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FULL PAPER

Analysis of total phenol, flavonoid, and chlorogenic acid content of robusta coffee leaves extract using nades (natural deep eutectic solvent)

Henny Sri Wahyuni[a,*] |Sri Yuliasmi[a] |Lia Laila[b] |Clara Claudia Jap[a]

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia*

[b]*Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia*

Robusta coffee (*Coffea canephora* L.) is a widely traded tropical commodity recognized for alkaloids and flavonoids found in leaves, primarily identified as polyphenols. The use of Natural Deep Eutectic Solvent (NADES) as a green solvent proves effective in extracting these compounds. Therefore, this study aimed to determine total phenol, flavonoid, and chlorogenic acid content of robusta coffee leaves extract using 96% ethanol and NADES. This study was carried out experimentally, including sample preparation, phytochemical screening, simplified extract characterization, NADES formulation, testing physicochemical properties of NADES, and preparing coffee leaves extract. NADES had a 2:1 ratio of choline chloride to citric acid and a 1:1 ratio of choline chloride to lactic acid. Simplified extract was extracted through maceration using a combination of 96% ethanol and NADES, with varying extraction times of 15, 30, 45, 60, 120, and 240 minutes. Phytochemical screening results showed the presence of alkaloids, flavonoids, tannins, saponins, glycosides, and triterpenoids/steroids. Simplified extract composition included 6% water, 17.35% water-soluble essence, 8.06% ethanol-soluble essence, 7.95% total ash, and 0.76% acid-insoluble ash. In coffee leaves extract with NADES2 (choline chloride:lactic acid) after 240 minutes, peak concentrations of total phenol (detected via the Folin-Ciocalteau method), total flavonoid (assessed with the AlCl3 reagent), and chlorogenic acid were identified. The levels obtained were 29.42 mg GAE/g extract for total phenol, 68.84 mg QE/g extract for total flavonoid, and 2.77 mg/g for chlorogenic acid at a concentration of 40.86. Notably, NADES exhibited superior extraction of total phenol and flavonoid compared to ethanol from coffee leaves.

***Corresponding Author:**
Henny Sri Wahyuni
Email: henny@usu.ac.id
Tel.: + 628126217358

KEYWORDS

Chlorogenic acid; flavonoid; HPLC; NADES; phenol; robusta coffee.

Introduction

Indonesia is the fourth largest coffee exporting country, with robusta coffee (*Coffea canephora* L.) as the primary commodity, followed by arabica coffee [1]. Robusta coffee is known for the caffeine content compared to other types of coffee, it has twice the caffeine

content compared to Arabica and possesses a distinctive nutty aroma with bitter taste [2].

Previous research found that robusta coffee beans contained alkaloids, tannins, saponins, and polyphenols [3]. Phenolic compounds present in coffee beans, categorized as flavonoids, shows the biological activity by disrupting bacterial cell walls. This occurs because of the difference in polarity between the lipid components that form DNA and the alcohol groups in flavonoid compounds. As a result, the bacterial cell wall is damaged, allowing the compound to enter the bacterial cell nucleus [4].

In Indonesia, coffee plantations predominantly grow robusta coffee. The country holds the third position in robusta coffee production, following Brazil and Vietnam. Production amounted to 5.82 million sacks in 2007, rising to 6.01 million sacks in 2008 [5].

One of the methods used in drug discovery from natural ingredients is the extraction technique, which is a process of withdrawing chemical compounds from plants, where these compounds will be dissolved in a suitable solvent. Maceration and remaceration are commonly used extraction methods in research due to the straightforward treatment approaches. Moreover, these methods do not necessitate expensive equipment, and the chemical content in simplified extract being extracted remains safe as heating is not involved. Maceration also known as cold extraction is an extraction method by soaking simplified extract powder in a filtered liquid without a heating process [6]. The use of chloroform, hexane, methanol, ethanol, and ethyl acetate as an organic solvent in the extraction process is to isolate phytochemical compounds found in plants. However, most organic solvents that extract these compounds are relatively toxic, increasing the risk of chemical compound residues in the crude extract obtained. Therefore, using environmentally friendly solvents (green solvents) is essential because it will minimize the damage that organic solvents cause [7].

