ABSTRACT

Objective: Melinjo (Gnetum gnemon L) seeds are known to contain resveratrol, which are classified as a phenolic compound of the stilbenoid. Melinjo seeds have high water content, making them unstable to be stored for prolonged periods with open packaging at room temperature. The present study aimed to explore the use of ultrasonic-assisted extraction with a betaine-based natural deep eutectic solvent (NADES) for resveratrol extraction from irradiated melinjo seeds.

Methods: The best betaine-based NADES component was identified among betaine-urea, betaine-lactic acid, and betaine-malic acid. Optimization of extraction methods was performed using the best NADES and extraction variables such as time of extraction, water percentage, and sample:solvent ratio. The outcome of extraction was evaluated by measuring resveratrol content using high-performance liquid chromatography and the results were analyzed using response surface methodology.

Results: The best betaine-based NADES was found to be betaine-lactic acid, yielding a resveratrol content of 0.3344 mg/g powder. The optimum extraction was achieved in 10 min with 60% water and a sample:solvent ratio of 1:10, yielding a resveratrol content of 0.227 mg/g powder.

Conclusion: Betaine-based NADES can be purposed as an alternative solvent for resveratrol extraction from irradiated melinjo seeds.

Keywords: Gnetum gnemon, Resveratrol, Natural deep eutectic solvent, Response surface methodology.
high-performance liquid chromatography (HPLC) grade (Merck, Germany), acetonitrile HPLC grade (Merck, Germany), and resveratrol standard (Wako, Japan) were used in the present study.

**Preparation of materials**
Melinjo seeds with dry brown skin were sorted to remove extraneous material. The seeds were dried in a drying cabinet followed by dry sorting to separate remaining contaminants. Seeds weighing 0.5 kg were placed in a plastic container, which were tightly closed to be irradiated at a dose of 5 kGy. The irradiation source used was cobalt 60 (60Co). The irradiation process was carried out at the Pusat Aplikasi Teknologi Isotop dan Radiasi, Pasar Jumat, South Jakarta. After the melinjo seeds were irradiated, they were ground until a fine powder was obtained.

**Preparation of NADES**
The NADES solvent used was a betaine base with combinations of four components; betaine: urea (1:1) [9,12,14], betaine: lactic acid (1:1) [12,15], and betaine: malic acid (1:1) [9,12]. There are three methods of preparation of NADES solvents, comprising heating and stirring methods, evaporation methods, and freeze-drying methods [16]. We used the heating method. First, betaine and its component mixtures were weighed. Second, the mixtures were stirred on a hotplate stirrer at 90°C and 500 rpm for 90 min. Third, water was added to dissolve the betaine in the mixture and the mixture was stirred again on the hotplate stirrer. Stable NADES was characterized by the formation of clear solutions. Finally, the solutions were stored in tightly closed vials.

**Extraction**
Melinjo seed extraction was performed using the UAE method. Melinjo seed powder (0.5 g) was dissolved with NADES at a different sample: solvent ratio. The sample solutions were extracted with an ultrasound apparatus and centrifuged at 4500 rpm for 17 min [12]. The obtained liquid extract was then filtered using filter paper and the volume was adjusted using water in a flask of suitable volume.

**Identification of the best betaine-based NADES components**
Screening to identify the best betaine NADES components was done using HPLC. Extracts made using different NADES preparations (betaine-urea, betaine-lactic acid, and betaine-malic acid) were injected into an HPLC. The area under the curve values of the chromatograms were plotted using linear regression to obtain the resveratrol content of each test sample. The sample with the highest resveratrol level was then used for determination of the optimum extraction conditions. A Shimadzu LC-20AT HPLC was used at a wavelength of 306 nm. The flow rate was 1.0 mL/min and the injected volume was 20 μL. The mobile phase used was aqua pro injection:acetonitrile (75:25 v/v) at pH 3, regulated with the addition of 0.01% acetic acid. The detector was used as a ultraviolet-visible detector and the column used was Inertsil 150 mm × 4.6 mm; 5 μm [17].

**Optimization of extraction conditions**
The experimental design for optimization of the extraction conditions was determined using RSM with Design Expert version 10.0 software and Box-Behnken as study design. The determination of optimal UAE was obtained by assessing resveratrol content from all samples. Data were entered into the software, which provided recommendations regarding the appropriate mathematical models. The recommended model was selected and further analyzed using ANOVA to determine the impact of each variable. The optimum level of each variable factor was determined on the basis of the desirability index, which has a value of 0–1. A desirability index value approaching one indicates that the study design is closer to the optimum condition. The optimum condition was demonstrated using a three-dimensional surface response graph.

The effect of temperature extraction on resveratrol level
The test was performed by extracting samples at optimum extraction conditions at different temperatures. The first sample was extracted using an ultrasonicator without heating, whereas the second sample was extracted using an ultrasonicator with heating. The extracts obtained from both samples were then analyzed using HPLC and the resveratrol content of each sample was calculated. The resveratrol level was then compared to determine the effect of the extraction temperature.

