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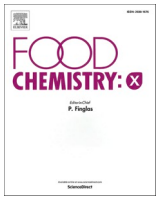

Proteomic-based prediction of functional bioactive peptides in proteins extracted from *Torreya grandis* using choline chloride-based deep eutectic solvents☆

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ABSTRACT

Five deep eutectic solvents were used to extract *T. grandis* nut protein, with water as comparison. ChCl:Urea was the most effective, preserving integrity and functionality of extracted proteins. Cold ethanol purification yielded highest protein recovery rate 6.89 % ± 0.12 %. Time, temperature, and solid-liquid ratio, were optimized using response surface methodology and a Box-Behnken design. Extracted proteins were characterized *via* SDS-PAGE to assess digestibility and functional properties. Proteins extracted by DES were further analyzed *via* LC-MS/MS. *De novo* sequencing identified 600 peptides with 235 proteins in DES extract, compared to 349 peptides and 143 proteins in the water extract. Furthermore, 87 functional oligopeptides in DES, compared to 41 in water. DES extract contain 63 antioxidant peptides, with three distinct peptides GGE, CME, and YIY. CME peptide had highest antioxidant activity. These findings highlighted DES for extracting smaller functional proteins and peptides from *T. grandis*, demonstrating potential in food industry applications for protein extraction.

1. Introduction

Alternate protein sources are important due to the increasing global population and the need for economically sustainable development. Plant proteins are getting common in human diet due to their sustainability and cheaper cost. It has sparked a lot of interest in the processing and manufacturing of these proteins in every form (Lucas et al., 2015). Tahergorabi and Hosseini described the role of proteins as physiological, chemical and nutritional aspect. Humans are dependent on animal protein for their daily diet that includes fish, dairy, meat and other items (Tahergorabi & Hosseini, 2017). By 2050, this demand is projected to double. However, transporting animal proteins poses significant challenges, limiting their availability and causing financial strain, thus emphasizing the importance of exploring natural alternatives. Such transportation brings various human diseases, depletion of fresh water and adverse climate change. Researchers are focusing on developing environmentally friendly and sustainable diets, driven by increasing awareness among humans about the value of diet rich in high-quality protein. In this regard, better methods have been developed for the extraction of proteins from plant which serves as a better substitute for animal-based proteins. The popularity of vegetarian diets is increasing in Western countries, with a trend toward completely replacing animal protein diets by 2050 (Tahergorabi & Hosseini, 2017).

The plant proteins are basically found in lentils and grains. Agriculture by-products are also important source of proteins with essential amino acids and nutritional values compared to animal proteins (Contreras et al., 2019). Plant proteins can be efficiently recovered using various extraction techniques tailored to different plant matrices,

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resulting in improved recovery efficiency. Conventional extraction of plant protein brings less yield and breakdown of protein structure. Temperature, pH, type of solvent and duration of extraction affects the breakdown of proteins.

Researchers are developing improved methods for extracting plant proteins, to increase extraction efficiency, prevent protein structure degradation, and preserve protein functional properties. Some researchers (Kumar et al., 2021), (Gençdağ et al., 2020), (Franca-Oliveira et al., 2021) provided clear explanations of the parameters influencing protein extraction. According to them, one crucial consideration is the type of solvent used. Therefore, efforts have been made to identify eco-friendly, sustainable and efficient solvents for protein extraction, in order to reduce experimental time and energy consumption.

Compared to conventional plant protein extraction techniques (alkaline, acid, and salt extraction), novel extraction techniques (such as ultrasound-assisted extraction, pulsed electric field, deep eutectic solvent, enzyme-assisted extraction, and combined methods) offer more benefits, including reduced energy consumption, fewer toxic residues, and environmental friendliness. Plant proteins have several drawbacks, including unpleasant odors, anti-nutritional elements, allergic components, low solubility, *etc.* To enhance their functional qualities and eliminate unwanted elements, appropriate modification techniques are needed (Tang et al., 2024). Plant proteins have been altered using a variety of techniques, including chemical, biological, and physical alteration. In order to maximize the functional performance of plant proteins, it is crucial to look into appropriate processing and modification conditions. In fact, using plant proteins rather than animal proteins has become popular in the food sector for applications such as bioactive peptides, food emulsion gels, active ingredient encapsulating materials, and food packaging films. Future research should focus on the development, identification, effectiveness, and use of plant proteins. To maximize the performance of various plant protein types for particular applications, it is critical to determine the primary parameters influencing their physicochemical stability. Furthermore, the range of uses for these plant proteins ought to be expanded. For example, their use in medicine delivery and the production of functional foods, *etc.*, that are intended to treat or prevent specific diseases through diet, should be taken into consideration (Tang et al., 2024).

Deep eutectic solvents (DES) are viewed as greener solvents due to their biodegradability, sustainability, lower toxicity, and ease of preparation, making them suitable for a variety of applications (Zainal-Abidin et al., 2017). Quaternary ammonium salt *i.e.*, Choline chloride (ChCl) with hydrogen bond donors (HBD) like sugars, polyols, acids are the most common DES (Y. Dai et al., 2015). The physicochemical characteristics of DESs are crucial fundamental attributes that require substantial research and reporting because they are novel agents with exciting potential in chemical engineering, green chemistry, and synthesis applications (Omar & Sadeghi, 2022). A comprehensive evaluation of DESs' sustainability as possible substitutes for traditional organic solvents should be conducted, taking into account their physicochemical characteristics. Notably, the molar ratio of the elements and the selection of HBD/HBA components have a significant impact on the physicochemical characteristics of DES. The density, viscosity, and ionic conductivity of the generated DES are also influenced by the alkyl chain lengths and contributing functional group types of the HBD and HBA components. Future development of innovative DESs and the assessment of their viability in particular industrial applications will greatly benefit from a thorough understanding of the contributions of these parameters to DES physicochemical characteristics. Standardizing testing techniques and accurately disclosing the results will guarantee reproducibility and allow for additional investigation of novel DES applications. Even though the physicochemical characteristics of DESs must be empirically characterized, the infinite variety of HBA and HBD components could be a barrier, as repeated laboratory studies are time- and cost-limited (Yeow et al., 2024). Variety of bioactive substances were extracted by DES in last few years. Some researchers (Mahmood et al., 2019) extracted polyphenols antioxidants from *Chlorella vulgaris*. (Cui et al., 2018) isolated and improved flavonoids extraction by DES from sea buckthorn leaves. (Bubalo et al., 2016) used DES to extract grape skin phenolics and showed that it is more effective than methanol. In addition to extracting bioactive compounds, DES are also being used for the extraction of both animal and plant-based proteins. (Bai et al., 2017) extracted and separated collagen peptides from cod skin using DES with better purity. A few researchers adopted polyethylene glycol-based DES aqueous biphasic systems for extraction of protein from Pumpkin (*Cucurbita moschata*) seeds and they also used Bovine serum albumin (BSA) (L. Wang et al., 2017); (Pang et al., 2017). (Grudniewska et al., 2018) used glycerol–choline chloride based DES for extraction of protein from rapeseed and primrose cake. While DES are being explored as substitutes for food protein extraction, there remain several methodological and technical challenges for their use in the food sector. Also, the impact of water and isomer analogues of components (*e.g.* hydrogen bond donor) on the formation, structure, and functionality of proteins extracted by DES is still unclear.

T. grandis seed nuts are nutritious and therapeutic. Bioactive chemicals present in *T. grandis* seed include tocopherols, sterols, polyphenols, vitamins (folic acid and nicotinic acid) and minerals (magnesium, calcium, zinc, and selenium) (Ni et al., 2015). The seed nuts have bioactive qualities that could be applied in traditional medicine, such as antiviral, antifungal, anti-inflammatory, anti-atherosclerotic, and antioxidant. Around 50–60 % of the dry mass weight of *T. grandis* consists of oil, which is enriched with unsaturated fatty acids like oleic and linoleic acid (Dong et al., 2014). China is consuming more *T. grandis* seed nuts due to its health benefits and high nutritional content (Ni et al., 2015). *T. grandis* nut meal has a protein level ranging from 10.3 % to 16.4 %, with amino acid content of 118.1 g/Kg (he et al., 2016; J. Wang et al., 2017).

In this study, we aimed at testing several DES which could be employed in the food industry for extraction of proteins from *T.grandis* seed nuts. DES includes *i.e.* ChCl: Urea; ChCl: Acetamide; ChCl:Glucose:H_2O; ChCl:Sucrose:H_2O; ChCl:Xylose:H_2O. Single-factor and response surface methodology (RSM) were employed to determine the optimal process parameters, including material-liquid ratio, extraction time and water temperature. Our study demonstrated the potential use of DES as an environmentally friendly solvent and served as an experimental reference for the industrial extraction of *T. grandis* seed nut proteins using DES.

2. Materials and methods

2.1. Chemicals, samples and instruments

The cracked *T. grandis* nuts were taken from a commercial garden in Xinchang September 2021, Zhejiang province, China (about 525 days after floret). Within 4 h, the seed nuts with shells were delivered to the lab. The shells were then manually removed. Following that, cakes were collected and the nuts oil was cold-pressed using an oil press (Qingdao AUCMA Consumer Electric Co., Ltd). n-hexane, ethanol, Petroleum ether (60–90 °C), HCl, H_2SO_4 and other reagents were purchased from Aladdin Industrial Corporation Suite 601, Shanda Building, No. 196 xinjinqiao Road, Pudong District, Shanghai 201,206, China.

TS − 1 table concentrator (Haimen Kylin-Bell Lab Instruments Co., Ltd.); AL104 thousandth Electronic Balance (Mettler-Toledo Instruments (Shanghai) Co., Ltd.); HH-4 thermostatic water bath (Shanghai Jixing Instrument Co., Ltd.); ZNCL-GS190 * 90 Magnetic heating mixer (Shanghai Yuezhong Instrument and Equipment Co., Ltd.); High-speed Crusher, (Tianjin Taisite Instrument Co., Ltd.); PowerPac Basic Power Supply basic electrophoresis instrument 164–5050 (Jinan Biobase Medical Equipment Co., Ltd.); EPOCH2 microplate reader (American Berten Instrument Co., Ltd. Beijing Representative Office).

2.2. Conventional extraction of protein from Torreya grandis seed nut using water

Protein extraction was performed using the previously described method (Yu et al., 2017) with a few minor modifications. The nuts (*T. grandis* Merrillii) were husked and then processed into flour. The flour was defatted at room temperature by mixing it with n-hexane/ethanol (9:1 *v*/v) in a 1:3 (*w/v*) ratio. After two hours of continuous stirring, the mixture was further separated using an aspirator filter pump (Zhenzhou Great Wall Scientific Industry & Co. Ltd.). The filtrate was discarded, and the remaining pellet was air dried at 25 °C in a laminar air flow (Shanghai Shengdai Laboratory Equipment Co. Ltd) until it was free of solvent odor (Yu et al., 2017). 500 g of dried powder was defatted *T. grandis* flour. The defatted *T. grandis* flour was dissolved in distilled water at a 1:10 (w/v) ratio while being stirred for two hours at 30 °C. After centrifuging the mixture at 10,000*g* for 20 min at 20 °C, the precipitate was redissolved in water at a ratio of 1:5 (w/v) and centrifuged once more at 10,000 rpm for 20 min at 20 °C. The two supernatants were mixed, and the pH was lowered to 4.0 with 2 M HCl in order to precipitate the protein isoelectrically. The mixture was then centrifuged at 10,000*g* for 20 min at 4 °C.

The precipitates were further dissolved and spin for an hour in distilled water after being redissolved and neutralized with 2 M NaOH. In distilled water, the precipitates were further dissolved and stirred for an hour. The protein extract was freeze-dried, vacuum-packed, and stored at −80 °C for later use. The protein content of the combination was determined using Thermo Scientific's Bovine Serum Albumin Kit (Quan et al., 2021). Using the conventional Kjeldahl method and AOAC method 984.13 (Horwitz & Albert, 2006), the protein content of *Torreya grandis* seed nuts extracted by DES and ultrasound-based extraction was calculated by measuring the total nitrogen content. A conversion factor of 6.25 was used to analyze the protein content of the *Torreya grandis* seed nut isolate.

2.3. Synthesis of deep eutectic solvents (DES)

By changing the molar ratio of hydrogen bond donors to the quaternary ammonium salt choline chloride (ChCl), deep eutectic solvents were created. The two ingredients were mixed using rotavapor in a water bath at 80–100 °C to create a homogenous, transparent liquid that could be used to create deep eutectic blends. After being allowed to cool to ambient temperature, all of the created deep eutectic solvents were vacuum-dried for 24 h at 55 °C. All of the deep eutectic solvents were kept in vacuum-sealed laboratory vials for further use (Toledo et al., 2019).