Deep eutectic solvents (DES), ionic liquids (ILs), subcritical water and supercritical fluids are frequently studied as green solvent alternatives with excellent properties. ILs and DES shared similar properties and considered popular as two of the most promising green solvent. However, ILs face several challenges such as high production costs, toxicity problems, long-term stability not yet known, and the relatively high viscosity of ILs for large-scale applications. Natural deep eutectic solvents (NADES) are a subset of environmentally friendly DES, composed of natural and safe primary metabolite compounds. These include amino acids, monosaccharides, disaccharides, polysaccharides, acetic acid, lactic acid, and choline chloride, combined in specific mole ratios [8,9].

In this research, NADES will be used as a replacement solvent for the organic solvents, concerning the superiority of being made from environmentally friendly natural ingredients. NADES are known to be more superior compared to other solvent because their constituent components can easily accessed in chemicals market, biodegradable and easily decompose, has low levels of toxicity, their extraction performance is as great as the conventional solvent, stable at high temperatures, and nonflammable. Due to their advantages, the use of NADES in pharmaceutical, food, and cosmetic industry is more preferable than the organic solvent and the emerging cases of environmental damage are one of the reasons why the use of NADES has a positive contribution to the environment because NADES can be easily biodegraded without producing toxic by-products. However, the NADES limitation is that it is not available directly in the form of NADES, unlike conventional solvents [7]. In addition, the suitability of various NADES, including fructose-glucose-sucrose (FGS), glucose-choline chloride (GCC), lactic acid-glucose

(LAG), 1,2-propanediol-choline chloride (PCC), proline-malic acid (PMA), sucrose-choline chloride (SCC), and sorbitol-choline chloride (SoCC), is shown for extracting phenolic compounds such as cartooning and carthamin from *Flos carthami* and the corolla of *Carthamus tinctorius* L. The extraction results showed the remarkable solubility of cartormin and carthamin in SCC, PMA, and LAG. The increased solubility of phenolic compounds in NADES further extends to other compounds present, including rutin, quercetin, cinnamic acid, taxol, and ginkgolide B [9].

The variety of bioactive compounds that can be dissolved in NADES, both macromolecules and micromolecules, underscores the considerable potential as a multi-component solvent suitable for nonpolar and polar compounds [10]. However, further research is needed on applying NADES as an environmentally friendly extraction solvent [9].

Results and discussion

Simplified extract characterization

The characterization results carried out on simplified extract robusta coffee leaves powder for various parameter observations are indicated in Table 1.

TABLE 1 Simplified extract characterization results

No.	Parameter	Results (%)
1.	Water Content	6.66
2.	Total Ash Content	7.27
3.	Acid Insoluble Ash Content	0.54
4.	Water Soluble Essence Content	27.47
5.	Ethanol Soluble Essence Content	23.76

The obtained water content results successfully meet the specified requirements, totaling 6.66%, which is lower than the established threshold of 10%. Maintaining low water content is important, as excess water can promote microbial growth and enzymatic reactions, potentially degrading active compounds [11]. The assessment of water content was conducted through the toluene distillation method. This method relies on the separation of compounds, using high pressure and a solvent, such as toluene, which can attract water but does not mix with it due to polarity differences. Water, with a BJ value of 1 g/cm^3 and polar properties, contrasts with toluene, which has nonpolar properties and a BJ value of 0.87 g/cm^3. This difference causes water in the simplified extract leaves to be attracted to the solvent, reaching the boiling point and generating steam. Subsequently, the steam travels through the condenser. For the cooling process, it will turn into water droplets. The collected water droplets are observed and counted to quantify the amount of water present in simplified extract [12].

The determination of total ash content serves as the mineral content identifier in simplified extract, yielding a result of 7.27%. Assessing acid-insoluble ash content aims to identify the levels of acid-insoluble compounds, such as silica and heavy metals, Pb, and Hg [11]. The results obtained from determining the insoluble ash content was 0.54%. Analysis of ethanol-soluble essence content reveals concentrations of both polar and nonpolar compounds. The results show that the water-soluble essence content is lower than the ethanol-soluble essence content, suggesting greater solubility of

chemical compounds in ethanol compared to water.

