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Oleanolic acid Separation From Grape Skins by Aqueous Two-phase Extraction and Estimate Its Antioxidant Activity

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Abstract The aim of the current study is to focus on separating the oleanolic acid (OA) from previously prepared aqueous two-phase extraction (ATPE) of grape skins. Several different influential extraction parameters, such as ethanol concentration (v/w), ammonium sulphate concentration (w/w), crude extract concentration (w/w), extraction temperature, and pH, were also investigated. The optimal differential partitioning of OA was achieved in a system (at pH4.0, temperature=25°C)composed of 23% (v/w) ethanol, 18% (w/w) ammonium sulphate, 8% (w/w) crude extract and 41% (w/w) water. The recovery of extracted OA from experiments was determined to be 93.54%. The antioxidant activity of the separated ATPE in relative to VC were, also, investigated in this study at the proper conditions.The ATPE extract showed a relatively high antioxidant ability compared with that of Vitamin C. This proposed extraction technique opens up new possibilities in extraction of other active ingredients in natural plants or biologic samples.

Keywords: *oleanolic acid, Aqueous two-phase extraction (ATPE), Ethanol/ammonium sulphate, Antioxidant activity*

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1. Introduction

Agricultural byproducts contain a variety of biologically active species which mostly go to waste. Particularly, most plant derived materials are rich in antioxidant compounds. An estimated 13% by weight of the grapes processed by the wine industry ends up as byproduct after pressing [1]. Grape skins are the primary main byproducts of viniculture and fermentation. In the last few years, increased attention has been focused on industrial wastes that are rich source of polyphenolic compounds, tannins, flavonoids, polysaccharides and triterpenoids [2,3,4,5]. Triterpenoids are important due to their high medicinal value; and oleanolic acid (OA) which is triterpenoid is major active substance. Figure 1 shows the chemical structure of oleanolic acid. Many researchers have studied the beneficial effects of OA in grapes and wines on human health [5]. These benefits include antioxidant activity [6], anti-inflammatory [7], anticancer activity [8], and others [9].

In recent years, industry has been seeking for efficient and economical downstream processes for partitioning and purification of OA. Several methods, such as extraction [10], precipitation, supercritical carbon dioxide purification [11] and column chromatographic purification [12], have been used for the fractionation of oleanolic acid from pomegranate flowers and Eucalyptus globulus bark. However, those methods are expensive, time-consuming, difficult for the recovery, reutilization of solvents, etc.

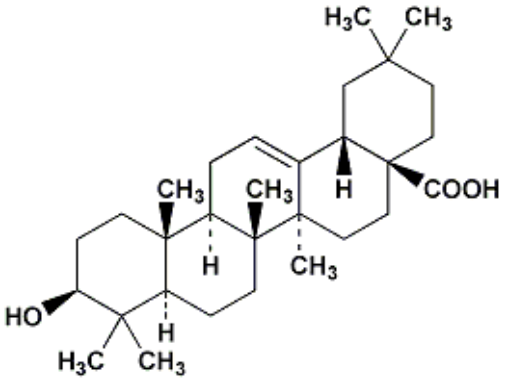

Figure 1. Chemical structure of oleanolic acid

Aqueous two-phase extraction (ATPE) is recognized as an effective, versatile and important emerging technique for the downstream processing of biomolecules. It will achieve high product purity as well as high yield, while maintaining the biological activity of the molecules, which has been widely applied in the separation of proteins, enzymes and antibiotics [13,14,15]. The aqueous two-phase system (ATPS) comprises of short chain

alcohols/hydrophilic organic solvents and inorganic salts, which have many advantages, such as high extraction capacity, mild conditions, low cost, short process time without back extraction, and the potential to achieve the desired purification and concentration of the product in a single step [16].