2.4. Ultrasound assisted Torreya grandis nut protein extraction using DES

The defatted *Torreya grandis* meal was combined with DES in a 100 ml bottle with a solid- liquid ratio of 1:10 (*w/v*) for ultrasonic-assisted protein extraction. In the sonnication bath, this reaction process was carried out for 20 min at 30 °C. The reaction combination had a maximum frequency of 20 kHz, 40 W of power, 50 % amplitude, and a pulse width of 20s on and 10s off. The mixture was then centrifuged at 10,000 rpm, the supernatant containing extracted proteins was collected and stored in sterile airtight polypropylene tubes pending further analysis(Liu et al., 2022).

2.5. Protein purification of Torreya grandis seed nut protein using protein dialysis bag, cold acetone and cold ethanol

The *Torreya grandis* seed nut protein was extracted using three distinct standard techniques. The mixture was placed in an ultrasonic water bath for many hours before being extracted. After adding the DES mixture and defatted *Torreya grandis* nut powder to the tube, the supernatant was collected by centrifuging the tube at 10,000 rpm. The mixture's protein content was determined by BCA method. Additionally, at 4 °C in a refrigerator, the extracted protein was added to the protein dialysis bag (500D). The protein was first stored in the dialysis bag using cold acetone, which involves pre-cooling the acetone and protein supernatant. Acetone and protein supernatant were combined in a 1:4 protein supernatant to acetone ratio. The precipitate is kept after the mixture is centrifuged once more. After being cleaned with acetone, the precipitate was put in a fume hood to dry. The precipitate was redissolved in distilled water after the acetone had entirely evaporated. The combination was then centrifuged at 10,000 rpm to extract the supernatant, and the BCA was used to measure the protein content.

In a different method, cold ethanol was added to the protein supernatant after it had already cooled. Ethanol and protein supernatant were combined in a 1:6 ratio. The precipitate was saved after the mixture was centrifuged at 10,000 rpm. After being cleaned with ethanol, the precipitate was put in a fume hood to dry. The precipitate was redissolved in distilled water after the ethanol had entirely evaporated. After centrifuging the mixture once more at 10,000 rpm, the supernatant was collected. The BCA method was used to determine the protein concentration.

We repeatedly added $(NH_4)_2SO_4$ to the extract in tiny increments until the concentration reached 70 % using the salting out procedure. To preserve the protein precipitate, centrifuge after that. Wash the protein with 70 % $(NH_4)_2SO_4$, and proceed with subsequent steps similar to those using acetone and ethanol.

2.6. Single factor experiments

For the *Torreya grandis* nut protein extraction, DES and water were used as single parameters for comparison. Inorder to study the effect of solid- liquid ratio (1:05, 1:10, 1:20, 1:40, 1:80, 1:160) on the protein extraction rate of *Torreya grandis* nut protein, with an Ultrasonic water bath temperature of 50 °C and 2 h and 0.2 g of protein. The protein samples were treated with a material-liquid ratio of 1:20 and a water bath temperature of 50 °C with 0.2 g of protein in order to investigate the impact of extraction times (1, 2, 3, 4, 5, 6) on the protein extraction rate of *Torreya grandis* nut protein. Lastly, the protein samples were handled with a material–liquid ratio of 1:20, an extraction period of 2 h, and 0.2 g of protein in order to investigate the impact of water bath temperature (40, 50, 60, 70, and 80 °C) on *Torreya grandis* nut protein. In contrast, all the parameters were maintained the same as in DES when the material-liquid ratio, extraction duration, and water bath temperature were studied using H_2O. All experiments were performed three times (S. Wang et al., 2024).

2.7. Response surface methodology

The variables chosen for the Response surface methodology of *Torreya grandis* seed nut protein were the solid-liquid ratio, extraction time and temperature of ultrasonic water bath. For the experimental investigation, Box-Behnken was used. In a single factor experiment, each of these variables had a nonlinear impact on the rate at which *Torreya grandis* seed nut protein was extracted. For the RSM experiment, these three factors were chosen. All experiments were performed three times (S. Wang et al., 2024).

2.8. Protein separation of DES extracted proteins in Torreya grandis using SDS-PAGE

In accordance with the Laemmli method, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed utilizing a discontinuous buffered system with 12 % resolving gel and 4 % stacking gel. The protein sample (12 mg/mL) was diluted using a sample loading buffer (1:1) that included 5 % (*v*/v) -mercaptoethanol (−ME) (Laemmli, 1970). Twenty liters of material were loaded into each lane, and prior to electrophoresis, the samples were heated to 95 °C for five

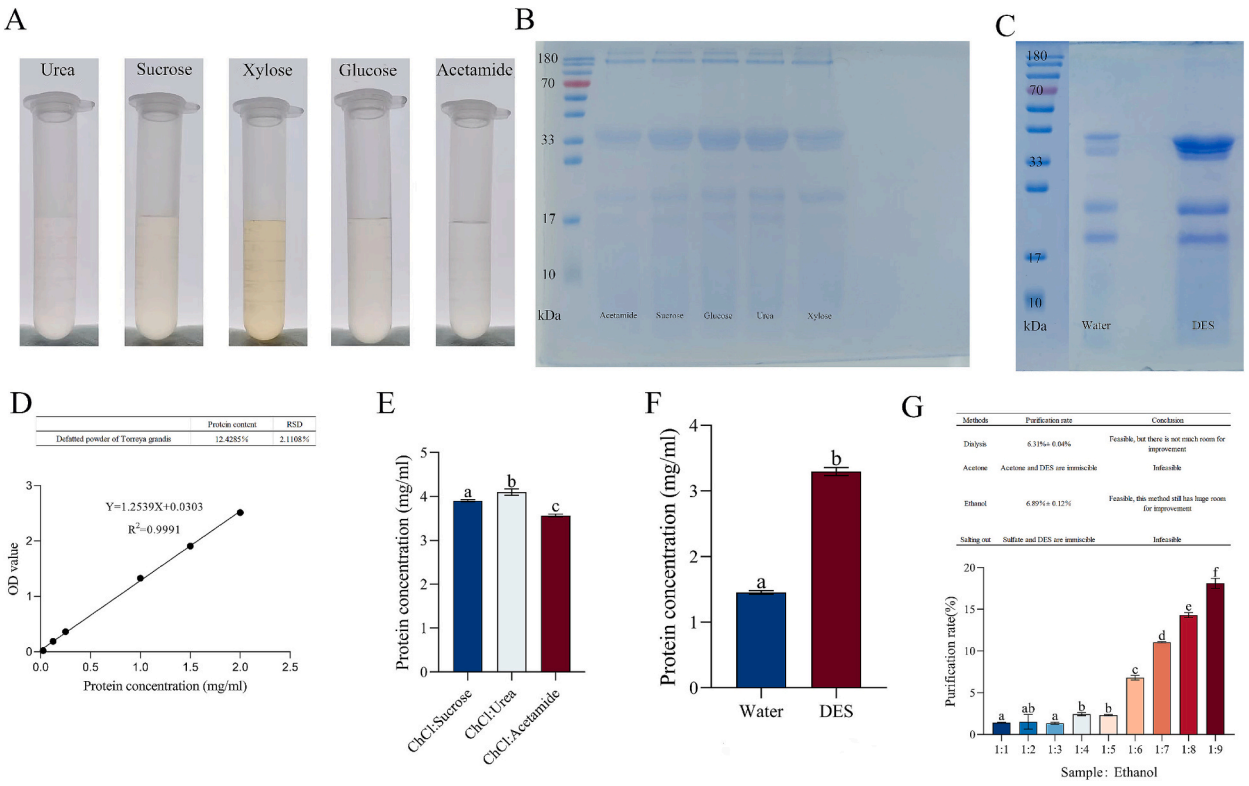


Fig. 1. DES based extraction of *Torreya grandis* seed nuts. 1 A Five types of DES used in this study. 1B. Separation of five types of DES on SDS-PAGE. 1C. Comparison of DES and Water based extraction of *Torreya grandis* seed nut protein on SDS-PAGE (DES with intense band). 1D. The protein content is measured by BCA method. 1E. Protein concentration of DES based *Torreya grandis* seed nut showed ChCl: Urea as the highest protein content. 1F. Comparison of water and DES based *Torreya grandis* protein extraction showed DES had the highest protein content. 1G. Measurement of Purification rate of DES based extracted protein using ethanol 1:9 as the best concentration. All values were represented as mean ± SD ($n = 3$). Different alphabetical letters on the error bar were significantly different ($p < 0.05$) according to Duncan's Multiple range Test (MRT). ^{ab} represents $p < 0.01$.

minutes. After two hours of staining with 0.1 % Coomassie Brilliant blue (R-250), the gel was de-stained for eight hours with a current set to 180 Ma using a de-color solution (methanol/acetic acid/water, 20/30/350) (Durrani et al., 2023).

2.9. Identification of DES extracted functional proteins from T. grandis nuts using liquid chromatography mass spectrometry (LC-MS/MS) and proteome sequencing

The protein extract from *T. grandis* was analyzed using label-free tandem mass tagging and LC-MS/MS (nanoLC-QE Thermo Fisher) (Shanghai Applied protein Technology Co., Ltd). The LC-MS/MS data was used to qualitatively identify the target protein and polypeptide molecules (Durrani et al., 2023). Prior to LC-MS/MS (nanoLC-QE) analysis of the digested protein samples, trypsin was utilized to break down the *T. grandis* protein and polypeptide. A mass spectrometry program such as MASCOT was used to identify the peptide sequence. Hydrolysis of the test product by enzymes: Following reduction and alkylation, trypsin (mass ratio: 1:50) was added to the protein sample, and the enzyme was then hydrolyzed for 20 h at 37 °C.

Following enzymatic hydrolysis, the solutions were heated and kept at 85 °C for 15 min in order to deactivate the enzyme. After centrifuging the mixture at 5000 g for 15 min at 4 °C, the supernatant was collected, processed using a Millipore ultrafiltration centrifugal tube, lyophilized, dissolved in a 0.1 % fluoroacetic acid solution, and stored at −20 °C for later use (Durrani et al., 2023). Mass spectrometry requirements: Solution B contains 0.1 % formic acid in 84 % acetonitrile, while Solution A is an aqueous solution with 0.1 % formic acid. The protein sample was added onto the Trap column by the autosampler after the Pierce's Zeba Spin column had been equilibrated with 95 % of solution A. Mass spectrometry data collection: The mass-to-charge ratios of the following peptide segments were determined: Twenty fragments (MS2 scan) were collected after each full scan spectrum. Data analysis: Proteome Discoverer 1.4 software was used to search and identify peptide sequences in a database (Durrani et al., 2023).

2.10. Anti-oxidative assay of DES extracted proteins

2.10.1. A, α-diphenyl-β-picrylhydrazyl (DPPH)

Sangon Biotech (Shanghai) Co., Ltd. kit was used for the DPPH, Trace total antioxidant activity measurement kit. 20 ml of the sample and 380 ml of the kit's working solution were mixed and was allowed to react for 20 min at room temperature. 96 wells were filled with 200 ml of mixing liquid, and the OD is measured at 515 nm. Further *in vitro* antioxidant activity was determined using the following formula, according to the kit's instructions: Total antioxidant activity (μmol Trolox / mg prot) = (△A + 0.0081) ÷0.7072*Vsample ÷ (Vsample÷Vtotal sample*Csample) =1.414*(△A + 0.0081)÷Csample (△A = Ablank-Asample).

2.10.2. ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

Sangon Biotech (Shanghai) Co., Ltd. kit was used the ABTS, Trace total antioxidant activity detection kit. Reagents 1 and 2 were included in the package. The two reagents were mixed for 20 min after adding 11 ml of Reagent 1 to each bottle of Reagent 2. After that, the supernatant was removed by keeping the mixing liquid stand. These were functioning liquids. On a 96-well microplate, combine 10 μl of sample and 190 μl of working solution. Measured the OD at 734 nm (the measurement procedure must be finished in 10 min). The *in vitro* antioxidant activity was determined using the following formula, according to the

Table 1
Deep eutectic solvents with solid-liquid ratio selected in our study.

