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ORIGINAL ARTICLE

Honey as a Solvent for the Green Extraction, Analysis, and Bioconversion of Daidzin from *Pueraria candollei* var. *mirifica* Root

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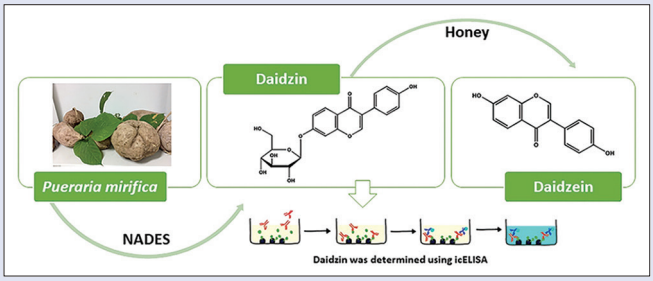
ABSTRACT

Background: Honey has been widely used as a traditional vehicle of herbal medicines. Honey behaves as a natural deep eutectic solvent (NADES) containing β-glucosidase; therefore, it can be used for the extraction and bio-activation of the bioactive compounds of herbs. **Objectives:** This study aims to apply honey (H-NADES) and a sugar-based NADES (S-NADES) for the extraction, analysis, and bioconversion of daidzin from *Pueraria candollei* var. *mirifica* (PM) root. **Materials and Methods:** Various concentrations of H-NADES and S-NADES (water:sucrose:glucose: fructose, 18:3:18:22 by weight) were used as solvents for extraction. Indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed and validated for monitoring the extraction efficacy. The catalytic reactivity against daidzin of β-glucosidase purified from honey was investigated. **Results:** Using NADESs as solvents, icELISA was suitable for the reliable determination of daidzin with high sensitivity (1.95–125 ng/mL), specificity (% cross-reactivity ≤ 2.60), and accuracy (98.3-106% daidzin recovery). Daidzin at a concentration of 75.8 ± 3.67 μg/mL was extracted using 50% (v/v) S-NADES, which was the most effective for the extraction compared to H-NADES, water and ethanol. In addition, daidzin was converted to daidzein by honey β-glucosidase. **Conclusion:** Both S-NADES and H-NADES were useful for the extraction, analysis, and bioconversion of daidzin, and β-glucosidase from honey might enhance the oestrogenic activity and bioavailability of PM phytochemicals.

Key words: Enzyme-linked immunosorbent assay, daidzein, daidzin, honey, *Pueraria mirifica*

SUMMARY

- The NADES able to extract the daidzin from PM that S-NADES has more effective than H-NADES.
- The icELISA appropriate for daidzin determination in NADES system
- The β-glucosidase from honey presented as catalytic for bioconversion from daidzin to daidzein.



Abbreviation used: NADES: Natural deep eutectic solvent; H-NADES: Honey-based NADES; S-NADES: Sugar-based NADES; PM: *Pueraria mirifica*; icELISA: Indirect competitive enzyme-linked immunosorbent; mAb: Monoclonal antibodies; cOVA: Cationized ovalbumin; HPLC-UV: High-performance liquid; chromatography-UV/Visible detectors; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS: Phosphate-buffered saline; PBS-T: Phosphate-buffered saline plus 0.05% Tween 20.

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INTRODUCTION

Honey is a unique and beneficial product containing complex chemicals, of which fructose and glucose are the main constituents.[1] In addition, minor components such as proteins, amino acids, organic acids, carotenoids, vitamins, minerals, Maillard reaction products, and aromatic substances have been reported.[1,2] In many countries, honey has been used for a long time in traditional medicines as an excipient and medium for the various recipes of herbs.[3] Honey is an important ingredient utilized for the traditional pill preparations of herbal recipes. The chemical character of honey places it in a class of natural deep eutectic solvents (NADESs), which have been shown to be useful solvents for the extraction because the solvent can solubilize both polar and nonpolar compounds. Therefore, honey may be used for the extraction of phytochemicals. Moreover, honey may

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affect the absorption, pharmacological activity, and bioavailability of phytochemicals. β-glucosidase is a honey component, and the enzyme can cleave glycoside compounds into aglycone forms.[4] Moreover, β-glucosidase-containing honey might increase the activity and bioavailability of daidzin by converting it to daidzein.[5] Therefore, using honey as a solvent for the extraction has the advantage of simultaneously facilitating the bioconversion of a specific target. Therefore, the extraction process using honey represents an effective green process that can activate the pharmacological activity of extracted substances.