The purpose of the present work was to demonstrate that ATPE novel and effective friendly technique formed by ethanol-ammonium sulfate can be successfully applied to the extraction and separation of OA from grape skins. To optimize the partition coefficient and recovery of OA using this system, the influence of various process parameters, including ethanol concentration, ammonium sulfate concentration, extraction temperature, and pH on OA partition capacity and recovery were studied. Under the optimal conditions, this novel method was indeed capable of purification of the target materials. Undoubtedly, this research may aid in the further utilization of renewable resources, and this method is worthy of further investigation for the extraction and enrichment of natural products from other industrial byproducts and primary materials.

2. Materials and Methods

2.1. Materials and Reagents

Grape (*Vitis vinifera* L.) skins (by products) were obtained from the Bin He food industry Co, Ltd at spring season. (Gansu, China). The chemicals 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), Ethanol, ammonium sulfate, and perchloric acid were purchased from the sixth Chemical Reagent Factory of Tianjin, China. OA (purity >98% HPLC grade) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Other materials used in this study were of analytical grade.

2.2. Preparation of Crude Extract

Grape skins powders(10g) were extracted twice with 200mL 95% ethanol at 60°C for 2 h. The extract was centrifuged at12,000×g for 10min and the supernatant was filtered through a 0.45um filter membrane, the ethanol was removed by vacuum evaporator and the samples was changed to dryness. The crude extract could be diluted by deionized water to obtain different concentrations and to use in the following experiments.

2.3. Aqueous Two-Phase Extraction (ATPE)

An aqueous two-phase system was prepared as follows: 1.5-2.8g of ammonium sulphate was dissolved in water, and then certain volumes of ethanol and crude extract of the grape skins were added into the ammonium sulphate solution, and mixed well to form two phases. After that, more water was added to make the total weight of the system equal to 10g. The mixture was vortexed thoroughly and held until the two phases were completely separated. The OA concentration in both the top and bottom phases was analyzed.

In order to obtain the optimum extraction conditions, the effects of ammonium sulphate on the differential partitioning of OA in ATPS (at fixed 23% (v/w) ethanol concentration and 8% (w/w) crude extract concentration at 25°C, no pH adjustment) were investigated with different amounts of ammonium sulphate (final concentration from 16 to 24% (w/w)). If the ethanol concentration was less than 18%, no two-aqueous phase could be formed, whereas when the concentration was above 32%, the ammonium sulphate would precipitate in the system. Similarly, the ATPS with varied ethanol concentrations (final concentration from 19 to 27% (v/w)), under constant other conditions, were studied to investigate the influence of ethanol concentration on partition behaviours. Other parameters of the ATPS were also studied: the sample concentration was tested from 6 to 14% (w/w), the temperature was studied in the range of 25°–50°Cwater bath, and the pH was tried in the range of 3–7 with 1mol/L HCl.All the experiments were carried out in triplicate.

The volume ratio(R) of the ATPS was estimated via the equation [17].

$$R = V_t / V_b; \quad (1)$$

where V_t and V_b were the volumes of top phase and bottom phase, respectively.

The partition coefficient (K)of the OA was calculated using the equation [17].

$$K = C_t / C_b; \quad (2)$$

where C_t and C_b were the equilibrium concentrations of the OA in the top phase and bottom phase, respectively.

The recovery(Y) was the ratio of the OA partitioned in the top phase. It was calculated using the following equations [17].

$$Y = RK / (1 + RK) \quad (3)$$

2.4. Determination of OA Concentration [18]

The OA concentration in the top phase and bottom phase was estimated using the colorimetric method of vanillin perchloric acid under the conditions of OA as a standard. To evaporate sample solvent, the sample residue was reacted with 0.3 mL 5%(w/v) vanillin in glacial acetic acid and 1.0 mL perchloric acid at 70°C in water bath for 25 minutes, the optical density was measured at 548 nm.

2.5. Antioxidant Activity

2.5.1. DPPH Assay [19]

Any substance that can donate a hydrogen atom (antioxidant) to the solution of DPPH can reduce the stable free radical and change the colour of the solution from violet to pale yellow. The non-reacted radical form of DPPH absorbs in the visible range, and spectroscopic method is based on the measurements of colour intensity at 517 nm. The radical scavenging activity was calculated as follows:

$$\%\text{Inhibition} = \left[1 - \left(A_i - A_j\right) / A_0\right] \times 100$$

where A_0 was the absorbance of the blank sample, A_i was the absorbance in the presence of the test compound at different concentrations and A_j was the absorbance of the blank reagent.

