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Research Paper

Extraction Optimization of Phenolic Compounds from Rimpang Lempuyang Gajah (*Z. Zerumbet*): Green Solvent (NADES-UAE) (Ultrasound-Assisted Extraction) and MAE (Microwave Assisted Extraction)

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Abstract
The Lempuyang plant is one of the medicinal plants in Indonesia. One of the plants of the Lempuyang family that has not been widely researched is Lempuyang Gajah *Zingiber zerumbet* (L). The commonly used method of extraction of phenolic compounds is maceration with organic solvents. The disadvantages are the number of organic solvents that must be used and the long extraction time (days). An environmentally friendly solvent that has been successfully developed in the 21st century is eutectic or known as Natural deep eutectic solvent (NADES). In addition, NADES can also be used in conjunction with other extraction methods such as (UAE), and microwave aid (MAE). The results obtained were the synthesis of NADES with HBA (Choline Chloride) and HBD variations (Glucose, Lactic Acid, and Ethylene Glycol) with ratios of 1:1 and 1:2. In the maceration process, variations in time (2, 4, 6 hours) and variations in extraction methods (maceration, UAE and UAE-MAE) are carried out. The best TFC (Total Flavonoid Content) value data was obtained by NADES D (choline chloride: glycerol 1:2) of 697.24 mg QE/g extract through a combined ultrasonic and microwave method (UAE-MAE) while the best TPC (Total Phenolic Content) was NADES C (choline chloride: glycerol 1:1) of 2491.88 mg GA/g extract through a combined ultrasonic and microwave method (UAE-MAE). Meanwhile, the characterization of NADES synthesis to see the interaction of hydrogen bonds through FTIR analysis, and the content of phenolic compounds and flavonoids was carried out through HPLC-DAD.

Keywords
Choline Chloride, Lempuyang Gajah (*Z. zerumbet*), Microwave-Assisted Extraction (MAE), NADES (Natural Deep Eutectic Solvent), Ultrasonic-Assisted Extraction (UAE)

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1. INTRODUCTION

Lempuyang Gajah, scientifically known as *Zingiber aromaticum* Val., is a species within the Zingiberaceae family commonly found in tropical regions such as Southeast Asia. This plant can be found in lowland areas or on hillside slopes (Koga et al., 2016; Jahiddin and Low, 2020). Lempuyang Gajah is well-known and proven as an herbal plant that can be used in traditional medicine. The most utilized part of this plant is its rhizome. In Indonesia, Lempuyang Gajah rhizomes are typically used as herbal remedies to treat various ailments such as inflammation, fever, diarrhea, bacterial infections, stomach cramps, poisoning, body allergies, digestive disorders, sprains, as an antirheumatic agent, and as a diuretic (Da'i et al., 2013; Moreira da Silva et al., 2018).

The plant has a distinctive aromatic scent, which is due to the complex mixture of monoterpenoids and sesquiterpenoids found in the essential oils of the rhizome (Chondhe et al., 2024). The Lempuyang Gajah plant has thick, knobby rhizomes that grow beneath the soil surface. The plant also has thin green leaves that sprout from the rhizomes, with lengths ranging from 25 to 35 cm. The flowers of this plant are green when young and change to white or red as they mature (Mukherjee et al., 2024). The rhizomes of Lempuyang Gajah are commonly used in traditional medicine to treat various ailments, such as inflammation, fever, diarrhea, bacterial infections, stomach cramps, poisoning, body allergies, digestive disorders, sprains, anti-rheumatic agents, and diuretics (Moreira da Silva et al., 2018). The plant contains secondary metabolites, such as polyphenols, terpenes, and alkaloids. The phenolic compounds have a biological activity higher than the other secondary metabolites (Bavesh et al., 2013; Murini et al., 2020).

