J Young Pharm, 2017; 9(4): 457-462
A multifaceted peer reviewed journal in the field of Pharmacy
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Original Article

Optimization of Ionic Liquid-Based Microwave Assisted Extraction to Obtain *Trans*-resveratrol from *Gnetum gnemon* L. Seeds

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ABSTRACT

Introduction: *Gnetum gnemon* L. (Melinjo) is popular in Indonesia as a source of nutrition. Its seeds contain *trans*-resveratrol, which has been shown to have multiple pharmacological activities. The application of ionic liquid [Bmim]Br as a selected solvent in the microwave assisted extraction (MAE) method was developed for extraction of *trans*-resveratrol from Melinjo seeds. MAE method with ionic liquid has also been applied to extract *trans*-resveratrol from the different plant with a high extraction yield value. **Methods:** The Box-Behnken design on four factors with response surface methodology were used to optimize extraction conditions for *trans*-resveratrol content from Melinjo seeds. For further, the various salt was applied to separate of the *trans*-resveratrol form ionic liquid and facilitate for extraction back using an organic solvent. **Results:** The optimum process for the ionic liquid-based microwave assisted extraction was below: [Bmim]Br concentration 2.5 mol/L; liquid-melinjo seeds powder ratio 15 mL/g; microwave power 10%; and extraction time 10 min. Under optimized conditions, the *trans*-resveratrol value was 1.34 mg/g. Moreover, the salt Na_2CO_3 0.01 mol/L with ethyl acetate were useful to the extraction back of *trans*-resveratrol from [Bmim]Br solution. **Conclusion:** This IL-MAE method was suitable to apply as an alternative technique to extract the active compound from the plant.

Key words: Ionic liquid, *Gnetum gnemon*, Melinjo, Microwave assisted extraction, *Trans*-resveratrol, Response surface methodology.

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DOI: 10.5530/jyp.2017.9.90

INTRODUCTION

Melinjo (*Gnetum gnemon* L.) is widely cultivated in Southeast Asia. In Indonesia, melinjo is very popular as a vegetable for consumed in soup or cracker snack (locally was known Emping). The seeds, fruits, and leaves are nutritious. Melinjo seeds are rich in resveratrol dimer derivatives (Gnetin C and its glucosides, Gnetin L) and resveratrol monomer derivatives (isorhapontigenin, *trans*-resveratrol (3,5,4-trihydroxy-*trans*-stilbene), and *trans*-pieced).[1,2] *Trans*-resveratrol is a polyphenolic compound (a group of stilbenes). Melinjo seeds contain trans-resveratrol reported to have multiple pharmacological effects, such as antioxidant,[3] lower uric acid,[4] tyrosinase inhibitor,[5] antitumor, lipase and α-amylase inhibitor, antibacterial, and immunomodulator.[6,7]

Microwave-Assisted Extraction (MAE) is a modern extraction technique that widely applied to extract bioactive compounds from the plant, such as alkaloids, phenolic, and essential oil. MAE is easy to use, rapid, efficient, effective, good selectivity, and economic expense. Conventional methods of extraction were used organic solvents, that has a problem when applied for extraction because of volatility, flammability, and toxicity.[8,9]

Ionic liquid (IL) is a pure salt compound consisting of ionic components (cations and anions). Recently, IL potential used in a various extraction process as an alternative for organic solvents because of the unique properties. IL is known as green solvents, nontoxic, negligible vapor pressure, good thermal stability, and non-flammable.[10] It makes IL better than organic solvent.

IL has good extractability for the various active compounds, such as from *Peperomia pellucida*,[11] *Dioscorea nipponicae*,[12] *Psidium guajava*, *Smilax china*,[13] *Curcuma longa*,[14] *Apocynum venetum*,[15] and *Lycium ruthenicum*.[16] The IL 1-n-butyl-3-methylimidazolium ([Bmim]Br) solution was well known to extract *trans*-resveratrol from *Polygoni cuspidatum*.[17] Moreover, the aqueous biphasic system (ABS) based on ionic liquid has been developed to separate the active compound from the water-miscible ionic liquid solvent. Thus, the ionic liquid can be recycled and reused.[18]

IL-based MAE has been developed as extraction method last few years, so now IL as solvents in the MAE more preferable. There are no study reports on IL-based MAE of *trans*-resveratrol from melinjo seeds. [Bmim]Br was chosen as IL solvent because of their ability to extract *trans*-resveratrol. The *trans*-resveratrol compound also will be evaluated using thin layer chromatography (TLC) densitometry.[19-22] In this work, the optimal extraction condition using IL-MAE would be investigated to obtain *trans*-resveratrol from melinjo seeds. Furthermore, application of various salt combined with an organic solvent for back extraction was also investigated for separated of the *trans*-resveratrol from the ionic liquid solution.

