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Natural deep eutectic solvents ultrasound-assisted extraction (NADES-UAE) of *trans*-cinnamaldehyde and coumarin from cinnamon bark [*Cinnamomum burmannii* (Nees & T. Nees) Blume]

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ABSTRACT: Natural deep eutectic solvents (NADES) have been shown to be more effective in extracting plant marker compounds than are organic solvents. In this study, mixtures of choline chloride or betaine with three combinations of sugar molecules (glycerol, xylitol, and sorbitol) and three combinations of organic acids (lactic acid, citric acid, and malic acid) were used as solvents in ultrasonic-assisted extraction (UAE) of trans-cinnamaldehyde and coumarin from cinnamon bark [*Cinnamomum burmannii* (Nees & T. Nees) Blume]. Optimization of extraction conditions to obtain the optimal trans-cinnamaldehyde content was performed by varying the percentage of water in NADES and extraction time. Efficiency of extraction was determined by conventional extraction performed by maceration, reflux, and the soxhlet method using 96% ethanol for comparison. Trans-cinnamaldehyde and coumarin contents were determined by high-performance liquid chromatography. The surfaces of samples before and after extraction were observed by scanning electron microscopy (SEM). The study results showed that choline chloride–citric acid (1:2) with 40% water addition for 30 minutes produced the highest trans-cinnamaldehyde content of 9.24 ± 0.01 mg/g dry weight. The highest trans-cinnamaldehyde content from conventional extraction using the maceration method was 1.93 ± 0.01 mg/g dry weight. SEM analysis of sample surfaces showed that the ruptured cells in the NADES-UAE method gave a higher trans-cinnamaldehyde yield than that from the shrunken and damaged cells in the conventional extraction. NADES-UAE provided higher extraction efficiency for trans-cinnamaldehyde and coumarin from *C. burmannii* than that of the conventional methods using 96% ethanol as solvent.

KEYWORDS: *Cinnamomum burmanii;* coumarin; cinnamaldehyde; extraction; NADES.

1. INTRODUCTION

Some organic solvents present as residual solvents in commercial extraction processes may not decompose quickly and thus cause environmental pollution, with potential toxic effects in humans [1]. These adverse effects of organic solvent usage have led to development of non-toxic "green extraction" methods for extraction of active compounds from natural materials. Natural deep eutectic solvents (NADES) is one approach to green solvents [2]. This technology reduces the use of harmful compounds by replacing them with a solvent system that is more environmentally friendly and has controllable physicochemical properties (polarity and viscosity). In the development of green extraction methods, identification of green solvents that can replace harmful organic solvents is an important research area [1].

NADES is a liquid mixture made from primary metabolites (such as sugars, alcohols, organic acids, amino acids, and amines) that are bonded by strong intermolecular interactions, especially hydrogen bonds [3]. Compared with conventional solvents commonly used for extraction, NADES has been proven to have many benefits related to volatility, polarity, and viscosity. From an economical perspective, NADES also

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provides considerable benefits in term of biodegradability, continuity, low cost, and ease of manufacturing [4]. A few studies have reported that NADES was successfully used to extract bioactive compounds from plants [5].

Cinnamon is a plant that is widely known as a spice and for its medicinal uses. Previous studies have shown that a few species of Cinnamomum have been considered to have some pharmacological effects in medical treatment [6]. The stem bark of *Cinnamomum burmannii* is known to have antidiabetic activity in pre-clinical and clinical studies [7]. The main compound in *C. burmannii* that has antidiabetic activity is cinnamaldehyde (C_9H_8O). *C. burmannii* also contains coumarin ($C_9H_6O_2$), a phenolic compound which has several activities such as anticancer, inflammatory, and coagulation inhibition activities. Coumarin also has antifungal, antibacterial, and antivirus activities [8].

In this study, the contents of trans-cinnamaldehyde and coumarin extracted by three conventional extraction methods using maceration, reflux, and the Soxhlet process with 96% ethanol as solvent were compared with the contents extracted by ultrasonic-assisted extraction (UAE) using NADES solvents with choline chloride and betaine. The conventional methods were based on the studies that have been conducted by Julianti et al [9], Wong et al [10], and Wardatun et al [11].

