Separation and Purification of Astragalus membranaceus Polysaccharides by Deep Eutectic Solvents-Based Aqueous Two-Phase System

Bangfu Liu 1 and Zhijian Tan 2,*

1 Hunan Electronic Information Industry Institute, Changsha 410012, China
2 Institute of Bast Fiber Crops & Center of Southern Economic Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, China
* Correspondence: tanzhijian@caas.cn; Tel.: +86-731-8899-8517

Abstract: (1) Background: Aqueous two-phase systems (ATPSs) have been widely used in the separation and purification of bioactive substances in recent years. (2) Methods: In this study, deep eutectic solvents (DESs)-based ATPSs were employed for the extraction and separation of Astragalus membranaceus polysaccharides (AMP). The optimal DES (choline chloride:urea = 1:1) was first screened to extract AMP, and the effect of DES concentration, solid–liquid ratio, pH, extraction temperature, and extraction time on the extraction yield of AMP were investigated. (3) Results: The maximum extraction yield was 141.11 mg/g under the optimum conditions. AMP was then preliminarily purified by ATPS, to further realize the recycling and reuse of DES. The effect of type of salts, salt concentration, and extraction temperature on extraction efficiency was investigated. The extraction efficiency was 97.85% for AMP under the optimum ATPS conditions. Finally, the obtained AMP was studied by molecular weight determination, infrared spectroscopy analysis, and monosaccharide composition analysis. (4) Conclusions: This ATPS extraction based on DESs is simple, environmentally friendly, low-cost, and has high extraction efficiency, which provides new ideas for the extraction of plant polysaccharides and other bioactive compounds.

Keywords: Astragalus membranaceus polysaccharides; deep eutectic solvents; solid–liquid extraction; aqueous two-phase system; separation and purification

1. Introduction

Astragalus membranaceus, a genus of leguminosae in dicotyledons, is a kind of traditional Chinese medicinal plant and an important raw material of health food [1]. Studies have shown that the bioactive components of Astragalus membranaceus include polysaccharides, saponins, flavonoids, amino acids, and other compounds [2]. Astragalus membranaceus polysaccharide (AMP) is an important active component that has the functions of bacteriostasis, anti-inflammatory, enhancing immunity, and anti-aging [3].

So far, the main methods of AMP extraction include aqueous extraction, enzyme-assisted extraction, ultrasonic extraction, and alkali extraction [4–8]. Aqueous extraction has the disadvantages of low extraction efficiency and is very time-consuming. Although the enzyme-assisted extraction method has high extraction efficiency, it has a high requirement for environmental conditions, so it is not suitable for industrial production. Ultrasonic extraction has high extraction efficiency, but the strong mechanical vibration of the ultrasonic procedure may also destroy the structure of the polysaccharides and reduce the activity of the polysaccharides [6]. The alkali extraction not only has low extraction efficiency but also reduces the bioactivity of polysaccharides.

In 2003, deep eutectic solvents (DESs) were first prepared by Abbott and his coworkers, using choline chloride as a hydrogen bond acceptor (HBA) and urea as hydrogen bond donor (HBD) [9]. In 2019, Coutinho et al. improved the definition of DES, which
provided a solid theoretical basis for further research and application of DES [10]. In the past, traditional organic solvents were widely used to extract bioactive substances. However, these organic solvents are volatile and toxic, which can cause environmental pollution [11]. With the increasing requirements of the public for “green” and “environmental protection”, green chemistry has become one of the research hotspots in recent years. As environmentally friendly solvents, most DESs have the advantages of low toxicity, easy preparation, and good stability [12,13]. Therefore, DESs can be used as good substitutes for traditional organic solvents. At present, DESs have been widely used in the extraction of various natural substances, such as alkaloids [14], polyphenols [15,16], flavonoids [17], hemicellulose [18], lignin [19], etc. DESs are widely used in the extraction of bioactive compounds, but it is difficult to separate DESs from the target products after extraction. Therefore, switchable DESs have been developed, but they still have the disadvantages of difficult preparation and high cost [20].