MATERIALS AND METHODS

Plant materials and reagents

Mature melinjo fruits (Figure 1) were purchased from local market. The seeds (Figure 1c) were separated from their shell and dried. The dried

(1a) (1b) (1c)

Figure 1: (a) Matured melinjo fruit; (b) The brown seeds shell, matured; (c) The seeds matured melinjo fruit.

seeds were ground to fine flour by using a commercial kitchen blender. The powder seed samples were stored in air tight container and refrigerated at 4°C. Standard *trans*-resveratrol was purchased from Sigma-Aldrich Pte. Ltd., (Singapore). Ionic liquid 1-butyl-3-methylimidazolium bromide ([Bmim]Br) (>99%) was bought from Chengjie Chemical Co., Ltd., (Shanghai, China). The salt Na_2CO_3, KH_2PO_4, NaCl, K_2HPO_4 were purchased from Merck. All reagents used for TLC 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).

Optimization IL-MAE by response surface methodology (RSM)

Response surface methodology (RSM) used Design-Expert 10 software (Statease Inc., Minneapolis, USA) was applied to study the interaction between factors and to optimize experimental conditions. Four factors with three levels Box-Behnken design (BBD) was developed (Table 1). The independent factors were IL concentration (0.5, 1.5, and 2.5 mol/L), liquid/solid ratio (10, 15, and 20 mL/g), microwave power (10, 30, and 50 %), and extraction time (5, 10, and 15 min). The response value was a *trans*-resveratrol yield (mg/g). The analysis of significance model of RSM used ANOVA.

Extraction of trans-resveratrol from melinjo seeds

One gram sample melinjo seeds powder was placed in a boiling flask. IL solution with different concentration was added into the flask to a certain liquid/solid ratio. The boiling flask was placed into a microwave. The extraction was performed under different power level and time. The extracts were centrifuged at 3000r/min for 15 minutes, and then the supernatant was diluted to 10 mL with aquabidestilata for analysis.

Determination of trans-resveratrol

The *trans*-resveratrol in the extract was determined semiquantitatively by thin layer chromatography (TLC)-densitometry. The 0.5 ml ethyl acetate was added to 1 ml of diluted supernatant, and then the mixture was a vortex for few second and will be formed into two phase. The samples (ethyl acetate phase) were spotted on a TLC silica gel aluminum plates 60 F_{254} (20x10 cm) using microcapillary tube 5 µL. The mobile phase consisted of n-hexane: ethyl acetate: acetic acid (1: 1: 0.01). The length of chromatogram run was 8 cm through glass chamber that saturated by the mobile phase. Subsequently, TLC plate was air dried. The scanning was performed with Camag TLC Scanner III in absorbance length at 313 nm and operated by winCATS software. The spot of trans-resveratrol in a sample was confirmed by comparing the spectrum and the Rf value of the spot of the standard.

Table 1: Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of Squares	Df	Mean Square	F-Value	p-Value
Model	3.21	14	0.23	5.14	0.0066
A	1.48	1	1.48	33.14	0.0002
B	0.24	1	0.24	5.34	0.0434
C	0.036	1	0.036	0.81	0.3880
D	0.10	1	0.10	2.26	0.1635
AB	0.23	1	0.23	5.06	0.0482
AC	0.20	1	0.20	4.54	0.0589
AD	0.18	1	0.18	3.96	0.0747
BC	6.250E-004	1	6.250E-004	0.014	0.9081
BD	0.018	1	0.018	0.41	0.5369
CD	1.225E-003	1	1.225E-003	0.027	0.8716
A^2	0.28	1	0.28	6.34	0.0305
B^2	0.026	1	0.026	0.59	0.4613
C^2	5.100E-003	1	5.100E-003	0.11	0.7422
D^2	0.017	1	0.017	0.38	0.5511
Residual	0.45	10	0.045		
Cor Total	3.66	24			