UAE is used to extract bioactive compounds from plants because of its high extraction efficiency that can be attained at relatively lower temperatures [12]. The use of a UAE method with a NADES solvent to extract the bioactive compounds from the bark of *Cinnamomum burmannii* (Nees & T. Nees) Blume is expected to give high extract yields of trans-cinnamaldehyde and coumarin compound, which would make it an alternative method for green extractions of these compounds.

2. RESULTS AND DISCUSSION

2.1. Method validation

Validation is an important process to confirm the performance characteristics of the method of analysis. The analytical method is a series of detailed directions, from preparation of test samples to reporting the results which that should being followed exactly to obtain the acceptable results [13]. Determination of trans-cinnamaldehyde and coumarin was performed by using High-Performance Liquid Chromatography, with UV detector at 280 nm. Method validation was done using parameters that refer to the Association of Official Analytical Chemist (AOAC) Guidelines 2002 [13] including system suitability tests, linearity, accuracy, precision, limits of detection (LOD), and limits of quantification (LOQ).

The results (Table 1) showed that the system was suitable, as indicated by a % Relative Standard Deviation (RSD) for area of 1.35%–1.92%, a %RSD for retention time of 0.35%–0.61%, a tailing factor ranging from 1.11–1.15, and a theoretical plate number ranging from 5450.17–6081.17. All obtained results were in the acceptable range. The intra-day %RSD and inter-day %RSD values were 0.89 and 1.15, respectively, for trans-cinnamaldehyde and 1.35 and 1.84, respectively, for coumarin. The results of the intra-day and inter-day precision experiments are also shown in Table 1.

Table 1. Validation parameters of the HPLC method.

Compound	Repeatability of Area (%RSD)	Repeatability of Retention Time (%RSD)	Tailing Factor	Theoretical Plate Number	Precision (%RSD)	
					Intraday	Interday
Trans-Cinnamaldehyde	1.92[a]	0.61[c]	1.11 ± 0.0041[e]	6081.17 ± 30.57	0.89	1.15
Coumarin	1.35[b]	0.35[d]	1.15 ± 0.0024[f]	5450.17 ± 69.87	1.35	1.84

[a-d] RSD values obtained from 6 replications.
[e-f] tailing factor was calculated from the HPLC using LabSolutions SW.

The LOD is when Signal to Noise Ratio (SNR) is 3. The LOQ is when Signal to Noise Ratio (SNR) is 10 [13]. For trans-cinnamaldehyde and coumarin, the LOD values were 1.11 and 1.07 mg/g, respectively, and the LOQ values were 3.69 and 3.58 mg/g, respectively (Table 2).

Linearity was shown by the calibration curve. Calibration curves were linear over a concentration range of 1.98–31.68 µg/mL for trans-cinnamaldehyde and 2.02–32.38 µg/mL for coumarin. The linear regression equation for coumarin was y = 63164x − 2542.4, with a correlation coefficient (r) of 0.99995. The linear

regression equation for trans-cinnamaldehyde analysis was y = 201982x + 26007, with a correlation coefficient (r) of 0.99995. The calibration curves exhibited good linearity (r ≥ 0.999) (Figure 1).

Table 2. The LOD and LOQ Result of trans-cinnamaldehyde and coumarin.

Analyte	LOD (mg/g)	LOQ (mg/g)
Trans-Cinnamaldehyde	1.11	3.69
Coumarin	1.07	3.58

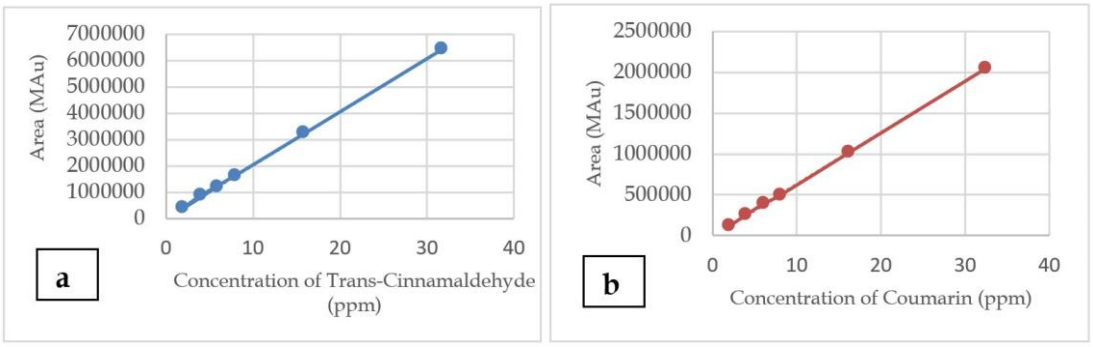

Figure 1. Standard calibration curves of trans-cinnamaldehyde (a) and coumarin (b).