An aqueous two-phase system (ATPS) is a mixed aqueous solution of either two hydrophilic polymers or a hydrophilic polymer and a salt, which will form two insoluble phases at appropriate concentrations [21,22]. When the target compounds are added to ATPS, they are separated into different phases due to the influence of surface properties and various interaction forces (such as hydrogen bond interaction and ionic bond interaction) [23,24]. Compared with the traditional oil–water solvent extraction systems, ATPS extraction has the advantages of mild operation conditions, excellent biocompatibility, and simple operation [25,26]. At present, ATPSs have been widely used in the extraction and separation of proteins [27,28], antibiotics [29], flavonoids [30], polysaccharides [31,32], and so on. DESs-based ATPSs have drawn more attention in the last few years, which were developed for the extraction of proteins [33], anthraquinones [34], DNA [35], 5-hydroxymethylfurfural (5-HMF) [36], and so on.

In this study, AMP was extracted by using DESs as the extractants to obtain the crude extract; the effect of DES concentration, solid–liquid ratio, pH, extraction temperature, and extraction time on the extraction yield of AMP was studied. Then, ATPS was constructed for the preliminary purification of AMP, and the effect of the type of salt, salt concentration, and extraction temperature on the extraction efficiency was investigated. Finally, AMP was separated from DES by recycling and reusing the DES.

2. Results and Discussion

2.1. Selection of the Optimal DES

Five DESs were selected as the extractants to extract AMP, and the extraction of AMP using water was used as a control. As shown in Figure 1, the extraction yield of AMP extracted with DES-1 is much higher than that of other DESs and water. This can be attributed to the fact that the HBD in DES-1 is alkaline urea, which is conducive to polysaccharide extraction. It is reported that the polarity, viscosity, and other properties of DESs may also affect the extraction efficiency of DESs; moreover, the hydrogen bond interaction, hydrophobic interaction, van der Waals force, and other interactions between DESs and the target materials can also affect the extraction ability of DESs [37,38]. Therefore, DES-1 was selected for the following studies.
when the extraction time is 30–90 min, the extraction yield of AMP increases significantly with the increase in the liquid–solid ratio. When the liquid–solid ratio is 40:1, the extraction was studied in this work. As shown in Figure 2b, the extraction yield continues to increase will increase the solution viscosity, which is also adverse for extraction. Therefore, 80 wt% yield reaches 153.07 mg/g. With the increase in the solvent, AMP can dissolve more in the solvent, while excessive solvent will cause waste. The results are similar to the reported weak when the DESs concentration is small. However, the high DESs concentration will increase the solution viscosity, which is also adverse for extraction. Therefore, 80 wt% DESs concentration was selected for further studies.

DES-1 was selected as the optimal extractant for AMP extraction. The effect of DESs concentration from 60 to 90 wt% on the extraction yield of AMP was studied. The results are shown in Figure 2a, and it can be seen that the extraction yield first increases and then decreases with the increase in DES concentration. When the concentration of DES is 80 wt%, the extraction yield reaches 135.92 mg/g. Because the hydrogen bonding forces are weak when the DESs concentration is small. However, the high DESs concentration will increase the solution viscosity, which is also adverse for extraction. Therefore, 80 wt% DESs concentration was selected for further studies.

The effect of the liquid–solid ratio from 20:1 to 40:1 on the extraction yield of AMP was studied in this work. As shown in Figure 2b, the extraction yield continues to increase with the increase in the liquid–solid ratio. When the liquid–solid ratio is 40:1, the extraction yield reaches 153.07 mg/g. With the increase in the solvent, AMP can dissolve more in the solvent, while excessive solvent will cause waste. The results are similar to the reported literature [39]. Therefore, a liquid–solid ratio of 40:1 was selected for the extraction.

