Application of Ionic Liquids based Microwave-Assisted Extraction to Bioactive Compounds Comfrey (*Symphytum officinale* L.) Leaves

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**A B S T R A C T**

Extraction of bioactive compounds from comfrey leaves (*Symphytum officinale* L.) was carried out by comparing organic solvents and ionic liquids between conventional and microwave-assisted extraction (IL-MAE) methods. The comfrey leaf powder was extracted under various conditions. The types of organic solvents used are 65% ethanol and ionic liquids. The extraction methods used are reflux and microwave-assisted extraction. The ionic liquid was extracted by microwave-assisted extraction with the following conditions: ionic liquid concentration of 1 mol/L, the solid-liquid ratio of 1:20 (g/mL), extraction time of 10 minutes, and power of 30% (270 Watt). The total phenolic content of the extract was analyzed with a microplate reader. Allantoin and retrorsine N-oxide levels were analyzed by thin-layer chromatography – densitometry. MAE extraction in a solvent containing 65% ethanol resulted in the highest total phenolic content of 1.038±0.012. The highest levels of allantoin were also obtained from MAE extraction with 65% ethanol solvent at 2.922 ± 0.123. The highest levels of retrorsine N-oxide were obtained by extraction of MAE with [BMIM]Br with a concentration of 0.049 ± 0.007. Extraction of comfrey leaves using the MAE method of 65% ethanol produced the highest total phenolic and allantoin content compared to other methods. [BMIM] Br extraction with MAE can attract the highest retrorsine N-oxide in comfrey leaves.

**Kata kunci:**

*Symphytum officinale* L.
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INTRODUCTION

Comfrey (*Symphytum officinale* L.) is a plant from the Boraginaceae family used as herbal medicine for more than 2,000 years. Comfrey has been traditionally used to treat muscle pain due to inflammation and joint problems, respiratory problems, gastro intestinal diseases, metrorrhagia, phlebitis, and tonsillitis (Kruse et al., 2019; Rode, 2002; Staiger, 2012; Trifan et al., 2018). Comfrey is used topically, mainly for treating wounds, joint disorders, and musculoskeletal injuries of all kinds (Nastic et al., 2020). Comfrey leaves, herbs, and root extract have long been used to treat musculoskeletal disorders, wounds, gout, hematomas, and thrombophlebitis (Salehi et al., 2019).

Comfrey contains various chemical constituents, including allantoin, rosmarinic acid, mucilage, and pyrrolizidine alkaloids. The pyrrolizidine alkaloids such as retrorsine *N*-oxide, symphytine, lycopsamine, intermediate, acetyllycambine, and acetylcopamine have been reported in comfrey (Trifan et al., 2018; Wynn & Fougère, 2007). In the other hand, pyrrolizidine alkaloids are metabolized in humans to hepatotoxic and pulmotoxic pyrrole derivatives in reactions catalyzed by CYP3A4 (Janès & Kreft, 2014). Comfrey’s total pyrrolizidine alkaloid concentration was determined to range 1.380-8.320 µg/g root and 15-55 µg/g leaf (Salehi et al., 2019). In another study, the symphytine and echimidine content in comfrey leaves varied considerably between suppliers (Oberlies et al., 2004). Comfrey’s anti-inflammatory and analgesic therapeutic properties are related to the compounds allantoin and rosmarinic acid, the molecular mechanism of action of these compounds has not been fully understood. Several cellular mechanisms, including targeting several intracellular signalling pathways triggered by inhibiting NF-κB signalling at two stages, have been presented to explain their mode of action (Seigner et al., 2019).

Rosmarinic acid is present in the family Boraginaceae. Rosmarinic acid has antioxidant, antiviral, and anti-inflammatory effects and shows a shallow potential for toxicity and antitumor effects (Savić et al., 2015). Allantoin is a heterocyclic purine derivative widely used, mainly because of its tissue repair properties, it is safe and non-toxic (Becker et al., 2010). Allantoin also has antioxidant, anti-inflammatory, pain-relieving, and gastroprotective effects. In addition, allantoin’s ability to reduce the inflammatory process is associated with restoring enzymatic and nonenzymatic antioxidant pathways (Aman et al., 2021; Becker et al., 2010; da Silva et al., 2018).

Modern extraction techniques using green solvents have been developed to effectively and efficiently extract bioactive substances. Various procedures include the use of green solvents such as ionic liquids. Ionic liquids are composed of relatively large organic cations and either single or many anions. Ionic liquids are gaining significant interest due to their unique features, which make them appropriate for a variety of applications, including the extraction of bioactive chemicals from plants. The structure of an ionic liquid has a substantial effect on its physicochemical characteristics and is therefore predicted to have an effect on the liquid’s extraction effectiveness for the target analyte. (Yang et al., 2011).

