Journal of Applied Pharmaceutical Science Vol. 8(06), pp 008-013, June, 2018
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2018.8602
ISSN 2231-3354

Ionic Liquid-based Microwave-Assisted Extraction (Il-MAE) of Oxyresveratrol from *Morus alba* Roots

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ARTICLE INFO

Article history:
Received on: 15/02/2018
Accepted on: 14/05/2018
Available online: 29/06/2018

Key words:
ionic liquid, oxyresveratrol, *Morus alba*, ionic liquid-based microwave-assisted extraction.

ABSTRACT

The objective of this study was to investigate the efficacy of using ionic liquid-based microwave-assisted (IL-MAE) approach for extraction of oxyresveratrol from *Morus alba* roots and to compare the result with conventional extraction. The roots were extracted with nine IL, 1-butyl-3-methylimidazolium chloride (BmimCl), 1-butyl-3-methylimidazolium bromide (BmimBr), or 1-butyl-3-methylimidazolium tetrafluoroborate ($BmimBF_4$) at concentration 1.5, 1.67, and 2 M for each IL, in a microwave at Power 30% for 10 minutes respectively. The extracts were filtrated and diluted with 30 ml of deionized water. Then, fractionated with ethyl acetate and salting out by addition of Na_2CO_3 or KH_2PO_4. The fractions were analyzed for oxyresveratrol contents with TLC-densitometer and HPLC. Then the result was compared with conventional methods. IL-MAE approach showed improvement extraction efficiency compared to the conventional ethanol and methanol macerations. The optimized solvent condition for extraction of oxyresveratrol from *Morus alba* root was 1.5 M BmimCl with 0.01 M KH_2PO_4 salt. Oxyresveratrol was separated by ethyl acetate fractionation, and the yield calculated was 3.53% from raw material. The study showed that IL-MAE was an efficient method for the extraction of oxyresveratrol from *Morus alba* roots.

INTRODUCTION

White mulberries (*Morus alba*) roots have been used since a long time ago as Chinese traditional medicine to cure inflammation diseases. The root of this plant contains terpenoids, flavonoids, and stilbene, including oxyresveratrol and resveratrol (Chan *et al.*, 2016). Oxyresveratrol has been known to inhibit mushroom tyrosinase activities with IC_{50} value of 1.2 µM (Kim *et al.*, 2002), was 32 times stronger than that kojic acid (Shin *et al.*, 1998). Ionic liquids (IL) are a group of non-molecular compounds, with a melting point below 100°C. Typically, they consist of big, asymmetric organic cation (e.g., imidazolium, pyrrolidinium, or pyridinium) and smaller organic or inorganic anion (e.g., tetrafluoroborate, bromide) (Justyna *et al.*, 2017). IL has been applied in an analysis, organic synthesis, and separation technic because it has good physical and chemical properties, including unvaporized, good stability, viscosity, solubility, and extraction ability to different organic compounds and easy to be controlled, if was compared to conventional solvents. Microwave-assisted Extraction (MAE) is a fast and easy sample preparation technique. IL is potentially applied as MAE solvent for various compounds from the crude sample because IL can absorb efficiently the microwave energy (Han and Row, 2010). IL interacts efficiently with microwave through the ionic conduction mechanism and rapidly heated without any significant pressure build-up. Therefore, safety concerns arising from over pressurization of heated sealed reaction vessels can be minimized (Vekariya, 2017). Recently, IL 1-n-butyl-3-methylimidazolium has been investigated as a solvent in the extraction of *trans*-resveratrol from *Polygonum cuspidatum* roots, that shows IL is potentially applied to MAE for some compound from the medicinal plant (Du *et al.*, 2007). Until now, there is no report about extraction of oxyresveratrol using ionic liquid. Therefore, this study aimed to investigate the effect ionic liquid-based microwave assisted extraction to increase oxyresveratrol yield of *Morus alba* and to compare this method with conventional extraction methods on literature.