**Comparison of the optimized method with the conventional extraction method**
The results of the optimized extraction conditions were compared with other extraction methods to verify whether the optimized method was more effective in extracting resveratrol from irradiated melinjo seeds. The resveratrol level obtained through the RSM method yielding the highest content was compared with that obtained through extraction using other conventional extraction methods such as maceration and reflux.

**RESULTS**

**Screening of the best betaine-based NADES components**
The betaine-lactic acid NADES solvent was found to be the best NADES component, yielding a resveratrol content of 0.3344 mg/g powder (Fig. 1).

**Optimization of extraction conditions**
The best extraction condition based on the results of data processing using Design Expert 10.0 Box-Behnken design was run 6, which used 10 min of extraction time, 60% water, and a sample: solvent ratio of 1:10, yielding a resveratrol content of 0.227 mg/g powder (Table 1).

**Effect of temperature**
Tests on samples extracted without heating resulted in an average resveratrol level of 0.111 mg/g powder. On the other hand, samples extracted by heating resulted in average resveratrol level of 0.153 mg/g powder (Fig. 2).

**Comparison of extraction results with conventional extraction methods**
Tests on sample run 6 resulted in average resveratrol content of 0.227 mg/g powder. The maceration sample yielded an average resveratrol content of 0.026 mg/g powder (Fig. 3).

**DISCUSSION**

Identification of the best betaine-based NADES components
Screening of betaine-based NADES components was done to select the NADES component yielding the highest resveratrol content. The comparison of each NADES used for extraction is shown in Fig. 1.

The results showed that betaine-lactic acid as an NADES component can extract phenolic compounds more effectively than betaine-urea and betaine-malic acid. The synthesis of NADES is easier and more effective in extracting resveratrol from irradiated melinjo seeds. The interreaction between betaine and

| Component NADES | Resveratrol Content (mg/g) |
|-----------------|----------------------------|
| Betaine-Urea    |                             |
| Betaine-Lactic Acid |                       |
| Betaine-Malic Acid |                         |

**Fig. 1: Screening of betaine-based natural deep eutectic solvent components**
resveratrol cannot be explained on the basis of the findings of the present study, and there are no supporting studies reporting such an interaction. Further, specific analysis such as molecular simulation analysis using heteronuclear Overhauser spectroscopy or nuclear Overhauser spectroscopy is needed [5]. However, NADES has a significant ability to extract phenolic compounds, which is associated through the hydrogen-bonding interactions formed between phenolic compounds and NADES molecules [15]. Resveratrol is also a phenolic compound, and it is predicted that reactions occurring between NADES and resveratrol are also associated with hydrogen-bonding interactions.

### Optimization of extraction conditions

Determination of the optimum extraction conditions using the UAE method was done on the basis of resveratrol yield data shown in Table 1.

On the basis of the results of data processing using Design-Expert software, a recommended research model was obtained. The recommended research model was Quadratic versus 2FI. This model was being recommended because it has a value of 0.0252. The "prob >F" <0.05 indicates significant influence, whereas a value >0.05 indicates an insignificant effect [18]. In addition, this research model has an insignificant "lack of fit" value of 0.4360.

The research model recommendation was then analyzed using ANOVA with the aim to assess the significance of the model. In addition, it was also used to determine the probability of interaction between the research model and its variables. The ANOVA results showed that the value of "prob >F" obtained from the recommended research model was 0.0020. This indicated that the extraction conditions had a significant influence on resveratrol yield. The "prob >F" values of the three factors extraction time, water percentage, and sample: solvent ratio also had a significant effect on the resveratrol yield. The "prob >F" of extraction time was 0.0188 that of water percentage was 0.0006 and that of sample: solvent ratio was 0.0003. A precise extraction time was required to ensure optimal extraction. The longer the extraction time, the better the extraction of polyphenol produced. Water addition to the NADES eutectic solution can reduce the viscosity of NADES. However, a high amount of water can also result in interactions between NADES and target compounds and improve polarity of extraction media; thus, water percentage can affect resveratrol yield. The sample: solvent ratio has been studied previously to improve extraction efficiency and reduce solvent usage. A high sample: solvent ratio did not show any significant improvement compared with a smaller sample: solvent ratio [15].

The RSM equation obtained for optimization of extraction conditions for resveratrol yield was as follows:

\[
\text{Resveratrol } (y) = -5.125 \times 10^{-3} A^2 + 0.011 B^2 - 0.019 C^2 - 0.250 \times 10^{-3} AB - 3.000 \times 10^{-3} AC + 6.500 \times 10^{-3} BC + 0.011 A + 0.025 B - 0.030 C + 0.18
\]

A = Extraction time  
B = Water percentage  
C = Sample: solvent ratio

The equation shows that the resveratrol yield increases with an increase in extraction time, water percentage, and interaction between water percentage and sample: solvent ratio. This is indicated by a positive constant value in the RSM equation obtained. The resveratrol level decreases with an increasing sample: solvent ratio, interaction between...
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extraction time and water addition percentage, and interaction between extraction time and sample: solvent ratio. This is indicated by a negative constant value in the RSM equation obtained.

On the basis of the results of ANOVA, an optimum experimental factor was obtained. This optimum point was determined on the basis of the desirability index with a value between 0 and 1. Therefore, the desirability index indicates the optimum conditions. The optimized research model is shown in Table 2.