Accuracy was defined as the difference between the reported value and the acceptable one. The requirement for natural sample accuracy is 90%–108% [13]. The accuracy for recovery of trans-cinnamaldehyde was 103.18% which met the requirements. Meanwhile the recovery of coumarin was 124.02%. The recovery value of coumarin was not met the requirements. This means this method could not separate the coumarin and its impurities, which means for coumarin analysis, it needs better separation method [13]. The accuracy results are shown in Table 3.

Table 3. The recovery of trans-cinnamaldehyde and coumarin (n=6).

Analyte	Analyte (µg/ml)	Amount of spike (µg/ml)	Total Analyte (µg/ml)	Recovery (%) (±SD)	%RSD
Trans-Cinnamaldehyde	3.0641	3.1615	6.3276	103.18± 1.90	1.84
Coumarin	3.0728	3.1615	7.1095	124.02± 1.13	0.92

2.2. Screening of optimum NADES

In this study, the UAE method using NADES choline chloride-citric acid (ChCl-CA) produced the highest trans-cinnamaldehyde content among all NADES combinations and gave 17.67 mg/g yield. Meanwhile, betaine-lactic acid (B-LA) produced the highest coumarin content among all NADES combinations and gave 15.33 mg/g yield (Figure 2). Trans-cinnamaldehyde and coumarin compounds can act as HBDs. Alcohol sugars and organic acids in this study also acted as hydrogen bond donors (HBD), which competitively interact with targeted compounds (trans-cinnamaldehyde and coumarin). An HBD can interact with negatively charged moiety from choline chloride and betaine as hydrogen bond acceptors (HBA) [14]. This result means that trans-cinnamaldehyde interacts more with ChCl-CA than other NADES. Meanwhile, the coumarin interacts more with B-LA than other NADES.

Addition of water to the NADES system can increase polarity, reduce surface tension, and reduce viscosity in NADES. The addition of water also increased the extraction yield significantly. However, excessive addition of water can damage the hydrogen bond between the HBA and HBD and reduce the interactions between the targeted compounds (trans-cinnamaldehyde and coumarin) and NADES, so it will reduce the efficiency of the extraction [15].

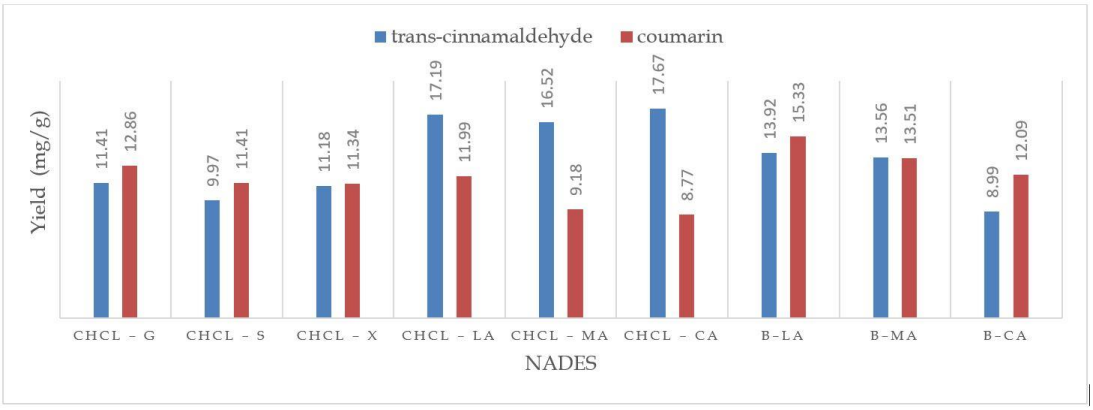

Figure 2. NADES's effect to trans-cinnamaldehyde and coumarin contents in extraction.

