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Original Article

Optimization of Imidazolium-Based Ionic Liquid-Microwave Assisted Extraction for Oxyresveratrol Extraction from *Morus alba* Roots

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ABSTRACT

Objective: The application of ionic liquids as solvent in the microwave assisted extraction (MAE) method has been developed for extraction of secondary metabolites from plants. However, oxyresveratrol extraction from *Morus alba* roots using IL-MAE method has never been reported. Purpose of this research was to get the optimum condition of imidazolium-based ionic liquid-microwave assisted extraction (IL-MAE) for oxyresveratrol extraction from *M. alba* roots. **Methods:** *M. alba* roots were extracted with 3 kinds of ionic liquids (1-butyl-3-methylimidazolium bromide ([Bmim]Br), 1-hexyl-3-methylimidazolium bromide ([Hmim]Br)and 1-butyl-3-methylimidazolium sulfate ($[Bmim]_2[SO_4]$), respectively by microwave with 3 different microwave power (P10%, P30% and P50%) for 10 min. Then, fractionated with ethyl acetate and salted out by additionof 4 different salts (Na_2CO_3, $NaHCO_3$, K_2HPO_4, and NaCl). Oxyresveratrol concentration of the extracts was analyzed with TLC-densitometry and HPLC. **Results:** Based on the results of this study, the most optimum condition of IL-MAE for oxyresveratrol from *M. alba* roots was using [Bmim]Br concentration 1.5mol/L with microwave power 10% for 10 min. The salt 0.01 mol/L $NaHCO_3$ was optimum for the back extraction of oxyresveratrol from [Bmim]Br solution. Under optimized conditions, oxyresveratrol concentration in *M. alba* roots extract using IL-MAE method was 735.25 ppm. **Conclusion:** This IL-MAE method was suitable to be applied as an alternative technique to extract oxyresveratrol from the plants.

Key words: Ionic liquid, Microwave-assisted extraction, IL-MAE, Oxyresveratrol, *Morus alba*, Roots extract.

Key message: Ionic Liquid-Microwave Assisted Extraction seem to be promising method to get optimum oxyresveratrol from *Morus alba* roots or other plants.

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INTRODUCTION

The roots of the mulberry plant (*Morus alba*) are known to have rich in oxyresveratrol and demonstrated a skin lightening effect. Oxyresveratrol is the main content of potential candidates to inhibit dopa oxidase activity from tyrosinase.[1] Oxyresveratrol is known to have the inhibitory activity of tyrosinase enzyme which is more effective than azelaic acid, curcumin, kojic acid, and mimosa. Oxyresveratrol with a concentration of 0.3 - 5μM showed inhibition of tyrosinase by 25-84%, in which 50% inhibition was shown at a concentration of 1 μM.[2] Generally, the root part of the mulberry plant is rarely used commercially. Methanolic extract of *M. alba* roots was reported to have the highest oxyresveratrol content compared to other plant parts of *M. alba*.[3] Therefore, the study uses mulberry roots.

The method to get the oxyresveratrol from mulberry roots generally used conventional extraction using organic solvents. However, this method has some disadvantages such as low extraction efficiency values, much-needed solvents, long extraction times, and less environmentally friendly. The microwave-assisted extraction is a fast, efficient and effective extraction method compared to conventional extraction methods such as maceration or reflux. The microwave-assisted extraction method is easier and cheaper if compared with other modern extraction methods.[4] MAE has been applied to obtains some active compounds from different plants.[4] Ionic liquid-MAE method on *Polygonum cuspidatum* roots showed it could obtain the *trans*-resveratrol content of 92.8% on first extraction stage, better than methanol-MAE (88%) or reflux (84.7 %).[4]

The method of extraction using ionic liquid-MAE is a way that has never been done to obtain the more optimum oxyresveratrol content from the mulberry roots. Ionic liquids have the advantages of non-flammable, non-toxic, biodegradable, and non-corrosive characteristic. The ionic liquid extraction method proved to be able to increase the yield of secondary metabolite extraction in plants efficiently.[5]

MATERIALS AND METHODS

Materials

The roots of *M. alba* were collected from Rumah Sutera, Bogor, West Java, Indonesia. The roots were washed and dried for six days. The dried roots were ground by using an industrial grinder. The powders were stored in airtight container. Standard oxyresveratrol was purchased from Tokyo Chemical Industry Co., Ltd. (TCI) (Japan). The ionic liquids, 1-Butyl-3-methylimidazolium bromide ([Bmim]Br); 1-Hexyl-3-methylimidazolium Bromide ([Hmim]Br); 1-Butyl-3-methylimidazolium sulfate ($[Bmim]_2[SO_4]$) were purchased from Chengjie Chemical Co. Ltd., (Shanghai, China). Na_2CO_3, $NaHCO_3$, K_2HPO_4, NaCl were purchased from Merck. All reagents used for HPLC analysis were analytical grade and purchased from a local distributor.

