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Determination of Allantoin Levels as A Result of Optimization of Ionic Liquid-Ultrasound Assisted Extraction of Comfrey Leaves by TLC-Densitometry

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Abstract

Allantoin is a compound contained in comfrey leaves. This research aims to obtain optimal condition parameters of ionic liquid-ultrasound-assisted extraction (IL-UAE) comfrey leaves to attract allantoin compounds. Comfrey leaves are extracted with eight ionic liquids by ultrasound-assisted extraction (UAE) and screened for allantoin levels. The best ionic liquid from the screening results is optimized response surface methodology (RSM) with Box-Behnken design with three factors and three levels to determine the optimum extraction conditions for allantoin levels. Determination of levels is carried out with TLC densitometry. The results of the IL screening obtained [HMIM]Cl as the best IL in the evaluation of optimization of extraction parameters. The results of optimization of extraction condition parameters [HMIM]Cl obtained the highest allantoin levels on the sixth run of 306.396 µg / g of powder at the ratio of solvents to powders of 10 mL / g, concentrations [HMIM] Cl 1 mol / L and extraction time of 30 minutes.

Keywords : Allantoin; Comfrey L; IL-UAE; [HMIM]Cl; Respon Surface Methodology; TLC-Densitometry

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1. Introduction

Comfrey with the latin name *Symphytum officinale* L. It is a widespread plant for therapy in South America, Europe, and parts of Asia. In ancient medicine, comfrey extract was sed topically, mainly or the therapy of joint disorders, wounds and muscle injuries [1], supported clinical[2]. The comfrey plant can also be used as a mouthwash, strep throat, dietary supplement[3], diarrhea treatment [4], metrorhagia[5] and as comfrey leaf tea[6]. According to the Research on Medicinal Plants and Herbs in 2017, comfrey leaves are used as anti-hypertensive drugs[7] and the treat of mental and emotional disorders however heir potential has not been proven[8].

The therapeutic properties are associated with several chemical compounds in the comfrey plant. Carbohydrates (29% mucus), purine derivatives, triterpenes, polyphenols (rosmarinate (0.2%), p-hydroxybenzoate, kafeic, chlorogenic, and p-coumarinic acid), and pyolytic acid (0.013-1.2%) are the five main groups of compounds found in comfrey. Purine derivatives are byproducts of the degradation of purines, purine bases, and purine nucleosides. Allantoin, a diurea of glylic acid, is the most abundant compound in this group, accounting for 0.6 – 4.7 % [9]. Allantoin and rosmarinic acid have been identified as active ingredients in comfrey[10], and have been linked to anti-inflammatory and analgetic effects [5]. Comfrey plant extract products have been promoted to various countries [11].

UAE is a faster and more effective extraction technique for extracting bioactive compounds from a variety of sources, when compared to other conventional and latest extraction techniques. As a substitute method for sample preparation, uae proposed. As well as more environmentally friendly methods that allow for high levels of efficiency, shorter repetitions, easier use, and reduced volume and temperature consumption organic solvents, as well as less energy input. However, one of the most difficult challenges is conventional solvents that can pollute the air and cause damage to the environment and human health. In the

last decade, ionic liquids (IL) have been used as a more friendly replacement solvent[12].

Ionic liquids (IL) are new alternative solvents with mixtures (cations and anions) with certain physical properties and solutions that have proven promising to substitute for traditional flammable, volatile, and toxic solvents in various extraction processes over the past decade. IL solvents are often used for the extraction of bioactive compounds from medicinal plants such as piperine compounds, caffeine, quercetin, gallic acid, apigenin, stilbel, coumarins, flavonoids, saponins, artemisinin, and other active components of medicinal plants [13],[14], and allantoin and retrorsin N-oxide [15]. In this study, a screening approach of various types of IL was carried out, optimization of the selected IL-UAE extraction conditions, and evaluation of the extraction performance of allantoin.

Therefore the importance of Comfrey in treatment with allantoin compounds. TLC densitometry is often used because analysis is fast, easy, simple, and has a high sensitivity[16], [17].