Preparation of NADES (Natural Deep Eutectic Solvent)

NADES produced are NADES1 (choline chloride: citric acid) and NADES2 (choline chloride: lactic acid), where these solvents are made by heating and stirring using a hotplate stirrer. NADES is made with a specific ratio between two compounds. The specification of the NADES preparation are presented in Table 2.

TABLE 2 NADES preparation and duration of the formation

Types of NADES	Molar Ratio	Code	Production Time	Temperature (°C)	Formation time
ChCl-citric acid	2: 1	NADES 1	60 minutes	80-90	Seven days
ChCl-lactic acid	1: 1	NADES 2	60 minutes	60-70	Three days

The above data indicate that stabilization time of NADES solvent is longer when formed with both solid components compared to when formed with one of the liquid components. Subsequently, a stable NADES is characterized by the colour changing to clear [13].

The number of hydrogen bond donors (HBDs), hydrogen bond acceptors (HBAs), spatial structure, and bond positions are influencing the formation and stability of NADES. Strong hydrogen bonding directly influences phase-transition temperature, stability, and solvent characteristics in the mixtures. The supramolecular structure of NADES undergoes changes with water dilution, primarily due to the gradual disruption of hydrogen bonds. Viscosity, conductivity, density, water activity, and polarity exhibit variations to some degree based on the chemical composition of the components [14].

NADES has demonstrated effectiveness in extracting phenolic compounds, providing an extract deemed safe for use in pharmaceutical, cosmetic, and food industries. The solvents utilized in NADES are recognized excipients in pharmaceutical preparations. NADES consist of a specific molar ratio of HBA and HBD that interact through hydrogen bonds, forming a eutectic mixture with a lower melting point than each constituent. This results in the production of a clear liquid extract [15].

By selecting the appropriate components and proportions, the solid-state components can be transformed into a stable liquid phase under environmental conditions. The change in the melting point is attributed to the formation of hydrogen bonds facilitated by hydroxyl, carboxyl, carbonyl, and amino groups in the mixture. Other than hydrogen bond interaction, electrostatic, dipole-dipole, and Van der Waals interactions are further plausible. A higher hydrogen bonding ability correlates with a decreasing melting point. Subsequently, NADES is part of the DES type III, including initial components such as quaternary ammonium salts and natural primary metabolites. The presence of organic acids in NADES can contribute to their toxicity. However, when compared to DES and conventional solvents such as methanol, ethanol, ethyl acetate, acetone, chloroform, and n-hexane, NADES are generally safer, with lower toxicity [16]. In this study, the NADES formulations used are combinations of choline chloride with citric acid and choline chloride with lactic acid, where choline chloride is a quaternary ammonium salt, and in the NADES formation, choline chloride plays a role in the HBA formation. Choline chloride is known to have low cytotoxic activity. Meanwhile, lactic acid contributes to the HBD formation in NADES and has shown safe results as a raw material for NADES. Citric acid is also used in the HBD formation in NADES and contributes

to increasing compound solubility. As for the benefits to the body, lactic acid can increase the body's ability to absorb iron and improved gut health, which are further benefits of citric acid. The safety of using NADES is due to their non-volatile nature, thermal stability, conductivity, and excellent solubility, which make them environmentally friendly and suitable for various applications [17-19]. In addition, NADES is a specialized designer solvent since physicochemical properties can be adjusted by manipulating the HBA and HBD. Variations in polarity, pH, solvency, viscosity, and stabilization depended on the molar ratio and initial components. The molar ratio plays a crucial role in shaping the hydrogen bond network, influencing factors such as polarity and viscosity [20].

NADES physico-chemical properties test

pH and viscosity

The physico-chemical properties measured by the pH and the viscosity test of the NADES. The results are summarized in Table 3.