Instrumentation

Centrifuge (Heraeus Christ GmbH, Osterode, Germany). Vortex (Wise-Mix VM 10, Daihan Scientific Co., Ltd. Korea). Microwave with

the system supplies 900 W for 100 % power (Modena MV-3002 with slight modification). TLC equipment – TLC silica gel aluminum plates 60 F_{254} (Merck, Darmstadt, Germany); Camag microcapillary tube 5 µL, Camag TLC scanner III (Muttenz, Switzerland), HPLC system (LC-20AT, Shimadzu, Kyoto, Japan).

Preparation of Ionic Liquid

Ionic Liquid (IL) was prepared by diluted each ionic liquid [Bmim]Br, [Hmim]Br,and$[Bmim]_2SO_4$ with deionized water 100 ml to make concentration 1.5 mol/L, 2.0 mol/L, and 2.5 mol/L respectively.

Determination the optimum IL Solution for IL-MAE

One gram of *M. alba* roots powder was placed in a boiling flask. 20 mL of each IL solution with different concentration was added to each flask. The boiling flask was placed into a microwave. The microwave set at power 10% and run for 10 min. The extract was filtered and diluted in 30 ml of deionized water.[4] This procedure was repeated three times. For analysis by TLC-densitometry, each 1 ml IL-MAE extracts was diluted with 1 ml ethyl acetate. The solution was vortexed for 20 sec, then centrifuged for 15 min at 3000 rpm. Two phases of solution appeared, and the upper layerwas taken for TLC-densitometry analysis.

Thin Layer Chromatography (TLC) – Densitometry Analysis

Standard ofoxyresveratrol solution was prepared by diluting the oxyresveratrol standard with 80% ethanol in concentration 1 mg/mL or 1000 ppm. Silica gel 60 F_{254} was used as a stationary phase and methanol: chloroform: acetate acid (10:88:2 %v/v) as a mobile phase. Ethyl acetate fractions were spotted 5µl into TLC plate. The length of chromatogram run was 8 cm through glass chamber that saturated by the mobile phase. Subsequently, the plate was removed from the chamber and air-dried. The plate was scanned by Camag TLC Scanner III in absorbance wavelength at 366 nm. The TLC-densitometry analysis was operated by winCATS software. Ionic liquid extraction condition with highest area under curve (AUC) spot of oxyresveratrol peak then continued to be analyzed by HPLC.

$$\text{Oxyresveratrol concentration} = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard volume spotted}}{\text{sample volume spotted}} \times \text{C standard}$$

Determination the optimum microwave power for IL-MAE with the chosen IL solution and concentration

Each one gram of *M. alba* roots powder was placed in a three boiling flask. The chosen IL solution with optimum concentration 20 mL was added to each flask. The boiling flask was placed into a microwave. The microwave set at power 10%, 30% and 50% and run for 10 min. The extracts were filtered and diluted with 30 ml of deionized water. This procedure was repeated three times. Then each 1 ml IL-MAE extracts was diluted with 1 ml ethyl acetate. The solution was vortexed for 20 sec, then centrifuged for 15 min at 3000 rpm. Two phases of solution appeared, and the upper layer was analyzed by the TLC-densitometry method as mentioned above.

Separation of Oxyresveratrol

Extract sample with optimum ionic liquid concentration and microwave power from the previous experiment subsequently was separated using salts. The salts used for separation were Na_2CO_3, $NaHCO_3$, NaCl, and K_2HPO_4solution with concentration each 1.0 mol/L, 0.1 mol/L, and 0.01 mol/L respectively. The IL extract and a certain of salt (1:1) were added to a tube. The oxyresveratrol was separated from the ionic liquid solution by back extraction using ethyl acetate in equal amount (1:1). The mixture was vortexed and two clear phases are formed. The top phase was organic phase containing oxyresveratrol, and bottom phase was an aqueous phase. The oxyresveratrol concentration in upper phase was determined by TLC-densitometry as mentioned above.