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MATERIAL AND METHODS

Samples

The roots of *Morus alba* were collected from Rumah Sutera, Bogor and has been identified in Research Center for Biology LIPI Bogor. The roots were washed with water, then air dried for six days at room temperature and protected from sunlight, and then ground.

Chemicals

The ILs, 1-butyl-3-methylimidazolium chloride (BmimCl), 1-butyl-3-methylimidazolium bromide (BmimBr) and 1-butyl-3-methylimidazolium tetrafluoroborate ($BmimBF_4$) were purchased from Shanghai Chengjie Chemical Co. Ltd., China. Standard *trans*-oxyresveratrol was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Deionized water, ethanol, methanol, chloroform, acetic acid, ethyl acetate, acetonitrile, Na_2CO_3, KH_2PO_4 were obtained from the local supplier. The extraction method used the microwave (Modena MV 3002 900-Watt), The analytical method used TLC silica gel 60 F_{254} with TLC scanner III Camag CATs system (Muttenz, Switzerland), and HPLC system (LC-20AT, Shimadzu, Kyoto, Japan).

Extraction IL-MAE

Nine Ionic Liquids (ILs) were prepared by diluting each ionic liquid BmimBr, $BmimBF_4$, and BmimCl with deionized water 100 ml to make concentration 1.5, 1.67, and 2 M, respectively. *M. alba* root powder (1 g) was dissolved in 20 ml ionic liquid solution in a flask for each concentration, then put into the microwave. The microwave set at 30% power (900-watt microwave) and run for 10 minutes. The suspension was filtered through filter paper and diluted ad 30 ml with deionized water. Each IL extraction was repeated three times and collected (Du *et al.*, 2007).

Ethyl acetate fractionation and separation of IL with salt

The separation of IL from the extracts was optimized by using Na_2CO_3 or KH_2PO_4 salts at different concentrations (0.01, 0.1, 0.5, and 1 M respectively). The IL extracts then were fractionated by ethyl acetate (EA). 1 ml IL-MAE extracts added with 1 ml salt of Na_2CO_3 or KH_2PO_4 and fractionated with 1 ml EA. The solution was vortexed for 10 seconds, then centrifuged for 10 minutes at 3000 rpm. Two phases of solution appeared, and the upper layer (EA fraction) was taken for further analysis (Ayuningtyas *et al.*, 2017). For comparison, the same procedure was repeated for separation without salt.

Thin layer chromatography (TLC) analysis

Standard *trans*-oxyresveratrol solution was prepared at concentration 1 mg/ml with 80% ethanol. TLC analysis was performed using silica gel 60 F_{254} (Merck, Darmstadt, Germany) as a stationary phase and methanol:chloroform:acetate acid (10:88:2% v/v) as a mobile phase. The samples were EA fractions (with salt and without salt) and standard. Samples were spotted 5 µl and then were placed in the chamber that was saturated with solvent before. After that, the plate was removed from the chamber and air-dried. The TLC spots were viewed under UV light at 366 nm wavelength (Soonthornsit *et al.*, 2017), and were analyzed by densitometry TLC scanner with detector 254 nm wavelength to give area under curve (AUC) values of *trans*-oxyresveratrol. EA fraction with highest AUC value then chose to continue as the pilot scale of extraction and analyzed by HPLC.

Pilot-scale of IL-MAE

The optimum IL and salting out extractions condition with highest AUC value was studied further to calculate *trans*-oxyresveratrol yield. 10 grams of root powder was added 200 ml IL solution, then was microwaved, filtered and added 300 ml of deionized water. The solution was added 300 ml salt and 300 ml ethyl acetate and was mixed at 1000 rpm for 10 minutes with a magnetic stirrer. The solution was centrifuged at 3000 rpm for 10 min to separate EA fraction. This procedure was repeated four times with fresh EA. The EA fractions then were collected and evaporated in vacuum rotary evaporator and the yield calculated as below:

$$\%\,\text{yield} = \frac{\text{ethyl acetate fraction mass}}{\text{crude sampel mass}} \times 100\%$$

High-performance liquid chromatography (HPLC) analysis of oxyresveratrol in the extract