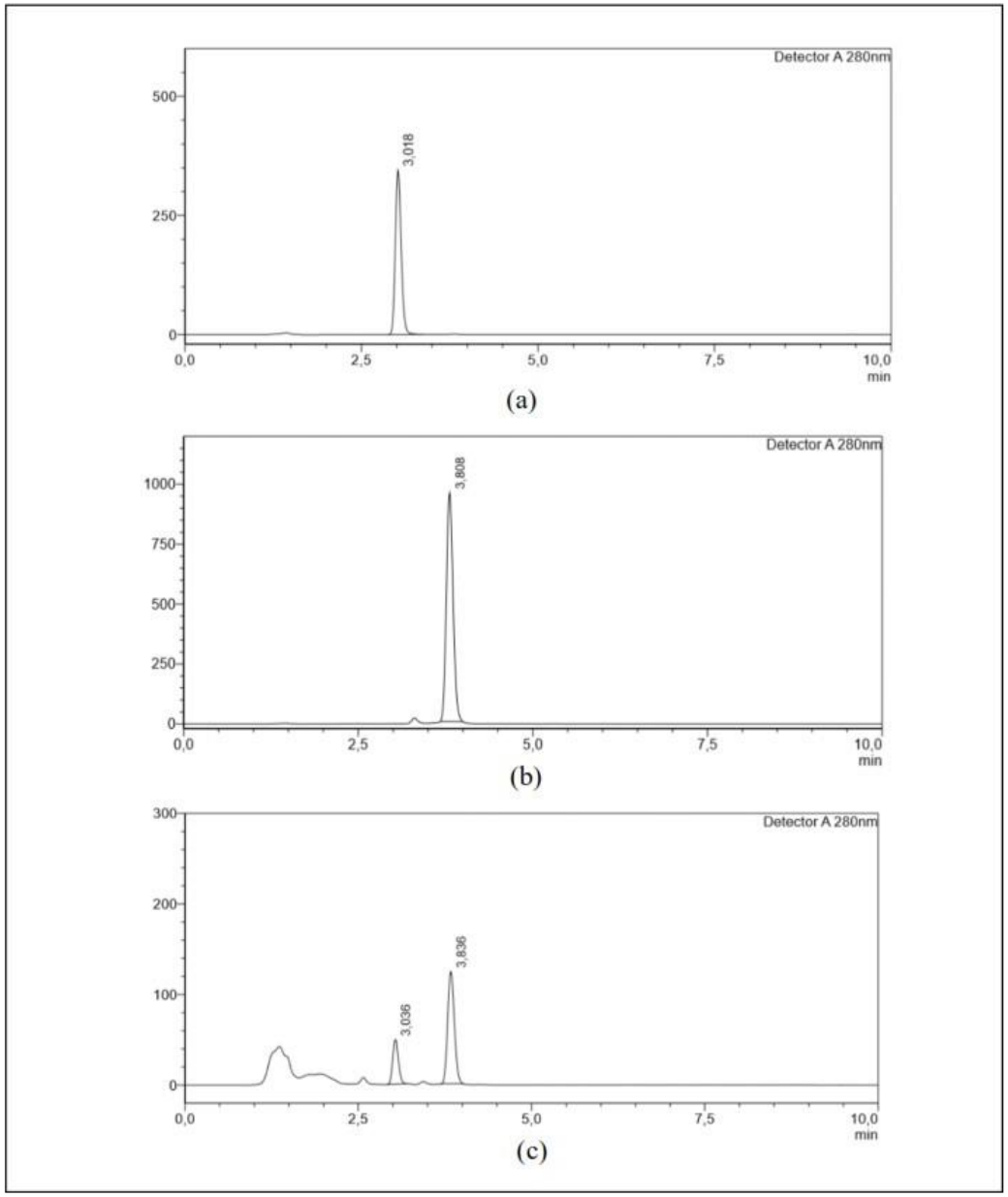

Figure 3. The chromatogram profile of coumarin standard (a); trans-cinnamaldehyde (b); and sample containing trans-cinnamaldehyde and coumarin.