In addition to its use in traditional medicine, Lempuyang Gajah has also attracted scientific interest due to its secondary metabolite content, which has the potential to provide health benefits. Secondary metabolites such as flavonoids, alkaloids, and terpenoids are known to possess various biological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties (Sekar et al., 2014). With the growing interest in natural medicines and phytopharmaceuticals, in-depth research on the chemical composition and biological activities of Lempuyang Gajah has become highly relevant (Minarno et al., 2024; Rahayu et al., 2020). The choice of extraction method and solvent type significantly influences the efficiency of isolating bioactive compounds from this plant, which in turn impacts its potential applications in health products and pharmaceuticals.

The method of extraction of metabolite compounds is generally maceration using organic solvents that are not environmentally friendly. Therefore, the development of environmentally friendly solvents is very necessary. An environmentally friendly solvent that has been successfully developed in the 21st century is eutectic or known as Natural Deep Eutectic Solvent (NADES). NADES can be made with a composition of compounds that are hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). The most common NADES are based on choline chloride (ChCl) compounds, carboxylic acid, and other hydrogen-bonded donors such as sugars, citric acid, succinic acid, amino acids, and glycerol which are commonly found in the cells of living organisms Bajkacz and Adamek, 2018.

DES can also be used in conjunction with other extraction methods such as Ultrasonic-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), etc. The UAE method can extract many bioactive compounds in a shorter extraction time. The main advantage of this technique is that the cell wall is disrupted by acoustic cavitation, thereby increasing the penetration of the solvent into the matrix. Also, it reaches low temperatures and hence it is more suitable for the extraction of unstable thermal compounds. UAE along with DES is an energetic method that can damage the cell wall structure and release intracellular bioactive compounds. This advanced approach has various advantages, including higher extraction efficiency, shorter time duration, and low solvent requirements compared to conventional extraction methods with organic solvents (Chemat et al., 2019; Bubalo et al., 2016). Similarly, DES has been used with MAE for the recovery of bioactive compounds and shows better results than conventional methods (Bubalo et al., 2016).

Research on the application of DES to the extraction of bioactive compounds (phenolic compounds) is still very small compared to extensive research on maceration extraction methods. Therefore, in this study, we will compare the extraction methods, namely maceration, ultrasonic (UAE), and UAE Microwave, and the use of NADES as a green solvent applied to extract phenolic and flavonoid compounds contained in Lempuyang Gajah plants.

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used are Lempuyang Gajah commercial, distilled water, KLT Silica GL plate, choline chloride, glycerol 85%, ethylene glycol, $FeCl_3$ 10%, chloroform, methanol, gallic acid, 96% ethanol, Folin-Ciocalteu's Phenol Reagent, natrium carbonate solution, quercetin powder (s), aluminum chloride solution, and DPPH. All materials were in analytical grade and obtained from Merck. Ethanol was obtained from Smartlab, aluminum chloride was obtained from Loba Chemie, acetic acid was obtained from Merck KGaA, whereas quercetin as the standard reference was obtained from Sigma Aldrich. The instruments used are Analytical balance, hot plate, UV-Vis Spectrophotometry, Fourier Transform Infrared Spectroscopy plat KBr from Thermo-Scientific Nicolet iS5-Id7, Panasonic Microwave NN-ST32HM modification, UV-VIS handle, and HPLC-DAD Chromaster Hitachi.

