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"Deep eutectic solvent extract of Pueraria lobata improves anti-aging and antimicrobial activity"

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Abstract: Deep eutectic solvents (DESs) are known for their lower melting points compared to their individual components, formed through the interaction between a hydrogen bond donor and acceptor. These solvents offer advantages such as lower manufacturing costs and an environmentally benign nature compared to conventional ionic solvents. However, despite the wide variety of deep eutectic solvents (DESs) reported in the literature, their application in cosmetics is limited due to safety and regulatory concerns.
In this study, a skin-friendly DES was formulated by mixing betaine and citric acid in a 2:1 molar ratio. The physicochemical properties of the betaine-citric acid DES were characterized by using Fourier Transform Infrared (FT-IR) spectroscopy and polarized optical microscopy.
The DES was applied to extract puerarin from *Pueraria lobata*, achieving a significantly higher yield (373 ppm) compared to the ethanol-based extract (294 ppm), as quantified by high-performance liquid chromatography (HPLC). Antimicrobial activity tests revealed that the DES-based *P. lobata* extract exhibited clear zones of inhibition against *S. aureus* (34 mm), *P. aeruginosa* (25 mm), *E. coli* (21 mm), and *C. albicans* (24 mm), showing greater efficacy against *S. aureus* than phenoxyethanol. Additionally, the DES extract demonstrated superior antioxidant activity compared to conventional solvents. Moreover, in the anti-glycation activity evaluation, the DES extract of *P. lobata* showed a 47% inhibition of advanced glycation end product (AGE) formation, underscoring its potential for enhanced anti-aging.
These findings suggest that the betaine-citric acid DES is a promising green solvent for the cosmetic industry, providing improved bioactive compound extraction, broad-spectrum antimicrobial activity, and anti-aging properties. This study highlights the potential for sustainable and innovative applications of DESs in cosmetic formulations.

1. Introduction

Conventional organic solvents have been commonly used for extracting bioactive compounds from plants due to their cost-effectiveness and efficiency. However, their toxicity and environmental impact have raised concerns, prompting interest in sustainable alternatives such as deep eutectic solvents (DESs) [1]. DESs, composed of hydrogen bond acceptors (e.g., choline chloride, betaine, proline) and hydrogen bond donors (e.g., sugars, alcohols, organic acids), are considered safer and more environmentally friendly than ionic liquids [2][3].

Kudzu (Pueraria lobata), native to East Asia including Korea, Japan, and China [4][5], is a medicinal plant rich in bioactive isoflavones such as puerarin, daidzin, and genistein [6]. Among these, puerarin has drawn attention for its antioxidant, anti-inflammatory, and cardiovascular effects [7–10].
This study introduces a green extraction system using biodegradable DESs (betaine and citric acid) to improve the extraction of puerarin from *P. lobata* and investigates its antimicrobial and anti-aging potential for skincare applications.

2. Materials and Methods

2.1 Preparation of betaine-citric acid based DES

Betaine-citric acid DESs were prepared by mixing the components in specified molar ratios. The three components (betaine, citric acid, and water) were mixed in molar ratios, as presented in Table 1. The mixtures were stirred at 300 rpm and a temperature up to 80°C using a temperature-controlled magnetic stirrier until a homogeneous liquid was formed.

Table 1. Description of the components in the Betaine-citric acid DESs.

Component			Molar ratio	Appearance
Betaine	Citric acid	Water	1 : 0.3 : 1	Solid paste
			1 : 0.6 : 1	Clear liquid at room temperature
			1 : 0.9 : 1	Solid paste

2.2 Preparation of *P. lobata* extracts

P. lobata was purchased from herb markets in Chungcheongbuk-do, Korea. Dried *P. lobata* (50g) was treated with 500g of 70% aqueous ethanol or betaine-citric acid DES under reflux 6 hours. The resulting extracts were filtered using filter paper.

2.3 Characterization of Betaine-citric acid DES by Fourier Transform Infrared (FT-IR) spectroscopy

The qualitative analysis of betaine-citric acid DES as characterized using Fourier Transform Infrared (FT-IR) spectroscopy to determine the changes in characteristic functional groups.