2. Materials and Methods

2.1 Material and Tool

Comfrey leaves, allantoin (Phytolab, Germany), Ionic Liquid [EMIM]Cl, [BMIM]Cl, [HMIM]Cl, [PMIM]Br, [OMIM]Br, [BMIM]MeSO4, [BMIM]HSO4 ¬and [BMIM]Ace (Cheng Jie Chemical, Shanghai), Butanol, Formic Acid, Methanol for analysis, GF254 silica gel TLC Plate (Merck, Germany), Aqua demineral and aquabidestilata. Analyte scale, Maserator, Ultrasonicator (Krisbow), Centrifuge EBA 200, Whatmann Paper (0.45 µm), Vortex VM-10, micropipet (Socorex), macropipet (Socorex), Chamber size 10 x 10 cm and 20 x 10 cm Camag, UV lamp Camag, TLC Scanner Camag with CATs system (Muttenz, Switzerland).

2.2 Methods

2.2.1 Extraction of Conventional Methods

a. Extraction by Maceration

The method of extraction of comfrey leaf powder is carried out by a modified maceration method from (Nastić et al., 2020). A total of 2 g of comfrey leaf powder plus 40 ml of methanol solvent 65% v/v. The mixture can macerate for 24 hours under stirring at room temperature. The extracted liquid extract is then filtered with Whatman 0.45 µm. Liquid extract is steamed in a rotary evaporator vacuum at 40-45 ^{o}C and concentrated in a water bath at 45 ^{o}C.

b. Extraction by Ultrasound Assisted Extraction (UAE)

As much as 2 grams of comfrey leaves are put in an erlenmeyer glass then 40 ml of methanol (65% v / v) is put into the ultrasonicator at a temperature of 40 ^{o}C for 30 minutes. The obtained liquid extract is filtered, evaporated and concentrated.

2.2.2 Screening ionic liquid - UAE

The method of screening ionic liquid with UAE is carried out based on the method from Zu et al., 2012 with minor modifications. Ionic fluid screening is carried out with a ratio of powder with solvent 1:20, extraction time for 30 minutes, IL concentration 1 M and temperature 40 ^{o}C. Eight types of ionic liquid used in screening include, [EMIM] Cl, [BMIM]Cl, [HMIM]Cl, [PMIM]Br, [OMIM]Br, [BMIM]$MeSO_4$, [BMIM] HSO_4 and [BMIM]Ace.

As much as 2 grams of komfrey leaf powder is added to 40 ml of IL in erlenmeyer, then put in an ultrasonicator at a temperature of 40 ^{o}C for 30 minutes. The obtained liquid extract is filtered with Whatman paper (a filter 0.45 µm). The liquid extract is supplemented with KH_2PO4 salt and re-extracted with dichloromethane in a ratio of 1:1:1, then evaporated and concentrated.

2.2.3 Validation of TLC- densitometry Allantoin Analysis

a. Instrumentation Condition Analysis

The analysis was carried out using TLC silica gel plate 60 GF_{254}. The sample/standard solution is spotted on the TLC plate of capillary pipes measuring 10 µl with the distance between the spots is 1 cm. The plate is diluted to a height of about 8.5 cm in a chamber that has been saturated with phase-mobile steam. The phase of mobile used is one of the mixture of butanol: methanol 50%: formic acid (66.5: 33.2: 0.3), methanol: acetone: formic acid : water (40:2: 1: 6) and butanol : methanol : water (75:12.5: 12.5), which can best separate the three compounds in the range of Rf 0.2-0.8. Densitometric scanning is performed at 200 - 780 nm with TLC-Scanner III equipped with CATS software. The gap dimensions are 8 mm x 0.4 mm and the scanning speed is 10 mm per second. In this study, the selected mobile phase was used butanol: methanol 50%: formic acid (66.5:33.2:0.3) with a wavelength of 200 nm. The analysis conditions are then carried out validation consisting of linearity, LOD and LOQ, and accuracy and precision.

b. Analysis of Allantoin in TLC-Densitometry

The sample is spotted on the TLC GF254 plate with a volume of 10 µL. TLC plates are developed in a mixture of butanol: 50% methanol: formic acid (66.5:33,2:0,3, V/V/V) in a saturated chamber. The TLC plate is then dried and analyzed with a densitometer at a wavelength of 200 nm [17]; [21].