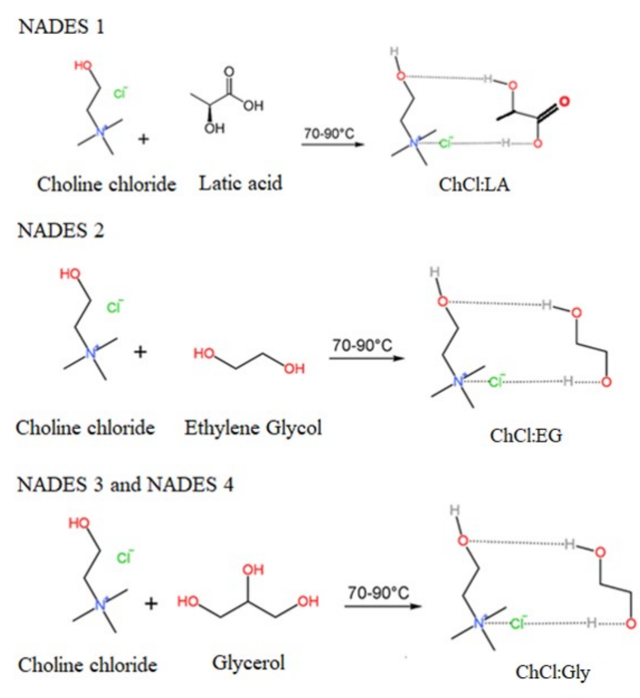


Figure 1. Hydrogen Bond Interaction between HBA and HBD in All of NADES

Table 1. Summary of Synthesis NADES

Sample	HBA	HBD	Ratio HBA: HBD	Temperature (°C)	Time (Second)	Total Volume (mL)
NADES 1	Choline Chloride	Lactic Acid	1:1	85	120	180
NADES 2		Ethylene Glycol	1:2	83	25	185
NADES 3		Glycerol	1:1	77	35	180
NADES 4		Glycerol	1:2	80	50	190

Table 2. Summary of FTIR Analysis All of NADES

Vibration of Bond	Wavenumber (cm^{-1})							Reference
	ChCl	Lactic Acid	Ethylene Glycol	Glycerol	NADES 1	NADES 2	NADES 3	
O−H Alcohol (Stretching)	3217.37	3238.86	3280.73	3277.93	3263.07	3290.29	3290.33	(Jurić et al., 2021)
C−H Alkane (Stretching)	2923.34	2942.82	2935.90	2935.35	2920.04	2935.02	2928.68	
C−H Methyl (Stretching)	1481.00	1441.72	1419.12	1416.66	1356.10	1478.36	1650.42	(Santana et al., 2019)
C−H Vinyl (Stretching)	3005.45	-	-	-	-	-	-	
C−H (Bending)	952.82	952.82	880.91	921.56	954.24	952.89	953.50	(Yudha et al., 2024)
C−N (Stretching)	1348.34	-	-	-	-	-	-	
C−O (Bending)	1083.20	1106.50	1031.64	1031.85	1074.31	1034.00	1203.97	

Table 3. Physical Characterization Data of NADES

Sample	Viscosity (mPa.s)	Density (g/ml)
ChCl: Al (1:1)	343.3300	1.3108
ChCl: Eg (1:2)	134.8920	1.2752
ChCl: G (1:1)	201.7700	1.2326
ChCl: G (1:2)	105.5700	1.2440

2.2 Methods

2.2.1 Synthesis of NADES

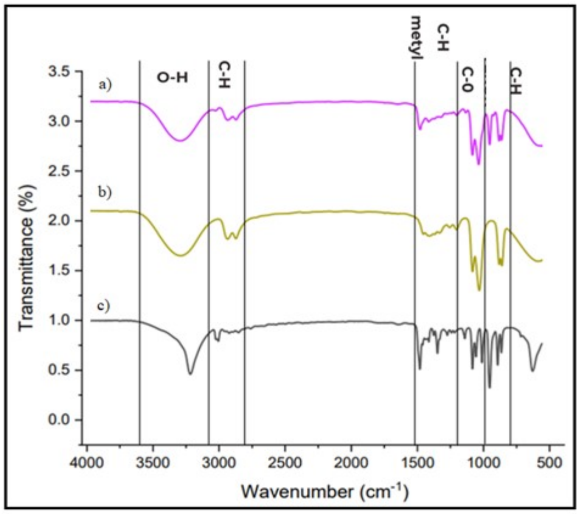
The NADES synthesis procedure refers to the research done by Vo et al. (2023). NADES was synthesized by applying the heating method. HBD and HBA were homogenized in the appropriate ratio. HBD used in this experiment is ethylene glycol, lactic acid, and glycerol. HBA is based on choline chloride. Next, the HBD and HBA solution was heated at 90 °C. This mixing was done while stirring using a stirrer. The synthesis of NADES was then characterized using FTIR and physical characterization such as viscosity, and density.