TABLE 3 NADES pH and viscosity

Physico-Chemical Properties			NADES1	NADES2
Viscocity	Rotor 1	6 rpm	999	81.9
		12 rpm	499.5	85.5
		30 rpm	199.8	86
	Rotor 3	6 rpm	1600	20
		12 rpm	1800	80
		80 rpm	1728	32
pH			1.4	1.9

Based on pH test results using pH meter, the result for NADES1 is 1.4 and NADES2 is 1.9, and this shows that NADES solvent produced is acidic. The pH value of NADES correlates to the chemical structure of HBD, confirming how HBD type influences NADES properties. High extraction yields on bioactive compounds of acid-based NADES solvents are caused by the capacity to dissolve effectively at low pH levels and due to stronger hydrogen bonding and dipole-dipole interactions [21]. This suggests a strong ability to donate protons and accept electrons, facilitating the dissolution of both polar and nonpolar compounds [22].

The viscosity test results show that for rotor types 1 and 3 with different speed variations (rpm) – 6, 12, and 30 – NADES1 has a higher viscosity value than NADES2. This suggests that NADES1 is thicker than NADES2. The higher the NADES viscosity, the less effective it will be in attracting compounds because of the low mass transfer and reduced extraction efficiency. Therefore, the lower the viscosity value, the easier the mass transfer and the better the properties of NADES in attracting compounds [23].

The solid molecular interactions of H-bonding, Van der Waals, and electrostatics contribute to the forming of a high viscosity of NADES. Carboxyl, hydroxyl, and amine are the functional groups involved in this molecular interaction. According to Table 3, the elevated viscosity of NADES1 is attributed to the stronger molecular interactions formed by citric acid structure compared to those formed by lactic acid [22].

In general, all NADES with different ratios have a higher density than water. The density of ChCl-based NADES ranges from 1.04 at room temperature with systems containing 1,3- butanediol to 1.26 for NADES with citric acid and fructose as HBD. Although solvent densities exhibit negligible differences, it can

be inferred that the increasing density in the components is influenced by the number of hydroxyl groups [24].

The results show that pH varies with the molar ratio of DES, viscosity, surface tension, and density are also observed to have a high value caused by the increased molar ratio. Therefore, the properties of the extracted material will be affected by the difference in the molar ratios in DES [25].

Results of total phenols of coffee leaves extract

The calibration methods used in determining the total phenols of coffee leaves extract was quercetin with the concentrations of 20, 30, 40, 50, and 60 µg/mL. The total phenols results are provided in Table 4.

TABLE 4 Results of total phenols of coffee leaves extract

Sample Name	Concentration (µg/mL)	Sample Volume (L)	Sample Weight (g)	Dilution Factor	Total Phenol (GAE/g extract)
Ethanol 96%	7.5690	0.025	0.025	1	7.5690
NADES1 15 m	16.9252	0.01	1	100	16.9252
NADES1 30 m	13.2564	0.01	1	100	13.2564
NADES1 45 m	14.4858	0.01	1	78.94	11.4351
NADES1 60 m	15.3088	0.01	1	93.75	14.3520
NADES1 2 h	18.3041	0.01	1	83.33	15.2528
NADES1 4 h	15.6772	0.01	1	107.14	16.7965
NADES2 15 m	15.5825	0.01	1	83.33	12.9849
NADES2 30 m	16.0963	0.01	1	88.23	14.2017
NADES2 45 m	17.9366	0.01	1	100	17.9366
NADES2 60 m	21.1829	0.01	1	100	21.1829
NADES2 2 h	20.8694	0.01	1	100	20.8694
NADES2 4 h	21.5724	0.01	1	136.36	29.4161

The highest total phenol results were obtained from coffee leaves extract with NADES2 solvent at a time variation of 240 minutes/4 hours, where the levels obtained were 29.4162 GAE/g extract. This shows that NADES2 solvent with an extraction time of 4 hours is the best solvent and time to extract the phenolic compounds in coffee leaves.

The DES capacity to extract bioactive compounds is correlated with physicochemical properties, including H-bond interactions, polarity, viscosity, and pH. The H-bonding interaction properties contribute to a higher extractability of phenolic acids, showing the significant role of DES polarity in its extraction efficiency. The results show that components of NADES can be selected to increase the biological activity of extracts [26].

Results of total flavonoids from coffee leaves extract

The calibration methods used in determining the total flavonoids of coffee leaves extract was gallic acid with a concentration of 20, 30, 40, 50, and 60 µg/mL. The total flavonoids results can be seen in Table 5.