Determination of Oxyresveratrol by High-Performance Liquid Chromatography (HPLC) Analysis

Oxyresveratrol content of the extract with optimum ionic liquid and concentration, optimum microwave power and optimum salt was analyzed using HPLC. Standard stock solution 1 mg/ml of oxyresveratrol in 80% ethanol. The HPLC analysis was used C_{18} bonded-silica gel column (YMC, 5µm, 150 × 4.6 mm, Phenomenex, Torrance, USA) and system (LC-20AT, Shimadzu, Kyoto, Japan), equipped with a UV-Visdetector (SPD-20A, Shimadzu, Japan) in the isocratic mode. The mobile phase was used acetonitrile: 1% acetate acid with ratio 25:75. The flow rate was 1 ml/min and run time set for 18 min. UV detector wavelength was 320 nm and the injection volume was 20 µl. The sample was prepared in 80% ethanol as solvent.[6] For comparison, the same sample without salt (only ethyl acetate fraction) was also analyzed.

$$\text{Oxyresveratrol concentration} = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard volume injected}}{\text{sample volume injected}} \times \text{C standard}$$

RESULTS

Determination the optimum IL Solution for IL-MAE

Determination the optimum IL Solution for IL-MAE was analyzed by TLC-densitometry to compare three kinds IL solution with three concentrations. Oxyresveratrol concentration from each sample shown in Table 1. IL solution [Bmim]Br with concentration 1.5mol/L show 595.61 ppm as the highest oxyresveratrol content among the samples.

Determination the optimum microwave power for IL-MAE

Subsequently, extractionof IL solution 1.5 mol/L [Bmim]Br with three different microwave power was analyzed by TLC-densitometry. Oxyresveratrol concentration from each sample shown in Table 2. Extraction using IL solution 1.5 mol/L [Bmim]Br with microwave power 10% for 10 min showed the highest oxyresveratrol concentration among the samples was 463.02 ppm.

Separation of oxyresveratrol from ionic liquid

Four types of salts, Na_2CO_3, $NaHCO_3$, NaCl and K_2HPO_4 solution with concentration 1.0 mol/L, 0.1 mol/L, and 0.01 mol/L respectively with ethyl acetate for back extraction, were studied to separated oxyresveratrol from the IL solution. The extract was obtained using IL concentration 1.5 mol/L [Bmim]Br solution. These result can be shown in Figure 1. The 0.01 mol/L $NaHCO_3$ had the best result to obtained oxyresveratrol among the other. Thus, the 0.01 mol/L $NaHCO_3$ was chosen as the optimum salt.

Determination of Oxyresveratrol by High-Performance Liquid Chromatography (HPLC) Analysis

The sample in optimum condition (1.5 mol/L [Bmim]Br, 0.01mol/ L$NaHCO_3$, microwave power 10% for 10 min) was analyzed by HPLC. As comparison,oxyresveratrol standard and sample without $NaHCO_3$ separation were also analyzed. The comparison of chromatogram and oxyresveratrol concentration between standard and samples were shown in Figure 2 and Table 3 respectively. Oxyresveratrol concentration on the sample with 0.01mol/L $NaHCO_3$ separation showed the highest result.

DISCUSSION

Oxyresveratrol is a substituted stilbene compound of hydroxyl groups found mostly in roots, stems, leaves and plant fruits of the Moraceae family, Liliaceae, and Gnetaceae.[7-8] Based on study above, [Bmim]Br was chosen as the optimum IL solution to obtain oxyresveratrol from *M. Alba* roots extract. IL solution could lower the surface tension of water and make it easier to penetrate the membrane to extract this compound.[9] The determination of optimum ionic liquid is influenced by anions and

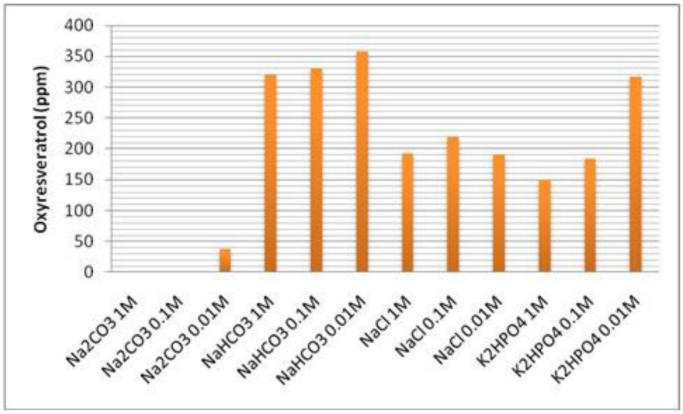

Figure 1: Comparison of oxyresveratrol concentration between sample using salts [source:original data].