DES	Molar ratios
ChCl:Urea	1:2
ChCl:Acetamide	1:2
ChCl:Glucose:H_2O	5:2:5
ChCl:Sucrose:H_2O	5:2:5
ChCl:Xylose:H_2O	5:2:5

kit's instructions: Total antioxidant activity (μmol / mg) = (△A + 0.0012)÷0.7021*Vsample ÷ (Vsample ÷ Vtotal sample * Csample protein concentration) =1.424*(△A + 0.0012) ÷ Csample (△A = Ablank-Asample).

2.10.3. FRAP (ferric reducing antioxidant power) assay

Sangon Biotech (Shanghai) Co., Ltd. kit was used for FRAP, Trace total antioxidant activity measurement kit. The working liquid were mixed (pre-warm at 37 °C before use) with the following ratios: reagent 1: reagent 2: reagent 3 (7: 1: 1). Reacting for 10 min after combining 180 ml of working liquid, 6 ml of sample, and 18 ml of distilled water. 96 wells were filled with 200 cc of mixing liquid, and the OD was measured at 734 nm. The *in vitro* antioxidant activity was determined using the following formula, according to the kit's instructions: Total antioxidant activity (μmol/mg) =34*× / C sample protein concentration (△A = Asample-Ablank).

2.11. Data processing and statistical analysis

Every experiment was carried out three times. For statistical analysis, Design Expert 13 and Graph Pad Prism 8.0 were utilized (La Jolla CA, USA). The mean of ±SD was used to express all experimental outcomes. To confirm the findings of Response surface approach, the tests were also conducted five times to calculate the protein extraction rate of *Torreya grandis* seed nut proteins.

3. Results and discussion

Proteins are usually denatured by heating or by radiation (Patra et al., 2023; Svigelj et al., 2021). DES are greener solvents that has advantages of using being non-toxic, non hazardous and quickly biodegradable with efficiency in protein extraction. Extraction of proteins by DES prevents toxicity, environmental contamination and denaturation of proteins during extraction process. The extraction capacity of proteins is determined by the hydrogen bonding in deep eutectic solvents. Selectivity in hydrogen bond adsorption is provided by the reversibility, directionality, and short-range nature of hydrogen bonding interactions, a weak interaction force field. By facilitating extraction through hydrogen bond adsorption, the limitations of existing protein separation and purification techniques can be lessened. Different hydrogen bond donors, however, give electronegative atoms unique functional groups, which also result in varying degrees of hydrogen bond localization (Patra et al., 2023; S. Wang et al., 2024).

3.1. Selection of suitable DES for Torreya grandis nut protein extraction

Extraction of proteins from *Torreya grandis* nut seeds was investigated for the first time using deep eutectic solvents. In the present study ChCl: Urea; ChCl: Acetamide; ChCl:Glucose:H_2O; ChCl:Sucrose:H_2O; ChCl:Xylose:H_2O as shown in Fig. 1A, were prepared for extraction of *Torreya grandis* seed nut protein. Table 1 showed the hydrogen bond acceptor (ChCl) and hydrogen bond donor (urea, acetamide, glucose, sucrose, xylose) with different molar ratios of DES. DES were selected on the basis of previous studies from (Bai et al., 2017; Yuntao Dai et al., 2013; Hernández-Corroto et al., 2020; Xu et al., 2015).

(Xu et al., 2015) used aqueous two phase system as a strategy for protein purification and showed that ChCl: glycerol was the best DES solvent. The protein extraction from *Torreya grandis* seed nuts with DES showed that using ChCl: Urea showed high concentration of protein content in mg/ml as shown in Fig. 1E. There were two factors influencing the results. Urea-containing DES have demonstrated their efficiency to extract proteins of naturally low solubility (Wahlström et al., 2017), indeed from our results the solubility of *Torreya grandis* nut proteins in water seems rather low. In addition that high urea concentration tends to conserve the integrity and functionality of bioactive systems (Costanzo, 2019). The reason explaining both these results could be due to the extraction mechanism of *Torreya grandis* nut protein in the DES might be polydisperse (forming aggregates of various sizes) and not microscopically homogenous. As a result the structures of extracted proteins were more restrained (decrease of degree of freedom) and thereby enhanced stability as well as solubility (Zeng et al., 2014). The Xylose and Glucose DES reacted with the working solution, so the protein concentration can't be calculated. The results showed that DES based extraction was the best method because protein remained stable for longer period as shown in Fig. 1F.

3.2. Purification of Torreya grandis nut protein

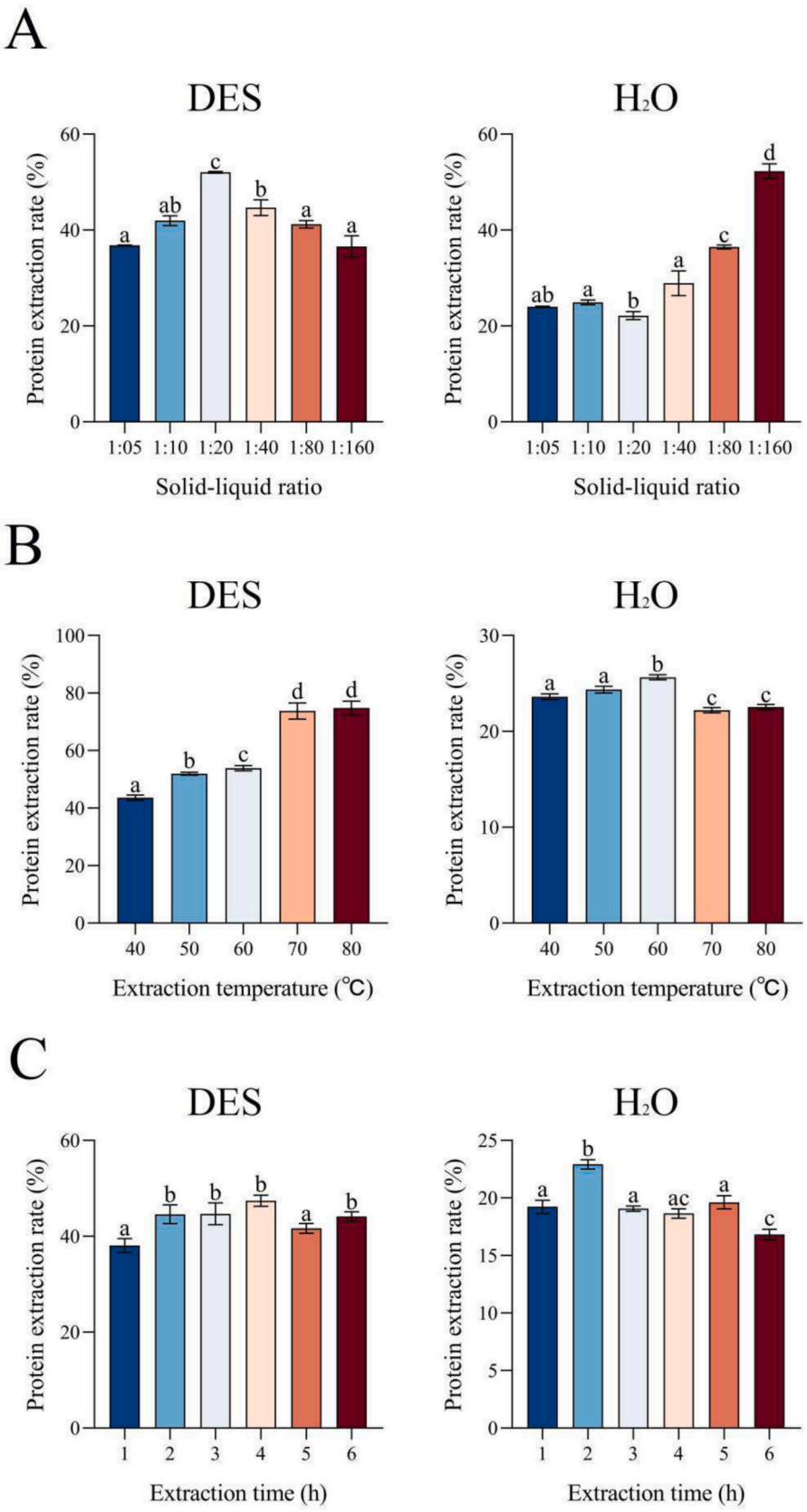
Pure DES reduce protein activity because the interactions between specific DES components are stronger than those between protein residues and DES. They are useful as solvents for the purification of enzymes and proteins because they maintain the function of macromolecules, such as membrane proteins and relatively smaller extent soluble macromolecules. DES can also serve as a crystallization medium for proteins (Katrak & Ijardar, 2024). In this experiment, three different methods were employed for the purification of *Torreya grandis* protein from DES. The first one was the use of dialysis bag (500D) and the purification was quite feasible. The rate of purification of protein using dialysis bag was 6.31 % ± 0.04 %. The second method was the use of cold acetone but the purification was infeasible as acetone and DES got immiscible. The third method was the use of cold ethanol. This method was quite feasible and the purification rate was 6.89 % ± 0.12 % as shown in Fig. 1G. The results showed that dialysis bag used for purification need improvement (Zhao et al., 2019).

To extract crude protein isolates, target molecules must be separated from the DES-rich top phase. By adding ethanol and adjusting the salt concentration, target proteins were back extracted, resulting in a new ATPS (aqueous two phase system) since the salt competed with water and DES. Since DES from the top phase was poor in phase development, ethanol was injected in the same volume as the DES-rich top phase to create new ATPS. In comparison to previous K_3PO_4 concentrations, the addition of 0.45 g/ml K_3PO_4 resulted in a considerably greater yield (86.24 ± 0.56 %) and extraction efficiency (60.47 ± 0.72 %) ($p < 0.05$). Protein molecules were back-extracted to the bottom phase with high salinity as a result of the strong electrostatic contact between proteins and K_3PO_4 and the weaker hydrogen bonding interaction between DES and proteins (Kinugasa et al., 1991). Our results were different from (Zhao et al., 2019) as they concluded that ATPS reverse extraction made it possible to effectively separate the target protein isolates from the DES.

When compared to traditional methods that used DES, current approaches usually fall short of the necessary purity levels and yield. Therefore, efforts must be made to increase the back extraction efficiency in order to improve protein purification (Katrak & Ijardar, 2024).

3.2.1. Characterization of DES extracted Torreya grandis seed nut proteins

Inorder to study the functional characterization of *Torreya grandis* seed nut protein, we separated the DES extracted proteins by SDS-PAGE. Additionally, we created a proteome database for functional oligopeptide groups that may be extracted, evaluated, and used in industry and for human health. The results showed that DES-based extracted proteins



(caption on next page)

Fig. 2. Single factor experiments to check the influence of solid-liquid ratio, temperature and extraction on the *Torreya grandis* seed nuts. A(DES). Variation of protein extraction rate on *Torreya grandis* seed nut with different solid-liquid ratio using DES. A(H_2O). Variation of protein extraction rate on *Torreya grandis* seed nut with different solid-liquid ratio using H_2O. B(DES). Variation of protein extraction rate on *Torreya grandis* seed nut with different water bath temperature using DES. B (H_2O). Variation of protein extraction rate on *Torreya grandis* seed nut with different water bath temperature using H_2O. C(DES). Variation of protein extraction rate on *Torreya grandis* seed nut with different extraction time using DES. C(H_2O). Variation of protein extraction rate on *Torreya grandis* seed nut with different extraction time using H_2O. Each Value represents the mean ± SD (n = 3). ^{ab} and ^{ac} represents $p < 0.01$. Different alphabetical letters on the error bar were significantly different ($p < 0.05$) according to Duncan's Multiple range test (MRT).

showed deep, intense bands compared to water-based extracted proteins, as illustrated in Fig. 1B and C. The weaker bands of *Torreya grandis* seed nuts with water-based extraction may be the result of weaker disulphide bonds. The results of the SDS-PAGE analysis of the extracted protein from *Torreya grandis* seed nuts were identical to those obtained using the water-based method. The SDS-PAGE results of DES extracted *T. grandis* nut protein showed that the molecular weight of proteins were between 20 and 21 kDa and 31 and 37 kDa. The possibility that these proteins serve as seed storage proteins in *T. grandis* nuts were highlighted by these significant bands. There were also a number of additional bands. The more noticeable major band, for example, is located close to 14.4 kDa. These proteins are small in *T. grandis* nuts and rely on the kind of protein separation technique employed, as demonstrated by the low molecular weight band. The protein concentration were measured by BCA as shown in Fig. 1D.

