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Solvent preextraction influenced to coumarin and glucose binding capacity of cinnamomi's extracts

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ABSTRACT

Cinnamon is one of the world's oldest spices that is also widely used as medicine for antimicrobe, anti-inflammation, and immune stimulant to now developed as an antidiabetic herbal medicine. Among its bioactive contents, the coumarin must have been controlled; since of its toxicities. Therefore, in this study aimed to reduce the amount of coumarin in the extracts by chloroform preextraction to dried powdered cinnamons and determine their glucose binding capacity. The extractions were used two methods by water infusion and ethanol soxhletation. To measure the coumarin's was used validated thin-layer chromatography (TLC)-densitometry, while for the chemical profiling of the extract was examined with liquid chromatography-mass spectrometry. The activity antidiabetic of the extracts was obtained by glucose binding. The TLC-densitometry method has been validated with silica gel 60F₂₅₄ and n-hexane: ethyl acetate (8:2, v/v) systems. The coumarin's spot was observed at a wavelength of 285 nm on retention factor (Rf) 0.33, with tailings factor 1. The intraday and interday linearities tests showed a linear response result. The recovery value, coefficient of variation, and detection and a quantitation limit were met the standard requirements, respectively. Moreover, the results were observed (1) the solvent preextraction may reduce the coumarin content, (2) the coumarin content in the ethanol extract was higher than in the infusion, and (3) the preextraction solvents would reduce the glucose-binding capacity in ethanol and water cinnamomi's extract. These results may be developed further and applied for producing cinnamon's free coumarin extracts.

Key words: Cinnamomum, coumarin, preextraction, thin-layer chromatography-densitometry, validation

INTRODUCTION

Cinnamomum burmanii is widely used in Asia not only as flavoring, but also as medicines to antioxidant,

anti-inflammatory, and antimicrobes.^[1] Moreover, it has been noted in the preclinical to clinical stages of its used for type 2 diabetes and obesity. The cinnamon's water extracts major compounds were cinnamaldehyde, coumarin, and proanthocyanidin type A.^[2] The cinnamaldehyde and coumarin are related compounds to its flavor, although the latter has detrimental effects since of its hepatotoxic, gene toxic, to carcinogenic, and mutagenic in the animal study. Therefore, the maximum level of coumarin in food and beverages is regulated, especially for Cinnamon

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containing products. The European Food Safety Authority has established the coumarin's tolerable daily dose of 0.1 mg/kg body weight.^[3,4]

For the purpose of its separation, coumarin can be extracted best with 80% methanol. For the purpose of the assay, the spectrophotometry can be used for its fairly selective, sensitive, and inexpensive method.^[5,6] The ultraviolet or fluorescent or NaOH spot viewer may be used.^[7] The previous study found that Cinnamomi's infusion and ethanol soxhletation provided the best yields and antioxidants capacity compared to ethanol extract with percolation methods as well as to their n-hexane and ethyl acetate fractions.^[8] Their antioxidant and antidiabetic potency were also better even compared to rutin and acarbose. Furthermore, noted that the Cinnamon infusion and extracts optimization to their α -glucosidase inhibition.^[2]

Therefore, the aim of this study was to continue the process of purifying cinnamon extract and infusion from coumarin by chloroform preextraction to dried plant. It was expected to found a simpler way to produce free or less coumarin extract, thereby reducing unwanted side effects and fulfilled the specified requirements.

MATERIALS AND METHODS

Materials and instruments

The dried plant of Cinnamomi (*C. burmannii*) Cortex was obtained from Tawangmangu (1200 ASL), determined and deposit at the Traditional Medicine Research Center Laboratory (Documented No. Cb-Tw100921), Widya Mandala Catholic University. The chemicals are provided by a local supplier, others cited was used as pro analysis grade. The thin-layer chromatography (TLC) silica gel 60F₂₅₄ coumarin (E Merck), methanol, chloroform, and ethanol 96% (RDH). The instruments consist of water bath, rotary vacuum evaporator, Multiskan Go microplate reader (Thermo Fisher), and TLC-densitometry (Camag and Switzerland), liquid chromatography-mass spectrometry (LC-MS) (ACQUITY UPLC® BEH).