2.2.2 Extraction Process

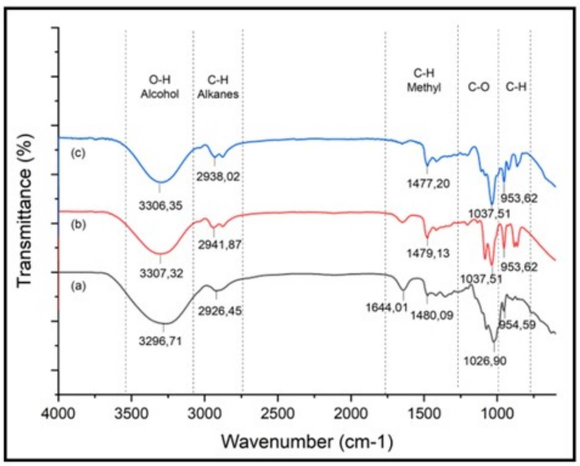
The extraction methods use a ratio of 1:10 (b/v) with variation of NADES, variation of time (2 h, 4 h, and 6 h), and variation of heat energy resources: Ultrasound and Ultrasound-Microwave (UMAE). The microwave oven was modified for the microwave-assisted extraction process. The extraction was conducted for 2 min. The extract was then concentrated and stored at room temperature for further analysis.

2.2.3 Quantitative Analysis

The extraction results were then analyzed quantitatively, namely: analysis of total phenolic content (TPC), analysis of total flavonoid content (TFC), and antioxidant by DPPH analysis. Characterization of the content of phenolic compounds and flavonoids was carried out using the HPLC-DAD method by Khuluk et al. (2021). The sample is dissolved in a vial and treated with methanol. The solution/suspension was sonicated for 10 minutes and homogenized. The solution/suspension is filtered with a filter membrane PVDF 0.22 µm. The filter was injected in HPLC with an injection volume of 50 µL. Condition: Column (Luna C18 250 × 4.6 mm × 5 µm), the temperature of the column is 30 °C. The mobile phase consisted of methanol (A) and 0.2% formic acid in water (B) with composition isocratic A/B 45:55 with a flow rate is 0.5 mL/min. We used an injection



(A) Spectrum: a) NADES 2, b) Ethylene Glycol and c) Choline Cloride



(B) Spectrum: a) NADES 1, b) NADES 2 and c) NADES 3

Figure 2. FTIR Analysis of (A). Spectrum Interaction Hydrogen Between HBA and HBD in NADES 2 (Choline Cloride: Ethylen Glycol) and (B). Spectrum All Variants of NADES

volume of standard and sample solutions of about 50 μL.

3. RESULTS AND DISCUSSION

NADES can be synthesized from HBD (Hydrogen Bond Donor) compounds, which play a role in donating hydrogen bonds, and HBA (Hydrogen Bond Acceptor) compounds that play a role in accepting hydrogen bonds so that intermolecular interactions will form in the form of hydrogen bond interactions, van der Waals forces, and electrostatic forces (Vo et al., 2023). The success of the NADES synthesis is characterized by a decrease in the melting point of the constituent compounds (HBD and HBA). In this study, the NADES synthesis method used a heating method accompanied by stirring. Heating in this synthesis serves to help both HBA and HBD compounds reach their melting points in a short time and at a temperature that is not too high around 70-90°C. The variation of HBD is done to see the correlation of the presence of the number of hydroxyl groups (OH) with its ability to extract phenolic compounds such as flavonoids. The following is a summary of NADES synthesis data results in Table 1.