Different *T. grandis* nut seed types have distinct protein patterns. Their distinct classifications were confirmed by their differing molecular weights. To distinguish between several *T. grandis* protein bands, SDS-PAGE was considered better protein separation technique. There was also a light band that was almost 10 kDa. The findings demonstrated that SDS-PAGE could be used to identify several *T. grandis* cultivars. The primary proteins in *T. grandis* nuts had great structural stability, as evidenced by the protein fraction with high band intensities being unchanged. Protein stability was also enhanced by disulfide bonds. In the denatured state, peptides containing tiny disulfide bonds may separate differently. Our findings were in consistent with those of (Yu et al., 2017) and (Durrani et al., 2023).

3.3. Extraction process of Torreya grandis seed nut total proteins by DES

3.3.1. Effect of extraction time on Torreya grandis seed nut protein

With a temperature of 50 °C and a material-liquid ratio of 1:2, we used five DES mentioned in our study to examine the extraction time of *Torreya grandis* seed nuts from 1 to 6 h. From 1 to 4 h, the rate of protein extraction increased gradually. But after 5–6 h, it progressively decline. As the protein sample had enough time to mix with the reaction mixture, the protein concentration began to rise between 1 and 4 h. Consequently, the optimal time frame for extracting proteins from *Torreya grandis* seeds from DES was determined between 1 and 4 h as shown in Fig. 2 C(DES).

The protein extraction time of bamboo shoots was examined by (S. Wang et al., 2024) utilizing a material-liquid ratio of 2:50, 40 % water content, and a water bath temperature of 50 °C. They discovered that the protein extraction rate increased to 23.1 % during a reaction time of three hours. After three hours, it started to decline. For further adjustment, they determined that the optimal extraction period for protein from bamboo shoots was between 2 and 4 h. Since we observed that the rate of extraction increased till four hours, our results were consistent with their findings (S. Wang et al., 2024).

In the same experiment, water was chosen for the extraction of *Torreya grandis* seed nut protein at 50 °C with a material-liquid ratio of 1:2. The rate of protein extraction rose for 1-2 h and then progressively fell after 3–4 h. Therefore, the optimal extraction period for extracting the protein from *Torreya grandis* seed nuts utilizing water was determined to be 1–2 h as shown in Fig. 2 C(H_2O).

3.3.2. Effect of temperature on Torreya grandis seed nut protein

In this experiment, we used DES and water to examine how temperature affected the protein in *Torreya grandis* nuts. DES was used to choose a temperature range of 40–80 °C while maintaining a material–liquid ratio of 1:20 and *Torreya grandis* seed nut protein for two hours in a water bath. The findings demonstrated that the protein concentration in DES increased with increasing temperature, reaching 4.60 mg/ml as illustrated in Fig. 2B(DES). This was due to the fact that the protein stayed stable in DES, demonstrating that DES was the most effective solvent for effectively extracting the protein from *Torreya grandis* nuts. (S. Wang et al., 2024) Wang et al. used DES, investigated how temperature affected the proteins in bamboo shoots between 10 °C and 60 °C. As the temperature rose, so did the extraction rate. Protein was combined with a material-liquid ratio at a higher temperature. The extraction rate was 23 % when the temperature hit 50 °C. They determined that 40 °C to 60 °C were the ideal temperatures. Lower protein extraction was the result of denaturation and higher temperatures. Our findings were in line with theirs since protein became permanently stable at higher temperatures when DES was used.

In the same experiment, the impact of temperature on the protein in *Torreya grandis* nuts was examined using water as a solvent. With a material-liquid ratio of 1:20, the temperature range of 40 °C to 80 °C for two hours was chosen. The protein content dropped to 1.3 mg/ml as the temperature rose as shown in Fig. 2B(H_2O). Since water is a polar solvent, proteins become denatured at higher temperatures. Therefore, it was evident from the results that water was not an appropriate solvent for effectively extracting the protein from *Torreya grandis* seed nuts.

3.3.3. Effect of solid-liquid ratio on Torreya grandis seed nut protein

Using DES and water, we examined how the solid-liquid ratio affected the protein in *Torreya grandis* nuts. The solid-liquid ratio was chosen from 1:05 to 1:160 using DES as the solvent. The average protein content increased to 12.8 mg when the material-liquid ratio was raised to 1:20. As shown in Fig. 2A(DES), the average protein concentration dropped to 8.3 mg when the material-liquid ratio rose to 1:160. As the solvent-material interaction increased, the larger material-liquid ratio caused a reaction. Addition of more deep eutectic solvent made it difficult to mix the protein with the solvent. (S. Wang et al., 2024) Wang et al. used DES to examine the impact of bamboo shoots. He demonstrated that a higher addition of bamboo shoots led to a higher extraction rate when the material-liquid ratio was 1:50. The rate of extraction decreased as more bamboo shoot protein was added. Adding more material liquid led to less effective contact with materials and poor solvent mixing. Therefore, they determined that the ideal material-liquid ratio for optimizing bamboo protein was 1:50 g/mL. Their findings and ours were comparable in that they demonstrated that raising the material-to-liquid ratio might raise the protein content.

Using water as a medium, the solid-liquid ratio was examined between 1:05 and 1:160. As the solid-liquid ratio rose, the average protein content increased to 12.7 mg. The average protein content increased as a result of the protein and water properly mixing, as seen in Fig. 2 A(H_2O). The findings showed that temperature, extraction time, and the solid-liquid ratio gradient level needed to be improved.

3.4. Selection of optimal conditions for the extraction of proteins from Torreya grandis seed nuts using DES

Traditionally, the optimization process has been conducted by examining the impact of a single element while holding the other factors constant. As is the case when protein isolates were extracted based on

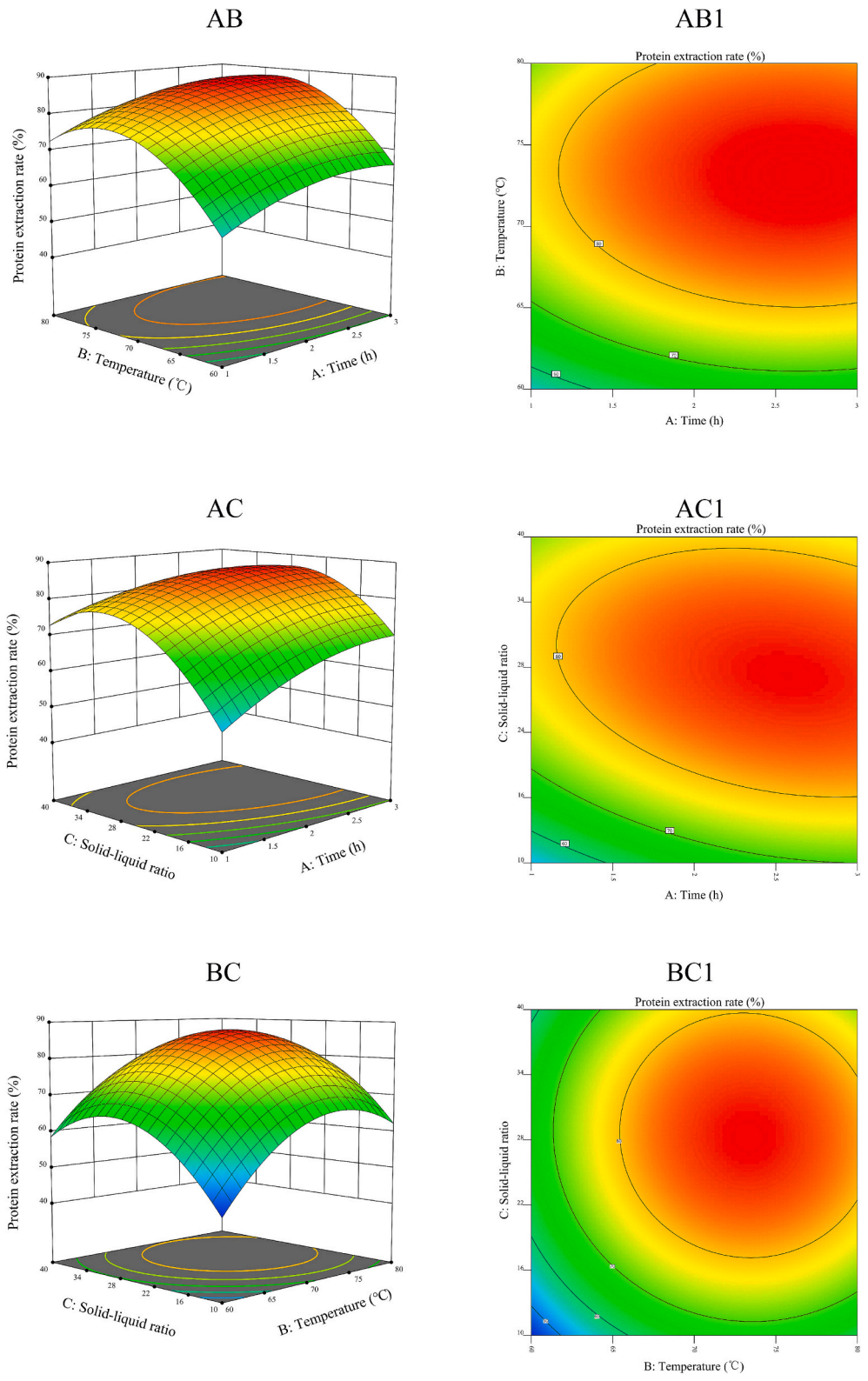


Fig. 3. Response Surface plot for *Torreya grandis* seed nut protein. AB. Effect of temperature and time on the protein extraction rate of *Torreya grandis* seed nut protein. AB1. Effect of time and temperature on the protein extraction rate of *Torreya grandis* seed nut protein. AC. Effect of solid-liquid ratio and time on the protein extraction rate of *Torreya grandis* seed nut. AC1. Effect of time and solid-liquid ratio on the protein extraction rate of *Torreya grandis* seed nut protein. BC. Effect of solid-liquid ratio and temperature on the protein extraction rate of *Torreya grandis* seed nut. BC1. Effect of temperature and solid-liquid ratio on the protein extraction rate of *Torreya grandis* seed nut. *The ABC in this picture corresponds to the ABC in Table 2, not the individual ABC.

Table 2
Levels of independent variables in response surface methodology design.

	−1	0	1
A(Time)	1	2	3
B(Temperature)	60	70	80
C(Solid-liquid ratio)	10	25	40

Table 3
Design and results of Box-Behnken design (BBD) experiment.[1]

Number	A (Time)	B (Temperature)	C(Solid-Liquid Ratio)	Protein extraction rate %
1	0	1	1	75.0355
2	−1	0	1	71.9554
3	0	0	0	85.4573
4	0	−1	-1	42.7365
5	0	0	0	83.2596
6	0	1	-1	61.6346
7	-1	1	0	71.7574
8	0	0	0	83.035
9	-1	-1	0	55.8598
10	**0**	**0**	**0**	**88.762**
11	1	0	1	74.7788
12	0	0	0	87.1578
13	-1	0	-1	54.6722
14	1	1	0	81.9682
15	1	0	-1	70.8427
16	1	-1	0	66.7475
17	0	-1	1	59.0756

[1] Mean of triplicate measurements, mean values with multiple letters in the same column shows statistical differences (Tukey test, $\alpha = 0.05$).

their varying pH-solubility, the evaluated response was typically influenced by a number of parameters and their interactions rather than just one. Response surface methodology (RSM) turns out to be an effective tool for optimizing the analytical process. It uses the fewest number of tests to deliver relevant data about a dataset in the shortest time. RSM has been utilized in various research to optimize the extraction of proteins from various sources (Baca-Bocanegra et al., 2021).

Five DES, as shown in Table 1, were successfully used in this experiment to extract the protein from *Torreya grandis* nut. Solid-liquid ratio 1: 05–1: 160, extraction period of 1–6 h, and ultrasonic water bath temperature between 40 and 80 °C were used in the single factor experiment as shown in Fig. 3 (AB) (AB1), (AC)(AC1), (BC),(BC1). The optimal gradient of the solid-liquid ratio varied considerably depending on the extraction temperature and time as shown in Table 2, according to the results of the single factor experiment. However, they were within the minimum range. As a result, the solid-liquid ratio, extraction duration, and temperature of the ultrasonic water bath were chosen as Box-Behnken design parameters. As shown in Table 3, the three ideal factors, three levels, and five times-centered parallel experiment were carried out. Design-Expert 13 was used to perform the quadratic response surface regression analysis. The quadratic response surface regression equation for *Torreya grandis* protein extraction rate was determined by fitting the regression equation and comparing it to the actual values of each individual factor. Regression eq.