Material preparation

Cinnamon bark obtained from Tawangmangu, Central Java, Indonesia. Identified and classified as *C. burmannii*. The bark was cleaned and then standardized, followed by national herbal pharmacopeia.

Extraction

The Cinnamon bark dried crumb was preextracted three times with chloroform to provide Cinnamon preextracted (P), whereas another named normal Cinnamon (N) for conventional dried Cinnamons. The P and N were then each proceed to infusion following the method by weighing 100 g, then were put into an infusion pot and added with 500 ml of distilled water, heated at 98°C

for 20 min, filtered and evaporated using a rotary vacuum evaporator, and water bath to obtained dried extract (PI and NI).^[2] While for soxhletation was used 100 g of Cinnamon's bark was objected to $\times 2.5$ circulation, which approximately 250 mL ethanol to produced preextracted (PS) and non preextracted (NS) ethanol extract. The water content of dried extracts was done by the gravimetric method.

The validation of coumarin analysis thin-layer chromatography-densitometry

The validation method followed USP 43, which includes selectivity, linearity, accuracy and precision, limit of detection (LOD), and limit of quantification (LOQ).^[9]

Selectivity

A coumarin standard, standard and matrix solutions were made on 3000 ppm and spotted on the TLC plate. The plate was eluted with three systems which were n-hexane: ethyl acetate (8:2), chloroform: methanol (8:2), and ethyl acetate: methanol: water (20:3:2). The spots were observed in their spectrum, wavelength, R_f, and tailing factor (T_f).

Linearity

A coumarin solution was made on 1500–4500 ppm and spotted on a TLC plate. The experiments were carried out for three times replication, in 3 consecutive days with a selected mobile phase. The regression equation and % coefficient of variation (CV) results were calculated in intraday and interday. The relationship can be declared linear if $r_{count} > r_{table}$.

Accuracy and precision

The solutions were made 2400, 3000, and 3600 ppm, then added 100 mg of the sample matrix and dissolved in 10 ml of solvents. The solutions were spotted on a TLC plate and eluted. Then, calculate % recovery for accuracy and % CV or relative standard deviation (RSD) for precision.

Limit of detection and limit of quantification

A standard solution was made on 500–1000 ppm, then spotted on a TLC plate and eluted with the selected mobile phase. The area of the selected wavelength is observed and the regression equation is calculated.

Determination of coumarin levels by thin-layer chromatography-densitometry

The infusion and extract were each made on 10,000 ppm and 10 μ L of the solution was spotted and eluted. The coumarin's spots are determined and counted for coumarin content.

Liquid chromatography-mass spectrometry/mass spectrometry

The extracts were then subjected (1 μ L) to LC-MS analysis with cinnamaldehyde and coumarin standards to the profile of their contents. The system was use waters Acquity UPLC I-Class dan XEVO G2-XS QToF ACQUITY UPLC®

BEH C18 1.7 μm 2.1 mm \times 50 mm, m/z 100–1200 mode ESI, Solvent A: H₂O + 0,1% formic acid (FA) Solvent B: ACN + 0,1% FA.

Glucose-binding capacity

The glucose absorption capacity was measured with slight modification to the Trinder reaction of glucose oxidase-peroxidase aminoantipyrine (Glory Diagnostic, Spain). The samples of extracts were prepared with dimethyl sulfoxide (DMSO) at 5000 ppm, whereas the reference of acarbose and rutin was at 1000 ppm. The blank of each sample was made to correct their origin absorption. To the sample was added glucose 1000 ppm. The glucose-binding capacity was calculated by remain glucose in the samples with spectrophotometer at λ 500 nm. The data were presented by mean and standard deviation values of triplicate experiments.

RESULTS

Cinnamon bark is widely used as raw material for the food and beverages, cosmetics to medicinal purposes. Well-known Cinnamon's cinnamaldehyde and coumarin contents are two major secondary metabolites related to its uses. Although latter should be controlled since of its negative effects to some organs. This study was conducted to determine the preextraction influenced to coumarin content in the water infusion and ethanol extract. The cinnamon bark used was observed macroscopically and identifies as *C. burmannii*. It has rolled shape and brown color with specific odor. It was ground into crumbs and subjected to extractor with water infusion and ethanol soxhletation; as a single to continuous extraction process comparison. The results then evaporate to produce free solvent extracts. Their yield is listed in Table 1. The continued extraction by soxhletation was produced a higher yield (19.63 ± 0.26 and $19.24 \pm 0.12\%$) than the water infusion (12.14 ± 0.06 and $11.89 \pm 0.05\%$). The soxhletation was known as exhaustive extraction; with repetitive cycles solvents from the siphon. On the contrary, the preextraction was produced slightly lower than normal infusion and soxhletation. This might cause by some compounds was extracted to chloroform.