The extraction pH from 7 to 11 was studied. The pH of the system was adjusted by a phosphate buffer solution. It can be seen from Figure 2c that the extraction yield did not change significantly with pH changing. Since the pH of the system itself is 9.2, so the system pH was not adjusted in the following studies.

Extraction temperatures from 40 to 80 °C were investigated. As shown in Figure 2d, the extraction yield increases significantly with the increase in temperature from 40 to 60 °C. When the extraction temperature is 60 °C, the extraction yield reaches 153.07 mg/g. The increase in temperature can reduce the viscosity of DESs and accelerate the mass transfer efficiency of polysaccharides, so the extraction yield increases. However, when the temperature is higher than 60 °C, the extraction yield has no obvious change. Therefore, 60 °C was selected as the optimal temperature.

The effects of extraction times from 30 to 150 min were studied. As shown in Figure 2e, when the extraction time is 30–90 min, the extraction yield of AMP increases significantly with the increase in extraction time. When the extraction time was 30 min, the extraction yield was 127.93 mg/g, and when the extraction time was 90 min, the extraction yield reached 157.78 mg/g. The extraction yield changes little with the further increase in extraction time. The longer extraction time will enhance the cost. Therefore, the extraction time of 90 min was selected.
The salt concentration is a very important factor in salting-out extraction, so it is necessary to investigate these factors.

2.3.1. Effect of Salt Type on ATPS Extraction

Two representative salts (K₂HPO₄ and K₃PO₄) were selected as salting-out reagents. As shown in the results, the AMP extraction efficiency of the DES/K₃PO₄ system (94.59%) is much higher than that of the DES/K₂HPO₄ system (56.44%). This can be attributed to the stronger alkalinity of K₃PO₄, which is conducive to polysaccharides extraction. Thus, the DES/K₃PO₄ system was chosen for further studies.

2.3.2. Effect of Salt Concentration on ATPS Extraction

The salt concentration is a very important factor in salting-out extraction, so it is necessary to study the effect of salt concentration on ATPS extraction. The effect of salt concentrations in the range of 33–41 wt% was investigated in this work. The results are shown in Figure 3, and it can be seen from the results that the extraction efficiency of AMP (94.14%) is the highest when the salt concentration is 41 wt%. The increase in salt concentration can improve the salting-out ability and facilitate phase separation. Since the salt solution was saturated, the salt concentration could not be increased further. Therefore, a salt concentration of 41 wt% was selected for subsequent studies.
solution was saturated, the salt concentration could not be increased further. Therefore, a salt concentration of 41 wt% was selected for subsequent studies.

2.3.3. Effect of Temperature on ATPS Extraction

The effect of temperature from 15 to 55 °C on ATPS extraction was investigated. The results in Figure 4 show that temperature has no significant effect on the extraction efficiency of AMP. Therefore, to facilitate the experiment, the follow-up experiments were conducted at room temperature.

2.4. Recycling Studies

The DES-rich phase was separated, the water in DES was removed by drying, and the AMP was extracted according to the optimal conditions. According to this procedure, the DES was recycled and reused for three cycles. As shown in Figure 5, the extraction yield and extraction efficiency of AMP decrease slightly after three cycles. The extraction yield and extraction efficiency of AMP in the third cycle are 131.65 mg/g and 97.51%, respectively. These results proved that DES has good recycling performance in the ATPS extraction process.
yield and extraction efficiency of AMP in the third cycle are 131.65 mg/g and 97.51%, respectively. These results proved that DES has good recycling performance in the ATPS extraction process.

Figure 5. The extraction yield (Y, mg/g) and extraction efficiency (%) of AMP in the recycling tests.