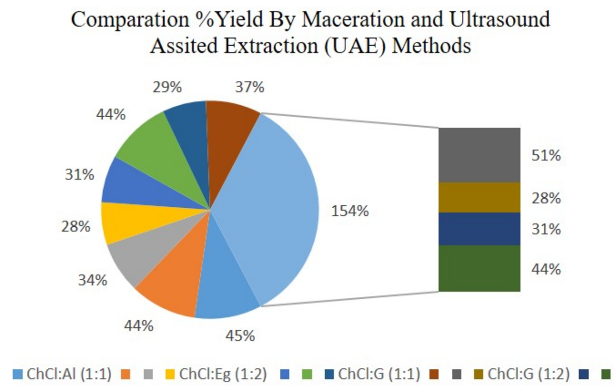


Figure 3. %Yield Comparison for Maceration and Ultrasound Assisted Extracted Extraction Methods

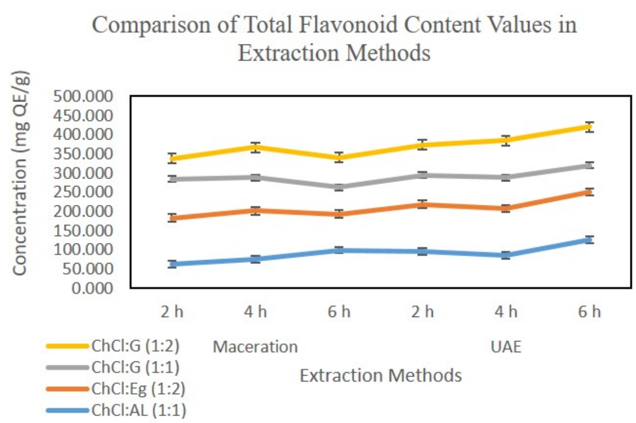


Figure 4. Comparison of Total Flavonoid Content (mg QE/g) From Extraction Methods (Maceration and UAE)

All NADES can dissolve easier because all the HBD is liquid and the HBA is solid and because liquid can also act as a solvent, so when the solid and liquid phases are mixed, it will form interactions with the solid which can also be assisted by heating and stirring so that the eutectic point or lower melting point can be formed. Hydrogen bond interaction between HBA and HBD in Figure 1.

It can be seen from the figure that there is a shift in intensity indicating that NADES was successfully synthesized which is marked by the delocalization between the alcohol groups from HBD with the charge of Choline Chloride through chloride

Table 4. Comparison of %Yield of Each Extraction Methods Performed

NADES	Time	Maceration	UAE	UAE-MAE
ChCl: G (1:1)	2 h	29%	59%	47%
	4 h	37%	23%	39%
	6 h	51%	40%	28%
ChCl: G (1:2)	2 h	28%	47%	51%
	4 h	31%	25%	40%
	6 h	44%	33%	53%

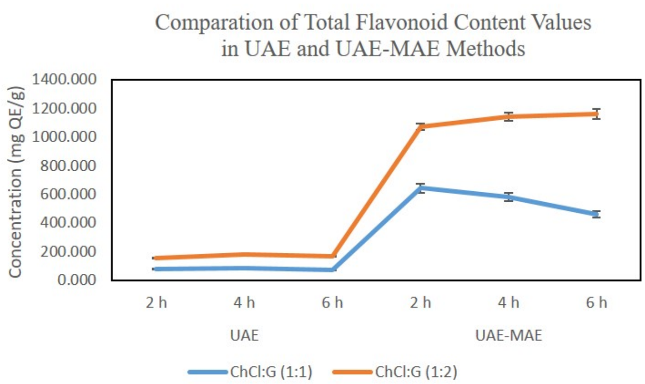


Figure 5. Comparation of Total Flavonoid Content Values in UAE and UAE-MAE Methods

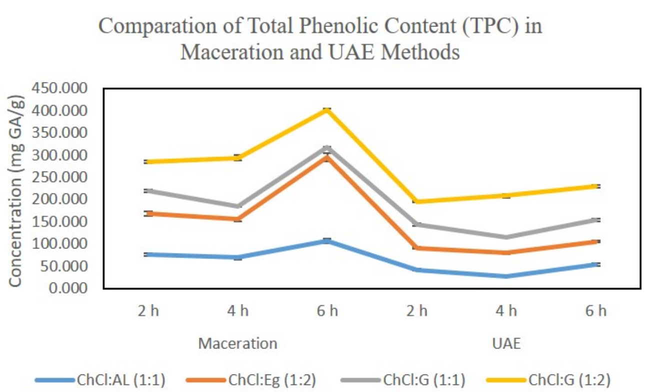


Figure 6. Comparation of Total Phenolic Content Values in Maceration and UAE Methods

ion interaction. The results of this FTIR data refer to (Yudha et al., 2024; Jurić et al., 2021; Santana et al., 2019). From the FTIR data above, the wave number values, and functional groups are obtained as follows in Table 2.