The TLC-densitometry was rapid and less expensive yet suitable for simultaneous coumarin analysis compared to other methods. The validation method for coumarin TLC-densitometry analysis was examined based on the USP 43. The validation method resulted as written in Table 2. The selectivity test aimed to determine the ability of a method to measure certain substances carefully and thoroughly in

the presence of other components in the sample matrix.^[10] Among the three systems, the n-hexane: ethyl acetate gave the best separation of purple spot, with no tailing, and at λ_{max} 285 nm Rf 0.33 ± 0.01 . Meanwhile, others resulted in Rf >0.8 . This showed that the prior system was optimum, which met the requirements for a Rf value of $0 < Rf < 1$ and the Tf 1 value.^[11]

The linearity test is carried out to ensure a linear relationship between analyte concentration and absorbance in such replications and days manners. This was done to obtain the random error and the nonuniformity of the analysis results from the test with the same conditions and at different times. The linearity test is shown in Table 2 in a range % CV intraday an interday of 0.2653–1.5936, consecutively. A linear relationship between intraday and interday experiments was obtained. The calibration curve for interdays did not give a different value R^2 was 0.99541 ($y = 4209.345 + 15.6712x$) and 2% CV.^[12] Based on these and the T statistical test showed not a significant difference.

The accuracy and precision tests are carried out to definiteness and exactness of the result values. The accuracy was expressed as percentage recovery of added analytes; thus determine the effect of the matrix (additive) on the analyte concentration. The precision was expressed as standard deviation or RSD (CV) for each calibration level.^[12] The results of the % recovery and % CV tests for infusion and ethanolic extract met the requirements as presented in Table 2. The SD and % CV were in the range of 0.32–1.30. These low values presented the method is precise and accurate.

The LOD and LOQ were carried out to determine the presence of the lowest coumarin content in the sample that can be detected and quantified accurately and thoroughly. The results can be seen in Table 2. This indicates that the Cinnamon's coumarin concentration in the sample can be observed versus signal noises.^[13] The extract's spectra purity was determined by scanned the spots at Rf 0.03 ± 0.01 to reference and by overlaying them at peak's initial, apex, and end positions. Figure 1 and Table 2 present the overlapped of coumarin's spectra in the extracts and its validation results.

The coumarin content in preextraction water extract cannot be detected; while counted in the ethanolic soxhletation. This suggested chloroform elimination coumarin; while in soxhletation method showed no significant difference between preextraction and non preextraction. Regarding

Table 1: The yield and coumarin content results of the extracts

Parameters (%)	PI	NI	PS	NS-E
Yield	11.89 \pm 0.05	12.14 \pm 0.06	19.24 \pm 0.12	19.63 \pm 0.26
Coumarin yield= $16,908.32 + 7035.89x$ ($r=0.9966$)	-	1.95 \pm 0.03	1.96 \pm 0.04	1.92 \pm 0.13

the result, the water infusion was more preferable than ethanol soxhletation. The water has polar characteristics so that the coumarin's spots in the infusion were hardly observed. As has been known that coumarin is very soluble in ether, diethyl ether, chloroform, oil, soluble in ethanol and methanol, and slightly soluble in water.

Moreover, the study compared the samples capacity to bind glucose at a lower concentration (5.55 mM) to rutin and acarbose [Figure 2]. The glucose-binding activity was showed that reference rutin [R] and acarbose [A] [Figure 3c and d] resulted in higher than all extracts as much as 33%–36% compare to 17%–31%. Thus the preextraction solvents eliminated some content which beneficial effect to bind glucose. Thereby have potential capacity in reducing glucose intestinal absorption in blood glucose. The previous finding obtained the *Caesalpinia bonducella* (Caesalpiniaaceae) had the highest capacity of 74% at 100 mM. The viscosity of soluble polysaccharides or phytochemical content from the extracts may influenced the delay of glucose absorption in the gastrointestinal tract.^[14,15]