2.3 Experimental Design and Statistical Analysis

The result of the extraction of the best ionic liquid from ionic solvent skrinig, then used Response Surface Methodology (RSM) Box-Behnken Design (BBD) to get the optimal value of three independent variables namely, comparison of the best solvent sample (X_1 = 10 – 30 mL / g), comparison of the best amount of ionic liquid concentration (X_2 = 0.5 – 1.5 mol / L), and extraction time (X_3 = 30 - 50 minutes) on three levels (-1, 0, +1) are displayed on table 1. Response Surface Methodology (RSM) is performed using Design Expert Ver 11.0.0. The number of experiments consists of 27 experimental points. The design results of RSM Box-Behnken, obtained 17 points that will be used to get optimal extraction conditions variations. The response from the experiment was observed from the number of extraction results in the form of allantoin levels.

Table 1. Box-Behnken experimental design for the best IL-UAE extraction parameters

Independent Variables	Level Factor		
	(-1)	(0)	(1)
Solvent ratio: powder (ml/g)	10	20	30
Konsentrase IL (mol/L)	0.5	1	1.5
Extraction Time (minutes)	30	40	50

3. Results and Discussion

3.1 Screening Ionic Liquid

The Ionic liquid is a solvent that has non-volatile properties. Separation of non-volatile and strongly bound compounds in extraction with IL, re-extraction is carried out using organic solvents using dichloromethane[19]. Comfrey extract IL is centrifuged to separate the liquid extract with powder. Ionic liquid is then filtered first using filter paper to avoid the presence of residue carried away. The ionic liquid phase is separated using KH_2PO_4 salt. The process of separation with salt helps to precipitate residues in the ionic liquid solution, resulting in a significant increase in extraction yield[20]. Dichloromethane is used in re-extraction, because in research conducted by Hasan, dichloromethane can attract allantoin compounds in the process of extraction back with IL [21].

Physicochemical properties are influenced by the IL Structure, which affects the extraction of target compounds[22]. In this study, IL used in screening allantoin levels included [EMIM]Cl, [BMIM]Cl, [HMIM]Cl, [PMIM]Br, [OMIM]Br, [BMIM]$MeSO_4$, [BMIM] HSO_4 and [BMIM]Ace. The types of anions and cations in their partners have an important role in effects that affect their physical properties, especially solubility in water[22]. The best withdrawal of anion pairs and cations against allantoin is obtained with [HMIM]Cl shown in figure 1.

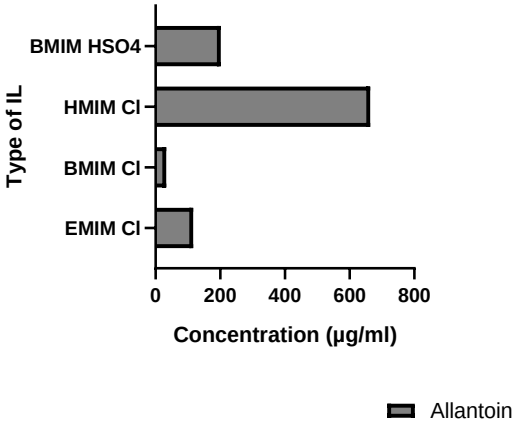


Figure 1. IL-UAE Screening Results Against Allantoin Levels

The results of the screening are known, allantoin compounds can be attracted with solvents [EMIM]Cl, [BMIM]Cl, [HMIM]Cl and [BMIM]HSO_4. The highest level of IL screening was obtained with [HMIM]Cl as much as 663.98 µg/g. These results indicate that the extraction efficiency increases significantly when the length of the alkyl chain is increased from ethyl to hexyl, specifically the Cl anion pair$^-$. The length of the alkyl chain is related to the solubility of water from IL and this property is related to extraction efficiency[22]. The results in the dengan screening took into account the effects of anions and cations, [HMIM]Cl was selected for further evaluation.

3.2 Comparison of IL-UAE with conventional methods

Comparison of extraction efficiency is carried out by various extraction methods, namely maceration with organic solvents, UAE with organic solvents and extraction with IL-UAE in extracting allantoin compounds in comfrey leaves. The approach taken with IL-UAE can reduce the extraction time compared to the maceration method, from 24 hours with maceration to 40 minutes with IL-UAE. Methanol 65% is used as an organic solvent in extraction by maceration and UAE, because in previous studies, methanol solvents can attract allantoin compounds more effectively in comfrey [17]). The results showed that IL-UAE extraction efficiency was slightly lower compared to conventional methods of maceration and UAE with methanol solvents, shown in table 2. However, the IL-UAE method is considered an economic method, as it saves time and the non-volatile nature of IL. On the other hand, IL-UAE is a safer and greener extraction process because no waste is discharged into the air during the extraction process.