2.6. Statistical Analysis

Data were reported as mean of triplicate determinations±SD (standard deviation). The statistical significances of process parameters were evaluated by analysis of variance using Microsoft excel. One-way ANOVA was applied to determine the statistic differences using SPSS statistical software (SPSS Statistics 17.0, Chicago, IL, USA). $P<0.05$ was considered as significantly different.

3. Results and Discussion

3.1. Effects of Ethanol Concentration on the Partition Coefficients and Recoveries of OA in ATPS

The effects of ethanol on the partition coefficients and recoveries of OA were further investigated. Figure 2 shows the curves of the partition coefficients and recoveries of OA versus the concentration of ethanol. With the ethanol concentration increasing from 19 to 27%.The partition coefficients and recoveries of OA were firstly enhanced. At a concentration of ethanol of 23% (v/w), the highest partition coefficient and recovery of the OA were achieved (10.53 and 91.04%, respectively). Such behavior can be attributed to an increase in the ethanol concentration in the top phase, which will favor partitioning towards the upper phase for OA soluble in alcohol. When the ethanol concentration was more than 23% (v/w), The partition coefficients and recoveries of OA decreased slightly. This may be because that the content of OA in top phase was closed to saturation, and the distribution in bottom phase was more than top phase, which reduced the OA partition coefficient and recoveries [16]. Thus, 23% (v/w) ethanol was used in further experiments.

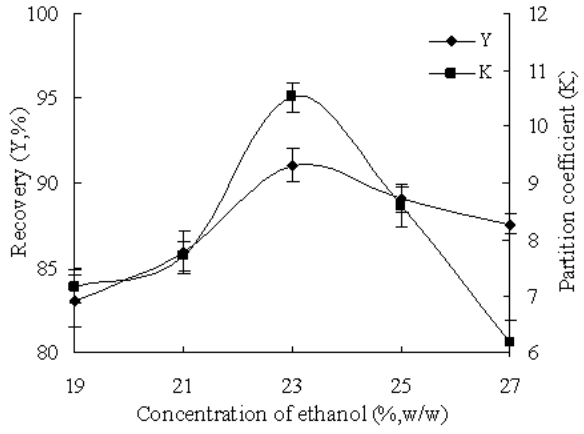

Figure 2. Effects of the ethanol concentration on the partition coefficients and the recoveries of OA

3.2. Effects of Ammonium Sulphate Concentration on the Partition Coefficients and Recoveries of OA in ATPS

The effects of ammonium sulphate concentration on the partition coefficients and recoveries of OA in ATPS were investigated (Figure 3). The partition coefficient and recovery of OA reached the maximum when the concentration of ammonium sulphate was 18% (w/w). This behavior can be related to the salting out effect. This is probably due to that the ''ion–dipole'' interaction between salt ions and water molecules leads to the hydration of ions, causing the phase-forming salts to dissolve in the lower phase, meanwhile, these interactions decrease the amount of free water molecules in the lower phase, leading to the exclusion of alcohol and the OA [20]. Therefore, 18% (w/w) ammonium sulphate was chosen for the following investigation.

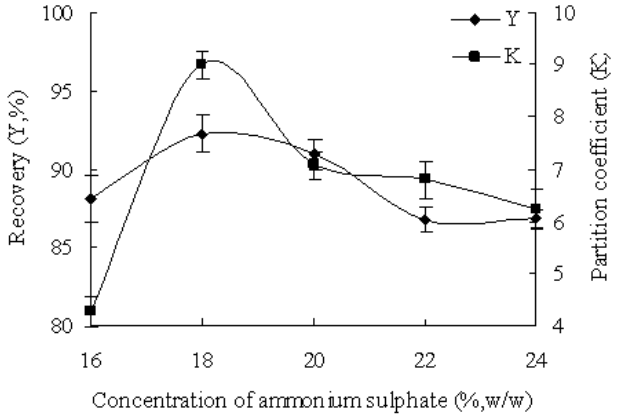

Figure 3. Effects of ammonium sulphate concentration on the partition coefficients and the recoveries of OA