Mean = 0.27
Std Dev = 0.21
CV% = 77.28
R-Squared = 0.8781
Adj R-Squared = 0.7074

Standard solutions were prepared by diluting the stock solution of trans-resveratrol (100 mg/L) with ethyl acetate in the concentration range 20–80 mg/L.

$$C_{trans-resveratrol} = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard volume spotted}}{\text{sample volume spotted}} \times C_{standard}$$

$$\text{Total } \textit{trans}\text{-resveratorl content (mg/g)} = \frac{\textit{trans}\text{-resveratol amount in extract (mg)}}{\text{melinjio seeds sample amount (g)}}$$

Comparison organic solvent extraction

Ethanol-MAE was chosen as the reference extraction process. Ethanol-MAE compared with IL-MAE to investigate their efficiency for extraction of trans-resveratrol. One g melinjo seeds powder was extracted with 10 mL of 90% ethanol at 30% power of microwave and 5 min extraction time. The extract was filtered through filter paper, and the filtrate analyzed by TLC-densitometry. The experiment for IL-MAE was operated under 2.5 mol/L [Bmim]Br solution and liquid/solid ratio 15 mL/g at the same power of microwave and extraction time.

Separation trans-resveratrol from ionic liquid solution

The trans-resveratrol in the IL solution was separated by ABS and liquid-liquid back extraction process (Figure 2). The IL extract and a certain of salt (1: 1) were added into a tube. The *trans*-resveratrol was separated from the ionic liquid solution by back extraction using ethyl acetate in equal amount (1: 1). The mixture was vortex and two clear phases are formed. The top phase was organic phase containing *trans*-resveratrol

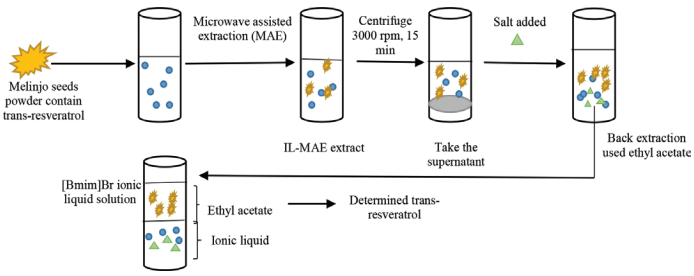


Figure 2: Separation process of trans-resveratrol from ionic liquid solution.

and bottom phase was an aqueous phase. The *trans*-resveratrol content in top phase was determined.

RESULTS

The model fitting and statictical analysis

Fitting the experimental data were analyzed using multiple regression to determine the model by following quadratic polynomial equation. The final equation for investigated relationship between independent variables and response variables was obtained, $Y = 0.11 + 0.35A + 0.14B - 0.055C - 0.092D + 0.24AB - 0.22AC - 0.21AD - 0.013BC - 0.068BD - 0.017CD + 0.32A^2 - 0.096B^2 + 0.042C^2 + 0.077D^2$, Y represented the response of *trans*-resveratrol yield (mg/g), A was IL concentration, B was liquid/solid ratio, C was microwave power, and D was extraction time. Subsequently, the quadratic polynomial models was performed ANOVA analysis test in Table 1.

The ANOVA showed that the model F-value of 5.14 and the p-value was 0.0066, implied the model was significant ($p<0.05$) and acceptable. Furthermore, the terms A and B, two interaction terms (AB), and quadratic terms (A^2) were significant models terms. The R-squared (R^2) was 0.8781, which implied that 87.81% of the variations could be explained by this model.

The 3D response surfaces for the interaction between two independent variables and response showed in Figure 3. Figure 3a, b, and c demonstrated relationship IL concentration and liquid/solid ratio, power, and time, respectively, which not showed the good result. Figure 3d and e showed that between solid/liquid ratio with power and time did not have influence toward trans-resveratrol, which was similar to the power and time in Figure 3f. So, in general, the graphic indicated that the relationship between two independent variables of the response not strong enough.