Table 2. Comparison of extraction efficiency with different extraction methods

Extraction Method	Allantoin Up (µg/g)	SD
Maceration MetOH 65%	11406.691	131.174
UAE MetOH 65%	7655.112	214.638
UAE [HMIM]Cl	663.980	60.672

3.3 Validation of Analysis Method

The mobile phase carried out optimization includes, butanol: methanol 50%: formic acid (66.5:33.2:0.3), methanol : acetone : Formic acid : water (40:2:1:6) and butanol : methanol : water (75:12.5:12.5) based on research conducted by kimel and Hasan. The best result is indicated by the phase of mobile of butanol: methanol 50%: formic acid (66.5:33.2:0.3) with Rf 0.51. These results are in accordance with the methods carried out by previous studies [17], [21] with the maximum wave event obtained is at a wavelength of 200 nm. The standard calibration curve of allantoin is made in 6 different concentrations (50 - 1000 µg/mL) at the value R = 0.9924, with regression equation y = 12.139x + 693.97. The smallest concentration of allantoin that can still be detected with a linear regression equation is 168.306 µg/mL and the quantification limit is 510.020 µg/mL**.** In this study, precision tests were conducted in conjunction with accuracy. The allantoin re-acquisition test in this study was 90.01% - 94.96% with rsd 0.709% – 1.682% as presented in table 3.

3.4 Optimization of IL-UAE Extraction Conditions

Variant analysis (ANOVA) is used to check the adequacy of the applied model and the results are summarized in tabel 4. Statistical analysis showed that the experimental value was insignificant with the model (p>0.05)

Table 3. Validation of allantoin analysis methods

Rf and Wavelength	Linieritas	Correlation coefficient	LOD (µg/mL)	LOQ (µg/mL)	Recovery (%)	SD
0.51 λ = 200 nm	y = 12.139x + 693.97	r = 0.9924	168.306	510.020	90.01% - 94.96%	0.709% – 1.682%

Table 4. Summary Variant analysis on optimization of IL-UAE extraction conditions

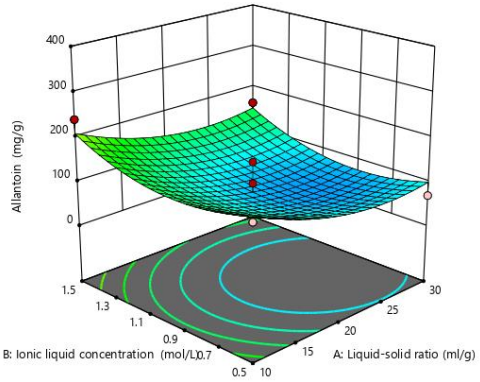
	Sum of Squares	df	Mean Square	F-value	p-value
Model	62142.72	9	6904.75	1.16	0.4342
A-Liquid-solid ratio	9317.49	1	9317.49	1.56	0.2519
B-Ionic liquid concentration	3425.60	1	3425.60	0.5732	0.4737
C-Extraction Time	25208.66	1	25208.66	4.22	0.0791
OFF	46.14	1	46.14	0.0077	0.9324
AND	2761.77	1	2761.77	0.4622	0.5185
BC	2534.47	1	2534.47	0.4241	0.5357
A^2	5648.45	1	5648.45	0.9452	0.3633
B^2	8541.70	1	8541.70	1.43	0.2708
C^2	2792.51	1	2792.51	0.4673	0.5162
Residual	41830.60	7	5975.80		
Lack of Fit	33282.89	3	11094.30	5.19	0.0727
Pure Error	8547.71	4	2136.93		
Total Color	1.040E+05	16			

and the F-value model of 1.16 implying an insignificant model relative to noise. There is a 43.42% chance that an F value of this magnitude may occur due to noise.