2.5. The Analysis of AMP

The molecular weight, monosaccharide composition, and FT-IR for AMP were analyzed. The structural characteristics of polysaccharides, including glycosidic bonds and functional groups, can be analyzed by FT-IR spectroscopy. The FT-IR is shown in Figure 6, which confirms the typical characteristic bands of AMP. The strong peak at 3315 cm$^{-1}$ is ascribed to the stretching vibration of O-H. The weak peak at 2960 cm$^{-1}$ is related to the stretching vibration of C-H. The absorption bands at 1733 and 1627 cm$^{-1}$ are caused by C=O asymmetric and symmetric stretching vibrations. The bond at 1478 cm$^{-1}$ is the symmetrical deformation vibration of C-H, and the absorption around 1192 cm$^{-1}$ is the stretching vibrations of the C-O-C and glycosidic bond [40]. The molecular weight of AMP is 4.86 kDa (Table S1 and Figure S1). The monosaccharide analysis of AMP is shown in Table 1 and Figure S2; it can be seen from the results that glucose, rhamnose, and fucose are the main monosaccharides of AMP.

Figure 6. The FT-IR spectra of AMP.
Table 1. The monosaccharide composition of AMP.

| Monosaccharide Composition | Percentage (%) |
|----------------------------|----------------|
| Mannose                   | 0.3352         |
| Ribose                    | 0.079652       |
| Rhamnose                  | 7.044621       |
| Glucuronic acid           | 4.45985        |
| Galacturonic acid         | 0.744248       |
| Glucose                   | 76.11869       |
| Galactose                 | 4.757241       |
| Xylose                    | 0.076693       |
| Arabinose                 | 0.982739       |
| Fucose                    | 5.41545        |

3. Materials and Methods

3.1. Materials and Reagents

Dried *Astragalus membranaceus* was purchased from a local drugstore in Changsha, Hunan Province. The dried *Astragalus* was crushed, sieved (60 mesh), and then stored on a dry and cool site. Choline chloride, urea, ethylene glycol, glycerin, oxalic acid, lactic acid, phenol, sulfuric acid, potassium phosphate, and dipotassium phosphate were all purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). All of the chemicals used in this study were of analytical grade.

3.2. Preparation and Characterization of DESs

The DESs were prepared by the heating method [12]. The HBDs and HBAs were stirred and heated at a certain molar ratio at 80 °C for 2 h to obtain a clear liquid. The details of these DESs are shown in Table 2. The characterizations of DESs (HBA: choline chloride) are shown in Figures S3–S7.

Table 2. The details of the prepared DESs (HBA: choline chloride).

| No.   | HBDs    | Molar Ratios |
|-------|---------|--------------|
| DES-1 | Urea    | 1:1          |
| DES-2 | Glycol  | 1:1          |
| DES-3 | Glycerol| 1:1          |
| DES-4 | Oxalate | 1:1          |
| DES-5 | Lactic acid | 1:1          |

3.3. Extraction of Polysaccharides Using DESs

In a 10 mL centrifuge tube, 0.2 g of dried *Astragalus* powder and a certain volume of DESs were added. AMP was extracted by ultrasonic-assisted heating. After centrifugation, the supernatant was withdrawn to determine the polysaccharide content. The content of polysaccharides was determined by the phenol–sulfuric acid method. The standard curve was obtained using glucose concentration as the abscissa and the absorbance at the 490 nm wavelength as the ordinate. The standard curve is shown in Equation (1). The standard glucose solution is in the concentration range of 2.5–12.5 µg/mL.

\[
y = 32.616x - 0.0104 \quad (R^2 = 0.9998)
\]

The extraction yield (Y, mg/g) of AMP is calculated by Equation (2).

\[
Y \text{ (mg/g)} = \frac{\text{Mass of determined AMP (mg)}}{\text{Mass of dried *Astragalus membranaceus* powder (g)}}
\]
3.4. Separation of Polysaccharides by ATPS

In a centrifuge tube, the crude polysaccharides extract and a certain amount of salt solution (K$_3$PO$_4$ or K$_2$HPO$_4$) were added, and the mixture was fully stirred. After centrifugation, two phases were formed. The volume of each phase was recorded, and the content of polysaccharides in the top and bottom phases were determined, respectively. The extraction efficiency (E, %) of AMP in the bottom phase was calculated by Equation (3).

\[
E(\%) = \frac{C_1 \times V_1}{C_2 \times V_2 + C_1 \times V_1} \times 100\%
\]

where $C_1$ and $V_1$ represent the AMP concentration and volume of the bottom phase, respectively. $C_2$ and $V_2$ represent the AMP concentration and volume of the top phase, respectively.