3.3. Effects of Crude Extract Concentration on the Partition Coefficients and Recoveries in ATPS

The effect of crude extract concentration on the partition coefficients and recoveries were presented in Figure 4. The partition coefficients and recoveries of OA showed an increased trend with increasing crude extract concentration from 6 to 8%(w/w). However, the partition coefficients and recoveries were slightly decreased with further increase of crude extract concentration from 8 % to 14% (w/w). This phenomenon could be attributed to the fact that increasing the crude extract concentration causes the aggregation of OA molecules and the enhancement of intra-molecular hydrogen bonds, which weaken the interactions between OA and water molecules. Moreover, excessive addition of crude extract caused accumulation at the interface, which negatively affected mass transfer [21]. These results suggest that the crude extract concentration should be 8%(w/w).

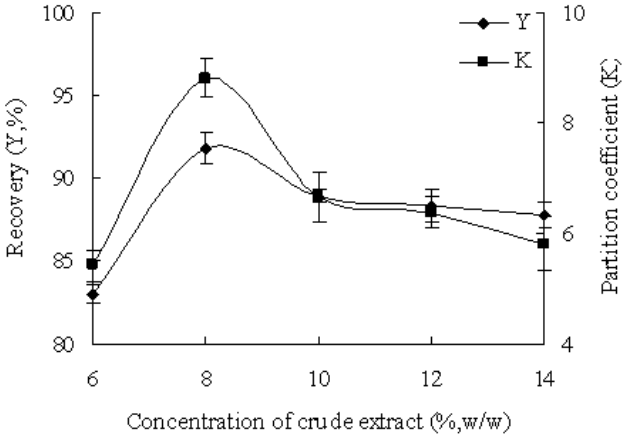

Figure 4. Effects of crude extract on the partition coefficients and the recoveries of OA

3.4. Effects of Extract Temperature on the Partition Coefficients and Recoveries in ATPS

The effects of temperature on partitioning behavior of OA in ethanol and $(NH_4)_2SO_4$ system were also investigated in the present study. The temperature in the range from 25° to 50°C water bath was investigated. As shown in Figure 5, The partition coefficient and extraction efficiency decrease with increase of temperature. It proves that this partition experiment should be done at a relatively low temperature. OA was more stable at lower temperature. The ethanol concentration and phase-forming ability of $(NH_4)_2SO_4$ decreased with increasing temperature. This result can be attributed to the transition of water from the ethanol -rich phase to the salt-rich phase [22], which decreased the salt-out effect in the salt-rich phase and reduced the recovery of OA. Furthermore, the OA was more stable at lower temperatures.

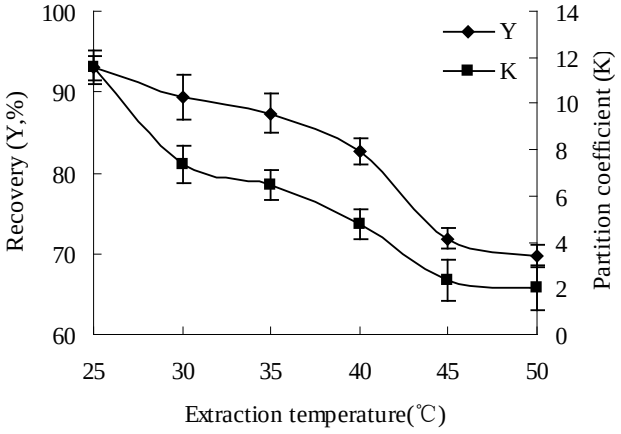

Figure 5. Effects of extract temperature on the partition coefficients and the recoveries of OA

To investigate the thermodynamics, extraction of OA can be regarded as a transfer process of the OA from the salt-rich phase to the ethanol-rich phase. The Gibbs energy change (ΔG) is related to the partition coefficient K can be calculated by the known Eq.(4).The enthalpy change (ΔH) and entropy change (ΔS) were obtained from the slope and intercept of the linear Eq. (5) between lnK and 1/T. Figure 6 shows the effect of temperature on the partition coefficient expressed through Van't Hoff plots.