2.3. Analysis of trans-cinnamaldehyde and coumarin compounds in samples

The analysis conditions in this study were those described by Ahmad et al [6], but the column was shorter. The retention time of coumarin was 3.0 minutes and that of trans-cinnamaldehyde was 3.8 minutes. The resolution value was calculated to assess the quality of compound separation. Based on the AOAC Guidelines [13], the minimum resolution value is ≥1.5. The average resolution was 4.5 for the sample extracted by NADES-UAE ChCl-CA with 40% water addition, extraction time 30 minutes, indicating that the coumarin and trans-cinnamaldehyde samples were well separated (Figure 3). Compared to the study by Bi et al [14], which has RSD value by 2.72-3.06%, the method that we used in this study has RSD value by 0.89-1.84%.

2.4. Optimization of extraction conditions

NADES that we used to be optimized was ChCl-CA, based on the validation method. This method was valid for trans-cinnamaldehyde determination, but not for coumarin determination. The optimum extraction conditions that gave the highest content of trans-cinnamaldehyde was NADES-UAE ChCl-CA with 40% water addition for 30 minutes, with yields of 9.24 mg/g trans-cinnamaldehyde and 11.60 mg/g coumarin (Table 4). In this study, Kruskal Wallis nonparametric analysis test results for trans-cinnamaldehyde and coumarin levels showed that the water addition of NADES (%) and extraction time (minutes) had a significant effect ($P < 0.05$). Water addition and extraction time had significant interactions with trans-cinnamaldehyde and coumarin content. The NADES solvents used for extraction of cinnamon bark were added with different levels of water. Water added to NADES at a certain amount can increase the extraction efficiency because it might reduce the solvent viscosity and enable a better mass transfer rate. However, at higher water contents (>50%) of NADES, extraction efficiencies of trans-cinnamaldehyde markedly decreased, which was due to the weak interaction between NADES and the target compound [14].

Table 4. The result of optimization of extraction condition.

Water addition (%)	Extraction time (min)	Trans-cinnamaldehyde content (mg/g)	Coumarin content (mg/g)
20	10	6.87 ± 0.02	8.94 ± 0.05
20	30	8.04 ± 0.00	10,11 ± 0.09
20	50	7.31 ± 0.01	9.68 ± 0.02
40	10	7.48 ± 0.08	9.29 ± 0.13
40	30	9.24 ± 0.01	11.60 ± 0.11
40	50	8.06 ± 0.03	10.53 ± 0.03
60	10	6.13 ± 0.02	7.51 ± 0.06
60	30	7.45 ± 0.03	9.19 ± 0.05
60	50	6.92 ± 0.05	8.23 ± 0.15

The trans-cinnamaldehyde and coumarin contents in NADES-UAE extract of cinnamon bark increased with longer extraction times and reached a maximum at 30 minutes and then decreased. Ultrasonic extraction is a process of diffusion and mass transfer, and before the process reaches equilibrium, the extraction yield can be increased. However, a long extraction time might cause the loss of volatile substances in essential oils, which causes a decrease in the levels of compounds from the extract [16]. The interaction between the water addition to NADES and the length of time of extraction also had a significant effect on trans-cinnamaldehyde and coumarin levels because at the optimum extraction time and optimum water addition of NADES, the process of diffusion and mass transfer was better, which increased the extracted trans-cinnamaldehyde and coumarin content.

2.5. NADES-UAE vs. conventional extraction

The highest trans-cinnamaldehyde content in conventional extraction (from highest to lowest) was achieved by using the maceration, then reflux method, and lastly the soxhlet method. The average levels of trans-cinnamaldehyde and coumarin in the maceration extract were 1.93 mg/g and 4.36 mg/g, respectively. The levels of trans-cinnamaldehyde and coumarin extracted using NADES ChCl-CA were 9.24 mg/g and 11.6 mg/g, respectively. The trans-cinnamaldehyde and coumarin contents extracted using the NADES-UAE were greater than those from the conventional extraction methods using 96% ethanol. The results of the extraction methods are shown in Table 5.

Table 5. Comparison of NADES-UAE and conventional extraction.