3.5. Determination of the Molecular Weight

The molecular weight of AMP was determined by HPLC-RID (LC-20, Shimadzu, Japan; RID-20, Shimadzu, Japan) equipped with an aqueous gel column (TSKgel GMPWXL, 7.5 mm × 300 mm, TOSOH, Tokyo, Japan). The parameter settings were as follows: the injection volume was 20 µL; the mobile phase was 0.1 mol/L NaNO$_3$ + 0.06% NaN$_3$ solution; the flow rate was 0.6 mL/min; the column temperature was 35 °C.

3.6. Analysis of the Monosaccharide

The Ultimate-3000 HPLC equipped with the Xtimate C$_{18}$ column (4.6 × 200 mm, 5 µm, Eka Nobel, Sweden) was used to analyze the monosaccharide constituents of the obtained AMP according to the reported method [41]. The parameter settings were as follows: a UV–Vis detector was used at the detection wavelength of 250 nm; the mobile phase was 0.05 mol/L potassium dihydrogen phosphate solution (pH = 6.7) and acetonitrile at a ratio of 83:17; the flow rate of 1.0 mL/min; the column temperature was 30 °C; the injection volume was 20 µL.

4. Conclusions

In this study, ATPS based on DESs has been developed for the extraction and preliminary purification of AMP. DES-1 (choline chloride:urea = 1:1) was selected as the optimal extractant. The extraction yield was 141.11 mg/g when the DES concentration was 80 wt%, the solid–liquid ratio was 1:40, the pH was not adjusted, the extraction temperature was 60 °C, and the extraction time was 90 min. Afterward, AMP was preliminarily purified by ATPS extraction. The extraction efficiency was 97.85% at the K$_3$PO$_4$ concentration of 41.0 wt% and extraction temperature of 25 °C. The extraction yield and extraction efficiency of AMP decreased slightly after three cycles, which proved that the DESs used in this study had good cyclic stability. The molecular weight determination, monosaccharide analysis, and FT-IR analysis of the obtained AMP were studied. This ATPS based on DESs can be effectively used for the extraction of AMP and allow for the recycling and reuse of extractants, which provides a new idea for the extraction of plant polysaccharides and other bioactive ingredients.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/molecules27165288/s1, Table S1: The molecular weight of AMP. Figure S1: High-performance gel permeation chromatograms for AMP. Figure S2: The HPLC chromatograms for monosaccharide analysis of AMP. Figure S3: The FT-IR spectra for DES-1. Figure S4: The FT-IR spectra for DES-2. Figure S5: The FT-IR spectra for DES-3. Figure S6: The FT-IR spectra for DES-4. Figure S7: The FT-IR spectra for DES-5.

Author Contributions: Z.T. conceived and designed the experiments; B.L. performed the experiments; Z.T. analyzed the data; B.L. and Z.T. wrote and revised the paper. All authors have read and agreed to the published version of the manuscript.
Funding: This research was funded by the Huxiang Young Talent Program from Hunan Province (No. 2021RC3116), the Training Program for Excellent Young Innovators of Changsha (No. kq2106073), the earmarked fund for the China Agriculture Research System (No. CARS-16-E24), and the Agricultural Science and Technology Innovation Program (No. ASTIP-IBFC08).

Institutional Review Board Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds *Astragalus membranaceus* are available from the authors.