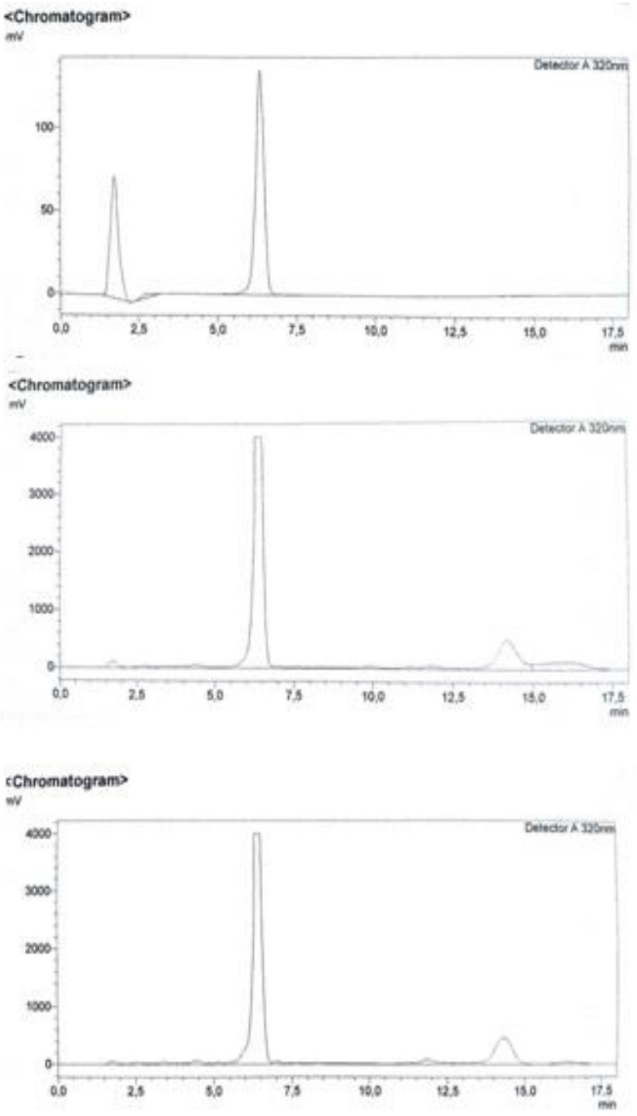

Figure 2: HPLC Chromatograms of standard oxyresveratrol 20 ppm (up), sample IL-MAE mulberry root extract with 0.01 mol/L NaHCO3 (middle) and sample IL-MAE mulberry root extract without salt (bottom) under detector 320 nm [source:original data].

Table 1: Oxyresveratrol concentration in Morus alba roots extract with different ionic liquid and concentration [source: original data]

No	Ionic Liquids	Concentration (mol/L)	Oxyresveratrol concentration (ppm)			Average (ppm)
			R1	R2	R3	
1	[Bmim]Br	1.5	402.99	623.46	760.37	595.61
2		2	301.58	499.2	460.39	420.39
3		2.5	241.01	324.15	235.29	266.82
4	[Hmim]Br	1.5	350.41	246.34	198.47	265.07
5		2	247.63	128.03	78.99	151.55
6		2.5	163.36	80.32	34.71	92.77
7	$[Bmim]_2SO_4$	1.5	199.35	189.93	279.1	222.79
8		2	16.08	34.82	45.63	32.18
9		2.5	29.48	18.24	nd	15.91

*nd : not detected; R1 : replication 1; R2 : replication 2; R3 : replication 3

Table 2: Optimization of microwave power for IL-MAE [source: original data]

No	Ionic Liquid 1.5 mol/L [Bmim]Br	Oxyresveratrol concentration (ppm)			Average (ppm)
		R1	R2	R3	
1	Power 10%	506.7	435.08	447.29	463.02
2	Power 30%	273.43	395.42	331.15	333,33
3	Power 50%	140.93	348.07	178.98	222.66

Table 3: HPLC Analysis result of oxyresveratrol concentration [source:original data]

