2015). Mulberry leaves have biologically active chemical compounds in the form of secondary metabolites such as alkaloids, terpenoids, tannins, and saponins; therefore, mulberry leaves can be used as traditional medicine (Mabruroh *et al.*, 2019). Based on research (Meng *et al.*, 2020), mulberry leaf extract is widely used to control blood glucose or as a supplement beneficial to health. Mulberry leaf extract also suppresses inflammatory mediators and oxidative stress, protects pancreatic β-cells, and modulates glucose metabolism in diabetic rats. The basic rule for

extraction is to break down the cell wall from the outer layer to the inner layer, and it is known that the location of polysaccharides in mulberry leaves is precisely in the inner epidermal cells located in adaxial leaves. In recent research, electroporation, ultrasound, and enzymes were applied to help break down the cell walls of mulberry leaves and fruit to help solubilize polysaccharides. The help of enzymes seems to be the most effective way (He *et al.*, 2018).

Conventional extraction methods such as maceration and percolation have the disadvantage of requiring a long time, using lots of solvents, and are not environmentally friendly. One of the methods developed for extraction based on green chemistry or green chemistry using green solvents is Natural Deep Eutectic Solvent (NADES). NADES has several advantages that conventional organic solvents don't have in terms of physical chemistry, one of which is that it is non-volatile, environmentally friendly, non-toxic, has stable properties at high temperatures, and is food grade.

Various conditions influence effective extraction; therefore, research is necessary to find the optimal extraction conditions. In the research conducted by (Kanyaprasit & Butkhup, 2021), a study was carried out to optimize the extraction of M.alba L. twigs by the MAE method with parameters of antioxidants and tyrosinase inhibitors with ethanol concentrations of 15%, 30%, 45%, 60%, and 75%. The most optimal results of antioxidant activity and tyrosinase inhibitors were obtained at a concentration of 45% (Kanyaprasit & Butkhup, 2021).

Optimization studies on microwave-assisted extraction were carried out by (Mustafa *et al.*, 2022), with parameters used percent yield of extraction, levels of total flavonoids and total phenols, total anthocyanin content, total sugar content, α-glucosidase inhibition, etc. Extraction effectiveness is influenced by microwave power (MP) and extraction time (ET).

One of the NADES compositions used for extraction is a combination of glycerin and urea (Astati *et al.*, 2019). The combination of urea glycerin was used to obtain the active substance oxyresveratrol from M.alba roots. This active substance is also found in the roots, stems, leaves, and fruits of plants from the Moraceae, Liliaceae, and Gnetaceae families. Urea acts as a hydrogen bond acceptor (HBA), and glycerin acts as a hydrogen bond donor (HBD). In NADES preparation, hydrogen bonds are formed due to intermolecular interactions and intramolecular hydrogen bonds. M. alba root extraction with urea-glycerin was able to obtain optimal active compounds (Alishlah *et al.*, 2019).

The application of urea-glycerin using the Ultrasonic Assisted Extraction method can increase the concentration of polyphenols in the extract (Jeong *et al.*, 2017). This extraction optimization study compares the effectiveness of extraction with ethanol solvent and urea glycerin combination solvent. Extraction effectiveness was assessed by conducting parameters, total phenol, and total flavonoid content.

2. METHODS

2.1. Material

The tools used are test tubes (Pyrex), dropper pipettes, volume pipettes (Iwaki), glass tools (Pyrex), analytical balances (OHAUS PPX224 Analytical Balance 220 g/0.1 mg), UV-Vis spectrophotometer (Shimadzu UV mini-1240), microwave (REWEZ), rotary evaporator (IKA), water bath, pH meter (Metler Toledo FE20-Kit FiveEasy),

The materials used are mulberry leaves (Morus alba L) from Kalioa village, Cirebon city, NADES solvent (Urea: Glycerin), 96% ethanol (PT. Brataco Indonesia), Quercetin, Aluminum Chloride, Sodium acetate, Aquadest (PT. Brataco Indonesia), Gallic acid, Barium Chloride.