2.4 Analysis of puerarin contents by HPLC analysis

The contents of isoflavone, puerarin, from *P. lobata* extract were determined quantitatively by HPLC. HPLC analysis was carried out by the modified method of Sakakibara et al. [11], using an Alliance Waters 2695 (Waters Co., Milford, MA, USA) system with a photodiode array detector (Waters 2998) and a CapcellPAK C18 column (250 x 4.6 mm, Osaka soda, Japan). The mobile phase was methanol (A) and 0.02% acetic acid (B), starting from 20% A to 60% A over 0-20 min, 60%-100% A over 5 min and 20% A over 5 min. The flow rate was 0.8 mL/min, detection was at 249 nm, and the column temperature was maintained at 35 °C. Samples were diluted with methanol and filtered through 0.45 μm PTFE syringe filters for injection into HPLC.

2.5 Disc diffusion assay

The disk diffusion method was used to evaluate antimicrobial activity of each extract. Pathogenic microorganism used in this study included *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and *Candida albicans* (ATCC 10231), obtained from the Korean Collection for Type Cultures (KCTC). *C. albicans* was cultured in Sabouraud Dextrose Broth (SDB) at 25 °C with shaking at 150 rpm for 48 hours, while the remaining bacterial strains were incubated in Tryptic Soy Broth (TSB) at 32 °C, 150 rpm for 24 hours.
Briefly, *S. aureus*, *P. aeruginosa*, and *E. coli* were inoculated on Tryptic Soy Agar (TSA) plates at a concentration of 10^6 CFU/mL, whereas *C. albicans* was spread on Sabouraud Dextrose Agar (SDA) plates at 10^5 CFU/mL. Sterile filter paper discs (6 mm in diameter) were loaded with 100 µL of each test sample and placed on the inoculated plates. The plates were incubated at 25 °C for *C. albicans* and 32 °C for the bacterial strains. After 24 to 48 hours of incubation, the diameter of the inhibition zones was measured.

2.6 DPPH Radical Scavenging Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity was evaluated according to the method of Blois, et al. with minor modifications [12]. DPPH solution (0.1 mM in methanol) was added to an equal volume of sample solution and allowed to react for 10 min at room temperature, after which the optical density was measured at 565 nm using a microplate autoreader (Multiskan GO, Thermo Fisher Scientific, USA).

2.7 *In vitro* glycation assay

The method of Sekiguchi et al(2016) was followed with slight modifications [13]. To each sample, an equal volume of double-concentrated PBS was added under low temperature conditions to prepare a neutralized solution. This solution was aliquoted in 50 µL volumes into a 96-well black plate and heated at 37 °C overnight to allow the collagen solution to gelate. To each well containing collagen gel, different concentrations (0.05–0.3 mM) of *P. lobata* extracts and aminoguanidine, a glycation inhibitor, and 500 mM glyceraldehyde were added and incubated at 37 °C for 24 hours to induce glycation [14]. The degree of glycation was assessed by measuring the intensity of AGEs specific fluorescence (at 370 nm excitation and 440 nm emission) on a fluorescence plate reader (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland).

2.8 Statistical Analysis

Statistical analysis was performed using the t-test. $p < 0.05$ based on at least three or more independent experiments was considered to be statistically significant.

3. Results

3.1 Fourier Transform Infrared (FT-IR) Spectroscopy Analysis

The formation of betaine-citric acid DES and the intermolecular interactions between the components were investigated using FT-IR spectroscopy.