Pueraria candollei var. *mirifica* (Airy Shaw and Suvat.) Niyomdham (PM) has been used in health products for its rejuvenating qualities. In Thai traditional medicine, PM has been prepared as honey-based traditional pills. According to clinical trials,[6,7] PM has been indicated to treat estrogen deficiency symptoms because the extract of its tuberous root exhibits oestrogenic activity. Among the bioactive compounds in PM is the isoflavonoid glycoside daidzin. The aglycone form of daidzin is daidzein, which is more hydrophobic and bioavailable than daidzin and has higher oestrogenic activity. Therefore, honey might influence the chemical profile and pharmacological activity of PM. Phytochemical standardization is the basic approach for chemical quality control of PM extract. Many methods have been developed to analyze the bioactive substances of PM. Although liquid chromatography with UV detection was reported as a sensitive method for daidzin analysis,[8-10] chromatographic techniques have suffered due to high organic solvent consumption. In this study, a green analytical method based on the indirect competitive enzyme-linked immunosorbent assay (icELISA) procedure was developed to overcome the analytical problems. We developed NADESs based on honey (H-NADES) and sugar (S-NADES) to extract daidzin from PM root. The icELISA was validated for accuracy, precision, and specificity for daidzin determination. In addition, the honey proteins were purified and then reacted with daidzin to evaluate the bioactivation potential. The processes of extraction, analysis, and bioconversion exemplified the strong potential of NADES as a green approach for PM extraction, quality control, and activation.

MATERIALS AND METHODS

Chemical and immunological reagents

Daidzin (≥99%), daidzein (≥97%), genistin (≥99%), and genistein (≥95%) were purchased from Fujicco Co., Ltd. (Kobe, Japan). s-Equol (≥98%) was provided by Daicel Corporation (Tokyo, Japan). Coumestrol (≥95%) and 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma-Aldrich (Steinheim, Germany). Miroestrol (≥90%) and Kwakhurin (≥90%) were extracted and purified from the root cortex of PM using a previously described method.[11] Glucose, fructose, sucrose, citric acid, sodium bicarbonate, sodium carbonate anhydrous, and sodium chloride were purchased from Ajax Finechem Laboratory Chemicals (NSW, Australia). Anti-mouse IgG secondary antibody (goat, horseradish peroxidase-conjugated form) was purchased from Abcam (Cambridge, UK). Monoclonal antibodies against daidzin (anti-daidzin mAb) and daidzin-cationized ovalbumin (daidzin-cOVA) conjugates were obtained from the previous research.[12] Skim milk and Tris-free base were supplied by Himedia Laboratories (India). Ethanol, acetonitrile, disodium hydrogen phosphate, and glacial acetic acid (analytical reagent grade) were supplied by RCI Labscan Limited (Bangkok, Thailand). Honey (Chitlada) was a commercial food product of the Royal Project. Other chemical and immunological reagents were commercial products of analytical grade.

Plant samples and extraction

PM roots were provided by DOD Biotech Public Company Limited (Samut Sakhon, Thailand). Six-year-old PM was cultivated by farmers in the area of Suphan Buri Province of Thailand. The roots were collected and sliced before drying under sunlight. The samples were additionally dried in a hot air oven at 60°C for 2 days before grinding into a fine powder. The efficacy of daidzin extraction using H-NADESs and S-NADESs was investigated using the following procedure. Ethanol, water, and their mixtures were used as the standard solvents. PM powder (150 mg) was extracted with each NADES (1.5 mL) via sonication-assisted extraction (37 Hz) for 1 and 3 h at ambient temperature. Then, centrifugation at 14,000 rpm for 10 min was performed to collect the supernatant extract. The extraction procedures were performed in triplicate. The concentration of daidzin was determined by icELISA, which was developed and validated using NADESs as the matrix. Moreover, the chemical profiles of the selected extract were investigated via high-performance liquid chromatography ultraviolet (HPLC-UV).

Development and validation of indirect competitive enzyme-linked immunosorbent assay

Initially, the effects of NADESs on anti-daidzin mAb binding against the daidzin-cOVA conjugate and free daidzin were determined. Previously, quantitative icELISA was developed and validated using ethanol as a solvent.[12] In this study, H-NADES and S-NADES were used as extraction solvents. Thus, the effect of NADESs on icELISA performance was evaluated to ensure that PM extracts prepared by NADESs can be analyzed directly. The effect of NADESs on the analytical performance (including sensitivity, specificity, accuracy, and precision) was then investigated to address the reliability of icELISA for daidzin quantitative analysis. In brief, the 96-well plate surface was coated with daidzin-cOVA conjugate and treated with skim milk protein. Next, the step of competitive binding of anti-daidzin mAb against immobilized daidzin-cOVA and free daidzin was conducted. Then, the steps of secondary antibody incubation and color development through substrate solution were successively performed. The absorbance values obtained were plotted versus the authentic daidzin concentrations. The linear range of the inhibitory effect through daidzin was set as the concentration range of the calibration curve. The half-maximal inhibitory concentration (IC_{50}) obtained by the inhibitory curve was set as the determinant of sensitivity. Then specificity and precision of icELISA were examined. The accuracy of icELISA was determined using a daidzin recovery experiment. Concentrations of daidzin (6.25–50 μg/mL) were spiked into solutions of PM extracts (20 mg/mL) dissolved in 70% (v/v) H-NADES and 50% (v/v) S-NADES. Then, the total daidzin concentrations of all nonspiked (NS) and spiked (SS) extracts were analysed using icELISA. The recoveries (%) of daidzin spiked into the samples were calculated using the following Eq. (1):