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TABLE 5 Results of total flavonoids from coffee leaves extract

Sample Name	Concentration (µg)	Sample Volume (L)	Sample Weight (g)	Dilution Factor	Total Phenol (GAE/g extract)
Ethanol 96%	38.2766	0.005	0.025	3.3333	25.5175
NADES1 15 m	36.7878	0.01	1	100	36.7878
NADES1 30 m	22.2653	0.01	1	100	22.2653
NADES1 45 m	32.4611	0.01	1	78.94	25.6248
NADES1 60 m	32.1475	0.01	1	93.75	30.1383
NADES1 2 h	36.9222	0.01	1	83.33	30.7673
NADES1 4 h	32.8669	0.01	1	107.14	35.2136
NADES2 15 m	36.0737	0.01	1	83.33	30.0602
NADES2 30 m	46.6614	0.01	1	88.23	41.1693
NADES2 45 m	42.9090	0.01	1	100	42.9090
NADES2 60 m	41.1515	0.01	1	100	41.1515
NADES2 2 h	53.5599	0.01	1	100	53.5599
NADES2 4 h	50.4848	0.01	1	136.36	68.8411

The highest total flavonoid results were obtained from coffee leaves extract with NADES2 solvent at a time variation of 4 hours, where the levels obtained were 68.8411 QE/g extract. This shows that NADES2 solvent with an extraction time of 4 hours is the best solvent and extraction time to extract the flavonoid compounds contained in coffee leaves compared to NADES1 and 96% ethanol, which are often used as solvents. It is known that NADES 2 with choline lactate is an outstanding flavonoid extractor [27].

Results of chlorogenic acid content from coffee leaves extract

The calibration curve aims to obtain the regression equation values used to determine the concentration of the samples.

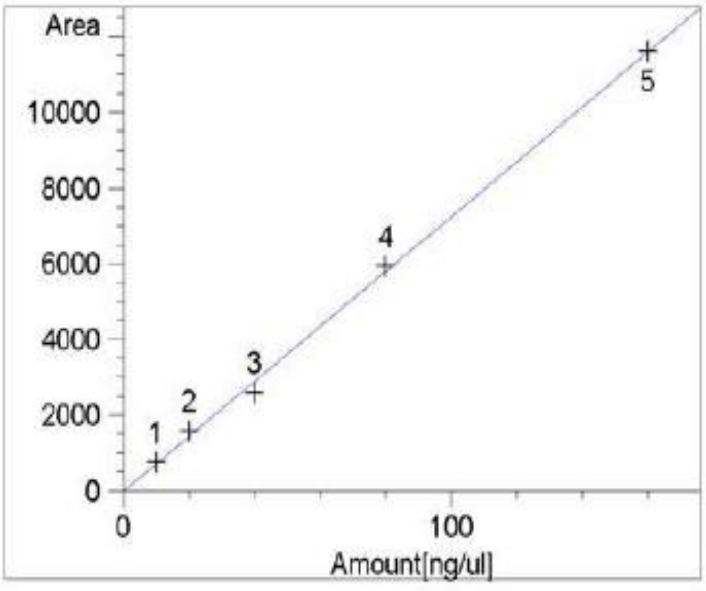

FIGURE 1 Chlorogenic acid calibration curve

The calibration curve obtained from the chlorogenic acid analysis can be seen in Figure 1. The calculation results and determination of the linearity of the calibration curve yield the regression equation and correlation coefficient for chlorogenic acid, given by Y =

72.70655X-10.03015 with a correlation coefficient (r) of 0.99935. The correlation coefficient (r) is used to determine the linear relationship between concentration and area under the curve. The relationship is considered perfect when the (r) value approaches one. The obtained correlation coefficient (r) is still within the acceptable limits, i.e. r = 0.995 [28].

The results of determining the chlorogenic acid levels in coffee leaves extract with NADES solvent are listed in Table 6.