2.2. Extraction

Mulberry leaves are obtained from mulberry plantations. Mulberry leaf samples collected were wet sorted, washed with running water, sliced , and then dried in an oven at 40 °C for 24 hours. Next, dry sorting is carried out and mashed. Samples were extracted using the maceration

method by soaking 1 part of the simplicia in 75 parts of the solvent (5 x 24 hours), stirring occasionally, then filtered and re-macerated with 25 parts of the solvent (2 x 24 hours) filtered to obtain a liquid extract. The extract was evaporated using a rotary evaporator followed by an evaporating cup. The solvent used was ethanol with a concentration of 50%, 60%, 70%, 80%, and 90%.

Extraction using the MAE method with NADES solvent was made with 2-time variables and two solvent comparison variables. Extraction time with MAE was 5 minutes and 10 minutes. A comparison of the various solvents used was 1 part of mulberry leaf simplicia added to 20 and 30 parts of NADES solvent (Urea (0.5 Molar): Glycerin (1.5 Molar)) and then extracted using Microwave-Assisted Extraction (MAE) at 60 °C for 5 minutes and 10 minutes. The extract obtained was filtered using a cloth and then evaporated using a water bath so that an extract that was not too liquid was obtained. The results obtained are stored in a tightly closed brown bottle (Kurnya *et al.*, 2019).

2.3. Specific Parameters Test

2.3.1. Organoleptic Test

The organoleptic test aims to determine the characteristics of mulberry leaf simplicia by direct observation based on shape, smell, color, and taste.

2.3.2. Determination of Water-Soluble Levels

A total of 1 g of extract was put into a plugged flask, and 25 mL of water-chloroform LP was added (2.5 mL of chloroform was put in a 1000 mL volumetric flask, and water was added up to the mark). Then let stand for 24 hours while shaking many times for the first 6 hours and left for 18 hours, then filtered. As much as 5 mL of the filtrate was evaporated to dryness in a flat-bottomed shallow cup that had been tarred. Then the residue is heated at 105 ℃ to constant weight. Levels in percent of water-soluble compounds were calculated for the initial extract (Kementerian Kesehatan Republik Indonesia, 2017).

2.3.3. Determination of Ethanol Soluble Levels

A total of 1 g of extract was put into a plugged flask, and added 25 mL of 96% ethanol was. Then let it stand for 24 hours while shaking it many times for the first 6 hours and leave it for 18 hours. Then filtered quickly to avoid evaporation of ethanol. As much as 5 mL of the filtrate was evaporated to dryness in a flat-bottomed shallow cup that had been tarred. Then the residue is heated at 105 ℃ to constant weight. Levels in percent of water-soluble compounds were calculated for the initial extract (Kementerian Kesehatan Republik Indonesia, 2017).

2.4. Non-Specific Parameters Test

2.4.1. Specific Gravity

Determination of specific gravity using a pycnometer that is clean and dry and has been calibrated by determining the weight of the pycnometer and the weight of water. The weight of the empty pycnometer minus the weight of the pycnometer that has been filled with the type of liquid extract is the result obtained by dividing the weight of the extract by the weight of water (Kementerian Kesehatan Republik Indonesia, 2017).

2.4.2. pH Test

pH testing is carried out using a pH meter. Prepared buffer solutions of pH 4, 7, and 10. Calibration was carried out on the pH meter based on the range of acids, bases, and neutrals. After calibration, the pH meter is inserted into the sample solution until a constant pH is measured. Repeat up to 3 times to get accurate results (Anhar, 2021).