$$\text{Recovery}(\%) = \frac{\text{total daidzin concentration in SS} - \text{total daidzin concentration in NS}}{\text{the spiked concentration of daidzin}} \times 100 \quad (1)$$

Purification of proteins from honey and their hydrolysis activity

The honey (250 mL) was dialyzed against 50 mM Tris-HCl buffer pH 7 containing 5% (v/v) glycerol (4°C for 24 h), and a dialysis membrane (molecular weight cutoff point 6000 Da, Membrane Filtration Products, Inc., TX, USA) was used. The protein residue of honey retained inside the dialysis membrane was collected. All obtained proteins were subjected to anion exchange resin (TOYOPEARL DEAE-650, 20 mL), which was initially equilibrated with 50 mM Tris-HCl buffer pH 7. Unbound protein was washed out using the same buffer. Next, the bound protein was eluted with a gradient concentration of NaCl (20

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to 500 mM in 50 mM Tris-HCl buffer pH 7, 100 mL of each). The β-glucosidase activity of each fraction was determined. The fractions exhibiting β-glucosidase activity were combined and concentrated for further purification using gel filtration chromatography. An ÄKTA Pure 25M liquid chromatography system (GE Healthcare, Uppsala, Sweden) and UNICORN software were used for size exclusion chromatography. The mobile phase was 150 mM NaCl in 50 mM Tris-HCl pH 7, and the flow rate was set at 0.8 mL/min for column equilibration (125 mL). The sample (100 µL) was then injected into a Superdex™ 75 10/300 GL size-exclusion column. The same buffer flowed (0.01 mL/min) to fractionate the proteins. The 0.5 mL fraction of the eluted protein solution was collected when the absorbance at 280 nm was over five mAU. These fractions were reacted with daidzin to reveal the β-glucosidase activity.

The hydrolysis reaction of daidzin with purified β-glucosidase from honey

Daidzin was used as the substrate, which was reacted with the purified proteins of honey. Daidzin solution (2.5 mM, 200 µL) was reacted with 800 µL protein fractions diluted (10-fold) in 50 mM citrate-phosphate buffer, pH 5. The reaction was performed by orbital shaking (80 rpm) of the solution at the room temperature for 12 h. In the control, daidzin was incubated with 50 mM citrate-phosphate buffer, pH 5. Next, to stop the reaction, the sample solutions (100 µL) were added to a mixture of 50 mM sodium carbonate buffer, pH 9.6 (300 mL), and ethanol (200 mL). The concentrations of daidzin and daidzein in the samples were then analyzed using HPLC-UV. The analytical method is detailed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The protein fraction of honey, including crude protein after dialysis, protein fractions obtained after anion exchange, and the proteins fractionated using gel filtration, were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were separated by 10% (w/v) SDS-PAGE under reducing conditions and then stained with Coomassie Brilliant Blue reagent (Loba Chemie Pvt. Ltd.). The molecular masses of the proteins in each fraction were estimated according to a standard protein marker (Enzmart Biotech, Thailand).

Statistical analysis

All experiments were performed in triplicate, and the values are presented as the mean ± standard deviation. Significant differences were analyzed using the one-way analysis of variance ($P < 0.05$) followed by Tukey's honestly significant difference test to determine the differences between the two groups of the study.

RESULTS

Method development and validation of indirect competitive enzyme-linked immunosorbent assay for daidzin determination in a natural deep eutectic solvent matrix

To evaluate the effect of NADES on anti-daidzin mAb reactivity against the daidzin-cOVA conjugate (2.5 µg/mL), each NADES was diluted, and then, they were incubated with anti-daidzin mAb (0.2 µg/mL). The low dilution rate of NADESs interfered with binding between anti-daidzin mAb and the daidzin-cOVA conjugate. The binding was significantly suppressed ($P < 0.05$) when using ≤ 1280-fold and ≤ 320-fold dilutions of H-NADES and S-NADES, respectively because the viscosity of H-NADES was higher than that of S-NADES. The higher water content with a lower viscosity reduced the suppression of binding between anti-daidzin mAb and the daidzin-cOVA conjugate. Therefore, these effects should be diminished by high dilution during the quantitative analysis of daidzin using icELISA. However, high dilution may result in insufficient concentrations of daidzin in the working solution for determination via the assay. The 1000-fold diluted H-NADES and S-NADES were selected as solvents for the preparation of the authentic daidzin and sample solution, and then, the effects of NADESs on binding between anti-daidzin mAb and free daidzin were determined.