Optimum extraction parameters

The 25 runs of four different experimental conditions were studied and the result for *trans*-resveratrol yield was shown in Table 2. The highest *trans*-resveratrol content was shown in run 14 with 2.5 M [Bmim]Br solution, 15 mL/g liquid/solid ratio, 10% microwave power, and 10 min extraction time. The experiment value was 1.34 mg/g, that close with the predicted value by Design Expert software, 1.10 mg/g, even slightly higher. The run 3 also showed the high value of trans-resveratrol, 1.32 mg/g, with the predicted value was 1.16 mg/g. Overall, the result showed that the model can be able to describe the expected optimization results.

Table 2: Experimental parameters of Box-Behnken design and resveratrol content

Run	A IL Concentration (M)	B Liquid/Solid Ratio (mL/g)	C Power (%)	D Time (min)	Resveratrol Yield (mg/g)	
					Experimental	Predicted
1	1.5	15	10	5	0.127	0.360
2	1.5	15	50	5	0.270	0.280
3	2.5	15	30	5	1.328	1.160
4	1.5	15	50	15	0.188	0.066
5	2.5	15	30	15	0.518	0.550
6	1.5	20	30	15	0.148	0.073
7	0.5	15	50	10	0.159	0.290
8	1.5	15	10	15	0.125	0.210
9	1.5	20	50	10	0.049	0.130
10	1.5	10	10	10	0.033	0.042
11	0.5	15	30	5	0.059	0.035
12	1.5	20	30	5	0.397	0.390
13	1.5	10	50	10	0.022	0.130
14	2.5	15	10	10	1.341	1.100
15	1.5	10	30	5	0.009	0.025
16	2.5	15	50	10	0.488	0.540
17	1.5	10	30	15	0.033	0.074
18	1.5	20	10	10	0.107	0.260
19	2.5	10	30	10	0.005	0.300
20	0.5	20	30	10	0.071	0.120
21	0.5	15	10	10	0.105	0.052
22	2.5	20	30	10	1.023	1.060
23	1.5	15	30	10	0.106	0.110
24	0.5	10	30	10	0.012	0.076
25	0.5	15	30	15	0.085	0.270

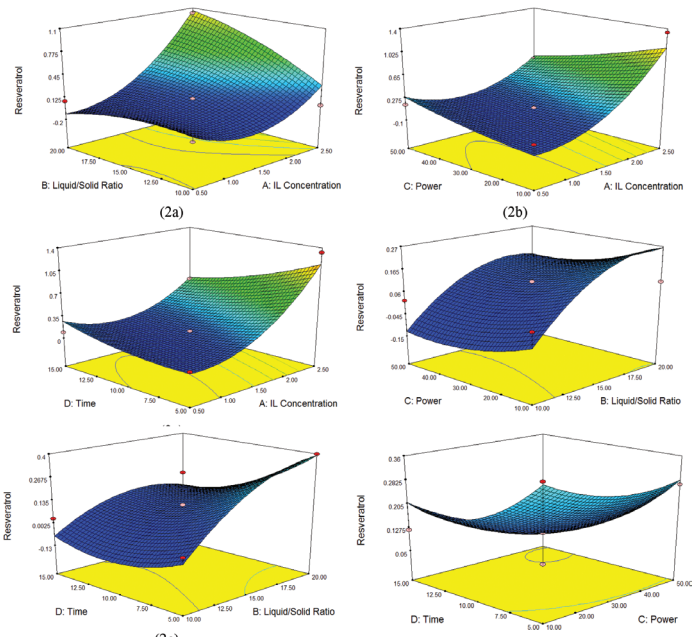
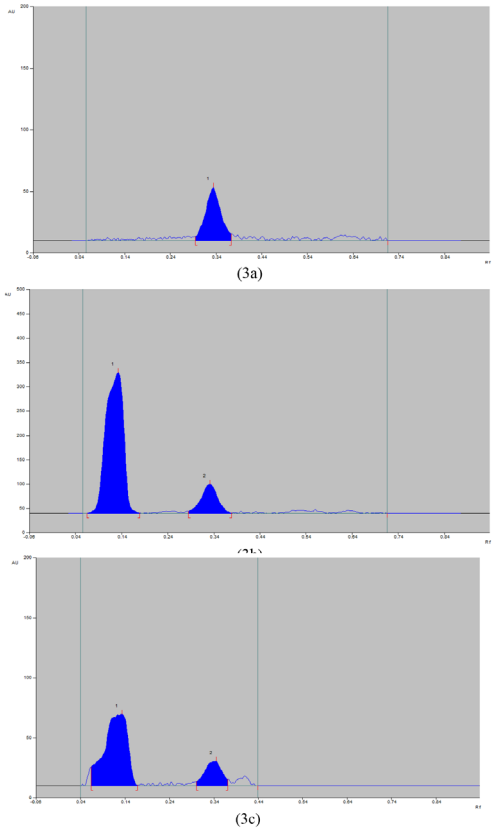