	Retention time	Area	Oxyresveratrol concentration (ppm)
Oxyresveratrol standard 20 ppm	6.376	2533153	20
Sample with 0.01 mol/L $NaHCO_3$	6.488	93124494	735.25
Sample without salt	6.504	87676311	692.23

cations. The previous study declared that anion Br^- is more efficient to attract *trans*-resveratrol than any other type of anion, Br> SO_4, because of the influence of acidity degree. Anion bromide (Br^-) make the ionic solution was slightly acid than another anion. The weak acid ionic solution could extract *trans*-resveratrol.[10] Oxyresveratrol is a hydroxystilbene compound with four hydroxy groups, while *trans*-resveratrol with three hydroxy groups.[2] Therefore oxyresveratrol as a derived compound of *trans*-resveratrol show the same result. Based on the type of cation, the efficiency of extraction increased by increasing the length of the alkyl chain from ethyl to butyl and starting decreases slowly from butyl to octyl, despite the hydrogen and hydrophobicity properties of hydrogen bonding increases.[11-12] Finally, the ionic liquid [Bmim] Br (1-butyl-3methylimidazolium bromide) is chosen as the solvent because of the bonding effect. It was also probably due to the stronger bonds including Π-Π, ionic charges, and hydrogen interaction between IL and active compound.[13]

Based on the previous study, the optimum concentration of [Bmim]Br solution to get *trans*-resveratrol from *Gnetum gnemon* seeds was 2.5 mol/L.[8]

The optimum IL concentration would make ionic liquid cleavage, penetrate into the cell membrane, and the bioactive compound would be dissolved.[14] In this study, [Bmim]Br solution with concentration 1.5 mol/L was chosen as the optimum concentration of IL solution to obtain oxyresveratrol from *M. alba* roots extract. The ILs concentration associated with viscosity that affects the ability to penetrate and damage the plant cell walls.[15] The lower concentration (1.5 mol/L) than other obtain the better oxyresveratrol content from *M. alba* roots extract probably due to the effect of viscosity. The ionic liquid solvent is a thick liquid. The higher concentration is related to the viscosity of the solvent which increases. The higher viscosity of solution would affect less ability to obtain the target.[16]

The higher microwave power may be effective to extract compounds from plant materials using MAE in a short time. Otherwise, the higher microwave power for a long time can cause damage the active compound because of the overheat. Therefore, the higher microwave power obtains less oxyresveratrol from *M. alba* roots. Based on the previous study, the optimum power and extraction time to obtain trans-resveratrol from *Gnetum gnemon* seed was 10% and 10 min.[8] This study showed the same result that optimum microwave power during IL-MAE in this study was 10% for 10 min.

The optimum salt for this extraction method was 0.01mol/L $NaHCO_3$ which obtain the highest oxyresveratrol concentration among others. One of the methods to separate active compound from ionic liquid was phase separation with the salting-out process. The salt would help to precipitate residue in the IL solution, thus oxyresveratrol would migrate into an organic solvent easier than using no salt.

The optimum condition of IL-MAE of *M. alba* roots extract was ionic liquid [Bmim]Br concentration 1.5mol/L with the 0.01mol/L $NaHCO_3$ salt solution with extraction value of oxyresveratrol concentration was 735.25 ppm or 163.389 mg oxyresveratrol/ g extract. If it is compared with maceration extraction, oxyresveratrol content from *M. alba* root with methanolic maceration extraction was 0.30 w/w by HPTLC and 0.27 % w/w with HPLC.[3]

The use of microwave-assisted extraction is known to cause a cells tissue interference by a microwave irradiation will contribute to the extraction process.[17] MAE was proved to increase the extraction efficiency due to rapid heat process and lower temperatures prevent the compounds damage.[18] MAE method was proven to be better in extraction efficiency, time, as well as quantity of solvent used.[19] MAE is suitable for a compound which is easily degraded by heat such oxyresveratrol. The use of modern extraction methods has proven to be more efficient and economical for extracting the active components of the plant by using ionic liquid microwave-assisted extraction (IL-MAE).[15]

CONCLUSION

Ionic liquid-based microwave assisted extraction is a rapid and effective method for extraction of *M. alba* roots to obtain the optimum oxyresveratrol. The optimum condition of IL-MAE of *M. alba* roots extract was ionic liquid [Bmim]Br concentration 1.5 mol/L using microwave power 10% with the 0.01 mol/L $NaHCO_3$ salt solution with extraction value of oxyresveratrol was 735.25 ppm, better than sample without using salt.

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CONFLICT OF INTEREST

The Authors have no conflict of interest to declare.

ABBREVIATIONS

IL: Ionic liquid; **MAE:** Microwave-assisted extraction; **TLC:** Thin layer chromatography; **HPLC**: High-performance liquid chromatography.

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