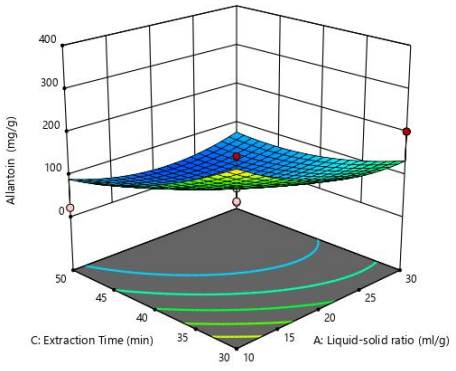
The result of extraction optimization of the 17 point extraction condition parameter [HMIM]Cl, it is known that allantoin compounds can be attracted by various extraction conditions, shown in table 5. In the 6^{th} run, it was found that allantoin was much interested in the content of 306.396 µg / g with solvent ratio conditions: powder 10 mL / g, IL concentration 1 mole / L and extraction time of 30 minutes. A comparison of allantoin levels of various extraction conditions is shown in figure 2.

Table 5. Allantoin values of independent variables for Box-Behnken design experimentally in obtained extracts

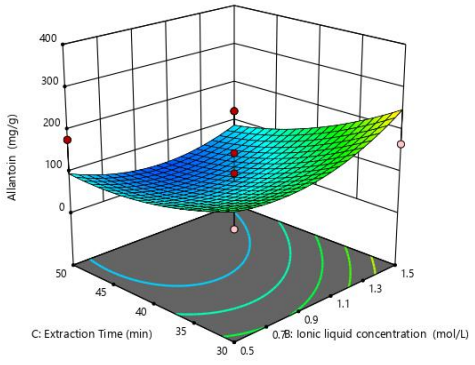
Run	Solvent ratio: powder (mL/g)	CONCENTRATION OF IL (mol/L)	Extraction Time (minutes)	Allantoin Up (µg/g)
1	10	0.5	40	161.927
2	20	1	40	37.135
3	20	1.5	50	124.536
4	30	1	50	24.019
5	20	0.5	30	119.811
6	10	1	30	306.396
7	20	1	40	68.605
8	30	0.5	40	68.893
9	30	1	30	203.575
10	30	1.5	40	161.303
11	20	1	40	96.742
12	10	1	50	21.735
13	20	1	40	34.667
14	10	1.5	40	240.752
15	20	0.5	50	177.725
16	20	1	40	145.609
17	20	1.5	30	167.309



(a)



(b)



(c)

Figure 3. Surface Response [HMIM]Cl with Allantoin; description: Relationship of allantoin levels with (a) solvent concentration and dissolving/powder ratio; (b) extraction time and solvent/powder ratio; (c) solvent concentration and extraction time

The factor that affects allantoin levels is the ratio of solvent to symplisia. This factor is used to ensure that all simplisia are submerged during the extraction process. Optimization of extraction conditions uses 3 different ratios 10:1, 20:1 and 30:1. The highest allantoin rate is at a ratio of 10:1, which indicates a lower solvent ratio in this study is sufficient to extract compounds in the symplicia of comfrey leaves and may more easily spread through ultrasonic waves throughout the powder during the extraction process[23].

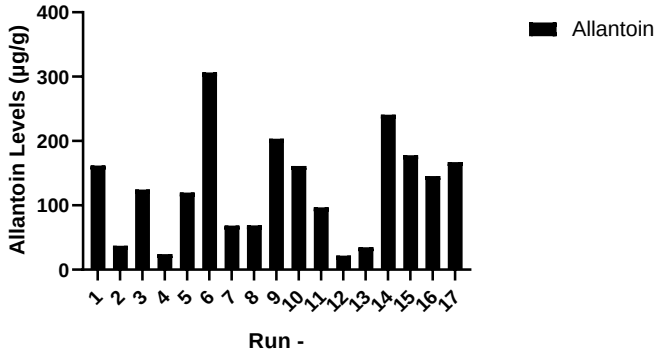


Figure 2. Graph of Allantoin levels in various parameters of the extraction condition [HMIM] Cl

A 3-dimensional image of RSM allantoin is shown in figure 3. The results of the response do not form a parabola, which indicates that the response value obtained in the analysis is not optimal. The approach in order to obtain optimum results in optimization needs to be done with condition parameters with the addition of a temperature factor, in order to be more detailed in optimizing the extraction conditions for the withdrawal of compounds in komfrey leaves.

4. Conclusion

Comfrey leaves extraction with [HMIM]Cl with UAE method can be applied and the highest condition in optimization is the ratio of solvent to powder of 10 mL / g, concentrations [HMIM] Cl 1, mol / L, extraction time 30 minutes with allantoin levels 306.396 µg/g powder.

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