The EA fraction was analyzed by HPLC to calculate oxyresveratrol content. A standard stock solution of oxyresveratrol was prepared at concentration 1 mg/ml in 80% ethanol and diluted into 5 different concentrations (from 0.0005 to 0.05 mg/ml) to make calibration curve and to determine linear regression. The stock solution of the EA fraction was diluted in 80% ethanol. The HPLC analysis was used C_{18} bonded-silica gel column (YMC, 5 µm, 150 × 4.6 mm, Phenomenex, Torrance, USA), the system (LC-20AT, Shimadzu, Kyoto, Japan) and equipped with a UV-Vis detector (SPD-20A, Shimadzu, Japan) in the isocratic mode. The mobile phase was used acetonitrile mixed with water in 1% acetic acid at ratio 1:3 with flow rate 1 ml/min and runtime set at 18 min. UV detector wavelength was 320 nm and the injection volume was 20 µl (Soonthornsit *et al.*, 2017).

RESULTS AND DISCUSSION

This research was to investigate, firstly, the effect of IL structures which determine their physical and chemical properties on extracting analysis. The others were to investigate the effect of salting out on IL water solutions and to analyze TLC spots and HPLC chromatograms of EA fractions of IL-MAE extracts. IL 1-butyl-3-methylimidazolium, as cation, with three kinds of anions, Br^-, BF_4^-, and Cl^-, were studied by IL-MAE. The salting out using Na_2CO_3 and KH_2PO_4 with different concentration, 0.01, 0.1, 0.5 and 1 M and with no salt were determined.

Thin layer chromatography (TLC) analysis

ILs BmimBr, BmimCl, and $BmimBF_4$ were used to extract *trans*-oxyresveratrol from *M. alba*. TLC analysis was performed to compare the effect of the ionic liquids on the oxyresveratrol constituent in *Morus alba* root extract. EA fraction was observed under UV light at 366 nm. Figure 1 showed the chromatogram of 1 mg/ml standard oxyresveratrol, EA fractions with 0.01 M Na_2CO_3, 0.01 M KH_2PO_4, and without salt.

All sample showed oxyresveratrol spot at Rf 0.16 and, while standard at 0.17. Densitometry under 254 nm resulted in

AUC value of this spot and showed the amount of oxyresveratrol as seen in Table 1. The highest AUC showed the highest amount of oxyresveratrol. The salting out procedure was proposed to observe the separation of IL from the extract and to compare the amount of oxyresveratrol in EA fraction with and without salt addition. The result showed that salting out with 0.01 M Na_2CO_3 did not increase AUC values for ILs BmimBr and $BmimBF_4$ than with no salt, while 0.01 M KH_2PO_4 increased AUC values significantly for all ILs. Fractionation with no salt showed 1.5 M BmimBr have the highest AUC among another ILs, while with Na_2CO_3, 1.67 M $BmimBF_4$ showed the highest AUC, and 1.5 M BmimCl for KH_2PO_4 salt. This result showed that different ILs has a different optimum condition for extraction of oxyresveratrol from plant sample. From AUC values, it shows that the optimum IL and salting agent to extract oxyresveratrol from *Morus alba* root was 1.5 M BmimCl with 0.01 M KH_2PO_4 salt among another sample tested which AUC value 8736.1.