2.5. Total Flavonoid Content

Determination of total flavonoid levels using quercetin as a comparison. The standard curve for quercetin was determined by making concentration solutions of 20 ppm, 30 ppm, 40 ppm, 50

ppm, and 60 ppm and measured at a wavelength of 434 nm. Measurement of total extract flavonoid content was carried out by making a solution of 2000 ppm ethanol extract and 20,000 ppm NADES extract. Take 1 ml of each extract, add 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M sodium acetate, and 2.8 ml of distilled water. Furthermore, the absorption of the solution is measured at a wavelength of 434 nm.

2.6. Total Phenolic Content

Determination of total phenol content using gallic acid as a comparison. The standard curve for gallic acid is determined by preparing a solution of concentrations of 50 ppm, 75 ppm, 100 ppm, 150 ppm, and 200 ppm and measuring the absorption at a maximum wavelength of 727nm. Determination of Total Phenol Content was carried out by making a solution of 2000 ppm ethanol extract and NADES extract. Each taken 0.5 ml added 2.5 ml of 10% Folin Ciocalteu reagent and 2.5 ml of 7.5% sodium carbonate. Furthermore, the solution was measured for its absorption at a maximum wavelength of 727nm.

3. RESULTS AND DISCUSSION

Mulberry leaves were extracted using the MAE method with a comparison of the various solvents used; 1 part of mulberry leaf simplicia added 20 and 30 parts of NADES solvent (Urea (0.5 Molar): Glycerin (1.5 Molar)) and then extracted using Microwave-Assisted Extraction (MAE) at 60 °C for 5 minutes and 10 minutes. The extract weights obtained are seen in **Table 1**.

Table 1. NADES extract weight

Simplicia + Solvent	Extraction Time	Comparison (Simplisia: Solvent)	Simplicia Weight (g)	Extract Weight (g)
Mulberry Leaf + NADES	5’	1:20	3.00	45.25
Mulberry Leaf + NADES	10’	1:20	3.00	44.16
Mulberry Leaf + NADES	5’	1:30	4.00	40.34
Mulberry Leaf + NADES	10’	1:30	4.00	39.70

In this study, the extraction process with green chemistry solvents was carried out under several conditions, namely a solvent ratio of 1:30 and 1:20 (Iswandana *et al.*, 2020). This solvent comparison refers to research (Alishlah *et al.*, 2019). With the difference in the amount of solvent, the amount also affects the penetration of the solvent in the simplicia matrix.

Based on **Table 1**, the NADES extract with a ratio of 1:30 has a lower extract weight than the NADES extract with a ratio of 1:20. Comparison of the amount of solvent simplicia affects the effectiveness of extraction with the total flavonoid parameter. This is to the research conducted by Aning, the addition of solvent ranges from 100 to 300 ml, and the optimal value is at 250 ml (Yulianingtyas & Kusmartono, 2016). MAE optimization studies conducted by (Ming Zhu *et al.*, 2020), total phenol extract content mulberry leaf with time parameter 8-24 minutes highest at 18 minutes. An isolation study of the active substance content of mulberry leaves conducted by (Alishlah *et al.*, 2019) found that the longer the extraction time, the higher the oxyresveratrol levels.

3.1. Specific Parameters Test

Extract identity is one of the most important specific parameters in preliminary testing as an initial introduction and the part of the plant used. The results of extract identity checks can be seen in **Table 2**.

Table 2. Identities of Nades Extract and Mulberry Leaf Ethanol Extract

Extract Identity	Results	
	NADES Extract	Ethanol Extract
Extract Name	Mulberry Leaf Nades Extract	Mulberry Leaf Ethanol Extract
Species	*Morus alba* L.	*Morus alba* L.

Part of the Plant Used	Leaf	Leaf
Indonesian Names of Plants	Mulberry Leaf	Mulberry Leaf

Examination of organoleptic extracts is one of the specific parameters determined using the five senses and aims to provide early recognition and identify extracts that indicate the characteristics of the extract in the form of shape, color, and smell (Gangga *et al.*, 2017). The results of the organoleptic examination can be seen in **Table 3**.