In the optimization of the research method, resveratrol yield was used as the criterion to determine the best extraction conditions. On the basis of the research model, 23 kinds of optimum extraction conditions were recommended and can be seen in Table 3.

The research models obtained had the highest desirability index of 0.988 and the lowest desirability index of 0.746. The model with desirability index 0.988 (run 6) was compared with resveratrol standard, and the chromatogram profile is shown in Fig. 4.

The surface response graph can be presented in three-dimensional form with different color gradations. However, the three-dimensional graphs produced in the present study were not optimal because the optimum point of the response of resveratrol level obtained did not lie in the curvature of the graph after the increase in response and before the decrease in response. The graphs are shown in Fig. 5.

Effect of temperature on resveratrol extraction

The effect of temperature on the optimum extraction conditions using RSM was then assessed. This was done by comparing two samples extracted using optimum RSM conditions, with one extracted using UAE with heating and the other one using UAE without heating. The extraction levels were then evaluated using HPLC and calculated by entering the area under the curve value obtained from the chromatograms into the existing regression equation. Each sample was tested three times and the result is shown in Fig. 2.

The level of resveratrol extracted at higher temperature was different compared with that at lower temperature. Increasing the extraction temperature can increase desorption and dissolution of secondary metabolites into the extraction solvents [15]. Thus, it can be concluded that the extraction temperature had an effect on the level of resveratrol extracted.

Comparison with conventional extraction methods

Extraction from irradiated melinjo seeds using NADES lactic acid with the optimum extraction method (run 6) yielded a higher resveratrol content compared with conventional maceration extraction methods using 70% ethanol. The result is shown in Fig. 3.

On the basis of these results, the use of NADES for resveratrol extraction from melinjo seeds was better than the use of conventional maceration extraction methods.
Fig. 5: Three-dimensional response surface graphs evaluating interaction between pairs of factors (a) water percentage: extraction time; (b) sample: solvent ratio: extraction time; and (c) sample: solvent ratio: water percentage
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Table 3: Optimum extraction conditions

| No. | Extraction time (min) | Water percentage (%) | Sample: solvent ratio (g/mL) | Resveratrol level (mg/g) | Desirability index |
|-----|----------------------|----------------------|------------------------------|-------------------------|--------------------|
| 1   | 13.37                | 60.00                | 13.36                        | 0.226                   | 0.988              |
| 2   | 13.32                | 60.00                | 13.36                        | 0.226                   | 0.988              |
| 3   | 13.29                | 60.00                | 13.26                        | 0.225                   | 0.988              |
| 4   | 13.21                | 60.00                | 13.48                        | 0.225                   | 0.988              |
| 5   | 13.58                | 60.00                | 13.54                        | 0.225                   | 0.988              |
| 6   | 13.30                | 60.00                | 13.07                        | 0.225                   | 0.988              |
| 7   | 13.64                | 60.00                | 13.45                        | 0.225                   | 0.988              |
| 8   | 12.72                | 60.00                | 12.96                        | 0.225                   | 0.987              |
| 9   | 14.31                | 60.00                | 12.93                        | 0.225                   | 0.986              |
| 10  | 14.41                | 60.00                | 13.43                        | 0.225                   | 0.986              |
| 11  | 12.24                | 60.00                | 13.57                        | 0.225                   | 0.986              |
| 12  | 14.13                | 59.95                | 13.27                        | 0.225                   | 0.985              |
| 13  | 14.72                | 60.00                | 13.12                        | 0.225                   | 0.985              |
| 14  | 11.93                | 60.00                | 14.00                        | 0.225                   | 0.984              |
| 15  | 14.90                | 60.00                | 13.06                        | 0.225                   | 0.984              |
| 16  | 11.53                | 60.00                | 13.65                        | 0.225                   | 0.982              |
| 17  | 14.92                | 60.00                | 14.24                        | 0.225                   | 0.982              |
| 18  | 14.47                | 60.00                | 11.61                        | 0.225                   | 0.982              |
| 19  | 11.15                | 60.00                | 14.55                        | 0.224                   | 0.979              |
| 20  | 14.99                | 60.00                | 10.96                        | 0.224                   | 0.976              |
| 21  | 13.79                | 60.00                | 10.02                        | 0.223                   | 0.971              |
| 22  | 15.00                | 60.00                | 20.68                        | 0.214                   | 0.895              |
| 23  | 15.00                | 40.00                | 13.04                        | 0.196                   | 0.746              |

CONCLUSION

The most effective betaine-based NADES component was found to be betain-lactic acid, yielding a resveratrol level of 0.334 mg/g powder. The optimal conditions for resveratrol extraction from irradiated melinjo seeds with optimum UAE were a time of extraction of 10 min, 60% water, and a sample:solvent ratio of 1:10, yielding a resveratrol level of 0.227 mg/g powder. It can be concluded that betaine-based NADES can be purposed as an alternative solvent for resveratrol extraction from irradiated melinjo seeds.

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CONFLICTS OF INTERESTS

The author has no conflicts of interest to declare.

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