Using icELISA, the reactivity of anti-daidzin mAb toward daidzin was evaluated. Standard daidzin was prepared in various concentrations. The results showed that anti-daidzin mAb reacted in a concentration-dependence of daidzin prepared in PBS-T, H-NADES, and S-NADES. The icELISA signal (A/Ao), Ao and A (the absorbance in the absence and presence of daidzin, respectively) were plotted against the

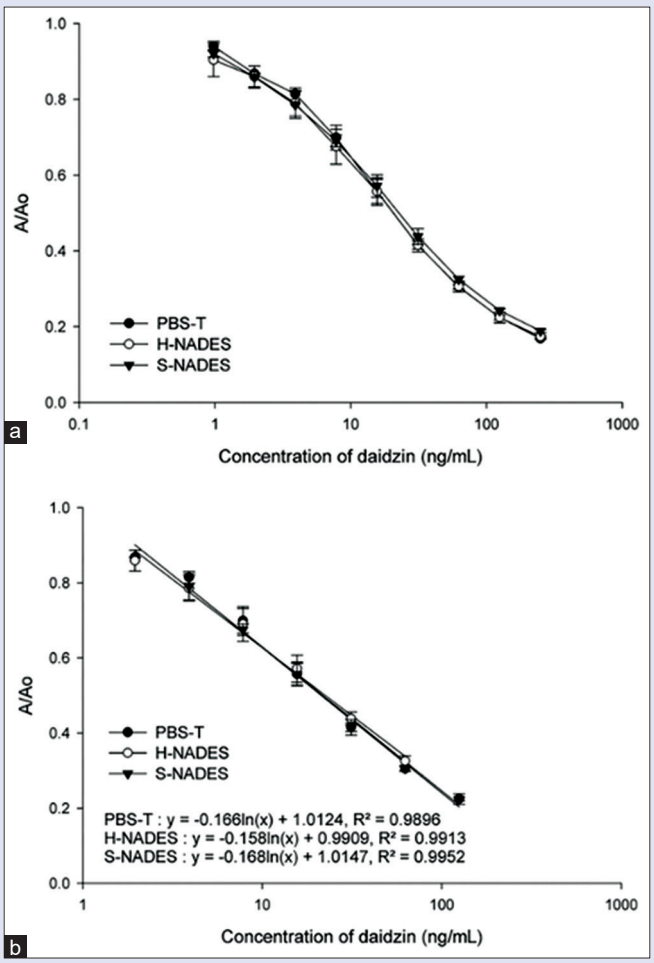

Figure 1: The inhibitory curve (a) of daidzin prepared in solvents of PBS-T, H-NADES and S-NADES and their calibration curves (b) by icELISA. With fixed concentrations of immobilized cationized ovalbumin-daidzin (2.5 µg/mL) and anti-daidzin mAb (0.2 µg/mL), the absorbance of icELISA reflects the inhibitory effect of free daidzin. A_0 and A are the absorbance values in the absence and presence of daidzin, respectively. The linearity of determination was 1.95–62.5 ng/mL for H-NADES and 3.90–125 ng/mL for S-NADES, for which standard deviations ($n = 3$) were indicated as error bars. icELISA: Indirect competitive enzyme-linked immunosorbent assay, S-NADES: Sugar-based natural deep eutectic solvent, H-NADES: Honey natural deep eutectic solvent

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concentrations of daidzin. When icELISA signals were compared within each concentration among the three solvents, the results showed that the signals were not significantly different [$P < 0.05$, Figure 1a]. Therefore, the 1000-fold-diluted H-NADES and S-NADES did not interfere with the binding between anti-daidzin mAb and free daidzin. The diluted NADES (1000-fold or more) is applicable as a working solvent for the quantitative analysis of daidzin by icELISA. The linearities of daidzin determination with PBS-T, H-NADES, and S-NADES are shown in Figure 1b. The linear ranges of daidzin determination using icELISA were 1.95-62.5 ng/mL ($R^2 = 0.9913$) and 3.91-125 ng/mL ($R^2 = 0.9952$) when daidzin solutions were prepared in 1000-fold diluted H-NADES and S-NADES, respectively. The coefficients of variation (CV) of the icELISA signal were <6%.