$Y = 85.53 + 5.01\ A + 8.25B + 6.37C\text{-}0.1692AB\text{-}3.34\ AC\text{-}0.7346\ BCE\text{-}4A^2\text{–}12.45B^2\text{–}12.47C^2$ was used to extract the protein from *Torreya grandis* nuts. The protein extraction rate of *Torreya grandis* nut protein in the regression model had $P < 0.0001$, according to the quadratic regression analysis table, indicated that the regression equation was reliable with accurate results. The lack of fit model $P > 0.05$ indicated that the model was well-fitted based on the pure error, which was not significant. The R2 and R^2_{adj} were 0.9871 and 0.9704, respectively. There was great degree of concordance established between actual and projected values. The experimental results were least affected by the response surface approach results. The extraction time and ultrasonic water bath temperature had *P*-values of 0.0004 and 0.0001, respectively, and F-values of 39.22 and 106.22 shown in quadratic regression analysis as shown in Table 4. The F value was 63.37 and the solid-liquid ratio *P* value was 0.0001. This showed that the temperature of the ultrasonic water bath had the greatest impact on the rate of protein extraction from *Torreya grandis* nut protein, followed by the extraction time and the solid-liquid ratio. (Guzmán-Lorite et al., 2022a) showed that using RSM for the successive extraction and recovery of pomegranate seed proteins, the model indicated that the following parameters would provide the best results for extracting proteins from pomegranate seeds: ultrasonic amplitude of 80 %, extraction duration of 15 min, temperature of 60 °C, and sample size of 27.8 mg. The anticipated protein yield in these circumstances was 13.3 g protein/100 g of defatted milled seed. Compared with their results *Torreya grandis* predicted protein extraction yield was 88.7 % as shown in Table 3.

The interaction between the two components is quite substantial in response surface box plots with a steeper surface. The contour lines' form reflects the interaction length. Smaller interactions were indicated by the box plot's circle, but larger interactions were indicated by the ellipse. According to our findings, the strongest correlation between extraction time and ultrasonic water bath temperature had the biggest impact on the rate of protein extraction from *Torreya grandis* nuts. This was followed by the solid-liquid ratio and ultrasonic water bath temperature.

3.5. Composition of total proteins from Torreya grandis seed nut extracted by DES and water

After the *Torreya grandis* nut protein was extracted from water and

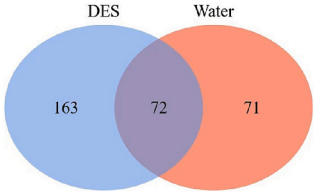
Table 4
Regression coefficients and ANOVA for the response surface variable in the second-order polynomial model.

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	P-value	Significance
Model	2736.34	9	304.04	59.35	< 0.0001	**
A	200.92	1	200.92	39.22	0.0004	**
B	544.11	1	544.11	106.22	< 0.0001	**
C	324.61	1	324.61	63.37	< 0.0001	**
AB	0.1146	1	0.1146	0.0224	0.8853	
AC	44.54	1	44.54	8.69	0.0214	*
BC	2.16	1	2.16	0.4213	0.537	
A^2	67.53	1	67.53	13.18	0.0084	**
B^2	652.26	1	652.26	127.33	< 0.0001	**
C^2	763.66	1	763.66	149.08	< 0.0001	**
Residual	35.86	7	5.12			
Lack of Fit	11.38	3	3.79	0.6197	0.6383	
Pure Error	24.48	4	6.12			
Cor Total	2772.199809	16				
R2 = 0.9871	RAdj2 = 0.9704	C.V. % = 3.17				

Note: "*"indicates the existence of a significant difference ($P < 0.05$), "**" indicates the existence of an extremely significant difference ($P < 0.01$).

Proteome

A



B Proteins(Top30)

DES(Accession)	Peptides	Molecular Weight	Abundances	Protein names	Water(Accession)	Peptides	Molecular Weight	Abundances	Protein names
107000_c0_g2	28	37.6	1.10E+11	Vicilin-like storage protein	107000_c0_g2	30	37.6	54565061247	Vicilin-like storage protein
106331_c2_g8	8	12.9	3.80E+10	2S seed storage protein	111713_c2_g2	11	14	28944908154	Bark protein-like protein
110283_c1_g1	8	12.2	3.76E+10	Bark protein-like protein	106331_c2_g8	7	12.9	18494754334	2S seed storage protein
105006_c1_g1	27	23.5	3.26E+10	Vicilin-like storage protein	108613_c0_g2	2	13.9	11669719056	Bark storage protein A
111713_c2_g1	6	15.9	2.08E+10	Bark protein-like protein	105006_c1_g1	25	23.5	11174598057	Vicilin-like storage protein
105006_c1_g2	12	23.9	1.72E+10	Vicilin-like storage protein	105006_c1_g2	14	23.9	9490328987	Vicilin-like storage protein
109715_c2_g1	5	12.9	1.52E+10	Bark storage protein A	109715_c2_g1	3	12.9	6046194303	Bark storage protein A
112970_c3_g2	5	14.5	1.49E+10	2S seed storage protein	118302_c3_g2	5	11.5	6034804277	2S seed storage protein
108613_c0_g1	6	17	1.47E+10	Bark storage protein A	109733_c1_g1	3	12	5997298134	Bark storage protein A
118302_c3_g2	6	11.5	1.44E+10	2S seed storage protein	110991_c1_g1	7	11.1	4829671808	Conglutin beta 2
105686_c2_g1	4	13.9	1.01E+10	Non-specific lipid-transfer protein	112970_c3_g2	4	14.5	4446183800	2S seed storage protein
108613_c0_g2	3	13.9	9.28E+09	Bark storage protein A	108613_c0_g1	3	17	3991172042	Bark storage protein A
114553_c2_g1	5	16.5	7.08E+09	Bark storage protein A	114553_c2_g1	4	16.5	3037211533	Bark storage protein A
110991_c1_g1	8	11.1	6.76E+09	Conglutin beta 2	102495_c1_g1	5	13.4	2524201654	2S seed storage protein
119119_c0_g4	7	21.6	6.32E+09	Mannose-specific lectin	119119_c0_g4	9	21.6	2438400888	Mannose-specific lectin
109733_c1_g1	3	12	6.31E+09	Bark storage protein A	105009_c3_g2	4	13.6	2058513560	Uncharacterized protein
117434_c2_g1	4	11.3	5.68E+09	Non-specific lipid-transfer protein	114850_c4_g2	4	11.6	1789917121	Bark storage protein A
109583_c1_g3	4	11.7	3.86E+09	Phosphoglucan-water dikinase	110283_c1_g1	9	12.2	1289081868	Bark protein-like protein
102495_c1_g1	5	13.4	3.82E+09	2S seed storage protein	113223_c2_g1	6	12	1207674048	Bark storage protein A
111068_c3_g1	6	16.8	3.66E+09	Vicilin-like antimicrobial peptides 2-2	105712_c0_g1	3	12.4	801377541.3	Sucrose-binding protein
112414_c0_g2	4	15	1.73E+09	2S seed storage protein	112414_c0_g2	5	15	771378638	2S seed storage protein
113168_c1_g1	3	11.9	1.19E+09	Bark protein-like protein	108551_c3_g1	6	11	718783546.2	Vicilin-like antimicrobial peptides 2-2
105009_c3_g2	4	13.6	1.14E+09	Uncharacterized protein	123001_c1_g3	1	75.5	513670048	Stromal 70 kDa heat shock-related protein
114850_c4_g2	4	11.6	9.22E+08	Bark storage protein A	105686_c2_g1	3	13.9	378627512	Non-specific lipid-transfer protein
105712_c0_g1	3	12.4	7.07E+08	Sucrose-binding protein	117434_c2_g1	3	11.3	220429600	Non-specific lipid-transfer protein
105075_c3_g1	1	17.2	6.40E+08	Uncharacterized protein	111068_c3_g1	6	16.8	217065767.6	Bark protein-like protein
108551_c3_g1	5	11	5.40E+08	Bark protein-like protein	104874_c1_g3	13	13.8	207352354	Vicilin-like antimicrobial peptides 2-2
118591_c0_g2	2	12.5	4.98E+08	Defensin 4	123793_c1_g1	7	13.5	192246672.3	Uncharacterized protein
124420_c1_g3	1	34.5	4.84E+08	Protein FIZZY-RELATED 2	124420_c1_g3	1	34.5	139643346.6	Protein FIZZY-RELATED 2
123793_c1_g1	6	13.5	3.59E+08	Uncharacterized protein	102580_c2_g2	6	44.5	99698188.5	Actin

C

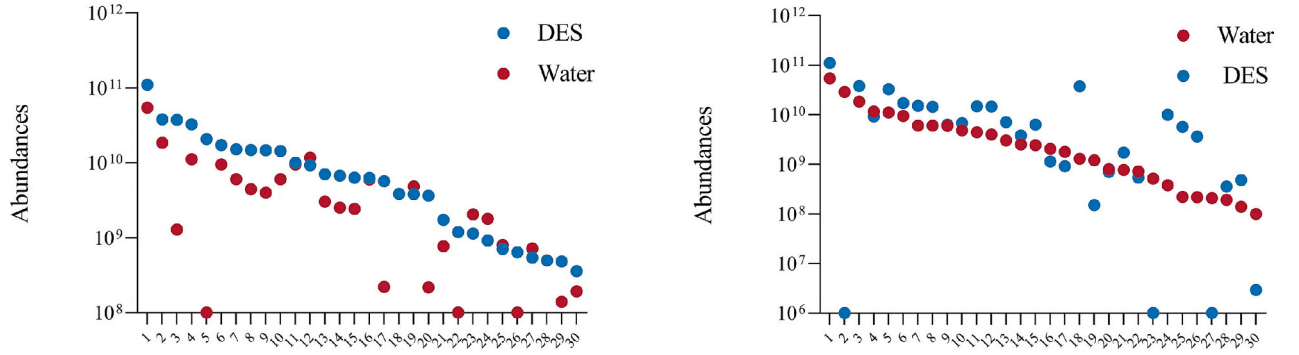


Fig. 4. Proteome of *Torreya grandis* seed nut protein extracted by DES and water. A. Protein in Venn diagram showed the number of peptides identified by *de novo* sequencing in DES and water. The blue color showed that peptides were both in DES and water and red color showed peptides were only in one group whereas purple color showed the peptides in both DES and water. B. Proteins (Top 30) Top 30 different distinct peptides found in *Torreya grandis* seed nut proteins after DES extraction. C. Abundances of peptides found in DES and water (the blue color showed peptides were found in both group and red color showed peptides were in one group). In Figure TOP30, the same background represents the same protein, the red characters on the white background represent that the protein does not exist in the other group, and the black characters on the white background represent that the protein exists in the other group but is not in the top 30. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DES, it was digested using trypsin and analyzed using LC-MS/MS (nanoLC-QE). Protein digestibility is a crucial factor in assessing its bioavailability because big molecules typically cannot pass through the intestinal lumen and must instead be broken down into smaller pieces, such as tiny peptides and amino acids (M. Guzmán-Lorite, M. L. Marina, & M. García, 2022b). *T. grandis* protein/polypeptide extract was digested with trypsin, and analyzed using LC-MS/MS (nanoLC-QE) generated a total of 600 peptides in DES with 235 proteins and 349 peptides in water with 143 proteins (Supplementary Protein File). Protein in Venn diagram Fig. 4A showed the number of peptides identified by *de novo* sequencing in DES and water with a total of 163 protein were found in DES and 71 in water with 72 common proteins found in both DES and Water. Numerous studies employed a range of bioactive peptides (BP) with different physiological effects in soybean seed storage protein. Since most studies focused on a single BP for the activity, there is little to no information available on BP for seed storage protein. The type of protein source, the enzyme used, and the processing method all affect each BP's biological activity. A single seed storage protein uses many enzymes and hydrolysis to release a large amount of BP. Many *in vivo* and animal studies are now being conducted to determine the bioactivities of seed storage proteins. Fewer clinical studies involving humans have been carried out, and it is less clear how such bioactive peptides from food-derived sources are distributed in the human digestive system *in vivo*. Further research would be helpful to determine whether regularly used grains and oilseeds produce bioactive peptides. Many bioactive peptides have been found to be reactive due to their low molecular weight; nevertheless, the precise mechanism by which they function is still unclear (Marambe & Wanasundara, 2012).