TABLE 6 Results of chlorogenic acid content from coffee leaves extract

Sample Name	Time (minute)	Sample weight (g)	Dilution Factor	Concentration (µg/mL)	Content (mg/g)
NADES1 15 m	15	1.0019	50	19.1018	0.9532
NADES1 30 m	30	1.0021	39.4736	26.1906	1.0316
NADES1 45 m	45	1.0033	46.8750	27.1231	1.2672
NADES1 60 m	60	1.0047	41.6667	32.9149	1.3650
NADES1 2 h	120	1.0039	53.5714	27.3445	1.4591
NADES1 4 h	240	1.0056	50	33.2243	1.6519
NADES2 15 m	15	1.0026	41.6667	27.6567	1.1493
NADES2 30 m	30	1.0054	44.1176	34.1857	1.5000
NADES2 45 m	45	1.0031	50	31.9150	1.5908
NADES2 60 m	60	1.0069	50	33.2642	1.6518
NADES2 2 h	120	1.0047	50	37.0080	1.8417
NADES2 4 h	240	1.0041	68.1818	40.8550	2.7741

The highest chlorogenic acid content obtained from coffee leaves extract using NADES1 solvent (choline chloride-citric acid) was observed at a time variation of 4 hours, with a concentration of 33.2243 and a content of 1.6519 mg/g. Meanwhile, the highest chlorogenic acid content obtained from coffee leaves extract using NADES2 solvent (choline chloride-lactic acid) was also at a time variation of 4 hours, with a concentration of 40.8555 and a content of 2.7741 mg/g. Among these two NADES solvents, NADES2 (choline chloride-lactic acid) exhibited the highest chlorogenic acid content. This suggests that NADES2 (choline chloride-lactic acid) excels in dissolving compounds due to the unique composition and properties, including the molar ratio, water content, and the ability to form hydrogen bonds and ionic interactions with chlorogenic acid. These factors contribute to its high solvation-free energy, enhancing the dissolution of chlorogenic acid that is known to be abundant in coffee [7,29].

Exmperimental

Materials

In this study, the materials used include beaker glass (Pyrex), blender (Philips), evaporating cup, funnel, measuring cup (Pyrex), HPLC (Shimadzu), measuring flask (Pyrex), analytical balance (Boeco), balance crude (Homeline), electric oven (Memmert), water bath, rotary vacuum evaporator (Stuart), pH indicator, pH meter, volume pipette (Pyrex), centrifuge, set of water content determination tools (Pyrex), sonicator (Branson), spectrophotometer UV-Visible (Shimadzu), test tubes, furnaces, vials, and viscometers. The chemicals used unless otherwise stated are of pro analytical quality, such as: quercetin (Sigma), aluminum chloride, amyl alcohol, acetic acid anhydride, hydrochloric acid, lactic acid, nitric acid, citric acid, sulfuric acid, benzene, iron (III) chloride, bismuth (III) nitrate, ethyl acetate, ethanol

96%, iodine, potassium iodide, chloral hydrate, chloroform, choline chloride, methanol, n-hexane, sodium acetate, sodium carbonate, mercury (II) chloride, magnesium powder, lead (II) acetate, and toluene and distilled water.

Methods

Sample preparation

Sampling was deliberately carried out without a comparison with a similar material from another region. The samples used were robusta coffee leaves. Coffee leaves were cleaned from dirt and washed with clean water. After draining, the wet weight was measured. The leaves were dried in a drying cabinet at ±40 ºC. The sorted dry leaves were weighed to determine the dry weight. The dried samples were ground into powder using a blender. The weight of the resulting powder was measured and stored in a plastic container containing silica gel to prevent moisture or other impurities from affecting the samples.

Simplified extract characterization

Analysis of water content

200 ml toluene was poured into a round-bottom flask, add 1-4 ml distilled water, the connecting tube was attached and collected, and then the bulb was colled and the mixture was distilled for 2 hours. After that, the distillation was stopped and cooled for 30 minutes. The water volume was measuerd in the receiving tube with a precision of 0.05 ml and record the result.

5 g of weighed sample was placed into a round-bottom flask containing saturated toluene inside and reheated for 15 minutes until the toluene boils. The drip rate was adjusted to 2 drops per second initially. As some water was distilled, the drip rate was increased to 4 drops per second. Once the whole water was distilled, the inside of the cooler was washed with toluene. The distillation was continued for 5 minutes and the receiving tube cool was kept at room temperature. Afterwards, the water and toluene were seperated, and then the water volume was measured with 0.05 ml accuracy, and water content was calculated in %v/w [12].