Ionic Liquids (IL) based Microwave-assisted Extraction (MAE) is a non-conventional extraction method, that has been developed where the principle of IL-MAE is identical to that of the MAE technique, except that the focus and kind of solvent utilized are different (Ahmad, 2018). Various analytical methods are used to discover bioactive constituents in medicinal plant materials (Shanaida et al., 2020). TLC detection of secondary metabolites from plant extracts is a widely used technique. For example, TLC densitometric techniques were employed to quantify allantoin and retrorsine *N*-oxide in a comfrey extract (Janès & Kreft, 2014; Kimel et al., 2019; Mroczek et al., 2006; Smyriska-Wieleba et al., 2017). Additionally, the 96-well microplate reader technique has been widely applied to determine the total phenolic content of medicinal plants (Islamudin Ahmad et al., 2017).

The purpose of this research was to examine the effectiveness of conventional and ionic liquid (IL) extractions of chemicals from comfrey leaves. Allantoin and retrorsine *N*-oxide contents in comfrey leaf extract were quantified using thin layer chromatography-densitometry. Additionally, we determined the total phenolic content using a 96-well microplate reader method. According to the study, phytochemicals from comfrey leaves may be extracted using an extraction process based on the IL-MAE method.

METHOD

**Chemicals and Materials**

The sample used is leaf of comfrey plant. Samples were obtained from farmers in Karanganyar, Surakarta, Central Java. Comfrey was determined macroscopically at the Center for Biological Research, Gadjah Mada University. Standard retrorsine *N*-oxide, allantoin (Phytolab, Germany) and gallic acid (Merck, Germany). As shown in Table 1, this study used a total of eight distinct kinds of ionic liquids. (Shanghai Cheng jie Chemical, China), Methanol pro analysis, Dichloromethane pro analysis, Ethanol pro analysis, Folin-Ciocalteu, Potassium dihydrogen phosphate (KH2PO4) pro analysis, Sodium carbonate (Na2CO3) pro analysis, Acetonitrile pro analysis, 25% ammonia solution (Merck, Germany), Aquadest, Ethanol, Dichloromethane (Chemestation Asia) and TLCsilica gel60 F254 (Merck, Germany), Aquabidestilata and deionized water were obtained from the local supplier.

**Instrumentation**

The equipment used in this study were analyte scales, reflux, hotplate, microwavewith modification (Modena MV 3002 900-Watt), Vacuum rotary evaporator (Buchi), a microfilter (Whatman 0.45 µm), Centrifuge EBA 200, Vortex WiseMix VM-10 (Daian Scientific, Korea), 20-200 µL micropipette, 100 – 1000 µl and 0.5 - 5ml macropipette (Socorex), Twin Chamber Camag, UV lamp Camag, TLC Scanner IIIWinCATs system (Muttenz, Switzerland), refrigerator, vacuum drying oven, 96-wellplate (Iwaki), microplate reader (Versamark Microplate Reader, USA).

**Conventional Reflux method**

The reflux extraction method of comfrey leaf powder was conducted, according to Trifan et al., 2018 with slight modification. Five grams of comfrey leaf powder were extracted with 500 ml of 65% ethanol for 2 hours under reflux. The liquid extract then filtered using Whatman paper no. 1 (0.45 µm) and evaporated in a vacuum rotary evaporator at 40–45 °C and concentrated in a water bath at...
Determination of Allantoin Content

Determination of Allantoin content was carried out by the thin layer chromatography (TLC) method from (Kimmel et al., 2017), with slight modifications. First, 5 mg of allantoin standard was dissolved in 5 ml of aquabidestilata to produce a standard solution of 1000 µg/ml. Allantoin standards are prepared in dilution concentrations of 50 - 1000 µg/ml. Standards or samples were spotted at 10 µl on a TLC plate and developed in the mobile phase of butanol: methanol (100:20:4) v/v/v as mobile phases as far as 8.5 cm in a saturated chamber. The TLC plate was evaluated using a TLC scanner equipped with a 220 nm wavelength to determine the area under the curve (AUC). Data was processed with the Camag WinCats 1.4.8 program. Each plate was measured three times.
50%: formic acid (66.5:33.2:0.3) v/v/v for 8.5 cm in a saturated chamber. The TLC plate was dried in the oven at 120 °C for 20 minutes. The TLC plate was evaluated using a TLC scanner equipped with a 200 nm wavelength to determine the area under the curve (AUC). Data was processed with the Camag WinCats 1.4.8 program. Each plate was measured three times.

Data analysis

The extract’s % yield was calculated using the formula:

\[
\text{Percentage yield (\%)} = \frac{\text{dry extract of comfrey}}{\text{dry powder of comfrey}} \times 100
\]

All observations were made in triplicate for this study, and results were presented as Mean ± Standard Error of Measurement. The data were analyzed using the Analysis of Variance (ANOVA) method (p< 0.05), and the means were separated using Bonferroni’s multiple comparison tests, both of which were performed using the program GraphPad Prism 8.