Conglutin β-2, 2S seed storage protein, vacillin-like storage protein, and bark protein-like protein were the most common proteins identified in both DES and water-based extracted proteins. As shown in Fig. 4B Proteins (Top30), the unique proteins include non-specific lipid transfer

Table 5
Proteome of DES extracted total proteins from *Torreya grandis* nut protein.

Functions	Proteins		Peptides		Peptide numbers	
	DES	**Water**	**DES**	**Water**	**DES**	**Water**
Anti-oxidant	Aspartic proteinase A1, Uncharacterized protein, Vicilin-like storage protein, Vicilin-like antimicrobial peptides 2–2, Chitin-binding protein,Conglutin beta 2, Uncharacterized protein, Probable glutathione S-transferase parC, Phosphoglucan-water dikinase, Uncharacterized protein, Bark storage protein A, Bark storage protein A, Bark storage protein A, Malate dehydrogenase, Nudix hydrolase 3, Proteasome subunit alpha type, 1-Cys peroxiredoxin, Bark protein-like protein, Desiccation-related protein PCC13–62-like protein, Bark protein-like protein, Mannosylglycoprotein *endo*-beta-mannosidase, Uncharacterized protein, Probable glutathione S-transferase parC, 4-hydroxy-4-methyl-2-oxoglutarate aldolase, Embryo-specific protein ATS3A, Uncharacterized protein, MD-2-related lipid-recognition protein ROSY1, Protein FIZZY-RELATED 2, 2S seed storage protein, Triosephosphate isomerase, Carboxyvinyl-carboxyphosphonate phosphorylmutase, Vicilin-like storage protein, Putative pentatricopeptide repeat-containing protein, Snakin-2, Alpha carbonic anhydrase 4, Superoxide dismutase, 2S seed storage protein, Isocitrate dehydrogenase, Gibberellin-regulated protein 8, Uncharacterized protein, SDD1 protein, Subtilisin-like protease SBT1.8, Alpha-mannosidase, Osmotin-like protein TPM-1, Thaumatin-like protein, Dihydrolipoyl dehydrogenase, Reactive Intermediate Deaminase A, Reactive Intermediate Deaminase A, STS14 protein,Pathogenesis-related protein 1C, Alpha-mannosidase, 6,7-dimethyl-8-ribityllumazine synthase, Glycoprotein endo-alpha-1,2-mannosidase-like protein, Cysteine proteinase inhibitor, Uncharacterized protein, Putative ubiquitin family protein, 18.2 kDa class I heat shock protein, Subtilisin-like protease SBT1.7, Subtilisin-like protease SBT1.1, Mannose-specific lectin, Ricin B-like lectin R40G3, Putative histone H1, Ricin B-like lectin R40G3, Band 7 protein, PLAT domain-containing protein 1, SDD1 protein, SDD1 protein, Probable UDP-arabinopyranose mutase 1, Protein STRICTOSIDINE SYNTHASE-LIKE 4, Uncharacterized protein, Bark protein-like protein, Bark storage protein A, Glyceraldehyde 3-phosphate dehydrogenase, Uncharacterized protein, Rhicadhesin receptor-like, Alpha-xylosidase 1, Nucleoside diphosphate kinase, Histone H3.2, Histone H3.3, Non-specific lipid-transfer protein-like protein, Actin	Chitin-binding protein, Uncharacterized protein, Bark storage protein A, Ricin B-like lectin R40G3, Superoxide dismutase, Vicilin-like storage protein, Uncharacterized protein,Uncharacterized protein, Subtilisin-like protease SBT1.7, Mannose-specific lectin, Uncharacterized protein, MD-2-related lipid recognition protein, ROSY1, Uncharacterized protein, Uncharacterized protein, Uncharacterized protein, Methylmalonate semialdehyde dehydrogenase, Actin, 4-hydroxy-4-methyl- 2-oxoglutarate aldolase, Bark storage protein A, Uncharacterized protein, Desiccation-related protein, PCC13–62-like protein, Uncharacterized protein, Protein fizzy-related 2, Uncharacterized protein, Non specific lipid-transfer protein-like protein At2g13820, Uncharacterized protein, Bark protein-like protein, Defensin 4	GGE, YLY, EAK, LLR, VYV, EYY, YIY, LHE, LPL, LHN, LWG, LWS, TDY, DHG, GAA, GPP, ACQ, YGLY, ADF, PEL, CME, SDF, LHD, PHR, IQY, LTC, TGC, AHK, FKK, HDH, AHH, HHY, QHH, YYR, YYL, YYG, AYY, EHH, LHA, LHF, LHL, LWD, LWK, LWT, PHA, PHD, PHG, PHI, PHV, VKP, LDY, GEC, QCL, YVE, DYK, SVL, LQL, YNL, VGDI, ELLT, IPAGV, AGDDAPR	GGE, YLY, EAK, LLR, VYV, EYY, YIY, LHE, LPL, LHN, LWG, LWS, TDY, DHG, GAA, GPP, ACQ, YGLY, KHH, LHI, LWF, PHY, VKV, TFE, NHAV, VAPEEHPV, GYCVSDNNCK	63	27

(*continued on next page*)

Table 5 (*continued*)

Functions	Proteins		Peptides		Peptide numbers	
Renin inhibitor	Alpha-mannosidase, SDD1 protein, Subtilisin-like protease SBT1.8, 1-Cys peroxiredoxin, Alpha-xylosidase 1, Malate dehydrogenase, Non-specific lipid-transfer protein-like protein	Non-specific lipid transfer protein-like protein At2g13820, Mannose-specific lectin	LPL, LALPA, LQL, YNL, IPAGV	LPL, LALPA	5	2
Opioid agonist	Bark protein-like protein	Bark protein-like protein	YGLF	YGLF	1	1
Anti-inflammatory	Ricin B-like lectin R40G3, Rapid alkalinization factor, Bark protein-like protein, Uncharacterized protein, Bark protein-like protein, Bark protein-like protein, Uncharacterized protein, Pathogenesis-related protein 1C, 1-Cys peroxiredoxin, Glucose regulated protein homolog 4, Probable glutathione S-transferase parC	Polyubiquitin 11	IPP, ANP, VPP, NLQ, LPF	IPP	5	1
Immunomodulating	4-hydroxy-4-methyl-2-oxoglutarate aldolase, Uncharacterized protein, Uncharacterized protein, Bark protein-like protein, Bark protein-like protein, Uncharacterized protein, Glyceraldehyde 3-phosphate dehydrogenase, Subtilisin-like protease SBT1.7, Thaumatin-like protein, Protein STRICTOSIDINE SYNTHASE-LIKE 4, Isocitrate dehydrogenase, Chitin-binding protein	4-hydroxy-4-methyl-2-oxoglutarate aldolase, Chitin-binding protein, Bark protein-like protein, Myosin heavy chain, Thaumatin-like protein Uncharacterized protein	YGG, YYP, YGLF, EAE, GVM, KEEAE, GLF	YGG, YYP, YGLF, EAE, GVM, KEEAE	7	6
Anti-thrombotic	Subtilisin-like protease SBT1.8, SDD1 protein, Dihydrolipoyl dehydrogenase, Bifunctional nitrilase/nitrile hydratase NIT4A, Uncharacterized protein, Histone H2A, Catalase isozyme 1, Aspartic proteinase A1, 1-Cys peroxiredoxin	Chitin-binding, Subtilisin-like protease SBT1.8	PGP, PPK, DDE	PGP, CRP	3	2
Pancreatic lipase inhibitor	Actin	Actin	AGDDAPR	VAPEEHPV	1	1
Anti-cancer	Thioredoxin, Vicilin-like storage protein, Vicilin-like antimicrobial peptides 2–2, Sucrose-binding protein, Conglutin beta 2, 6,7-dimethyl-8-ribityllumazine synthase, Uncharacterized protein, Uncharacterized protein, Superoxide dismutase	Vicilin-like storage protein, Vicilin-like antimicrobial peptides 2–2,Sucrose-binding protein, Conglutin beta 2, Uncharacterized protein	VVV, WTP	VVV	2	1

In this Table the peptides marked in blue represent the presence of both groups, and the peptides marked in red represent the absence of such peptides in the other group.

proteins, mannose-specific lectin, water-phosphoglucan dikinase sucrose binding protein, protein FIZZY-RELATED 2, and bark storage protein A. Another seed storage protein with anti-inflammatory and diabetes-preventive qualities was conglutin β-2. After trypsin digestion, we sequenced the amino acid sequence to further explain the functional characteristics of other identified peptides.

3.5.1. Proteome of DES extracted total proteins from Torreya grandis nut protein

Following the sequencing of the peptides from *Torreya grandis* nuts, the digested peptide sequences were looked up in the BIOPEP database. According to the findings, DES contained 87 functional oligopeptides, including 63 antioxidant peptides, 5 renin inhibitor peptides, opioid agonist 1, anti-inflammatory 3, immunomodulating 7, anti-thrombotic 3, pancreatic lipase inhibitor 1, and anti-cancer 2. 41 functional oligopeptides identified in water, 27 were antioxidants, renin inhibitors, opioid agonists, anti-inflammatory, immunomodulating, anti-thrombotic, pancreatic lipase inhibitors, and anti-cancer (Table 5). The peptides indicated in red showed the lack of such peptides in the other group, as seen in Fig. 4C, the blue indicates that the peptides were present in both groups. All of these functional oligopeptides belonged to eight functional groups in both DES and water as shown in peptide summary Fig. 5B. According to (Luo et al., 2021), the protein from *Torreya grandis* nuts has antioxidant properties. In both groups, the *Torreya grandis* nut protein included the blue-marked peptide IPP (Ile-Pro-Pro), which has anti-inflammatory properties. Additionally, this anti-inflammatory peptide was discovered by (Chakrabarti et al., 2015). The greater solubility and affinity of DES for the *Torreya grandis* nut protein was the primary cause of the higher peptide identification in the DES group. Another possible explanation is that peptides were more stable in DES. This could lessen the loss of peptides throughout the extraction procedure. When compared to other functional peptide groups, such as anti-cancer and pancreatic lipase inhibitors, the antioxidant peptide had the highest relative abundance of all functional oligopeptides. As illustrated in Fig. 5C antioxidant, the relative abundance of distinct peptides, GGE was greater than CME and YIY.

3.5.2. Antioxidative activity of Torreya grandis total nut protein in DES and water

Reactive oxygen species (ROS) can be neutralized by a bioactive peptide (BP) with antioxidant activity, which also prevents lipid peroxidation and preserves the structure and functionality of mitochondria and cells. This kind of bioactive peptide can prevent many dangerous diseases and slow the aging process. Other properties of this antioxidant