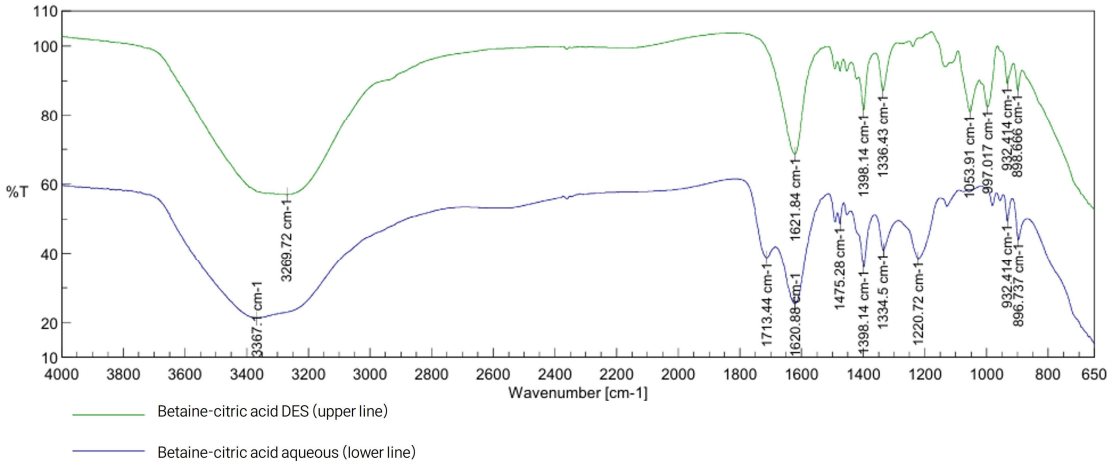
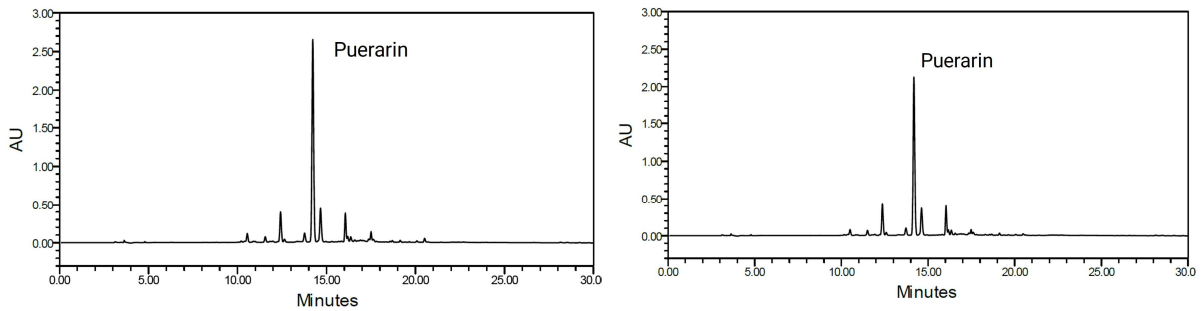


Figure 1. FT-IR spectra of betaine-citric acid system for confirming eutectic formation. The upper line represents the DES, and the lower line shows the aqueous mixture. After eutectic formation, broadening of the –OH stretching band (~3269 cm^{-1}) and a shift of the C=O band from 1713 cm^{-1} to 1621 cm^{-1} were observed, indicating enhanced hydrogen bonding and physical interactions between betaine and citric acid.

Figure 1 presents the FTIR spectra of betaine-citric acid DES and its aqueous mixture. In the DES, the O–H stretching band appeared at 3269.72 cm^{-1}, shifted from 3367 cm^{-1} in the aqueous solution, suggesting the presence of stronger and more organized hydrogen bonding. The C=O stretching band also showed a shift from 1713.44 cm^{-1} (aqueous) to 1621.84 cm^{-1} (DES), indicating changes in the electronic environment of the carboxyl group due to enhanced molecular interactions between the two components. Furthermore, the region between 1300–1000 cm^{-1} displayed more intense and defined peaks in the DES, attributed to C–O stretching and C–H bending vibrations. These spectral changes collectively confirm that the DES was successfully formed through strong hydrogen bonding and molecular interactions, distinguishing it from its aqueous counterpart.

3.2 HPLC analysis of *P. lobata* extracts

The content of puerarin, a key marker compound of *P. lobata*, was quantified in extracts obtained using the eutectic solvent and compared to those extracted using ethanol to evaluate differences in extraction efficiency.



(**a**) (**b**)

Figure 2. HPLC chromatograms of *P. lobata* extracts measured at 249 nm: (a) extract using DES, (b) extract using ethanol. Puerarin was detected at a retention time 14.2 min, with a content of 373 ppm in DES extract and 294 ppm in ethanol extract.

The HPLC analysis was performed to quantify puerarin in *P. lobata* extracts using DES and ethanol. As shown in Figure 2, both extracts exhibited a prominent peak at a retention time of approximately 15 minutes, corresponding to puerarin, when detected at 249 nm.
The DES-based extract (Figure 2a) showed a higher puerarin content of 373 ppm, while the ethanol-based extract (Figure 2b) exhibited a content of 294 ppm. This indicates that the use of DES as an extraction medium was more effective in extracting puerarin compared to ethanol.

3.3 Antimicrobial activity of betaine-citric acid DES based *P. lobata* extract

To evaluate the antimicrobial activity of the DES-based *P. lobata* extract, a disc diffusion assay was performed against *S. aureus*.