RESULTS AND DISCUSSION

The extraction method was carried out by conventional methods with reflux and non-conventional by MAE, and the yield of all extracts is shown in Figure 1. When compared to other methods, the microwave-assisted extraction (MAE) method is the most acceptable for using an ionic liquid solvent. Non-conventional approaches, on the other hand, have been shown to be more successful and efficient in extracting the active constituents of plants (Ahmad et al., 2017). The different extraction methods aim to obtain an optimal extraction method with secondary metabolite targets. The extraction results obtained differences in the yields of extract. The highest yield was obtained from the [HMIM][BF₄] at 92.28% and the lowest IL from [BMIM]Br at 2.38%. On the other hand, the 65% ethanol conventional solvent yield using the reflux method was 19.63% and with MAE, 8.28%. Due to the molecular structure of its anion and cation, ionic liquid is an enhanced extraction solvent. The length of the alkyl chain of the ionic liquid cation has a beneficial effect on the extraction yield but increases the hydrophobicity of the ionic liquid as the alkyl chain length increases. As a consequence of the correct hydrogen bonding and hydrophobic interactions, the solvation interactions with bioactive chemicals were improved (Xu et al., 2012).

Total Phenolic Content

A 96-well microplate reader was used to produce standard calibration curves. Based on linear regression analysis, \( Y = 0.0086x + 0.0418 \) and a correlation coefficient (\( R^2 \)) of 0.9995 (Figure 2). \( Y \) is absorbance of gallic acid and, \( X \) is the standard concentration or sample. Equations are used to calculate the total phenolic content of comfrey leaf extracted using a variety of solvents and techniques.

The yields of total phenolic content varied according to the absorbance measured on the sample (shown in Table 2). The highest total phenolic content yield were produced using the MAE extraction method with 65% ethanol (1.038 ± 0.012 mgCAE/g dried comfrey). Extraction using MAE with 65% ethanol to the total phenol content obtained was higher than the reflux solvent of 65% ethanol. This result was possible due to the suitable ratio of solvent and powder during microwave extraction, which resulted in inhomogeneous and effective heating (Chan et al., 2011). The MAE extract produced with all IL had much fewer or no phenolic compounds than ethanol. In this study, the separation of ionic liquids was carried out with dichloromethane as a solvent, which is less polar than ethanol. This result also confirmed that phenolic contents are more attracted to ethanol solvents than less polar solvents such as dichloromethane (Nastić et al., 2020). When selectivity and solubility are highly composition-dependent, these characteristics are also affected by the extraction temperature and microwave irradiation (Canales & Brennecke, 2016).
confirmed that allantoin content with ethanol is effective for ethanol (2.922 ± 0.123 mg/g dried comfrey). This result produced using the MAE extraction method with 65% presented in Table 2. The highest allantoin yields are obtained with the [BMIM] cation, the anions Br– and Tos– were more efficient than other anions in the MAE of phenolic contents from comfrey leaves. The studies suggested that increasing the length of the alkyl chain has an effect on the extraction of the same Br–. While the hydrogen bond acidity of the three cations increased proportionally as the alkyl chain length went from ethyl to hexyl at the site of the 1-alkyl-3-methylimidazolium ring, the hydrophobicity did not. [BMIM]Br’s hydrogen bonding and hydrophobic contacts resulted in enhanced solvation interactions with phenolic compounds, which resulted in better extraction yields when compared to [EMIM]Br and [HMIM]Br (Du et al., 2009).