Analysis of total ash content

2 g of a carefully weighed and crushed sample was placed into a pre-ignited and tared silicate crucible, and then it was flatten. Slowly heat the crucible until charcoal was depleted, incandescence occurring at 600 °C for 3 hours. The crucible was cooled down and weighed until a constant weight was achieved. The residue and filter paper were heated in the same crucible. After that, filtrate was obtained, evaporated, stired to a constant weight, and re-weighted. The ash content was determined for materials dried in the air [26].

Analysis of ash insoluble content in acid

The residue from the ash content analysis was subjected to boiling with 25 ml diluted hydrochloric acid for 5 minutes. The part insoluble was collected in acid and filtered using ash-free filter paper of a known weight, and then the residue underwent a process of heating, cooling, and weighing until a consistent weight was achieved. The ash content insoluble in acid was calculated against the weight of the simplified extract in %w/w [30].

Analysis of water soluble essence content

A 5 g sample powder, previously weighed, underwent maceration with 100 ml of chloroform-saturated water in a sealed flask. The mixture was shaken periodically for 6 hours and subsequently left undisturbed for 18 hours. After filtration, the first 20 ml filtrate was evaporated to dryness in a heated, flat-bottomed shallow dish. The resulting residue was then heated in an oven at 105 °C

until a consistent weight was achieved. The percentage of water-soluble essence content was calculated based on the dried material [31].

Analysis of ethanol soluble essence content

A total of 5 g simplified extract powder was weighed, and macerated with 100 ml ethanol (96%) using a stoppered flask for 24 hours. The mixture was agitated for the initial 6 hours, and then allowed to stand for an additional 18 hours. The strained solution was then partially evaporated by heating 20 ml filtrate in a tarred flat-bottomed shallow dish. The remaining solution was then heated in an oven at 105 °C until a consistent weight was achieved. The percentage of soluble essence content in ethanol was determined in relation to the dried material [30].

Preparation of NADES Solvent

NADES1 (Choline Chloride: Citric Acid)

NADES1 was produced with a ratio of 2:1. A total of 279.24 g choline chloride was added to 192.124 g citric acid, followed by heating on a hotplate in the temperature range of 70-80 °C. After complete melting in the solution, the mixture was stirred until achieving homogeneity and stability, typically by the 7th day.

NADES2 (Choline Chloride: Lactic Acid)

NADES2 was produced with a 1:1 ratio. A total of 279.24 g choline chloride was added to 180.156 g lactic acid, followed by heating on a hotplate at 60-70 °C. After complete melting in the solution, the mixture was stirred until achieving homogeneity and stability, typically by the 3rd day.

NADES Physico-Chemical Properties Test

Test pH

A buffer solution of pH 4.7 and 10 was prepared, and calibration was carried out, based on the acidic, basic, and neutral ranges. After calibration, the pH was added to the sample solution and record the pH value. The increase of the pH solution was continued until a constant pH reading is obtained.

Sample Extraction

96% ethanol extract

A total of 50 g simplified extract powder was added with 96% ethanol for 500 ml, stirred for 6 hours, and then it was kept for 24 hours. After that, the ethanol extract was filtered and evaporated to obtain coffee leaves extract.

NADES Extract 1

A total of 1 g coffee leaves simplified extract powder was added with 30 ml NADES1, and then sonicated based on time variations, namely 15, 30, 45, 60, 120, and 240 minutes. Subsequently, the sonication results were centrifuged for 20 minutes at 25 °C 4500 rpm. Centrifuge results are filtered to facilitate the extraction of coffee leaves using the NADES 1 solvent.

NADES Extract 2

A total of 1 g coffee leaves simplified extract powder was added with 30 ml of NADES2, and then sonicated based on time variations, namely 15, 30, 45, 60, 120, and 240 minutes. Subsequently, the sonication results were centrifuged for 20 minutes at 25 °C 4500 rpm. Centrifuge results are filtered to facilitate the extraction of coffee leaves using NADES2 solvent.