Cross-reactivity experiments revealed the binding specificity of anti-daidzin mAb prepared in diluted NADESs. For H-NADES, the IC_{50} values of daidzin and daidzein were 25.9 and 996 ng/mL, respectively; thus, the percentage of CR of daidzein was 2.60%. Other compounds, including genistin, genistein, miroestrol, kwakhurin, s-equol and coumestrol, which are found in PM root, have IC_{50} values greater than 15 μg/mL. For S-NADES, the IC_{50} values of daidzin and daidzein were 20.7 ng/mL and 1.13 μg/mL, respectively, and the %CR of daidzein was 1.84%. Other compounds investigated showed an IC_{50} of >15 μg/mL. The recovery experiment, which reflected the accuracy of icELISA, was examined, and the results are shown in Table 1. Both H-NADES and S-NADES were used as solvents to evaluate the accuracy. PM extract (20 mg/mL) prepared in 70% (v/v) H-NADES and 50% (v/v) S-NADES produced daidzin concentrations of 24.4 and 40.2 μg/mL, respectively. The results indicated that daidzin is more soluble in 50% (v/v) S-NADES than in 70% (v/v) H-NADES. The extract solutions of H-NADES and S-NADES were spiked with 6.25-50.0 μg/mL authentic daidzin. Then, the total concentrations of daidzin in the spiked extracts were determined. The percentage of daidzin recoveries was in the ranges of 99.7%–101% and 98.3%–106% when extract solutions were prepared in H-NADES and S-NADES, respectively. The CV of daidzin determination in these sample extracts reached a maximum of 9.98%.

Extraction of daidzin using natural deep eutectic solvents

The water content added to the NADES affected the extractability. The high viscosity results in slow mass transfer.[13] In this study, 10%–30% (v/v) H-NADES and 10%–20% (v/v) S-NADES gave a high content of daidzin after the 1st h of extraction. The higher concentration of NADESs with a longer extraction improved the extractive yield of daidzin. In this study, S-NADES containing sucrose, fructose, and glucose in the same amounts of honey sugars was used to compare the extraction efficiency. Sugar-based NADESs are simple, inexpensive, and edible. Daidzin was extractable using H-NADES and S-NADES in various concentrations from 10% to 80% (v/v) in water [Table 2]. With 1 and 3 h of extraction using sonication assistance, the results showed that each concentration of S-NADES yielded a higher extraction of daidzin concentration than H-NADES with the same water content ratio. The S-NADES produced an extract of PM with daidzin concentrations of 31.6–75.8 μg/mL, and H-NADES extracted daidzin with concentrations of 4.15–41.8 μg/mL. The solvent with 10% (v/v) H-NADES produced the highest concentration of daidzin in the 1st h of extraction. A reduced content of water (30%–80% H-NADES) decreased the extraction efficacy. This result was attributed to optimal NADES viscosity. The extraction capacity of H-NADES is higher than that of water, but it is less than that of 50% (v/v) ethanol. During the 1st h of extraction using 30% (v/v) S-NADES as the solvent, it produced the highest concentration of daidzin; additionally, 20%–40% (v/v) S-NADES yielded a higher daidzin concentration than water, 50% (v/v) ethanol, 80% (v/v) ethanol and absolute ethanol, which were used as conventional solvents. When the extraction process was extended to 3 h, S-NADES afforded higher daidzin content than extraction for 1 h, except in the case of 10%–20% (v/v) S-NADES. The 50% (v/v) S-NADES gave the highest daidzin concentration (75.8 μg/mL), which was higher than that of all the control solvents. After 3 h of extraction, the daidzin concentrations extracted by H-NADES (40%–80%) were higher than those obtained by 1 h of extraction. The higher-viscosity H-NADESs required a longer duration for penetration and extraction. The longer duration of extraction decreased the concentration of daidzin in the extract using 10%–20% S-NADES and 10%–30% (v/v) H-NADES; the reasons have not yet been investigated. This outcome might result from the saturation and precipitation of daidzin. These NADESs had a high water content, within which the solubility of daidzin may be limited. The longer extraction time improved the extraction efficiency of the higher-viscosity NADES; however, long extraction periods were not needed for NADES with high water content.

Purification and activity of proteins from honey

The β-glucosidase activity of each fraction of proteins eluted from the anion exchange resin was determined using p-nitrophenyl-β-glucopyranoside (p-NPG) as a substrate. The results indicated that the fractions eluted with 80, 100, 250, and 500 mM NaCl in 50 mM Tris-HCl buffer pH 7 (100 mL of each) exhibited β-glucosidase activity (data not shown). The fractions were combined and concentrated to 5 mL using Amicon® Ultra Centrifugal Filters (50 kDa cut-off) before further purification by gel filtration chromatography. The proteins were fractionated into seven fractions using gel filtration [Table 3]. When the catalytic reactivity against daidzin was determined for proteins from all steps of purification, the results indicated that the crude protein from honey showed slight hydrolysis reactivity toward daidzin. When β-glucosidase was concentrated via anion exchange resin, the catalytic activity was highly improved. After gel filtration chromatography, the fractions 1 – 3, containing the expected band of β-glucosidase (approximately 77 kDa) in SDS-PAGE [Figure 2], exhibited high catalytic reactivity in the