Table 3. Organoleptic of Nades Extract and Mulberry Leaf Ethanol Extract

Organoleptic Extract	Results	
	NADES Extract	**Ekstrak Etanol**
Form	Slightly Thick	Very thick
Color	Light green	Dark Green, Black
Smell	Typical Nades Mulberry Leaf Extract	Typical Mulberry Leaf Ethanol Extract
Picture		

Examination of water and ethanol-soluble essences was carried out to provide an initial description of the number of compounds that can be extracted with water and ethanol solvents (Ladeska & Dingga, 2019). The results of testing the water-soluble essence and ethanol extract of ethanol and NADES can be seen in **Table 4**.

Table 4. Determination of Water-Soluble Extract Content and Ethanol Soluble Extract Content

Extract	Water Soluble Extract Content	Ethanol Soluble Extract Content
Etanol 50%	31%	42%
Etanol 60%	29%	41%
Etanol 70%	29%	40%
Etanol 80%	28%	37%
Etanol 90%	26%	36%
NADES 1:20 5’	21%	85%
NADES 1:20 10’	22%	85%
NADES 1:30 5’	24%	86%
NADES 1:30 10’	24%	86%

3.2. Non-Specific Parameters Test

Density is the ratio of the density of a substance to the density of water with the volume value of a mass unit. Determination of specific gravity aims to describe the chemical content dissolved in the extract. Specific gravity relates to how to determine the purity of a substance is determined by its specific gravity (Syukri *et al.*, 2020). The results of testing the specific gravity of the NADES extract and the ethanol extract of mulberry leaves can be seen in **Table 5**.

Table 5. Specific gravity and pH of Nades Extract and Mulberry Leaf Ethanol Extract

Extract	Specific Gravity (g/mL)	pH
Ethanol 50%	0.95 g/mL	7.01
Ethanol 60%	0.92 g/mL	6.94
Ethanol 70%	0.87 g/mL	6.91
Ethanol 80%	0.84 g/mL	6.88
Ethanol 90%	0.82 g/mL	6.90
NADES 1:20 5’	1.08 g/mL	7.59
NADES 1:20 10’	1.09 g/mL	7.67

NADES 1:30 5’	1.16 g/mL	7.85
NADES 1:30 10’	1.18 g/mL	7.89

3.3. Total Flavonoid Content

Figure 1 shows that the absorbance of quercetin has a linear relationship with the concentration of quercetin; this is indicated by the regression value showing a value of 0.9863, which is close to 1 so that it can be said that the absorbance value is directly proportional to the concentration.