Analysis of Total Flavonoid Content

To create a calibration curve, concentrations of 20, 30, 40, 50, and 60 µg/mL were used for quercetin. Ethanol extract of coffee leaves with a concentration of 1000 µg/mL was diluted with methanol until a concentration of 300 µg/mL was obtained. Meanwhile, NADES

coffee leaves extract was placed in a 25 mL flask filled with aquabides, and then 5 mL was pipetted into a 10 mL flask then filled with methanol.

The 2 ml test solution was inserted in the vial, adding 0.1 mL of 10% AlCl3 (aluminum chloride), 0.1 mL of 1 M CH3COONa, and 2.8 ml distilled water and incubated for 30 minutes and the absorbance of the solution was measured against the quercetin calibration standard using a UV-visible spectrophotometer at the maximum wavelength (436 nm). The measurement results were expressed as the equivalent weight of quercetin per sample weight.

Analysis of total phenol content

To create a calibration curve, gallic acid was used with a concentration of 5, 10, 15, and 20 µg/mL. Ethanol extract of coffee leaves was obtained with a concentration of 1000 µg/mL. Meanwhile, the NADES coffee leaves extract was placed in a 25 ml flask filled with aquabides, and then 5 ml was pipetted into a 10 ml flask and filled with methanol.

A test solution of 0.1 ml was taken and mixed with 7.9 ml distilled water and 0.5 ml Folin-Ciocalteau reagent, and then 1.5 mL of 8% Na_2CO_3 was added and incubated for 90 minutes. The solution absorbance was measured against the gallic acid calibration standard with a UV-Vis spectrophotometer at the maximum wavelength (744 nm). The measurement results were expressed as the equivalent weight of gallic acid per sample weight.

The following is how to calculate the levels of total phenol and total flavonoid.

Phenol regression equation: y = 0.03945 X + 0.008

Flavonoid regression equation: y = 0.01265 X - 0.0037

Added NADES: 30 ml

Weighed coffee grounds: 1 g

The volume of extract is sufficient: 25 ml

Sufficient volume after pipetting: 10 mL

The volume of extract obtained: different

Pipetted volume: 5 mL

Total dilution factor:

$$\frac{\text{NADES added x Extract sufficient volume x Sufficient volume after pipetting}}{\text{Extract volume after filtering x Pipetted volume}}$$

$$\text{Rate} : \frac{Concentration\left(\frac{\mu g}{ml}\right) x\ Sample\ Volume\ (L)}{Sample\ Weight\ (g)}\ x$$

Dilution factor

Analysis of the content of chlorogenic acid

For the preparation of the calibration curve, chlorogenic acid with concentrations of 10, 20, 40, 80, and 160 µg/mL is used. Coffee leaves extracts with NADES1 or NADES2 solvents, which have been filtered, were placed in a 25 mL flask, and shaken with aquabides. Subsequently, samples were taken from each filtration result to be injected into HPLC. The mobile phase used is a 0.1% acetate gradient in aquabides:acetonitrile (minutes 0-10 90:10; minutes 11-20 80:20; and minutes 20-30 90:10).

Conclusion

To sum up, NADES has proven to be effective in extracting secondary metabolite compounds from robusta coffee leaves. The highest total phenol, flavonoid, and chlorogenic acid content were obtained from robusta coffee leaves extract using NADES2 (choline chloride: lactic acid) solvent with a 1:1 ratio and an extraction time variation of 4 hours. The resulting levels were 29.4162 GAE/g extract for total phenol, 68.8411 QE/g extract for total flavonoid content, and 2.7741 mg/g for chlorogenic acid at a concentration of 40.8555.

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Authors' Contributions

Conceptualization, H.S.W; methodology, analysis, H.S.W. and L.L; investigation, H.S.W. and S.Y; writing-orginal draft preparation, H.S.W, L.L. and S.Y; writing-review editing, H.S.W. and C.C.J; supervision, H.S.W; funding acquisition, H.S.W.

Conflict of Interest

All authors stated that there are no conflicts of interest

Orcid:

Henny Sri Wahyuni:*
https://orcid.org/0000-0002-7646-2928
Lia Laila:
https://orcid.org/0000-0002-9343-5518
Clara Claudia Jap:
https://orcid.org/0009-0007-6363-8334

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