Figure 3: Response surface interaction of two independent variables on trans-resveratrol content.TLC: silica gel 60 F_{254}, mobile phase: n-hexane: ethyl acetate: acetic acid (1: 1: 0.01), at 313 nm.



TLC: silica gel 60 F_{254}, mobile phase: n-hexane: ethyl acetate: acetic acid (1: 1: 0.01), at 313 nm.

Figure 4: TLC Chromatograms of standard *trans*-resveratrol 60 mg/L (4a), extract from melinjo seeds by [Bmim]Br solution (4b), and extract of melinjo seeds by ethanol (4c)

Comparison of organic solvent for extraction

Ethanol-MAE was used to extract *trans*-resveratrol from melinjo seeds. The result was compared to the *trans*-resveratrol yield of IL-MAE. The *trans*-resveratrol yield was 0.035 mg/g by using ethanol and 1.32 mg/g by using IL. The IL-MAE method showed the higher extraction yield than ethanol. Therefore, the IL could be an alternative solvent of volatile organic solvent in the MAE method.

The method for analysis of *trans*-resveratrol was used TLC-densitometry by analyzing standard and samples. Estimation *trans*-resveratrol yield in a sample was determined by comparison of Rf value and area under the curve of sample and standard. The Rf value of *trans*-resveratrol was obtained at 0.34. Moreover, all extract showed predominant peaks at Rf value 0.13, that was probably of another resveratrol derivative or the other compound in the sample. The TLC chromatograms of spectrum standard and sample showed in Figure 4.

Separation of trans-resveratrol from ionic liquid

Four types of salts, Na_2CO_3, KH_2PO_4, NaCl, and K_2HPO_4, with ethyl acetate for back extraction, were studied to separated *trans*-resveratrol from the IL solution. The extract was obtained using IL concentration 0.5 mol/L [Bmim]Br solution. The salt concentration is used 0.01 mol/L and the saturated salt. These result can show in Figure 5. The 0.01 mol/L Na_2CO_3 had the best result to obtained *trans*-resveratrol among the other, whereas the K_2HPO_4 had no ability to separation *trans*-resveratrol from the IL solution, which is not indicated by the trans-resveratrol spot on the TLC chromatogram (data not shown). Thus, the Na_2CO_3 0.01 mol/L was chosen as the optimal salt.

DISCUSSION

Trans-resveratrol is a polyphenolic compound (a group of stilbenes). The *trans*-resveratrol from melinjo seeds extract was high enough if compared with trans-resveratrol from another plant, such as in grape skin was 0.065 – 0.169 mg/g,[23] peanut was 0.00003 – 0.00014 mg/g,[24] *Smilax china* was 0.531 mg/g,[25] or *Polygonum cuspidatum* was 1.6 mg/g.

The [Bmim]Br was hydrophilic ionic liquid, which had a strong interaction with *trans*-resveratrol. The *trans*-resveratrol is a nonpolar compound that insoluble in water, but IL solution would be lower the surface tension of water and make it easier to penetrate the membrane to extract these compound. Therefore, the [Bmim]Br had good extraction efficiency to obtain *trans*-resveratrol. The alkalinity level of cation (1-butyl-3-methylimidazolium) influenced by anion that binds through hydrogen bonds. Anion bromide (Br^-) can make the ionic solution slightly acidity than another anion. The weak acid ionic solution gives an advantage for extract *trans*-resveratrol.[17] The efficiency of extraction also influenced by long-chain alkyl cations.[14]

The concentration optimum of [Bmim]Br solution was 2.5 M, that was appropriate concentration to make ionic liquid easier to disrupt membrane structure, and then the target compound in the cell membrane can be dissolved in the solvent. The efficiency extraction increased with the concentration of solvent enhancement. However, when the concentration of [Bmim]Br solution over enhances, the viscosity of solution also increased. Thus, the efficiency extraction was affected and the extraction rate would be decreased.[16] The liquid/solid ratio influence efficiency extraction because the more solvent is added will make it easier for the active components to be extracted.[9] The optimum liquid/solid ratio was 15 mL/g.