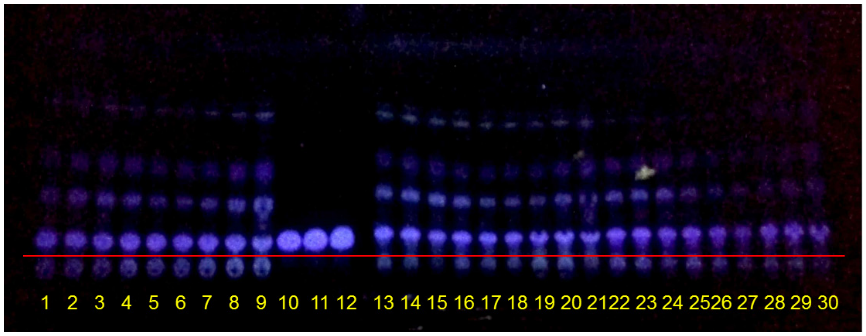

Fig. 1: TLC fingerprint analysis of *Morus alba* root extract under UV 366. From left to right, 1-3: 1.5, 1.67, 2 M BmimBr with 0.01 M KH_2PO_4; 4-6: 1.5, 1.6, 2 M BmimCl with 0.01 M KH_2PO_4; 7-9: 1.5, 1.6, 2 M $BmimBF_4$ with 0.01 M KH_2PO_4; 11-12: standard oxyresveratrol; 13-15: 1.5, 1.67, 2 M BmimBr with no salt; 16-18: 1.5, 1.6, 2 M BmimCl with no salt; 19-21: 1.5, 1.67, 2 M $BmimBF_4$ with no salt; 22-24: 1.5, 1.67, 2 M BmimBr with 0.01 M Na_2CO_3; 25-27: 1.5, 1.6, 2 M BmimCl with 0.01 M Na_2CO_3; 28-30: 1.5, 1.67, 2 M $BmimBF_4$ with 0.01 M KH_2PO_4.

Table 1: AUC Value of Oxyresveratrol on Fractionation with Salt and No Salt Addition.

IL	Concentration (M)	No Salt	Salt							
			Na_2CO_3				KH_2PO_4			
			0.01 M	0.1 M	0.5 M	1 M	0.01 M	0.1 M	0.5 M	1 M
BmimBr	1.5	6341.6	5891.1	214.6	0	0	8374.8	7934.5	6552.5	6852.8
	1.67	5800	6574.5	209.6	0	0	8570.8	7793.3	8502.9	8733.1
	2	4472.7	4761.8	0	0	0	8103.6	7357.1	6956.6	7877.4
BmimCl	1.5	5563.9	5339.4	498.3	444.2	0	8736.1	7177.6	6561.6	6903.4
	1.67	4993	4490.8	469.4	318.3	0	7660.5	7993.1	7015.9	7882.5
	2	4716.8	2828.7	389.6	265.3	295.6	6197.6	6607.7	5873.8	5820.2
$BmimBF_4$	1.5	4629.8	6742.6	6759.3	905.4	942.3	7044.6	7399.6	806.5	7990.7
	1.67	6092.8	8652.7	6132.5	919	1016.1	7700.8	6930	783.5	4918.9
	2	5060.4	7348	4851.2	979.1	1489.3	7907.3	5362.5	588.6	6143.6

Compared the results of three ILs, with the same cation at the same concentration (1.5 M) where analyzed by TLC-densitometry, salting out with 0.01 M Na_2CO_3 did not increase AUC values for ILs BmimBr and $BmimBF_4$ than with no salt, while 0.01 M KH_2PO_4 increased AUC values significantly for all ILs. This means that Na_2CO_3 cannot separate oxyresveratrol optimally from IL solution, while KH_2PO_4 salt successfully interacts and increases the separation. Fractionation with no salt showed 1.5 M BmimBr have the highest AUC among another ILs, while with Na_2CO_3, 1.67 M $BmimBF_4$ showed the highest AUC, and 1.5 M BmimCl for KH_2PO_4 salt. This result showed that different ILs has a different optimum condition for extraction of oxyresveratrol from plant sample.

The solution of ILs in water could greatly enhance the extraction yields of oxyresveratrol from *M. alba* roots. These can be explained that ILs had high solvation power and interactions with oxyresveratrol that contribute to raising the solubility of oxyresveratrol. That interactions influence by physical structures and chemical bonds, especially hydrogen bonding, polarity, π-π, and ionic/charge–charge. Therefore, the characteristic of ionic liquids to influence the conformation of carbohydrates through hydrogen bonding and can be considered as viable substituents to be value-added solvents. On the other hand, ILs can absorb and transfer microwave energy efficiently and consequently make the solvent and the sample warming rapidly. Both of them improved the efficiency of oxyresveratrol extraction from *M. alba* roots (Zeng *et al.*, 2010; Ahmad *et al.*, 2017).