Table 1: Recovery of daidzin from *Pueraria mirifica* extracts determined by indirect competitive enzyme-linked immunosorbent assay

Spiked concentration (μg/mL)[a]	H-NADES		S-NADES	
	Measured concentration (μg/mL)	Recovery (%)	Measured concentration (μg/mL)	Recovery (%)
0	24.4±1.44	-	40.2±2.51	-
6.25	30.7±0.420	99.7	46.8±3.10	105
12.5	37.0±1.11	101	52.5±2.79	98.3
25.0	49.5±4.94	100	65.4±3.33	101
50.0	74.6±3.70	100	93.0±3.30	106

The values are presented as the mean of three replicates±SD (mean±SD). [a]PM extract was dissolved in 70% (v/v) H-NADES and 50% (v/v) S-NADES, then the authentic daidzin concentrations were spiked into the extract. Finally, the total concentrations of daidzin were determined. SD: Standard deviation; PM: *Pueraria candollei* var. *mirifica*; NADES: Natural deep eutectic solvent; H-NADES: Honey based NADES; S-NADES: Sugar based NADES

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Table 2: The concentrations of daidzin extracted using honey based and sugar based natural deep eutectic solvents with various water content

Solvents	Daidzin concentration (μg/mL) with 1 h extraction		Daidzin concentration (μg/mL) with 3 h extraction	
	H-NADES	S-NADES	H-NADES	S-NADES
80% (v/v)	4.15±0.360	32.9±0.530	7.44±0.882	45.8±1.10
70% (v/v)	5.44±0.440	44.5±2.92	7.98±0.691	52.9±4.00
60% (v/v)	7.12±1.16	48.8±4.35	9.01±0.865	55.5±6.10
50% (v/v)	11.0±1.46	50.2±5.05	17.9±0.958	75.8±3.67
40% (v/v)	17.4±3.34	59.8±1.93	23.9±1.95	65.7±7.07
30% (v/v)	25.3±2.48	65.5±2.64	23.8±2.86	69.0±3.34
20% (v/v)	39.9±3.25	59.6±6.19	27.4±1.88	51.2±3.10
10% (v/v)	41.8±1.07	50.8±0.00	20.2±2.15	31.6±1.30
Water	22.1±1.25		3.44±0.409	
50% (v/v) ethanol	52.0±3.79		66.1±8.08	
80% (v/v) ethanol	26.1±0.990		34.9±2.66	
Absolute ethanol	6.88±0.624		9.31±0.663	

The values are presented as the mean of three replicates±SD (mean±SD). SD: Standard deviation; NADES: Natural deep eutectic solvent; H-NADES: Honey based NADES; S-NADES: Sugar based NADES

Table 3: The concentration of daidzin and daidzein from the reaction by purified protein from honey

Code	Protein fraction	Daidzin (μM)	Daidzein (μM)
Control	50 mM citrate-phosphate buffer pH 5	496±1.76	17.4±0.389
1	Crude protein after dialysis	469±1.91	43.1±0.379
2	Protein fraction after anion exchanger	4.05±0.328	412±1.42
3	Gel filtration fraction 1	145±5.89	301±1.49
4	Gel filtration fraction 2	6.78±0.361	405±2.67
5	Gel filtration fraction 3	11.6±0.558	403±2.49
6	Gel filtration fraction 4	367±10.6	137±3.47
7	Gel filtration fraction 5	339±2.58	146±0.853
8	Gel filtration fraction 6	422±2.35	81.1±0.930
9	Gel filtration fraction 7	496±4.45	22.1±0.581

The values are presented as the mean of three replicates±standard deviation (mean±SD). SD: Standard deviation

conversion of daidzin to daidzein.

DISCUSSION

Development and validation of indirect competitive enzyme-linked immunosorbent assay for daidzin determination in the natural deep eutectic solvent matrix