In addition to chemical characterization to ensure NADES was successfully synthesized, physical characterization was carried out, namely measurement of density and viscosity. The summary of viscosity and density of all NADES with all variants in Table 3 below.

Physical characterization can be used as a reference for the effectiveness of the extraction process. ME method that is carried out at room temperature only relies on the diffusion and osmosis phenomena. As a result, the extraction process using the ME method takes longer, even up to 2-3 days normally (Stéphane et al., 2021). UAE can increase the mass transfer rate in the extraction process due to the cavitation phenomenon in the material. The cavitation that occurs causes the formation of high-pressure micro jets that will damage the plant cell wall, as a result, the compounds inside will be released into the medium (solvent) (Prasetyaningrum et al., 2022; Pingret et al., 2013). Based on the data obtained, it was concluded that the UAE method was more efficient in obtaining higher extraction yields of Lempuyang Gajah than the ME method (Figure 3).

On the other hand, in this research, we use a combination of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) methods called UMAE. In this study, UAE was used as sample pre-treatment and the extraction was followed by irradiation with microwave (MAE). Comparison of %Yield of each Extraction Method performed was collective in Table 4 below.

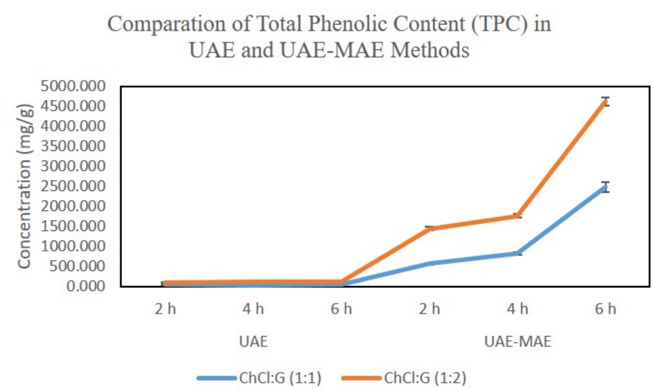


Figure 7. Comparation of Total Phenolic Content Values in Maceration and UAE Methods

The result agrees with several studies that state that the non-conventional extraction methods such as ultrasound-assisted extraction, microwave-assisted extraction, and ultrasound microwave assisted extraction produced higher extraction yield compared with conventional extraction methods (Dhanani et al., 2017; Cheng et al., 2023). Moreover, other studies have proven that the UMAE method is effective in increasing extraction yield compared to single extraction methods (UAE and/or MAE) (Liew et al., 2016; Wang et al., 2020).

To see a comparison of extraction methods carried out on Lempuyang Gajah plants, a quantitative analysis was carried out, namely the measurement of total flavonoids and total phenolic through the spectroscopic method. TFC analysis using quercetin as a standard is summarized in the graph Figure 4.

The best TFC averages were obtained when using NADES-

Table 5. Summary of Comparative Data of TFC Values in Various Extraction Methods

NADES	Maceration			UAE			UAE-MAE		
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
ChCl: AL	62.618	75.706	99.235	96.294	84.676	125.029	-	-	-
ChCl: Eg (1:2)	120.176	125.912	93.485	122.382	122.382	125.250	-	-	-
ChCl: G (1:1)	101.501	86.000	70.412	76.441	81.000	69.235	643.118	581.941	460.324
ChCl: G (1:2)	53.407	79.676	77.471	78.500	96.882	100.559	426.618	557.088	697.235