Extraction method	Trans-cinnamaldehyde content (mg/g)	Coumarin content (mg/g)
NADES-UAE	9.24 ± 0.01	11.6 ± 0.11
Reflux	1.47 ± 0.00	3.94 ± 0.03
Soxhlet	0.71 ± 0.00	4.25 ± 0.05
Maceration	1.93 ± 0.01	4.36 ± 0.05

These results were supported by analyzing the surface image of the cinnamon bark before and after extraction (NADES-UAE and conventional extraction). The surface image of a dried cinnamon bark powder before extraction is shown in Figure 4a. It can be seen that the cell walls of dried cinnamon bark powder are dense and well ordered. The cell wall damage in cinnamon due to the UAE extraction can be seen in Figure 4e. In the UAE method, there is a cavitation phenomenon indicated by the formation of bubbles when ultrasonic waves pass through the extraction solvent. These bubbles develop until they reach a critical size, then the bubbles burst violently and release a large amount of energy. As a result, the cell walls are damaged, and penetration of extraction solvents into a greater among of cell material is increased in a shorter time than occurs in conventional methods [17]. In addition, NADES is known to increase destruction of plant cells through fiber dissolution, so the target compound is extracted more quickly from plant cells [18]. With the destruction of plant cells, trans-cinnamaldehyde compounds will be greater.

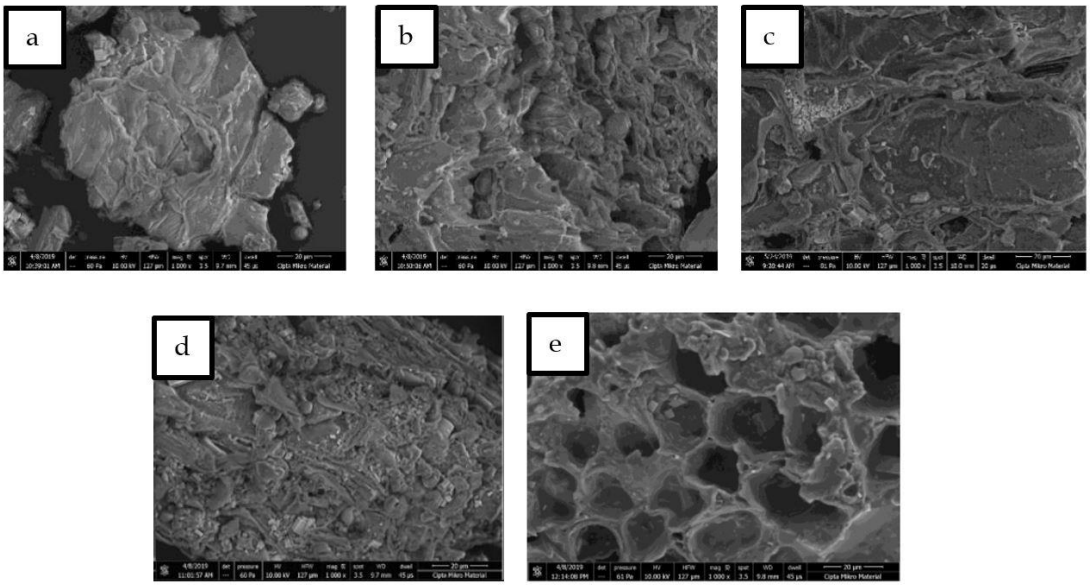

Figure 4. The surface image of cinnamon bark using scanning electron microscopy 1000x magnification; dried powder (a); after maceration extraction (b); after reflux extraction (c); after Soxhlet extraction (d); after extraction using NADES-UAE ChCl-CA (e).

An image of cinnamon cell surfaces that have shrunk after maceration can be seen in Figure 4b. An image of the cinnamon cell surfaces that were only slightly damaged after being extracted by the reflux and Soxhlet methods can be seen in Figure 4c and Figure 4d. In the maceration method, the process of dissolving or binding an active substance is based on the substance's solubility in a solvent ("like dissolves like"). Extraction in the reflux and Soxhlet methods occurs via the process of heat transfer by conduction and convection that damages plant cells, but the damage is less that that by UAE methods that use ultrasonic energy [18]. The lower damage to cinnamon cells causes the trans-cinnamaldehyde and coumarin content to be lower in conventional methods than that in the UAE method. It can be concluded that the NADES-UAE extraction method is more effective and faster for extracting trans-cinnamaldehyde compounds from cinnamon bark.