The concentration of salt also influences the separation ability, where salts (Na_2CO_3 and K_2HPO_4) at concentration 0.01 M give AUC higher than at concentration 0.1, 0.5, and 1 M. The previous study, at separation IL solution, 0.01 M Na_2CO_3 had the best result to obtain *trans*-resveratrol among the other, whereas the K_2HPO_4 had no ability on separation *trans*-resveratrol from the IL solution (Ayuningtyas *et al*., 2017). This showed that salt added at low concentration help to enhance separation of ionic liquid and recovery of chemical compounds from crude extract. On other works, IL-MAE with the addition of KH_2PO_4 salt obtained gamma oryzanol higher than Na_2CO_3 salt and without salt which is caused by the interaction of solutes (ion of inorganic salts and ionic liquid) and solvents (Trinovita *et al*., 2017).

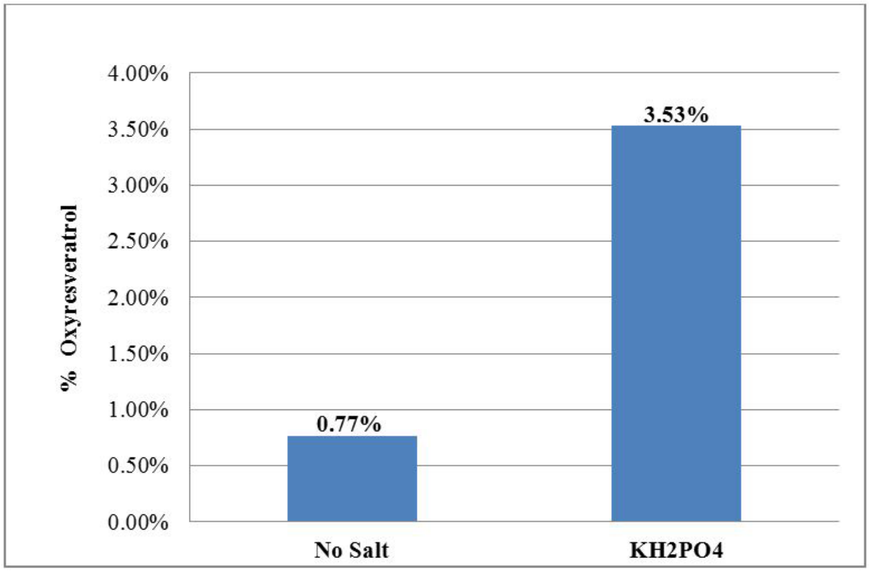

Fig. 2: The yield of oxyresveratrol obtained by 1.5 M BmimCl EA fractions with 0.01 M KH_2PO_4 salt and no salt addition.

Table 2: Percentage of Oxyresveratrol from *Morus alba* using Different Extraction Methods.

No	Methods	Sample	Extract yield	Fraction yield	Oxyresveratrol (w/w)	Reference
1	Methanol	Branch	5.8%	1.89%		Chen *et al*., 2013
2	Methanol	Barks, Roots			0.26%, 0.27%	Ayinampudi *et al*., 2011
3	Ethanol	Stems	5.13%		17.87 ± 0.61%	Zeng *et al*., 2010
4	IL-MAE	Roots		3.53%	79.5%	

Separation of oxyresveratrol

This study showed that the highest oxyresveratrol was separated from 1.5 M BmimCl with 0.01 M KH_2PO_4 salt with EA fractionation and was repeated four times and compared with the solution without salt, then calculated. The yield of EA fraction from solution with salt and without salt was 3.53% and 0.77% from 10-gram raw material respectively as shown in Figure 2.