Table 2: Results of coumarin's validation method

Parameters	Systems	Results
Selectivity (Rf)	n-hexane:EA (8:2) (0.88)	0.33
	CHCl ₃ :MeOH (8:2)(4.33)	0.89
	EA:acetone:	0.92
	water (20:3:2) (4.85)	
Linearity (percentage CV)	Intraday	1.1048
		1.3345
	Interday	0.9187
		1.5936
Accuracy and precision, mean±SD	Infusion	100.70±1.30
	Ethanolic extract	100.53±0.32
LOD dan LOQ	Y=4209.345+15.67116x	
	R ² =0.99541	
	LOD=52.74 µg/mL	
	LOQ=175.81 µg/mL	

LOD: Limit of detection, LOQ: Limit of quantification, SD: Standard deviation, CV: Coefficient of variation

Table 3: The LC-MS Summary results

Compounds name	PI			NI			PS			NS		
	m/z	RT	DCounts	m/z	RT	DCounts	m/z	RT	DCount	m/z	RT	DCount
2H-1 benzopyran-2-one	147.04	4.26	132,740	147.04	4.24	269,092	147.04	4.37	463,713	147.04	4.34	545,072
Aschantin							401.16	4.65	56,297	401.16	4.62	64,870
Cinnamaldehyde							133.07	5.17	33,851	1,330,646	5.14	59,438
Catechin-(4α→8)-catechin	579.15	2.54	108,150	579.15	2.51	68,341						
Paenolide	483.15	3.13	60,889	483.15	3.11	54,652	483.15	3.24	53,774	483.15	3.21	46,941
Candidate mass C ₉ H ₈ O				131.05	4.72	26,554						
Candidate mass C ₁₇ H ₁₆ O ₁₀	381.08	0.50	53,459									
Candidate mass 865.1984 type A proanthocyanidin	865.20	2.96	615,637	865.20	2.94	635,220	865.20	3.06	206,592	865.20	3.04	96,586

DCounts: Detector counts, Rt: Retention time, LC-MS: Liquid chromatography-mass spectrometry

The LC-MS analysis aimed to examine the extract's profile further; regarding their coumarin and cinnamaldehyde contents as seen in Table 3 and Figure 4. The LC-MS profile of the extracts obtained coumarin content in all extracts at varied levels; meanwhile, the cinnamaldehyde was only observed in ethanol (PS and NS) extracts. The LC-MS analysis obtained different results regarding coumarin content compare to TLC-densitometry analysis. The LC-MS has better sensitivity than TLC-densitometry. The PI and NI profiles were similar to reported by Erвина et al.^[2] that highlighted the *C. burmanii*'s peaks. They have proposed compounds such as coumarins and A-type proanthocyanidins that major content in and characterized of *C. burmanii* compare to others species.^[16] There were observed similar patterns of PI and NI; PS and NS. The coumarin at Rt 4.33 m/z 147.0440 was observed in all extracts. Importantly noted that the preextraction reduced coumarin 20%–50% to both methods (NI to PI 269092; 132740; NS and PS 545072; 463713, respectively). The cinnamaldehyde volatile oil was observed in PS and NS but not in PI and NI. This phenomenon was also observed to previous results.^[2] The catechin-(4α→8)-catechin with m/z 579.1498 was detected on PI and NI but not in PS and NS. The compounds at m/z 865 were recorded on all extracts, which suggested the type A proanthocyanidin.^[2] The type A

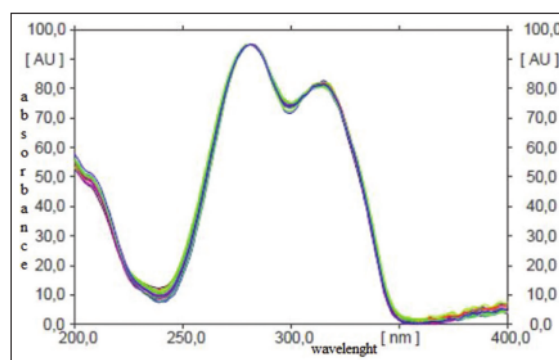


Figure 1: The overlay spectrum of coumarin's spots on Rf 0.33 in infusion and extract

proanthocyanidin and catechin were shared a similar structure base as polyphenol flavan-3-ol [Figure 3a and b], which has been reviewed *in vitro* and *in vivo* to clinically used for the prevention and management on type 2 diabetic. The compound possibly has a molecular mechanism by promoting functions and viability of pancreatic β -cells, and insulin signaling pathway for improving glucose transport in muscle and adipose tissue, enhancing the incretin effect, and decreasing of endogenous glucose production.^[17]