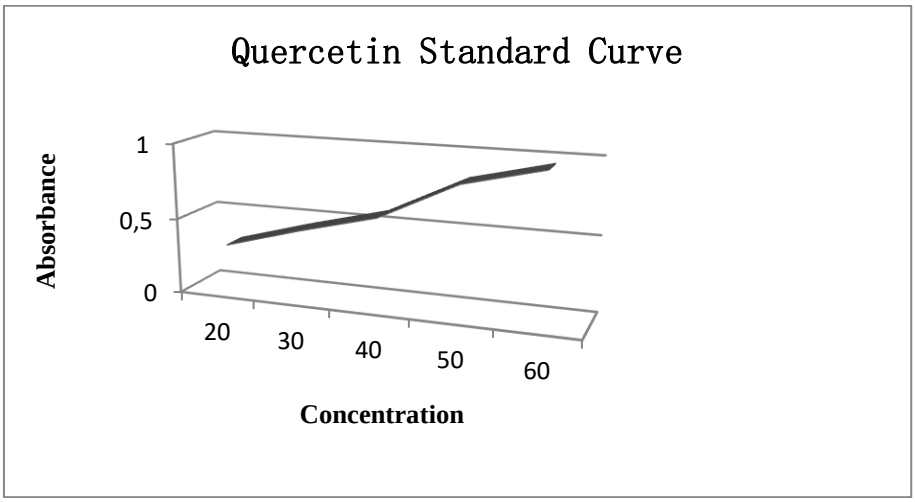

Figure 1. Quercetin Standard Curve

Based on Table 6, each solvent shows an average yield of flavonoid levels in ethanol concentrations from 50% to 70% indicating higher flavonoid content results, while at concentrations higher than 70% ethanol, namely 80% and 90%, decreased levels of flavonoids. This is in line with research conducted by (Suhendra *et al*., 2019), where the ethanol concentration from 40% to 70% increased the levels of flavonoids in thatch rhizomes, while at ethanol concentrations above 70% there was a decrease in total flavonoid levels so that it can be said that the ethanol concentration above 70% is less effective for dissolving flavonoid compounds. A mixture of ethanol and water solvents is the best solvent for use in the extraction of low molecular weight compounds such as flavonoids. Ethanol concentration can affect the polarity of the solvent resulting in a change in the solubility of bioactive compounds such as flavonoids (Suhendra *et al.*, 2019).

Table 6. Average of Total Flavonoids Content

Extract	Mean ± SD Total Flavonoid Content (%)
Ethanol 50%	0.73±0.04
Ethanol 60%	0.90±0.01
Ethanol 70%	1.32±0.08
Ethanol 80%	1.18±0.01
Ethanol 90%	0.77±0.01
NADES 1:3 M (1:30 5’)	0.28±0.03
NADES 0.5:1.5 M (1:20 5’)	0.36±0.03
NADES 0.5:1.5 M (1:20 10’)	0.4±0.02
NADES 0.5:1.5 M (1:30 5’)	0.44±0.03
NADES 0.5:1.5 M (1:30 10’)	0.48±0.01

NADES extract of mulberry leaves was extracted using the MAE method with two types of NADES ratio, namely 1:3 M (urea: glycerin) and 0.5:1.5 M (urea: glycerin) with a simplicia:solvent ratio (1:30) extracted for 10 minutes. Of the two types of solvent ratios, each has an average level of flavonoids of 0.2766% and 0.4833%; the results indicate that a solvent ratio of 0.5:1.5 M is more effective in extracting flavonoid compounds in mulberry leaves

compared to a ratio of 1:3 M. This is because the ratio of solvents can cause a change in solvent polarity thereby affecting the solubility of flavonoid compounds in mulberry leaf simplicia. From the Comparison of NADES 0.5:1.5 M, variations of the extract were made with simplicia:solvent ratios of 1:20 and 1:30 using MAE times of 5 minutes and 10 minutes, respectively. The results of the flavonoid content of NADES extract 1:20 at 5 minutes were 0.3566%, NADES extract 1:20 at 10 minutes 0.3966%, NADES extract 1:30 at 5 minutes 0.4366%, and NADES 1 extract: 30 in 10 minutes 0.4833%. The total flavonoid levels obtained from the NADES extract of meniran leaves (Yulianita *et al., 2022*) and the NADES Flos sophorae extract (Nam et al., 2015) were 1.4161% and 12.67%, respectively. When compared, the total flavonoid content of the NADES extract of meniran leaves and Flos sophorae was higher than the NADES extract of mulberry leaves with a concentration of 1:30 10', which obtained an average yield of 0.4833%. This can happen due to differences in the types of plants, types and concentrations of solvents, and the methods used. As for the total flavonoid content of the ethanol extract carried out by (Radojković *et al., 2012*), it was obtained at 3.3303% from Morus alba L. and 6.7369% from Morus nigra L., while from research (Zainab, 2019) the levels were obtained total flavonoids from Morus nigra L. by 0.3644%. The results obtained from the three data were varied compared to the average total flavonoid content of 70% ethanol extract with a yield of 1.3166%. This can happen due to differences in the type of plant, the source and location of the plant, the type, and concentration of solvent, and the method used. The absorbance of gallic acid stabilized at 45-50 minutes.