High-performance liquid chromatography (HPLC) analysis of oxyresveratrol in the extract

The amount of oxyresveratrol of EA fraction determined and analyzed by HPLC. EA fraction of BmimCl IL-MAE and a standard solution of oxyresveratrol were diluted in 80% ethanol and were analyzed under 320 nm. The chromatograms of EA fraction were compared with standard oxyresveratrol as shown in Figure 3. A series of standard oxyresveratrol solutions at five concentration range from 0.05 to 0.0005 mg/ml were prepared to make calibration curve and determine the linearity of this method for analyzing oxyresveratrol in *M. alba* roots. The calibration curve, as seen in Figure 4, showed regression linear by $y = 94383016x + 36277$ and regression coefficients (r) = 0.99949. The calculated amount of oxyresveratrol from 1.5 M BmimCl (IL-MAE method) with 0.01 M KH_2PO_4 salt was 60.212 µg/ml with yield 79.5%.

While previous studies, on *trans*-resveratrol content has been extracted from optimum IL-MAE condition 2.5 M BmimBr solution, 15 mL/g liquid/solid ratio, 10% microwave power, and 10 min extraction time (Ayuningtyas *et al*., 2017), on the yield of total polyphenolics content, IL-MAE 0.7 M BmimCl (14 ml/l liquid-solid ratio, and 270 Watts microwave power for 10 minutes) better than 0.7 M BmimBr (14 ml/l liquid-solid ratio, and 270 Watts microwave power for 15 minutes) (Ahmad *et al*., 2017).

This result was compared with the previous study of oxyresveratrol extraction on literature, as seen in Table 2 EA fraction of *M. alba* branch yield was 1.89% (Chen *et al*., 2013). Oxyresveratrol from barks and roots methanolic extract calculated with HPTLC method was 0.27%, and 0.30%, and was not identified in leaves, fruits, or xylem (Ayinampudi *et al*., 2011) and from stems ethanolic extract calculated with HPLC was 17.87 ± 0.61% with yield 5.13% (Zeng *et al*., 2010).

The efficiency of the IL-MAE method was much higher than the maceration method that caused damage to the cell wall surface of the sample matrix by microwave irradiation interference (Ahmad *et al*., 2017). IL-MAE method and salting out an improved yield of oxyresveratrol from *M. alba* roots significantly.