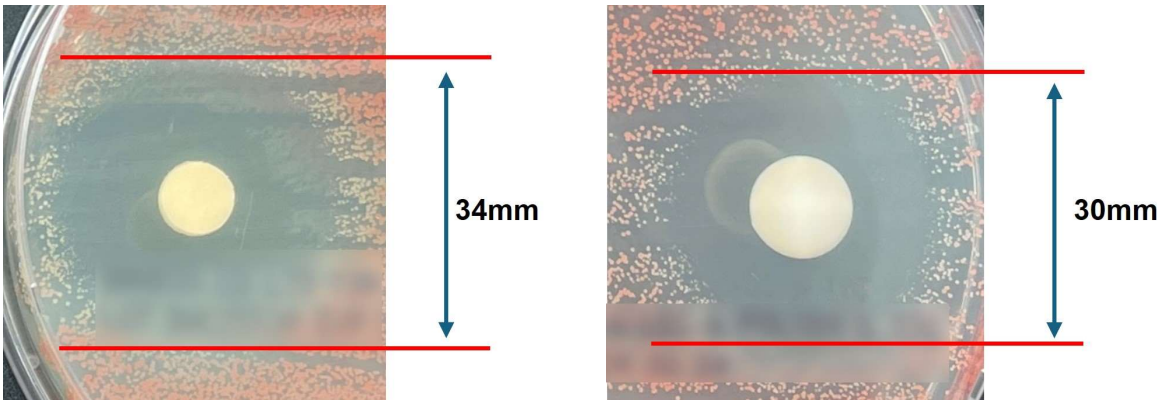


Figure 3. Antimicrobial activity against *Staphylococcus aureus* evaluated by disc diffusion method. The inhibition zone of the DES-based *P. lobata* extract (a) measured 34 mm, while that of the positive control phenoxyethanol (b) was 30 mm.

As shown in Figure 3, both DES-based extract and phenoxyethanol exhibited clear inhibition zones, indicating antibacterial properties. The inhibition zone diameter of the DES-based extract (Figure 3a) was measured to be 34 mm, while that of phenoxyethanol (Figure 3b), used as a positive control, was 30 mm. These results suggest that the DES-based *P. lobata* extract demonstrates strong antibacterial activity against *S. aureus*, potentially due to the enhanced bioavailability of active compounds within the DES matrix.

3.4 DPPH radical scavenging activity of *P. lobata* extracts

Figure 4 presents the results of evaluation of the scavenging activities of *P. lobata* extracts against DPPH assay.

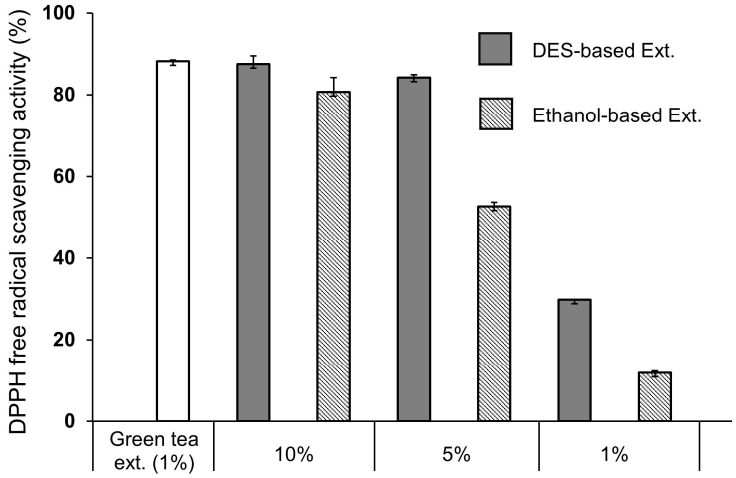


Figure 4. Anti-oxidative activities of *P. lobata* extracts. DES-based and ethanol-based *P. lobata* extracts at various concentrations (1-10%). Green tea ext. (1%) was used as a positive control. Both extracts showed dose dependent activity, with DES-based extract exhibiting superior antioxidant activity compared to ethanol-based extract.

As shown in Figure 4, both DES-based extract and ethanol-based *P. lobata* extracts demonstrated concentration-dependent DPPH radical scavenging activity. At a concentration of 10 %, the DES-based extract scavenged 87.5 %, while the ethanol-based extract showed 80.7 %. At 5 %, the DES-based extract showed 84.2 %, compared to 52.7 % for the ethanol-based extract. These results indicate that the DES-based extract consistently exhibited more pronounced antioxidant efficacy.