References

1. Li, X.; Qu, L.; Dong, Y.; Han, L.; Liu, E.; Fang, S.; Zhang, Y.; Wang, T. A Review of Recent Research Progress on the Astragalus Genus. *Molecules* 2014, 19, 18850–18880. [CrossRef] [PubMed]
2. Zheng, Y.; Ren, W.; Zhang, L.; Zhang, Y.; Liu, D.; Liu, Y. A Review of the Pharmacological Action of Astragalus Polysaccharide. *Front. Pharmacol.* 2020, 11, 349. [CrossRef] [PubMed]
3. Tang, Z.; Huang, G. Extraction, structure, and activity of polysaccharide from Radix astragali. *Biomed. Pharmacother.* 2022, 150, 113015. [CrossRef]
4. Seedevi, P.; Moovendhan, M.; Sudharasan, S.; Sivasankar, P.; Sivakumar, L.; Vairamani, S.; Shanmugam, A. Isolation and chemical characteristics of rhamnose enriched polysaccharide from Grateloupia lithophila. *Carbohydr. Polym.* 2018, 195, 486–494. [CrossRef]
5. Cheng, Z.; Song, H.; Yang, Z.; Liu, Y.; Liu, Z.; Hu, H.; Zhang, Y. Optimization of microwave-assisted enzymatic extraction of polysaccharides from the fruit of Schisandra chinensis Bail. *Int. J. Biol. Macromol.* 2015, 76, 161–168. [CrossRef]
6. Panadare, D.C.; Gondaliya, A.; Rathod, V.K. Comparative study of ultrasonic pretreatment and ultrasound assisted three phase partitioning for extraction of custard apple seed oil. *Ultrason. Sonochem.* 2020, 61, 104821. [CrossRef]
7. Yu, J.; Ji, H.; Yang, Z.; Liu, A. Relationship between structural properties and antitumor activity of Astragalus polysaccharides extracted with different temperatures. *Int. J. Biol. Macromol.* 2019, 124, 469–477. [CrossRef]
8. Teng, C.; Qin, P.; Shi, Z.; Zhang, W.; Yang, X.; Yao, Y.; Ren, G. Structural characterization and antioxidant activity of alkali-extracted polysaccharides from quinoa. *Food Hydrocoll.* 2021, 113, 106392. [CrossRef]
9. Abbott, A.P.; Boothby, D.; Capper, G.; Davies, D.L.; Rasheed, R.K. Deep eutectic solvents formed between choline chloride and carboxylic acids: Versatile alternatives to ionic liquids. *J. Am. Chem. Soc.* 2004, 126, 9142–9147. [CrossRef] [PubMed]
10. Martins, M.A.R.; Pinho, S.P.; Coutinho, J.A.P. Insights into the Nature of Eutectic and Deep Eutectic Mixtures. *J. Solut. Chem.* 2019, 48, 962–982. [CrossRef]
11. Duan, L.; Dou, L.L.; Guo, L.; Li, P.; Liu, E.H. Comprehensive Evaluation of Deep Eutectic Solvents in Extraction of Bioactive Natural Products. *ACS Sustain. Chem. Eng.* 2016, 4, 2405–2411. [CrossRef]
12. Chen, J.; Li, Y.; Wang, X.; Liu, W. Application of Deep Eutectic Solvents in Food Analysis: A Review. *Molecules* 2019, 24, 4594. [CrossRef]
13. Paetzold, M.; Siebenhaller, S.; Kara, S.; Liese, A.; Syladt, C.; Holtmann, D. Deep Eutectic Solvents as Efficient Solvents in Bio catalysis. *Trends Biotechnol.* 2019, 37, 943–959. [CrossRef]
14. Jiang, Z.M.; Liu, W.J.; Li, Y.; Liu, J.; Wang, H.Y.; Li, P.; Liu, E.H. Eco-friendly Deep Eutectic Solvents Contribute to Improving the Separation of Isoquinoline Alkaloids in Supercritical Fluid Chromatography. *ACS Sustain. Chem. Eng.* 2020, 8, 13777–13783. [CrossRef]
15. El Kantar, S.; Rajha, H.N.; Bousseda, N.; Vorobiev, E.; Maroun, R.G.; Louka, N. Green extraction of polyphenols from grapefruit peels using high voltage electrical discharges, deep eutectic solvents and aqueous glycerol. *Food Chem.* 2019, 285, 165–171. [CrossRef] [PubMed]
16. Dabetic, N.; Todorovic, V.; Panic, M.; Redovnikovic, I.R.; Sobajic, S. Impact of Deep Eutectic Solvents on Extraction of Polyphenols from Grape Seeds and Skin. *Appl. Sci.* 2020, 10, 4830. [CrossRef]
17. Skarpalezos, D.; Detsi, A. Deep Eutectic Solvents as Extraction Media for Valuable Flavonoids from Natural Sources. *Appl. Sci.* 2019, 9, 4169. [CrossRef]
18. Colombo Dugoni, G.; Mezzetta, A.; Guazzelli, L.; Chiappe, C.; Ferro, M.; Mele, A. Purification of Kraft cellulose under mild conditions using choline acetate based deep eutectic solvents. *Green Chem.* 2020, 22, 8680–8691. [CrossRef]
19. Chen, Z.; Ragauskas, A.; Wan, C. Lignin extraction and upgrading using deep eutectic solvents. *Ind. Crops Prod.* 2020, 147, 112241. [CrossRef] [PubMed]
20. Pollet, P.; Eckert, C.A.; Liotta, C.L. Switchable solvents. *Chem. Sci.* 2011, 2, 609–614. [CrossRef]
21. Asenjo, J.A.; Andrews, B.A. Aqueous two-phase systems for protein separation: A perspective. *J. Chromatogr. A* 2011, 1218, 8826–8835. [CrossRef] [PubMed]
22. Sinha, J.; Dey, P.K.; Panda, T. Aqueous two-phase: The system of choice for extractive fermentation. *Appl. Microbiol. Biotechnol.* 2000, 54, 476–486. [CrossRef] [PubMed]
23. Ebrahimi, T.; Shahriari, S. Extraction of Betanin Using Aqueous Two-Phase Systems. *Bull. Chem. Soc. Jpn.* 2016, 89, 565–572. [CrossRef]
24. Kaplanow, I.; Goerzgen, F.; Merz, J.; Schembecker, G. Mass Transfer of Proteins in Aqueous Two-Phase Systems. Sci. Rep. 2019, 9, 1–6. [CrossRef] [PubMed]