The higher microwave power may be effective to extract compounds from plant materials using MAE in a short time. MAE directly heat solvent and sample, thus resulted in rupture of the cell membrane and

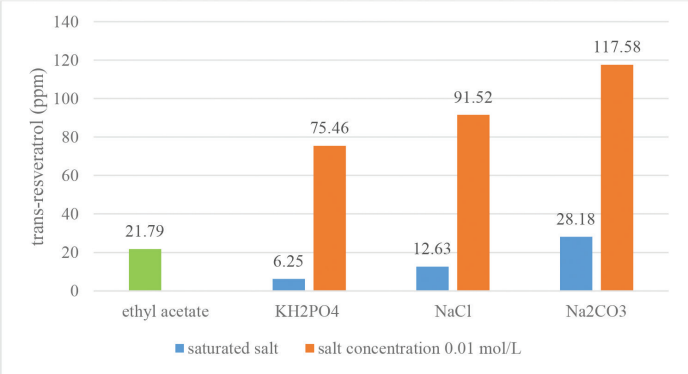


Figure 5: Effect of *trans*-resveratrol using various salt with ethyl acetate for back extraction.

release intracellular product into the solvent without damage. The longer extraction time with a high power can cause damage to the active compound because of the heat, that might result in the isomerization of *trans*-resveratrol in the extract.[25] Accordingly, the optimum power and extraction time in this study was 10% and 10 minutes.

One of the methods for separated active compound and recycling of ionic liquid was induced phase separation with the salting-out process. IL aqueous solution with the addition of cosmotropic salt can form aqueous biphasic systems (ABS) that induce the phase separation, that is ionic phase and salt phase. Organic solvent extraction methods also can perform to extract various compounds from IL.[10] In the experimental, the Na_2CO_3 can not form ABS with aqueous [Bmim]Br solution. But, the salt would help to precipitate residue in the IL solution, which makes *trans*-resveratrol more easy to migration into an organic solvent (liquid-liquid back extraction).

Some organic compounds could be extracted with an organic solvent from IL. The organic solvent must immiscible with water, but quite polar to extract most of the compounds.[26] Ethyl acetate was chosen because they have hydrogen bonding interaction with *trans*-resveratrol and water molecules, resulting in the trans-resveratrol and water migrating to the organic solvent from the ionic liquid.[15] The miscibility of IL with water also had a positive influence on recovery analytes and the possibility to regenerate IL by evaporation organic solvent.[26]

CONCLUSION

Ionic liquid based MAE was a simple, rapid, effective, and used to be alternative for extraction method of *trans*-resveratrol from melinjo seeds. The optimum condition was ionic liquid [Bmim]Br concentration 2.5 mol/L; liquid/solid melinjo seeds powder ratio 15 mL/g; microwave power 10%; and extraction time 10 min with extraction value of *trans*-resveratrol was 1.34 mg/g (0.134%). The ABS-back extraction with 0.01 mol/L Na_2CO_3 and ethyl acetate were used to separate trans-resveratrol from IL solution.

ACKNOWLEDGMENT

This work was financially supported by Directorate Research and Community Engagement, Universitas Indonesia via Hibah PITTA 2016.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ABBREVIATION USED

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

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Article History: Submission Date : 31-06-2017 ; Revised Date : 18-07-2017; Acceptance Date : 27-08-2017.
Cite this article: Ayuningtyas IN, Rahmawati M, Sutriyo, Mun'im A. Optimization of Ionic Liquid-Based Microwave Assisted Extraction to Obtain *Trans*-resveratrol from *Gnetum gnemon* L. Seeds. J Young Pharm. 2017;9(4):457-62.