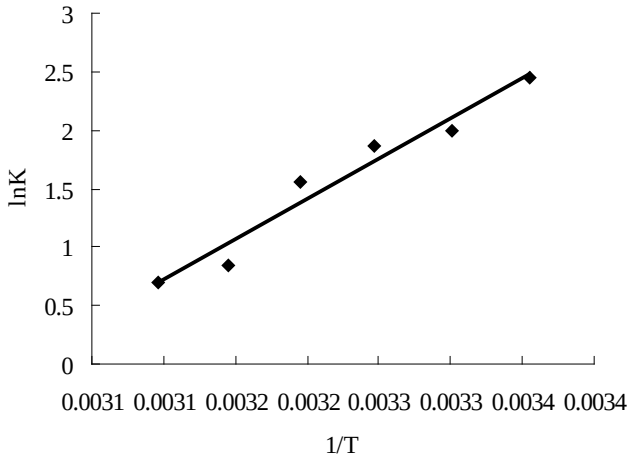

Figure 6. Van't Hoff plot of lnK versus 1/T produces a straight line

Table 1. The Transfer Thermodynamic Properties for OA from the Salt-rich Phase to the Ethanol-rich Phase in ATPS

T	K	ΔG(KJ/mol)	TΔS(KJ/mol)	ΔH(KJ/mol)
298	11.6	-6.07	-51.02	-57.09
303	7.35	-5.02	-52.07	-57.09
308	6.47	-4.78	-52.31	-57.09
313	4.76	-4.060	-53.03	-57.09
318	2.34	-2.247	-54.843	-57.09
323	2.01	-1.874	-55.216	-57.09

$$G = -RT\ln K \quad (4)$$

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (5)$$

The resultant ΔG, ΔH and ΔS values obtained by linear least-square analysis are showed in Table 1. The ΔG values are negative, indicating that the extraction of OA is spontaneous and preferential partitioning in the ethanol -rich phase as indicated by the K>1 within this temperature range. Partitioning of OA is marked by negative values for ΔH and TΔS with the enthalpy change being greater in value than the entropy change. Thus it can be concluded that partitioning of OA involves an exothermic, spontaneous process. This result indicated that temperature had great influence on the distribution behaviour of OA in the experimental range. In following studies, all extraction experiments were carried out at room temperature.

3.5. Effects of pH on the Partition Coefficients and Recoveries in ATPS

As seen in Figure 7, the partition coefficient of OA increased from 3.44 to 7.52, and the recovery varied in the range of 73.77-93.54% with the pH increasing from 3 to 7. OA is weak acid (HA). An ionization equilibrium of $HA \leftarrow H^+ + A^-$ happens at different pH values. OA exist in the form of molecules under acidic condition and tend to stay in ethanol-rich phase. To make this equilibrium go toward left, an acidic environment should be created. Interestingly, the inherent pH of the ATPS (23%(w/w) ethanol, 18%(w/w) ammonium sulphate, 8%(w/w) curde extract and 41%(w/w) water) was observed to be 4.0, which showed the highest recovery of OA in the top phase. In summary, the pH of ATPS had an important influence on the partition behaviour and pH 4.0 was shown to be the optimal condition.