25. Darani, S.F.; Ahsaie, F.G.; Puzuki, G.; Abdolrahimi, S. Aqueous two-phase systems based on thermo-separating copolymer for partitioning of doxorubicin. J. Mol. Liq. 2021, 322, 114542. [CrossRef]

26. Ahmed, T.; Yamanishi, C.; Kojima, T.; Takayama, S. Aqueous Two-Phase Systems and Microfluidics for Microscale Assays and Analytical Measurements. Anal. Rev. Anal. Chem. 2021, 14, 231–255. [CrossRef]

27. de Barros, D.P.C.; Campos, S.R.R.; Azevedo, A.M.; Baptista, A.M.; Raquel Aires-Barros, M. Predicting protein partition coefficients in aqueous two phase system. J. Chromatogr. A 2016, 1470, 50–58. [CrossRef]

28. Asenjo, J.A.; Andrews, B.A. Aqueous two-phase systems for protein separation: Phase separation and applications. J. Chromatogr. A 2012, 1238, 1–10. [CrossRef]

29. Zakrzewska, M.E.; Nunes, A.V.M.; Barot, A.R.; Fernandez-Castane, A.; Visak, Z.P.; Kiatkittipong, W.; Najdanovic-Visak, V. Extraction of antibiotics using aqueous two-phase systems based on ethyl lactate and thioulsalt. Fluid Phase Equilibria 2021, 539, 113022. [CrossRef]