3.5 Inhibitory effect of *P. lobata* extracts on protein glycation

The formation of AGEs occurs through multiple processes related in part through ROS [15]. Interestingly, *in vitro* exposure of AGEs to UVA irradiation leads to formation of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals [16]. To evaluate the inhibitory effects of *P. lobata* extracts on protein glycation, a glycation-inducing reaction system with collagen and glyceraldehydes was used for rapid detection of fluorescent AGEs. The fluorescence intensity was measured, and aminoguanidine was used as a known AGEs inhibitor.

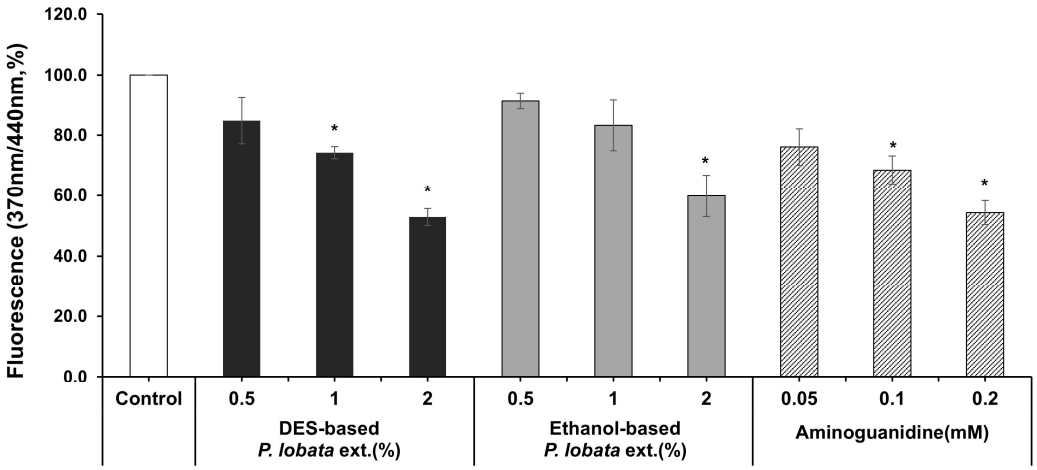


Figure 5. Antiglycation effects of *P. lobata* extracts on glyceraldehyde-derived AGE formation. DES-based and ethanol-based *P. lobata* extracts (0.5-2%) and aminoguanidine (0.05-0.2 mM) were evaluated for their inhibitory effects on AGE formation. AGE levels were quantified by fluorescence measurement (Ex 370 nm/Em 440 nm). The DES-based extract exhibited a higher inhibitory effect (15.2-47.2%) than the ethanol-based extract (8.8-40.1%).

P. lobata extracts, particularly the DES-based extract, significantly inhibited AGE formation in a dose-dependent manner. As shown in Figure 5. the DES-based extract showed a higher inhibitory effect (15.2-47.2%) compared to the ethanol-based extract (8.8-40.1%). These findings suggest that DES-based extraction enhances the antiglycation efficacy of *P. lobata,* supporting its applicability as a natural anti-glycation agent.

4. Discussion

This study confirmed the effectiveness of a betaine–citric acid based deep eutectic solvent (DES) as a green alternative for extracting bioactive compounds from *P. lobata*. FT-IR analysis verified strong hydrogen bonding between DES components, and HPLC revealed a higher puerarin content in the DES extract compared to ethanol extract.

Biological assays demonstrated that the DES-based extract exhibited superior antioxidant, antimicrobial, and antiglycation activities. It showed greater antibacterial activity against *Staphylococcus aureus* than phenoxyethanol, and demonstrated strong radical scavenging activity at lower concentrations as well as more effective suppression of AGE formation compared to the ethanol-based extract. These results suggest that the DES not only improves extraction efficiency but also enhances the functional properties of the extract.

5. Conclusion

The betaine–citric acid based DES proved to be a sustainable and efficient solvent for extracting functional compounds from *P. lobata*. The DES-based *P. lobata* extract showed enhanced biological activities, highlighting its potential as a natural active ingredient for skincare products targeting oxidative stress, bacterial growth, and glycation-related aging.

6. References

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