In a previous study of anti-daidzin mAb, the icELISA that was developed using 20% (v/v) ethanol as the solvent showed a linear range from 1.56 to 100 ng/mL daidzin.[12] In this study, the NADES components did not alter the sensitivity of icELISA, which provided a linear range of 1.95–125 ng/mL when daidzin solutions were prepared in 1000-fold diluted NADESs. The icELISA is highly sensitive compared with HPLC-UV, which was previously reported to have LOQ values of 2.21 μg/mL and 15.15 ng/mL.[8,9] Therefore, icELISA is an effective method for analyzing daidzin extracted from PM extract due to its requirements of small sample amounts and lower volumes of organic solvent, which make the analytical process safe for personnel and the environment. NADESs are effective extraction solvents for phytochemical extraction from PM root. NADESs are quite viscous; thus, sample pretreatment is also needed to diminish the interference in HPLC-UV analysis. Dilution-based sample treatment may not be applicable for the HPLC-UV method, which has a low sensitivity. Therefore, this high-sensitive ELISA was simple for direct analysis of the sample without complicated pretreatment procedures. The cross-reactivity results indicated that anti-daidzin mAb was high specific for daidzin analyses using icELISA. The cross-reactivity pattern of anti-daidzin mAb was similar to that of a previous investigation,[12] for which the maximum CR observed toward daidzein was 1.57%. Therefore, the NADESs did not alter the binding specificity of the anti-daidzin mAb. The daidzin concentration of the sample can be specifically determined. The recovery experiment results indicated that the icELISA exhibited high accuracy for daidzin determination when NADESs were used as the solvent.

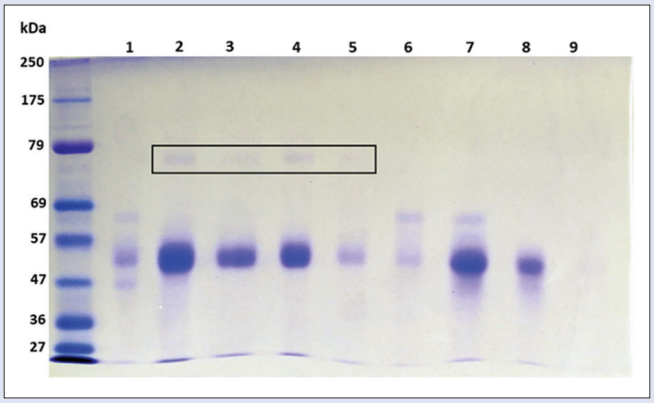

Figure 2: SDS-PAGE of purified β-glucosidase from honey. The first left lane is protein molecular mass markers; lane 1, crude proteins after dialysis of honey; lane 2, protein fraction from anion exchanger; lane 3-9, protein fractions No. 1–7 from gel filtration. The black square frame of lane 2, 3, 4, and 5 indicated β-glucosidase (77 kDa molecular mass)

Extraction of daidzin using natural deep eutectic solvent matrixes

The results indicated that the water content was an essential factor for adjusting the viscosity of NADESs; in addition, it influences their polarity. The viscosity of NADESs relates to the mass transfer, dispersion, solubility, and stability of the solute, which all affect the extraction efficiency. Other research also indicated that the extraction yield was improved with decreased NADES viscosity,[13-15] which is consistent with this study. For S-NADES, the higher water content causes a lower viscosity, resulting in a high extraction efficiency; however, adding too much water (10%–20% (v/v) S-NADES) in the system might disrupt the hydrogen-bonding interactions between NADESs and the targets of extraction[16] and decrease the extraction capacity. The S-NADES showed higher extractive capacity than conventional solvents such as 50% and 80% and absolute ethanol. Conventionally, daidzin was extracted from PM using ethanol, and 95% ethanol exhibited the highest extraction capacity compared with 50% and 75% ethanol.[17] In the reported studies,[14] sugar-based NADES could extract genistin, genistein, and apigenin with greater efficacy than organic-acid-based NADES. In addition, the sugar-based NADES was more effective for hydroxytyrosol extraction than 70% (v/v) ethanol and water.[18] However, in several studies,[19,20] using S-NADES as the solvent for extraction afforded a lower yield than that of other NADESs and conventional solvents. In this study, both S-NADESs and H-NADESs provided considerable numbers of hydroxyl groups. In addition, H-NADES has amine and carboxyl groups from other honey components, so both the hydroxyl and amine groups of the NADES were involved hydrogen in bonding between the

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target compound and NADES molecules, thus affecting the solubility of the compounds. Comparing the 1- and 3-h extractions, the daidzin content was substantially decreased with 10%–20% (v/v) H-NADES under the longer extraction time. This result may be explained by daidzin bioconversion by honey components. β-glucosidase, the enzyme reported to be present in honey, can cleave O-glycosidic bonds between sugar moieties and aglycones. The cleavage of daidzin resulted in its conversion to the aglycone daidzein. In the 30%–80% (v/v) H-NADES, the extract is more viscous; the enzyme activity should be slowed, as reported previously.[21] Therefore, in the recovery experiment, where daidzin was prepared in 70% (v/v) H-NADES, the accuracy of determination was high because the rate of the enzymes was suppressed. The results indicated that both H-NADESs and S-NADESs are appropriate solvents for the extraction of daidzin. The factors such as the water content, viscosity, and extraction time appeared to influence the extraction efficacy. The water content played an important role in adjusting the viscosity and extraction capacity of daidzin from PM materials. S-NADES is known for its composition; in addition, it highly solubilizes daidzin. Uncertainties related to unidentified components (such as enzymes) that may affect the analytical reliability can be avoided. Therefore, S-NADES is appropriate for the analytical process. Consistent, analytical reliability can be obtained. The price of S-NADES is also lower than that of honey. In general, honey is mainly composed of sugars, amino acids, organic acids, and other compounds.[22,23] Previously, NADESs were proven to increase the bioavailability, dissolution rate, and solubility of the polyphenol rutin and to change the pharmacokinetic profile of this compound. Therefore, isoflavones of PM are interesting to study, specifically regarding whether NADESs affect their bioavailability.