30. Yang, S.; Liu, B.; Tang, M.; Yang, J.; Kuang, Y.; Zhang, M.; Zhang, C.; Wang, C.; Qin, J.; Guo, L.; et al. Extraction of flavonoids from Cyclocarya paliurus (Juglandaceae) leaves using ethanol/salt aqueous two-phase system coupled with ultrasonic. J. Food Processing Preserv. 2020, 44, e14469. [CrossRef]

31. Cheng, Z.; Song, H.; Cao, X.; Shen, Q.; Han, D.; Zhong, F.; Hu, H.; Yang, Y. Simultaneous extraction and purification of polysaccharides from Gentiana scabra Bunge by microwave-assisted ethanol-salt aqueous two-phase system. Ind. Crops Prod. 2017, 102, 75–87. [CrossRef]

32. Zhang, X.; Teng, G.; Zhang, J. Ethanol/salt aqueous two-phase system based ultrasonically assisted extraction of polysaccharides from Lilium davidiiivar. unicolor Salisb: Physicochemical characterization and antiglycation properties. J. Mol. Liq. 2018, 256, 497–506. [CrossRef]

33. Li, N.; Wang, Y.; Xu, K.; Huang, Y.; Wen, Q.; Ding, X. Development of green betaine-based deep eutectic solvent aqueous two-phase system for the extraction of protein. Talanta 2016, 152, 23–32. [CrossRef]

34. Deng, W.W.; Zong, Y.; Xiao, Y.X. Hexafluoroisopropanol-Based Deep Eutectic Solvent/Salt Aqueous Two-Phase Systems for Extraction of Anthraquinones from Rhei Radix et Rhizoma Samples. ACS Sustain. Chem. Eng. 2017, 5, 4267–4275. [CrossRef]

35. Xu, P.L.; Wang, Y.Z.; Chen, J.; Wei, X.X.; Xu, W.; Ni, R.; Meng, J.J.; Zhou, Y.G. A novel aqueous biphasic system formed by deep eutectic solvent and ionic liquid for DNA partitioning. Talanta 2018, 189, 467–479. [CrossRef] [PubMed]

36. Yu, X.; Li, M.; Yagoub, A.E.A.; Chen, L.; Zhou, C.; Yan, D. Switchable (pH driven) aqueous two-phase systems formed by deep eutectic solvents as integrated platforms for production-separation 5-HMF. J. Mol. Liq. 2021, 325, 115158. [CrossRef]

37. Wang, M.; Wang, J.; Zhou, Y.; Zhang, M.; Xia, Q.; Bi, W.; Chen, D.D.Y. Ecofriendly Mechanochemical Extraction of Bioactive Compounds from Plants with Deep Eutectic Solvents. ACS Sustain. Chem. Eng. 2017, 5, 6297–6303. [CrossRef]

38. Bi, W.; Tian, M.; Row, K.H. Evaluation of alcohol-based deep eutectic solvent in extraction and determination of flavonoids with response surface methodology optimization. J. Chromatogr. A 2013, 1285, 22–30. [CrossRef]

39. Wang, M.; Wang, J.; Zhou, Y.; Zhang, M.; Xia, Q.; Bi, W.; Chen, D.D.Y. Ecofriendly Mechanochemical Extraction of Bioactive Compounds from Plants with Deep Eutectic Solvents. ACS Sustain. Chem. Eng. 2017, 5, 6297–6303. [CrossRef]

40. Ren, L.; Wang, X.; Li, S.; Li, J.; Zhu, X.; Zhang, L.; Gao, F.; Zhou, G. Effect of gamma irradiation on structure, physicochemical and immunomodulatory properties of Astragalus polysaccharides. Int. J. Biol. Macromol. 2018, 120, 641–649. [CrossRef]

41. Cai, C.; Wang, Y.; Yu, W.; Wang, C.; Li, F.; Tan, Z. Temperature-responsive deep eutectic solvents as green and recyclable media for the efficient extraction of polysaccharides from Ganoderma lucidum. J. Clean. Prod. 2020, 274, 123047. [CrossRef]