3. CONCLUSION

This study shows that the NADES-UAE method under optimum conditions gave higher trans-cinnamaldehyde and coumarin content than those obtained from conventional methods (reflux, soxhlet and maceration) using organic solvents (96% ethanol). The optimum conditions for NADES-UAE used ChCl-CA with 40% water addition and a 30-minute extraction time to extract trans-cinnamaldehyde (9.24 ± 0.01 mg/g) and coumarin (11.6 ± 0.11 mg/g) from cinnamon bark (*C. burmannii* Blume). Further investigation should be done to explore the pharmacological activity of the trans-cinnamaldehyde and coumarin from cinnamon bark.

4. MATERIALS AND METHODS

4.1. Plant materials and reagents

Cinnamon bark obtained from the main market area of Bogor, Indonesia was identified by the Indonesian Institute of Sciences (LIPI), Bogor Botanical Gardens, Indonesia with voucher code 030/IPH.1.01/If.07/I/2019. Other materials used in this study were glycerol (Molex Ayus Pharmaceutical, Indonesia), sorbitol (Dow Chemical, Singapore), xylitol (Zhejiang Huakang Pharmaceutical, China), lactic acid citric acid (Brataco, Indonesia), malic acid, choline chloride (Xi'an Rongsheng Biotechnology Co., Ltd., China), betaine (Shandong Ruihong Biotechnology, China), 96% ethanol, trans-cinnamaldehyde standard (Sigma-Aldrich, China), coumarin analytical grade (Sigma-Aldrich), methanol HPLC grade (Merck, Germany), glacial acetic acid (Merck), acetonitrile HPLC grade (Merck), double-distilled water (Ikapharmindo Putramas, Indonesia), and demineralized water (Brataco, Indonesia).

4.2. Instrumentation

The instruments used in this stufy were a Shimadzu high-performance liquid chromatography (HPLC) with an LC-20AT pump (Shimadzu, Japan), C-18 Inertsil ODS-3 column, 4.6 x 150 mm, 5-µm particle size (GL Sciences, Japan); Shimadzu SPD-20A ultraviolet- visible detector (Shimadzu, Japan); ultrasonic bath (Krisbow, China); 0.45-µm microporous membrane (Agilent, Germany); magnetic stirrer, centrifuge (Hettich Zentrifugen Universal 320), HPLC 100-µl syringe (Agilent, USA), hotplate stirrer (IKA® C-MAG HS7, Germany), 10-, 100-, and 1000-µl micropipettes (Socorex, Switzerland), grinding tools (Ji Mo Shandong, China), and a rotary vacuum evaporator (Buchi Switzerland, Switzerland).

4.3. NADES preparation

Choline chloride, betaine, glycerol, sorbitol, xylitol, citric acid, malic acid, and lactic acid were weighed according to each molar ratio as shown in Table 6. Choline chloride or betaine as a HBA was mixed with glycerol, sorbitol, xylitol, citric acid, malic acid, or lactic acid as a HBD and stirred on a hotplate stirrer at 80°C at a constant speed for 1–4 hours to form a eutectic liquid. Then, demineralized water (according to the determined water percentage) was added to the eutectic liquid and stirred again until a clear liquid was formed. For a mixture of choline chloride with xylitol, stirring was performed on a hotplate stirrer at 50°C. For a mixture of betaine with citric acid, demineralized water was added first and stirring was performed for 1 hour on a hotplate stirrer at 80°C at constant speed until a clear solution was formed. The solutions were kept in a tightly closed vial to prevent oxidation.

Table 6. Component of NADES.

NADES	Hydrogen Bond Acceptor (HBA)	Hydrogen Bond Donor (HBD)	Molar Ratio
ChCl – G	Choline Chloride	Glycerol	1 : 2 [5]
ChCl – S	Choline Chloride	Sorbitol	1 : 2 [5]
ChCl – X	Choline Chloride	Xylitol	4 : 1 [5]
ChCl – LA	Choline Chloride	Lactic Acid	1 : 1 [5]
ChCl – MA	Choline Chloride	Malic Acid	1 : 1 [5]
ChCl – CA	Choline Chloride	Citric Acid	1 : 1 [5]
B–LA	Betaine	Lactic Acid	1 : 1 [4]
B–MA	Betaine	Malic Acid	1 : 1 [4]
B–CA	Betaine	Citric Acid	1 : 1 [19]

4.4. Extraction process

4.4.1. Extraction using UAE

One gram of cinnamon bark powder was mixed with NADES with a ratio of sample to the solvent (b/v) 1:10 and extraction time according to each condition (Table 6). The extract was centrifugated and the supernatant was obtained. The supernatant was stored in a vial, protected from the light for further analysis.