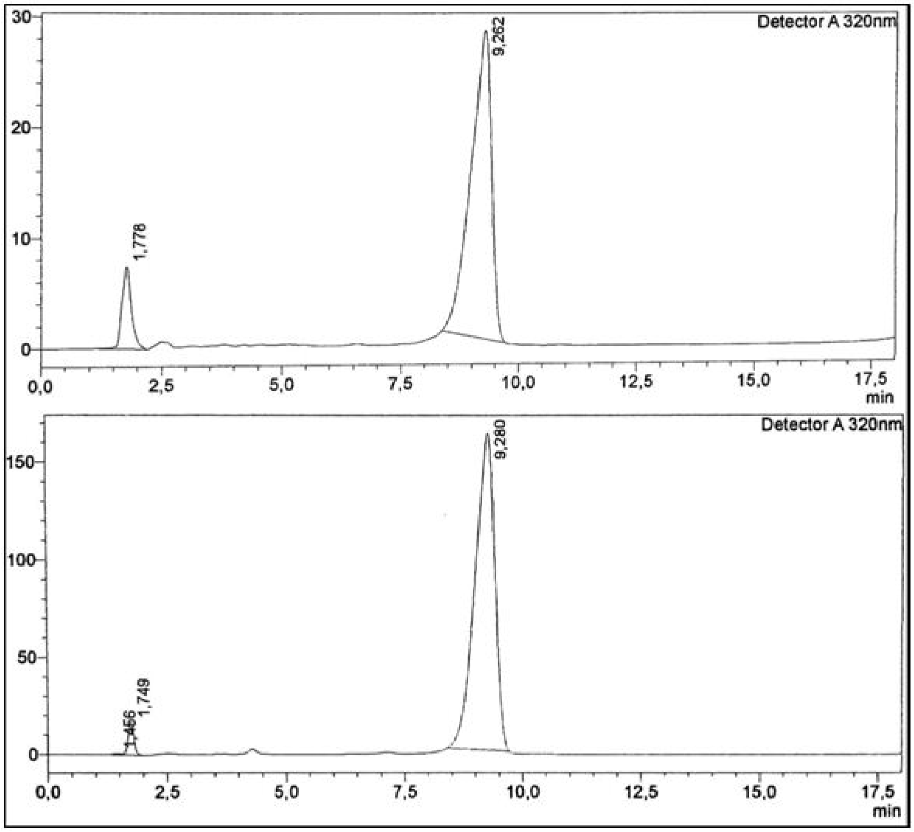

Fig. 3: HPLC chromatograms of EA fraction of IL-MAE root extract (above) and standard oxyresveratrol (below) 50 ppm under detection of 320 nm.

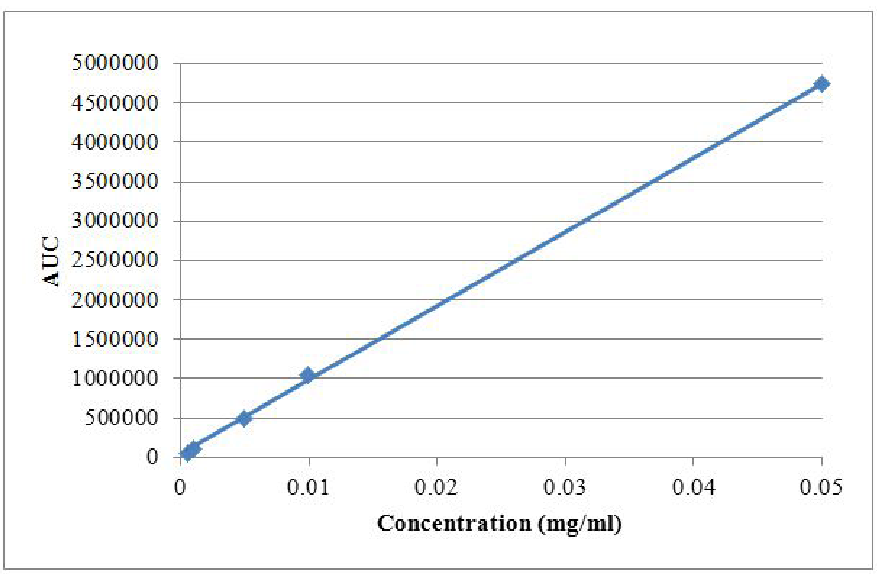

Fig. 4: Calibration curve of oxyresveratrol.

CONCLUSION

Ionic liquid-based microwave-assisted extraction (IL-MAE) would be an option to extract oxyresveratrol from *Morus alba* compared with the ethanolic and methanolic maceration. This research showed the optimum condition of IL-MAE to extract oxyresveratrol from *M. alba* root was 1.5 M BmimCl with 0.01 M KH_2PO_4 salt solution. The adding salt improved extraction yield of EA fraction of *M. alba* roots from 0.77% to 3.53%. The calculated oxyresveratrol from EA fraction was 79.5%.

ACKNOWLEDGMENT

This study was supported by Universitas Indonesia for Hibah PITTA 2017.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ABBREVIATIONS

AUC:	Area Under Curve
BmimCl:	1-butyl-3-methylimidazolium chloride
BmimBr:	1- butyl-3-methylimidazolium bromide
$BmimBF_4$:	1-butyl-3-methylimidazolium tetrafluoroborate
EA:	ethyl acetate
HPLC:	High performance liquid chromatography
KH_2PO_4:	Potassium dihydrophosphate
IL:	Ionic Liquid
IL-MAE:	Ionic Liquid-based Microwave Assisted Extraction
MAE:	Microwave Assisted Extraction
Na_2CO_3:	Sodium carbonate
TLC:	Thin Layer Chromatography.

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How to cite this article:
Fadhila M, Mun'im A, Jufri M. Ionic Liquid-based Microwave-Assisted Extraction (Il-MAE) of Oxyresveratrol from *Morus alba* Roots. J App Pharm Sci, 2018; 8(06): 008-013.