3.4. Total Phenolic Content

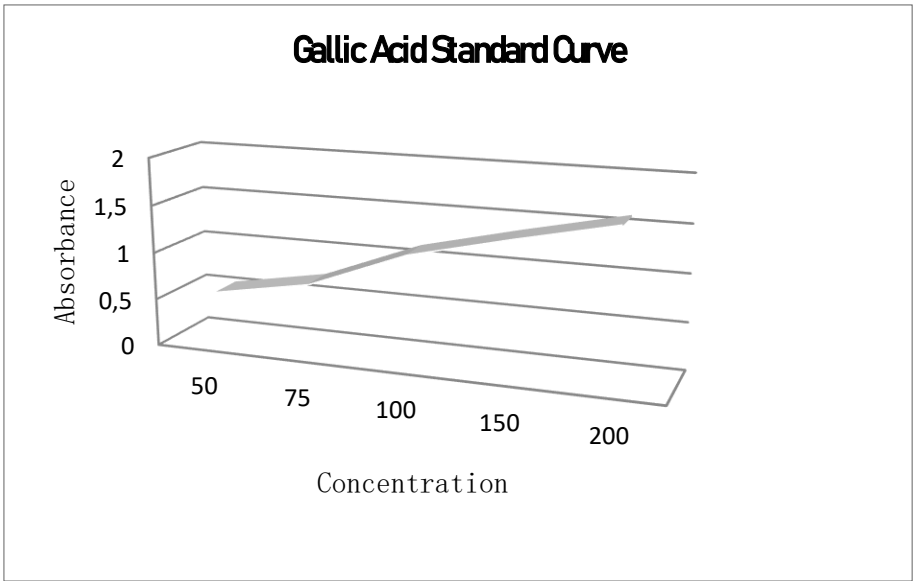

Figure 2. Gallic Acid Standard Curve

Based on the gallic acid standard curve (**Figure 2**), a linear regression equation was obtained, namely y=0.00711 x + 0.258, with a value of r = 0.971.

Table 7. Results of Determination of Total Phenol Content

Extract	Mean ± SD Total Phenol Content (mg/g)
Ethanol 90 %	93.366 ± 3.92
Ethanol 80%	116.643 ± 3.26
Ethanol 70%	126.465 ± 5.60
Ethanol 60%	71.262 ± 0.16
Ethanol 50%	96.789 ± 0.64
NADES 1:20 (5')	36.099 ± 0.69
NADES 1:20 (10')	75.621 ± 1.48

NADES 1:30 (5')	61.884 ± 0.68
NADES 1:30 (10')	121.237 ± 0.85

Based on the **Table 7** results of determining the total phenolic content of 70% ethanol extract, mulberry leaves had the highest total phenolic content compared to NADES extract; this was due to the similar solubility properties of phenolic compounds and ethanol, making it easier to withdraw phenolic compounds by ethanol solvent. The use of NADES solvent with the MAE method in extracting mulberry leaves is quite effective because with a short extraction time and the number of simplicia with a small amount of solvent can produce total phenol levels which are not much different from the ethanol extract with maceration extraction which takes several days and uses a solvent. and a lot of simplicity.

4. CONCLUSION

Based on the results of the study, it can be concluded that the total levels of flavonoids and total phenolic levels indicate that the optimal conditions for mulberry leaf extract are found in 70% extract and NADES extract with extraction time variables (5 and 10 minutes) and ratio variables (1:20 and 1:30). Extraction results using the maceration method and the MAE (Microwave Assisted Extraction) method showed different extract qualities, this was seen from the specific parameter test, namely the water-soluble extract content test and the ethanol soluble content test. In the water-soluble content test, the percentage yield of ethanol extract was greater than that of the NADES extract, while in the ethanol solubility test, the percentage yield of NADES extract was greater than that of the ethanol extract.

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6. CONFLICT OF INTEREST

The author states that there is no conflict of interest in conducting this research.

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