4.4.2. Extraction using reflux

One hundred and fifty grams of cinnamon bark powder was mixed with 96% ethanol. The solution was refluxed for an hour. The extract was filtered to obtain the residue. The residue was then extracted for 2 more times with the same solvent. The combined filtrate was concentrated using a rotary vacuum evaporator until thick extract was obtained. The extract was then stored for further analysis.

4.4.3. Extraction using soxhlet

Cinnamon bark powder was mixed with 96% ethanol with a ratio of sample to the solvent (b/v) 1:10. The extraction time was 8 hours. The extract was concentrated using a rotary vacuum evaporator until thick extract was obtained. The extract was then stored for further analysis.

4.4.4. Extraction using maceration

Cinnamon bark powder was mixed with 96% ethanol with a ratio of sample to the solvent (b/v) 1:10. The mixture was mixed using a shaker for an hour and then was reserved for 24 hours. The extract was filtered and was concentrated using a rotary vacuum evaporator until thick extract was obtained. The extract was then stored for further analysis.

4.5. Validation of HPLC method

Validation of HPLC method was carried out by determining parameters set by AOAC 2002. The parameters were system suitability test, linearity, LOD, LOQ, accuracy and precision. The system suitability test was performed by using trans-cinnamaldehyde and coumarin standards at 100 ppm. Each standard solution of trans-cinnamaldehyde and coumarin was analyzed by HPLC, with six injections each. The %RSD values of the peak areas, retention times, number of theoretical plates, and tailing factors from the HPLC chromatograms were calculated. Linearity was determined by using the correlation factor of calibration curves of trans-cinnamaldehyde and coumarin standards. The calibration curves were prepared by using trans-cinnamaldehyde and coumarin at 2, 4, 6, 8, 16 and 32 ppm. The peak areas were plotted against concentrations, and linear regression equations, which were further used to calculate the levels of trans-cinnamaldehyde and coumarin in the sample. The LOD and LOQ were also determined by using the linear regression equations of the calibration curve. The accuracy or recovery test was performed by using the spiking method in which trans-cinnamaldehyde and coumarin standards were added to the sample solution. One gram of cinnamon bark powder was added to the optimum NADES combination [choline chloride–citric acid (ChCl-CA), sample to solvent ratio of 1:10 with 40% addition of water and extracted for 30 minutes by the UAE method]. This step was performed six times until six samples were obtained.

4.6. HPLC determinaton of trans-cinnamaldehyde and coumarin contents

A 400-ppm cinnamon bark extract was used as test solution. The extracts were obtained from the optimum NADES-UAE conditions and conventional methods (maceration, Soxhlet and reflux). Coumarin and trans-cinnamaldehyde concentrations were calculated from calibration curve equations and calculated in mg/g. Determination of the level of the test solution was performed in triplicate.

The mobile phase was prepared by mixing water and acetonitrile each with 0.04% (v/v) glacial acetic acid at a ratio of 40:60 (v/v). The mixture was then filtered through a 0.45-µm membrane filter. HPLC analysis was performed by using a C18 Inertsil ODS-3, 4.6 x 150 mm, 5-µm particle size column at a flow rate of 1 ml/minute, with an injection volume of 20 µl and detection at 280 nm.

4.7. Optimization of extraction condition and data analysis

A two-variable, three-level, factorial experimental design was used. The first variable was the water addition (30%, 50%, and 70%) and the second variable was the extraction time (20, 40, and 60 minutes). Data analysis was performed by using Kruskal Wallis nonparametric analysis to determine which variables

influenced the content of trans-cinnamaldehyde and coumarin. Each sample content was analyzed by HPLC in triplicate.

4.8. Scanning Electron Microscopy Analysis (SEM)

Analysis using SEM was done before and after extraction with NADES-UAE and conventional methods. This analysis was done to explore how each solvent can destroy the cells of cinnamon bark. We used SEM FIE Quanta 650 with LFD detector. The experiments were done at 20°C and Relative Humidity (RH) 55-70%. The pressure was 59-61 Pa and HV 10.00 kV.

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