Table 6. Summary of Comparative Data of TPC Values in Various Extraction Methods

NADES	Maceration			UAE			UAE-MAE		
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
ChCl: Al (1:1)	76.377	69.130	107.536	41.594	27.681	53.913	-	-	-
ChCl: Eg (1:2)	91.594	85.797	187.246	49.565	52.464	51.739	-	-	-
ChCl: G (1:1)	51.232	30.580	22.971	51.957	35.652	47.971	565.507	819.130	2491.884
ChCl: G (1:2)	66.232	108.261	82.899	52.464	92.319	75.652	878.551	938.696	2123.768

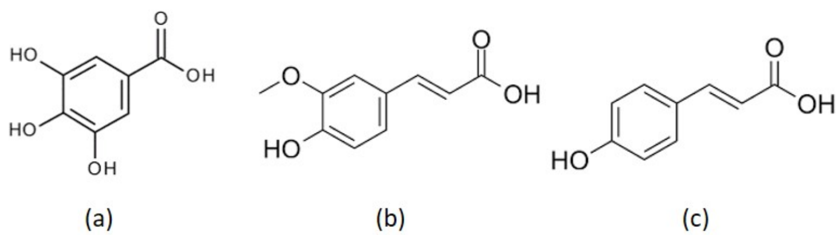


Figure 8. Simple Phenolic Compounds such as Gallic Acid (a), Ferulic Acid (b), and p-coumaric Acid (c)

B (choline chloride: ethylene glycol 1:2) and NADES-D (choline chloride: glycerol 1:2) (Figure 5). The effectiveness of the extraction process using NADES as a solvent is influenced by the viscosity value. The lower the viscosity value, the easier it will be to transfer time. In addition, the polar interaction between NADES containing hydrogen bonds and polar groups such as hydroxide (−OH) will interact with polar groups of phenolic compounds and flavonoids dominated by hydroxide (−OH) groups. There was an increase in TFC values when the extraction method was carried out using ultrasonic radiation. One important factor in extraction is temperature. The temperature must be hot enough to dissolve the compound in the solvent, but it also needs to be controlled so that compound degradation does not occur (Pingret et al., 2013). In this study, the ultrasonic bath was fixed at 40°C. The temperature set was still within the safe temperature range in the flavonoid extraction process.

When using the UMAE method, there was a significant increase in the TFC value. This is because the heat from microwave irradiation can open the cell walls of plants. In addition, the flavonoid compounds contained in the elephant Lempuyang plant have the characteristics of resistance to high temperatures (60-80 °C). The following is a comparative summary of TFC values from different extraction methods. The summary of comparative value of TFC in Table 5.

When compared to the study conducted by Bavesh et al. (2013) showed that the total flavonoid content value is equivalent to 198±2.65 mg/ml quercetin through the maceration method, then the use of NADES with choline chloride and glycerol composition through UAE-MAE heating results in better TFC values (Figure 6). As for the total content of phenolic compounds, there was a decrease in the number when using the UAE method. This can indicate that the phenolic compounds contained in the elephant Lempuyang plant are thermolabile. At a temperature of > 60 °C, it is likely to deteriorate.

However, the interesting thing is that when optimization is carried out using microwave irradiation, there is an increase in TPC value (Figure 7). Possible indications are some flavonoid compounds that will degrade when the extraction temperature is increased, in this case, the microwave irradiation temperature is recorded in the range of 60-80 °C. Based on the basic framework of flavonoid compounds containing two hydroxylated aromatic rings ($OH{-}C_6{-}C_3{-}C_6{-}OH$), when degradation occurs, it will produce simple phenolic compounds such as gallic acid, coumarin acid, and ferulic acid. The existence of this compound will be confirmed through HPLC-DAD analysis. Comparative summary of TPC values from different extraction methods in Table 6.