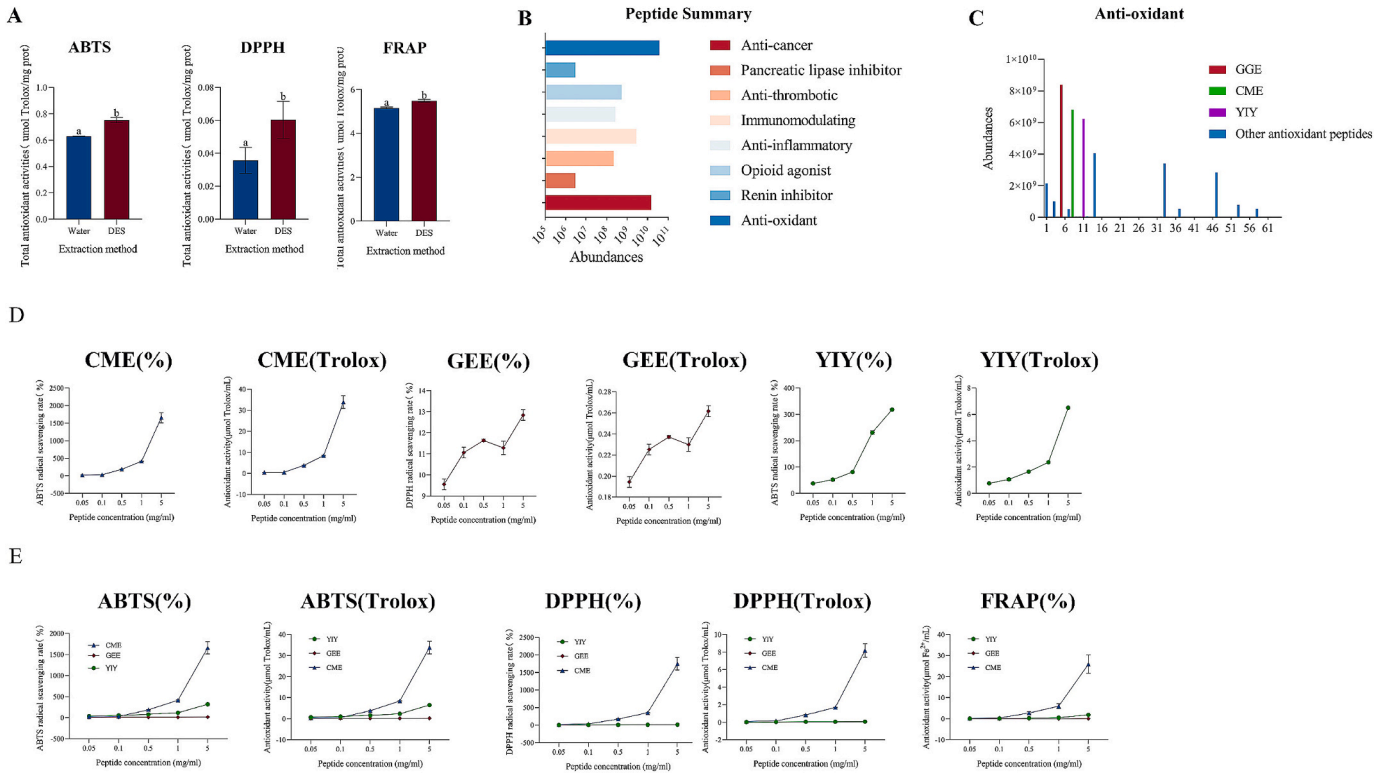


Fig. 5. Figure. The antioxidant activity of protein and peptides from *Torreya grandis* seed nut. A. Comparison of *in vitro* antioxidant activity of proteins extracted by DES or water. B.(Peptide Summary). Abundances of different functional peptides in the proteome of *Torreya grandis* seed nut. C.(Anti-oxidant). Abundances of antioxidant peptides in the proteome of *Torreya grandis* seed nut. D. *In vitro* antioxidant activity of three antioxidant peptides with the highest content in *Torreya grandis* seed nut (separation). E. *In vitro* antioxidant activity of three antioxidant peptides with the highest content in *Torreya grandis* seed nut (comparison). Each Value represents the mean ± SD (n = 3). Different alphabetical letters on the error bar were significantly different ($p < 0.05$) according to Duncan's Multiple range test (MRT).

BP include safety, reduced foaming, and water solubility. As a result, these peptides are well-known for their use and market value. The structure—that is, the content, molecular weight, and conformation of the amino acid sequence—determines the molecular mechanism of antioxidant BP. Aldehyde quenching, metal chelation, electron or hydrogen transfer reduction, and radical scavenging are some of the ways that BP demonstrates antioxidant activity in combination with parent protein (Durand et al., 2021).

In the food industry, antioxidant capacity is a highly valued attribute. Furthermore, the human body is significantly impacted by antioxidant molecules (Guzmán-Lorite et al., 2022b).(Quan et al., 2021) The antioxidant activity of *Torreya grandis* has already been determined, however, little is known about *Torreya grandis* nut peptides' antioxidant capacity. After trypsin digestion, proteome sequencing data showed the existence of anti-oxidative oligopeptides. Thus, as illustrated in Fig. 5A we used the Ultrasonic water bath extraction method and DES to test the antioxidant activity of *Torreya grandis* nut protein. Using DES had a greater ABTS+ of 0.751471375 (umol Trolox/mg prot) than water bath ultrasonic extraction, which had 0.629726964 (umol Trolox/mg prot). Moreover using DES, had a greater 0.060338633 (umol Trolox/mg prot) DPPH activity than Ultrasonic water bath extraction 0.035767973 (umol Trolox/mg prot). In contrast to Ultrasonic water bath extraction 5.159848825 (umol Trolox/mg prot), the FRAPH activity was high in DES 5.488029126 (umol Trolox/mg prot). Overall, the results demonstrated that proteins were more stable in DES and they could better sustain protein activity during the extraction process.

The amino acid content and sequence of the protein play a major role in determining the presence of antioxidant peptides with required bioactivities. The amino acid sequence of the protein can be identified in databases such as UniProtKB and NCBI if the protein source is examined. It was found that such protein's hydrolysate had specific bioactivity (Tejano et al., 2019). (Darewicz et al., 2016) used the UniProtKB database to determine the amino acid sequence of the Carp protein in order to anticipate potential antioxidant peptides. Alternatively, proteomic approaches can be used to examine and describe the amino acid sequences of unknown proteins from new source materials (Harvian et al., 2019). Different proteins from food components can be identified and determined using proteomics, a mass spectrometer (MS)-based technique (Hunag et al., 2015; Panjaitan et al., 2018; Tejano et al., 2019). BLAST analysis can be used to further observe the sequence and verify the homology of the detected protein by aligning the sequence from MS methods with comparable proteins from other materials retrieved from the NCBI database. BLAST analysis generates information about the "identities," "positives," and "gaps" between two or more aligned proteins (Panjaitan et al., 2018).

We chose three distinct antioxidant peptides—predicted CME, GGE, and YIY. Their antioxidant activity was assessed using a kit that employed two separate units (μmol Trolox/mL) and the radical scavenging rate (%) to measure the antioxidant activity of Fe^{+2} chelation, DPPH (2,2-diphenyl−1-picrylhydrazyl), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS+) as shown in Fig. 5D. As seen in Fig. 5E we found out that CME peptide had more antioxidant activity; nevertheless, this approach was unable to quantify FRAP.

Fig. 6. showed the structure of the peptides and comprehensively explained the source of their antioxidant capacity through functional groups and structure-activity relationships. In Fig. 6 Structures of various colors stand in for different amino acid residues. The adjacent black text explains how the residues' functional groups contribute to antioxidant capacity, while the red text represents the residues' abbreviations. Tyrosine residues' phenolic hydroxyl groups were the main

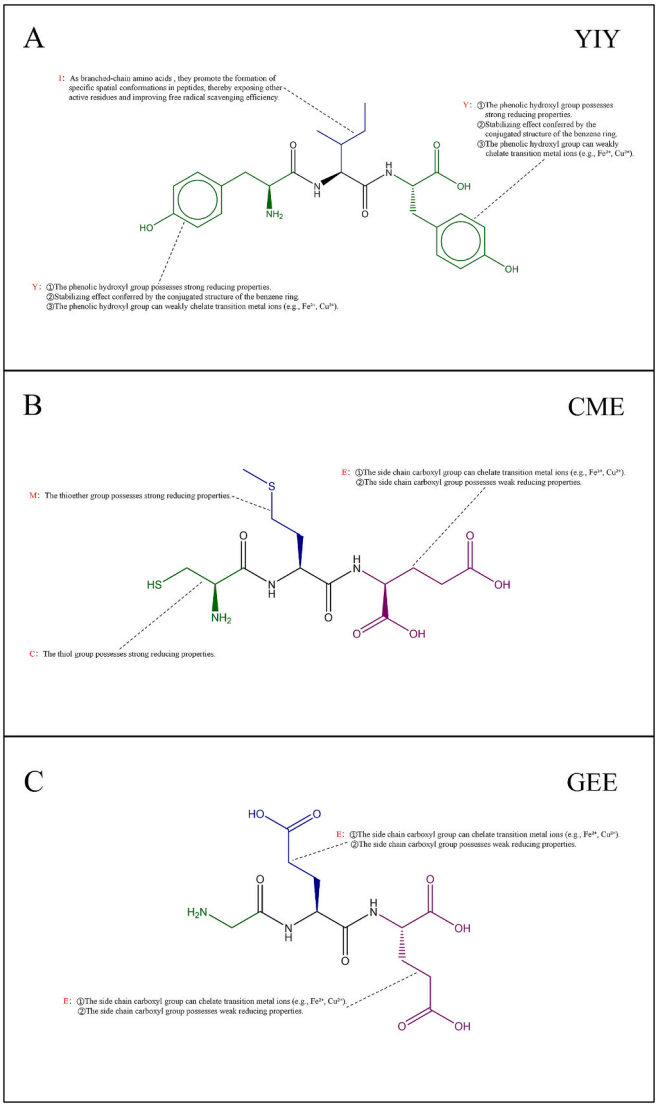


Fig. 6. Structure of the peptides and the source of their antioxidant capacity through functional groups and structure-activity relationships.

source of YIY's antioxidant activity. Strong reducing qualities that can neutralize free radicals and stop free radical chain reactions are possessed by these phenolic hydroxyl groups. At the same time, the phenolic hydroxyl groups' conjugated benzene ring structure can stabilize free radical intermediates, stopping oxidation processes from spreading and improving antioxidant effectiveness. Additionally, YIY's isoleucine residue may help generate a particular spatial conformation by functioning as a branched side chain. Two tyrosine phenolic hydroxyl groups may be exposed more easily thanks to this structural configuration, allowing for their cooperative interaction. The antioxidant activity of CME was significantly influenced by the residues of all three amino acids. Strong reducing qualities allow the thioether group in methionine and the sulfhydryl group in its cysteine residue to counteract free radicals and have antioxidant effects. Its glutamate residue's side chain carboxyl group can also chelate free transition metal ions, such as Fe^{2+} and Cu^{+}, which prevents oxidative chain reactions, the Fenton reaction, and the production of free radicals. The glutamate residues in GEE are the main source of its antioxidant potential. The two glutamate residues have poor reducing qualities and high chelating capabilities toward transition metal ions due to their side chain carboxyl groups. Together, these pathways give GEE its antioxidant properties. These findings demonstrated that average antioxidant capacity, average DPPH radical scavenging rate, and average ABTS radical scavenging rate all increased with peptide content.

4. Conclusion

This study investigated a more sustainable extraction method using deep eutectic solvents (DES) to enhance both protein content and yield from *Torreya grandis* seed nuts. Proteins from *T. grandis* were extracted using five different choline chloride-based DES and compared to water extraction. Among the DES tested, Choline chloride:Urea demonstrated superior performance in preserving protein integrity and functionality, achieving a purification rate of 6.89 % ± 0.12 % with cold ethanol precipitation. Optimization of extraction conditions, including solid-liquid ratio, temperature, and time, was carried out using the Box-Behnken design and response surface methodology. The functional properties and digestibility of the proteins were assessed *via* SDS-PAGE, which confirmed the advantage of DES extraction over water extraction. Notably, DES-extracted proteins yielded a twofold increase in recovery. Following trypsin digestion, the protein samples were analyzed using LC-MS/MS, revealing 600 peptides from 235 proteins in DES extracts, compared to 349 peptides from 143 proteins in water extracts. *De novo* sequencing identified 87 functional oligopeptides in DES extracts *versus* 41 in water, with unique peptides such as GGE, CME, and YIY showing higher relative abundance and antioxidant activity. Among these, CME exhibited the strongest antioxidant activity. Future research is necessary to evaluate the functional performance, potential toxicity, and residual content of *T. grandis* protein extracts before incorporating DES-extracted proteins into the food industry as a novel protein ingredient.

CRediT authorship contribution statement

Rabia Durrani: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Hou Bowen:** Methodology. **Erwann Durand:** Writing – review & editing. **André Delavault:** Writing – review & editing. **Hammad Ullah:** Formal analysis. **Zeyuan Ling:** Software. **Gao Fei:** Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Song Lili:** Resources, Project administration, Investigation, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102691.

Data availability

Data will be made available on request.