Furthermore, the results obtained using the MAE technique with eight different types of IL showed that [BMIM] Br is capable of extracting much greater levels of phenolic content (0.01672 ± 0.001 mg/g dried comfrey) than other types of ionic liquids. According to the extraction yields with the [BMIM] cation, the anions Br– and Tos– were more efficient than other anions in the MAE of phenolic contents from comfrey leaves. The studies suggested that increasing the length of the alkyl chain has an effect on the extraction of allantoin from comfrey leaves. The allantoin content was determined by TLC and analyzed by densitometry. The curve of the linear and chromatogram of allantoin is shown in Figure 3. Based on the analysis, the allantoin Rf value is 0.56 with linear regression obtained with the equation $Y = 12.13x + 693.97$. The correlation coefficient ($R^2$) is 0.9848 (Figure 3a). $Y$ is the area of allantoin and $X$ is the standard or sample concentration. Equations were applied to determine the allantoin content of comfrey leaf extract using various solvents and extraction methods.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Extraction Method</th>
<th>Total Phenolic Content (Mg GAE/ g dried comfrey)</th>
<th>Allantoin Content (mg/g dried comfrey)</th>
<th>Retrorsine N-oxide Content (mg/g dried comfrey)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[EMIM]Br</td>
<td>MAE</td>
<td>0.00664±0.001</td>
<td>0.869 ± 0.113</td>
<td>0.018±0.001</td>
</tr>
<tr>
<td>[BMIM]Br</td>
<td>MAE</td>
<td>0.01672±0.001</td>
<td>1.335 ± 0.243</td>
<td>0.049±0.007</td>
</tr>
<tr>
<td>[HMIM]Br</td>
<td>MAE</td>
<td>0.0003±0.001</td>
<td>1.070 ± 0.240</td>
<td>-</td>
</tr>
<tr>
<td>[EMIM]BF₄</td>
<td>MAE</td>
<td>0.0094±0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[BMIM]BF₄</td>
<td>MAE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[HMIM]BF₄</td>
<td>MAE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[BMIM]Tos</td>
<td>MAE</td>
<td>0.01092±0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>[BMIM]PF₆</td>
<td>MAE</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>65%Etanol</td>
<td>Reflux</td>
<td>0.891±0.008</td>
<td>1.989 ± 0.574</td>
<td>-</td>
</tr>
<tr>
<td>65%Etanol</td>
<td>MAE</td>
<td>1.038±0.012</td>
<td>2.922 ± 0.123</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2. A summary of the extraction processes and a comparison of the levels**

**Allantoin Content**

Standard calibration curves were performed using TLC-densitometry. The curve of the linear and chromatogram of allantoin is shown in Figure 3. Based on the analysis, the allantoin Rf value is 0.56 with linear regression obtained with the same Br–. While the hydrogen bond acidity of the three cations increased proportionally as the alkyl chain length went from ethyl to hexyl at the site of the 1-alkyl-3-methylimidazolium ring, the hydrophobicity did not. [BMIM]Br’s hydrogen bonding and hydrophobic contacts resulted in enhanced solvation interactions with phenolic compounds, which resulted in better extraction yields when compared to [EMIM]Br and [HMIM]Br (Du et al., 2009).
of ionic liquids. The cations, particularly the anions, of IL had an effect on the extraction, and ILs electron-rich aromatic π-system facilitated in extraction (Du et al., 2009).

**Retrorsine N-oxide Content**

Retrorsine N-oxide standard calibration curve was performed using TLC-densitometry. The curve of the linear regression analysis of retrorsine N-oxide is shown in Figure 4.

**Figure 4:** (a) Curve of linear regression analysis of retrorsine N-oxide (b) Chromatogram of standard retrorsine N-oxide

- Retrorsine N-oxide content was determined by TLC and analyzed by TLC scanner. Retrorsine N-oxide yields of each extract are presented in Table 2. Retrorsine N-oxide yields were only obtained from extracts of [BMIM]Br (0.049 ± 0.001 mg/g sample) and [EMIM]Br (0.018 ± 0.001 mg/g sample). The extraction efficiency of retrorsine N-oxide obtained with [BMIM]Br aqueous solution was significantly greater than the other IL, indicating that [BMIM]Br was more effective than the other IL-MAE of retrorsine N-oxide. This is probably because [BMIM]Br exhibited more interactions with alkaloids, including π-π, ionic/charge-charge, and hydrogen bonding. Additionally, [BMIM]Br exhibited a little greater acidity, which may aid in the extraction of the target pyrrolizidine alkaloid from comfrey leaves (Ma et al., 2010). The separation of the ionic liquid is carried out with dichloromethane as a solvent, which has fewer polar properties than ethanol. Pyrrolizidine alkaloid compounds, one of which is retrorsine N-oxide, can be extracted using chloroform or dichloromethane as a solvent. This is confirmed in previous studies, the use of dichloromethane resulted in higher extraction of less polar or non-polar compounds than ethanol solvents (Kopp et al., 2020; Nasti et al., 2020). The ethanol extract using MAE and the reflux method did not show any retrorsine N-oxide content. This is possible because the retrorsine content of the ethanol extract is very small, so it cannot be detected using the TLC-densitometry method. The pyrrolizidine alkaloids in the previous study were extracted with methanol/ethanol as a solvent, which was added with an acid such as tartaric acid, ascorbic acid, and hydrochloric acid, or by the addition of a base such as ammonia (Kopp et al., 2020).

**CONCLUSION AND SUGGESTION**

The results obtained concluded that the extraction of comfrey leaves with 65% ethanol solvent MAE method produced the highest total phenolic and allantoin content compared to other methods. In addition, extraction of ionic liquid [BMIM]Br with MAE resulted in the highest content of retrorsine N-oxide compared to other methods.

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**Conflict of interest**

This research does not include any conflict of interest.

**REFERENCES**