Table 7. Summary of HPLC-DAD Analysis Results

Sample	TPC /TFC values	RT (min)	λ (max), nm	Phenolic and Flavonoid Compound based on Reference (Kaeswurm et al., 2021)
Microwave (NADES 3) 6h	2491.8841	2.825	273	Gallic acid
		3.017	282	Procyanidin dimer
Microwave (NADES 2) 6h	187.2464	2.862	355	Isothamnetin-3-O-rutinoside
		3.038	263	Isorhamnetin
		3.222	289	Phlorizin
		3.320	351	Quercetin-3-rhamnosylglucoside
Ultrasonic (NADES 4) 4h	92.3188	2.777	200	Phenol
		2.865	274	Gallic acid
		3.037	225	p-coumaric
		3.230	355	Isothamnetin-3-O-rutinoside
		3.337	289	Phlorizin
		5.513	256	Isorhamnetin
Microwave (NADES 4) 6h	697.2353	6.075	328	chlorogenic acid
		5.650	228	p-coumaric
		6.035	233	Ferulic acid
Microwave (NADES 2) 4h	125.9118	2.858	200	Phenol
		3.032	226	p-coumaric
		3.128	280	(-) catechin
Ultrasonic (NADES 2) 6h	125.2500	2.853	275	Gallic acid
		3.032	271	Gallic acid
		3.198	258	Quercetin
		3.305	286	Phlorizin

When compared to the research conducted by Bavesh et al. (2013) which showed that the TPC value was equivalent to 331.93±1.23 mg/ml gallic acid. So, the use of NADES accompanied using heat from Ultrasound and Microwave (UAE-MAE) can increase the TPC value. To see the content of phenolic compounds and flavonoid compounds contained in the Lempuyang Gajah plant, an HPLC-DAD analysis was carried out. The results of the data obtained were then referred to the results of research conducted by Kaeswurm et al. (2021). The results are summarized in Table 7.

Referring to the data analysis of HPLC-DAD results, it was shown that there was a degradation of flavonoid compounds into simple phenolic compounds such as gallic acid (1), ferulic acid (2), and p-coumaric acid (3) when using heat from ultrasound bath or ultrasound-microwave combination (UAE-MAE) as a Figure 8.

4. CONCLUSIONS

The extraction of phenolic compounds and flavonoids from the Lempuyang Gajah Plant has been successfully carried out through a variety of extraction methods, namely maceration, ultrasound, and combined ultrasound-microwave (UAE-MAE). Optimization is carried out using an environmentally friendly solvent, namely NADES (Natural Deep Eutectic Solvent). NADES synthesis was carried out by varying HBD in a ratio of 1:1 and 1:2. The results obtained were that the eutectic point for all NADES synthesized ranged from 77–85°C, with a dissolution time range of 25–120 seconds. The formation of hydrogen bonds between HBA and HBD seen in the FTIR analysis (the O–H peak at a wavelength of 3290 cm^{-1}) shows that the synthesis of NADES was successful. Viscosity measurements from NADES obtained the lowest viscosity values of 105.5700 mPa·s (choline chloride: glycerol 1:1) and 134.8920 mPa·s (choline chloride: ethylene glycol 1:2). The resulting density value ranged from 1.2–1.3 g/mL. The yield of the extract produced for each NADES variation used ranged from 23%–62% with the best %yield being choline chloride: ethylene glycol (1:2) as much as 62% and choline chloride: glycerol (1:1) as much as 59% in the ultrasound method (UAE). The effectiveness test of the extraction process was carried out by total flavonoid content (TFC) analysis and total phenol content (TPC) analysis. The best TFC value was obtained when using NADES D (choline chloride: glycerol 1:2) of 697.24 mg QE/g. Meanwhile, the best TPC value was obtained when using NADES C (choline chloride: glycerol 1:1) of 2491.88 mg GA/g. Both through a combined extraction method between ultrasound and microwave (UAE-MAE). Referring to the data analysis of HPLC-DAD results, it was shown that there was a degradation of flavonoid compounds into simple phenolic compounds such as gallic acid (1), ferulic acid (2), and p-coumaric acid (3) when using heat from ultrasound bath or ultrasound-microwave combination (UAE-MAE).

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