Purification and activity of proteins from honey

Because the daidzin concentration extracted with 10%–30% (v/v) H-NADES was decreased after the long extraction process, the β-glucosidase component of honey was expected to mediate the daidzin transformation. Anion exchange and gel filtration are compelling methods for the purification of β-glucosidase from honey. The protein fractions from the above methods were monitored for β-glucosidase using its reaction with p-NPG and daidzin as substrates. SDS-PAGE [Figure 2] showed two major proteins obtained from the anion exchange resin (lane 2). Proteins from gel filtration showed three patterns of protein components in SDS-PAGE. Gel filtration fractions 1, 2, and 3 (lanes 3, 4 and 5, respectively) contain the expected band of β-glucosidase (77 kDa) and royal jelly protein (49 kDa).[24] Previously, β-glucosidase (72 kDa) was purified from honey bees.[4] Fractions 4 and 5 (lanes 6 and 7, respectively) contained royal jelly protein and another protein with a molecular mass of approximately 65 kDa that matches α-glucosidase, as determined in a previous study.[24] Gel filtration fractions 6 and 7 comprise only royal jelly protein. High β-glucosidase activity against daidzin was observed from fractions 1–3, while fractions 4–7 showed less activity in that order. From these results, it can be concluded that the protein band at 77 kDa is β-glucosidase [Figure 2]. A previous study reported that honey contains many protein types such as β-glucosidase, α-glucosidase, diastase, invertase, glucose oxidase, catalase, and acid phosphatase.[25] The major protein is royal jelly protein, which is used as a protein marker of authenticity and quality control of honey.[26] The molecular mass of the major royal jelly protein is 49 kDa,[24] which is present in all SDS-PAGE lanes. The β-glucosidase present in lanes 2, 3, 4, and 5 has a molecular mass of approximately 77 kDa and was confirmed to have β-glucosidase activity, consistent of a previous study.[4]

After ingestion of the isoflavonoid glycoside daidzin, it is hydrolysed to daidzein by intestinal microflora before absorption.[27] The presence of β-glucosidase-producing *Lactobacillus* and *Bifidobacterium* in the human intestine varies among individuals,[27] resulting in variations in the beneficial effects of isoflavonoids. *In vitro* transformation of daidzin to daidzein was developed using microbial β-glucosidase. In such a case, daidzin and isoflavonoids were extracted, and then, the enzymatic reaction was performed. The process was useful for enhancing the absorptivity and oestrogenic activity of soybean isoflavones.[28] In this study, H-NADES showed bimodal functions as an effective solvent for extraction and bioconversion. Both processes could be performed simultaneously. Therefore, the overall processes were shortened. Additionally, the interaction between honey and herb phytochemicals was revealed in this research, extending the application potential of honey.

CONCLUSION

Both S-NADES and H-NADES were useful for daidzin extraction from PM root. The extraction parameters (viscosity of solvents, extraction time, and water content) affect the extraction efficiency. The water content of NADESs influences the required duration of extraction. The results showed that 1 h of extraction was sufficient for NADESs with high water content, while 3 h of extraction was required for NADESs with low water content. When 50% (v/v) S-NADES was used as a solvent, it yielded a higher daidzin concentration than other NADESs, water and aqueous ethanol. According to icELISA validation, all parameters, including sensitivity, specificity, accuracy and precision, were proved to be satisfactory for daidzin determination in the NADES matrix. Purification of proteins from honey using anion exchange and gel filtration revealed the β-glucosidase constituent in honey and its catalytic activity towards daidzin. H-NADES not only resulted in high extraction efficiency but also activated daidzin to an active form (daidzein) that enhanced the oestrogenic activity and bioavailability. Both H-NADES and S-NADES function as green extraction solvents due being organic solvent-free, thereby resulting in safety for both the consumer and the environment. The PM extracts prepared by H-NADES and S-NADES can be consumed directly. The beneficial effects can be obtained from both PM and honey. Overall, the process is a zero-waste process.

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Conflicts of interest

There are no conflicts of interest.

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