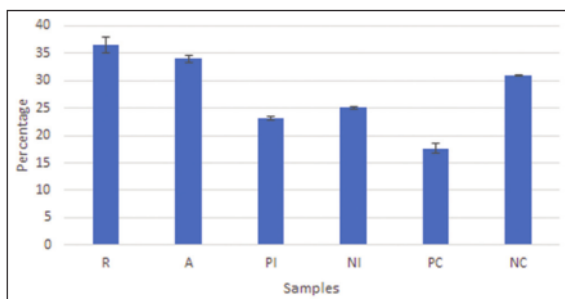


Figure 2: The glucose-binding activity of Cinnamomi's extracts and reference

CONCLUSION

The research found that (1) The TLC-densitometry method has met the validation requirement for coumarin analysis that resulted in purple spot at λ_{max} 285 nm, Rf 0.33; obtained intra- and inter-day linear relationship and %CV in a range of 0.2653–1.5936; LOD and LOQ on 52.74 and 175.81 $\mu\text{g/ml}$, respectively, (2) the chloroform preextraction may reduce coumarin 20%–50% content in the water and ethanolic of Cinnamomi Cortex extracts, and reduced their glucose binding capacity. These results may use further to produce Cinnamomi's free coumarin extract.

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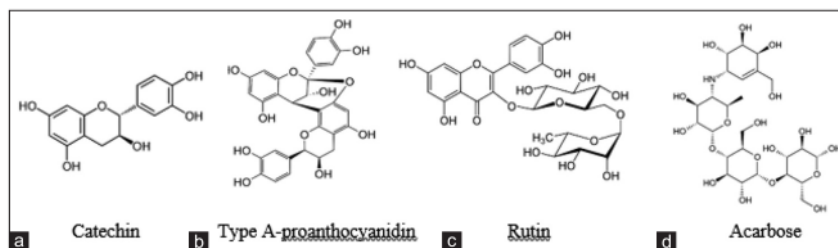


Figure 3: The structure of *Cinnamomum burmannii* contents and reference compounds

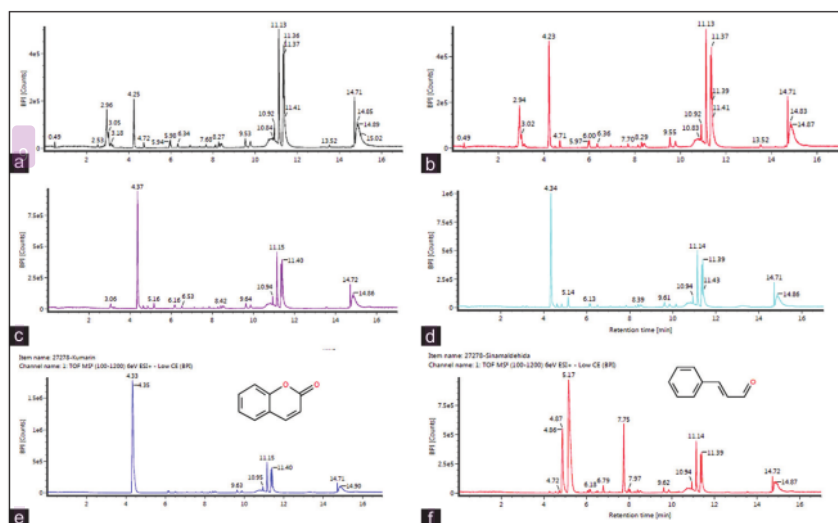


Figure 4: The LC results of the PI, NI infusions, PS, NS Soxhlet extraction, coumarin, and cinnamaldehyde (a) PI, (b) NI, (c) SP, (d) NP, (e) coumarin, and (f) cinnamaldehyde

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Conflicts of interest

There are no conflicts of interest.

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