References

Baca-Bocanegra, B., Nogales-Bueno, J., Hernández-Hierro, J. M., & Heredia, F. J. (2021). Optimization of protein extraction of oenological interest from grape seed meal using Design of Experiments and Response Surface Methodology. *Foods, 10*(1). https://doi.org/10.3390/foods10010079

Bai, C., Wei, Q., & Ren, X. (2017). Selective extraction of collagen peptides with high purity from cod skins by deep eutectic solvents (DESs). *ACS Sustainable Chemistry & Engineering, 5*. https://doi.org/10.1021/acssuschemeng.7b01439

Bubalo, C., Marina, Ć., Natka, T., Marina, K. G., & Karin, & Radojčić Redovniković, Ivana.. (2016). Green extraction of grape skin phenolics by using deep eutectic solvents. *Food Chemistry, 200*, 159–166. https://doi.org/10.1016/j.foodchem.2016.01.040

Contreras, M., del Mar, L.-M., Antonio, M. G.-P., José, E., Francisco, M., & Manuel, & Castro, Eulogio.. (2019). Protein extraction from Agri-food residues for integration in biorefinery: Potential techniques and current status. *Bioresource Technology, 280*, 459–477. https://doi.org/10.1016/j.biortech.2019.02.040

Costanzo, J. (2019). Overwintering adaptations and extreme freeze tolerance in a subarctic population of the wood frog, Rana sylvatica. *Journal of Comparative Physiology B, 189*. https://doi.org/10.1007/s00360-018-1189-7

Cui, Q., Liu, J.-Z., Wang, L.-T., Kang, Y.-F., Meng, Y., Jiao, J., & Fu, Y.-J. (2018). Sustainable deep eutectic solvents preparation and their efficiency in extraction and enrichment of main bioactive flavonoids from sea buckthorn leaves. *Journal of Cleaner Production, 184*, 826–835. https://doi.org/10.1016/j.jclepro.2018.02.295

Dai, Y., Witkamp, G. J., Verpoorte, R., & Choi, Y. H. (2015). Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chemistry, 187*, 14–19. https://doi.org/10.1016/j.foodchem.2015.03.123

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica Chimica Acta, 766*, 61–68. https://doi.org/10.1016/j.aca.2012.12.019

Darewicz, M., Borawska-Dziadkiewicz, J., & Pliszka, M. (2016). Carp proteins as a source of bioactive peptides – An in silico approach. *Czech Journal of Food Sciences, 34*, 111–117. https://doi.org/10.17221/300/2015-CJFS

Dong, D., Wang, H., Xu, F., Xu, C., Shao, X., & Li, H. (2014). Supercritical carbon dioxide extraction, fatty acid composition, oxidative stability, and antioxidant effect of Torreya grandis seed oil. *Journal of the American Oil Chemists' Society, 91*(5), 817–825. https://doi.org/10.1007/s11746-014-2419-0

Durand, E., Beaubier, S., Ilic, I., Fine, F., Kapel, R., & Villeneuve, P. (2021). Production and antioxidant capacity of bioactive peptides from plant biomass to counteract lipid oxidation. *Current Research in Food Science, 4*. https://doi.org/10.1016/j.crfs.2021.05.006

Durrani, R., Meiyun, Y., Yang, B., Durand, E., Delavault, A., Bowen, H., & Fei, G. (2023). Identification of novel bioactive proteins and their produced oligopeptides from Torreya grandis nuts using proteomic based prediction. *Food Chemistry, 405*, Article 134843. https://doi.org/10.1016/j.foodchem.2022.134843

Franca-Oliveira, G., Fornari, T., & Hernández-Ledesma, B. (2021). A review on the extraction and processing of natural source-derived proteins through eco-innovative approaches. *Processes, 9*, 1626. https://doi.org/10.3390/pr9091626

Gençdağ, E., Görgüç, A., & Yılmaz, F. M. (2020). Recent advances in the recovery techniques of plant-based proteins from Agro-industrial by-products. *Food Reviews International, 37*, 447–468.

Grudniewska, A., Melo, E., Chan, A., Gniłka, R., Boratyński, F., & Matharu, A. (2018). Enhanced protein extraction from oilseed cakes using glycerol-choline chloride deep eutectic solvents: A biorefinery approach. *ACS Sustainable Chemistry & Engineering, 6*. https://doi.org/10.1021/acssuschemeng.8b04359

Guzmán-Lorite, M., Marina, M. L., & García, M. C. (2022). Successive extraction using natural deep eutectic solvents and pressurized liquids for a greener and holistic recovery of proteins from pomegranate seeds. *Food Research International, 161*, Article 111862. https://doi.org/10.1016/j.foodres.2022.111862

Harvian, Z., Ningrum, A., Anggrahini, S., & Setyaningsih, W. (2019). In silico approach in evaluation of Jack bean (Canavalia ensiformis) Canavalin protein as precursors of bioactive peptides with dual antioxidant and angiotensin I-converting enzyme inhibitor. *Materials Science Forum, 948*, 85–94. https://doi.org/10.4028/www.scientific.net/MSF.948.85

Hernández-Corroto, E., Plaza, M., Marina, M. L., & García, M. C. (2020). Sustainable extraction of proteins and bioactive substances from pomegranate peel (Punica granatum L.) using pressurized liquids and deep eutectic solvents. *Innovative Food Science & Emerging Technologies, 60*, Article 102314. https://doi.org/10.1016/j.ifset.2020.102314

Horwitz, W., & Albert, R. (2006). The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. *Journal of AOAC International, 89*, 1095–1109. https://doi.org/10.1093/jaoac/89.4.1095

Hunag, B.-B., Lin, H.-C., & Chang, Y.-W. (2015). Analysis of proteins and potential bioactive peptides from tilapia (Oreochromis spp.) processing co-products using proteomic techniques coupled with BIOPEP database. *Journal of Functional Foods, 629*.

Katrak, V., & Ijardar, S. (2024). Redefining the landscape of protein extraction and separation from various sources using deep eutectic solvents. *Trends in Food Science & Technology, 153*, Article 104733. https://doi.org/10.1016/j.tifs.2024.104733

Kinugasa, T., Tanahashi, S., & Takeuchi, H. (1991). Extraction of lysozyme using reversed micellar solution. Distribution equilibrium and extractions rates. *Industrial & Engineering Chemistry Research*, 30. https://doi.org/10.1021/ie00059a017

Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., & Kennedy, J. F. (2021). Advances in the plant protein extraction: Mechanism and recommendations. *Food Hydrocolloids, 115*, Article 106595. https://doi.org/10.1016/j.foodhyd.2021.106595

Liu, A., Jing, H., Xiaojing, D., & Chaoyang, ma, & Wang, Hongxin.. (2022). Efficient ultrasonic-assisted aqueous enzymatic method for pecan nut kernel oil extraction with quality analysis. *Journal of Oleo Science, 71*, 1749–1760. https://doi.org/10.5650/jos.ess22119

Lucas, M. M., Stoddard, F., Annicchiarico, P., Frias, J., Martinez-Villaluenga, C., Sussmann, D., & Pueyo, J. (2015). The future of lupin as a protein crop in Europe. *Frontiers in Plant Science, 6*, 705. https://doi.org/10.3389/fpls.2015.00705

Luo, X., Wu, S., Xue, J., Hu, H., He, Z., Liu, X., & Wu, F. (2021). The bioactive peptide screening from Torreya grandis meal protein hydrolysates. *Food Bioscience, 44*, Article 101419. https://doi.org/10.1016/j.fbio.2021.101419

Mahmood, W., Asyraf, W. M., Lorwirachsutee, A., Theodoropoulos, C., & González-Miquel, M. (2019). *Polyol-based deep eutectic solvents for extraction of natural polyphenolic antioxidants from Chlorella vulgaris*. ACS Sustainable Chemistry & Engineering.

Marambe, P., & Wanasundara, P. (2012). *Seed storage proteins as sources of bioactive peptides*.

Ni, Q., Gao, Q., Yu, W., Liu, X., Xu, G., & Zhang, Y. (2015). Supercritical carbon dioxide extraction of oils from two Torreya grandis varieties seeds and their physicochemical and antioxidant properties. *LWT - Food Science and Technology, 60*, 1226–1234. https://doi.org/10.1016/j.lwt.2014.09.007

Omar, K. A., & Sadeghi, R. (2022). Physicochemical properties of deep eutectic solvents: A review. *Journal of Molecular Liquids, 360*, Article 119524. https://doi.org/10.1016/j.molliq.2022.119524

Pang, J., Sha, X., Chao, Y., Chen, G., Han, C., Zhu, W., & Zhang, Q. (2017). Green aqueous biphasic systems containing deep eutectic solvents and sodium salts for the extraction of protein. *RSC Advances, 7*(78), 49361–49367. https://doi.org/10.1039/C7RA07315A

Panjaitan, F., Gomez, H. L., & Chang, Y.-W. (2018). In silico analysis of bioactive peptides Released from Giant grouper (Epinephelus lanceolatus) roe proteins identified by proteomics approach. *Molecules, 23*, 2910. https://doi.org/10.3390/molecules23112910

Patra, A., Venugopal, A., & Pandiselvam, R. (2023). Deep eutectic solvent: An emerging trend for extraction of plant proteins. *Journal of Molecular Liquids, 389*, Article 122887. https://doi.org/10.1016/j.molliq.2023.122887

Quan, W., Zhang, C., Wang, Z., Zeng, M., Qin, F., & he, Zhiyong, & Chen, Jie.. (2021). Assessment antioxidant properties of Torreya grandis protein enzymatic hydrolysates: Utilization of industrial by-products. *Food Bioscience, 43*, Article 101325. https://doi.org/10.1016/j.fbio.2021.101325

Svigelj, R., Dossi, N., Grazioli, C., & Toniolo, R. (2021). Deep eutectic solvents (DESs) and their application in biosensor development. *Sensors, 21*, 4263. https://doi.org/10.3390/s21134263

Tahergorabi, R., & Hosseini, S. V. (2017). Chapter 2 - proteins, peptides, and amino acids. In C. M. Galanakis (Ed.), *Nutraceutical and functional food components* (pp. 15–38). Academic Press.

Tang, J., Yao, D., Xia, S., Cheong, L., & Tu, M. (2024). Recent progress in plant-based proteins: From extraction and modification methods to applications in the food industry. *Food Chemistry: X, 23*, Article 101540. https://doi.org/10.1016/j.fochx.2024.101540

Tejano, L., Peralta, J., Yap, E. E., Panjaitan, F., & Chang, Y.-W. (2019). Prediction of bioactive peptides from Chlorella sorokiniana proteins using proteomic techniques in combination with bioinformatics analyses. *International Journal of Molecular Sciences, 20*, 1786. https://doi.org/10.3390/ijms20071786

Toledo, M., Pereira, M., Freire, M., Silva, J., Coutinho, J., & Tavares, A. (2019). Laccase activation in deep eutectic solvents. *ACS Sustainable Chemistry & Engineering, 7*. https://doi.org/10.1021/acssuschemeng.9b02179

Wahlström, R., Rommi, K., Willberg-Keyriläinen, P., Ercili-Cura, D., Holopainen, U., Hiltunen, J., & Kuutti, L. (2017). High yield protein extraction from brewer's spent grain with novel carboxylate salt - urea aqueous deep eutectic solvents. *ChemistrySelect, 2*, 9355–9363. https://doi.org/10.1002/slct.201701492

Wang, L., Liu, F., Wang, A., Yu, Z., Xu, Y., & Yang, Y. (2017). Purification, characterization and bioactivity determination of a novel polysaccharide from pumpkin (Cucurbita moschata) seeds. *Food Hydrocolloids, 66*, 357–364. https://doi.org/10.1016/j.foodhyd.2016.12.003

Wang, S., Liu, Q., Xu, C., Gao, Y., & Yu, X. (2024). Response surface optimization for deep eutectic solvents extraction of bamboo shoots proteins. *Advances in Bamboo Science, 7*, Article 100080. https://doi.org/10.1016/j.bamboo.2024.100080

Xu, K., Wang, Y., Huang, Y., Li, N., & Wen, Q. (2015). A green deep eutectic solvent-based aqueous two-phase system for protein extracting. *Analytica Chimica Acta, 864*, 9–20. https://doi.org/10.1016/j.aca.2015.01.026

Yeow, A. T. H., Hayyan, A., Hayyan, M., Junaidi, U. M., Mohd, S., Jehad, J. B., … Mahar.. (2024). A comprehensive review on the physicochemical properties of deep eutectic solvents. *Results in Chemistry, 7*, Article 101378. https://doi.org/10.1016/j.rechem.2024.101378

Yu, M., Qin, F., & He, Zhiyong, & Chen, Jie.. (2017). Physicochemical and functional properties of protein extracts from Torreya grandis seeds. *Food Chemistry, 227*. https://doi.org/10.1016/j.foodchem.2017.01.114

Zainal-Abidin, M. H., Hayyan, M., Hayyan, A., & Jayakumar, N. S. (2017). New horizons in the extraction of bioactive compounds using deep eutectic solvents: A review. *Analytica Chimica Acta, 979*, 1–23.

Zeng, Q., Wang, Y., Huang, Y., Ding, X., & Kaijia, X. (2014). Deep eutectic solvents as novel extraction media for protein partitioning. *The Analyst, 139*. https://doi.org/10.1039/c3an02235h

Zhao, R.-t., Dong, P., Yu, P.-l., Wei, J.-t., Wang, N.-l., Di, D.-L., & Liu, Y.-w. (2019). Aqueous two-phase systems based on deep eutectic solvents and their application in green separation processes. *Journal of Separation Science, 43*. https://doi.org/10.1002/jssc.201900991