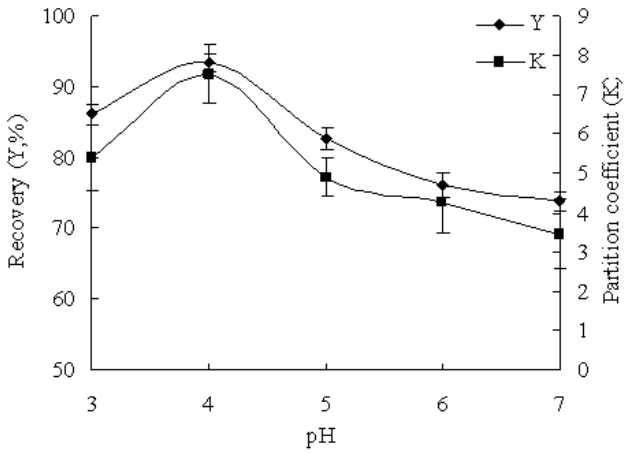

Figure 7. Effects of pH on the partition coefficients and the recoveries of OA

3.6. Antioxidant Activity of OA

The OA obtained by the ATPE was evaluated for the ability to scavenge DPPH radicals in relative to V_C. Figure 8 showed an increase in scavenging ability with increasing the concentration of the OA. It was also observed that a dose–response relationship was found in the free radicals scavenging ability. The DPPH scavenging rate reached 88.3% at a concentration of 0.08%(w/v) of ATPE extract, whereas the Vc scavenged 87.3% of DPPH radical at a concentration of 0.1%(w/v). The result indicated that the scavenging ability of the ATPE extract exhibited good antioxidant activity, which was higher than Vc.

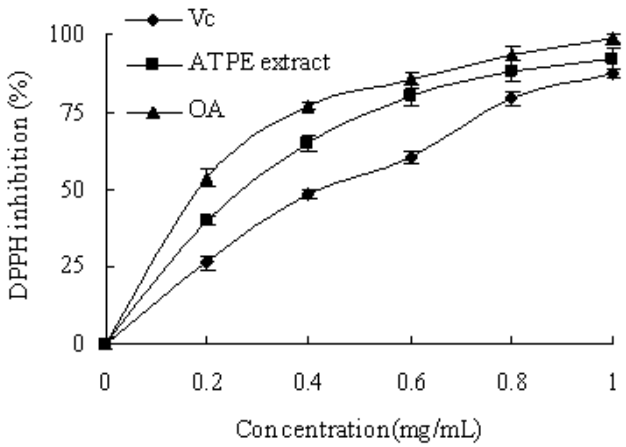

Figure 8. Scavenging effect of ATPE extract on DPPH radicals compared with these of Vitamin C (standard control) and OA

4. Conclusion

The extraction, antioxidant activity of OA from grape skins were reported in the present study. The optimal differential partitioning of OA was achieved by ATPE carried out at pH 4.0 and 25°C, using 23% (v/w) ethanol, 18% (w/w) ammonium sulphate, 8% (w/w)curde extract and 41% (w/w) water, where 93.54% of OA were partitioned to the top phase. Furthermore, the OA showed a relatively high antioxidant activity compared with that of Vitamin C (standard control). ATPS is more efficient and low-cost , and it will be widely used in isolation of other natural active compounds or bio-products.

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References

[1] Torres, J.L.,Varela, B., García, M.T., Carilla, J., Matito,C., Centelles, J. J., and Bobet, R., "Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content," *Journal of Agricultural and Food Chemistry*, *50* (26). 7548-7555. Nov. 2002.

[2] Ky, I., Crozier, A., Cros, G., and Teissedre, P. L., "Polyphenols composition of wine and grape sub-products and potential effects on chronic diseases," *Nutrition and Aging*, *2* (2). 165-177. Jun. 2014.

[3] Azuma, A., Yakushiji, H., Koshita, Y., and Kobayashi, S., "Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions," *Planta*, *236* (4).1067-1080. May. 2012.

[4] Li, Z., Pan, Q., Cui, X., and Duan, C., "Optimization on anthocyanins extraction from wine grape skins using orthogonal test design," *Food Science and Biotechnology*, *19* (4).1047-1053. Aug. 2010.

[5] Pensec, F., Pączkowski, C., Grabarczyk, M., Woźniak, A., Bénard-Gellon, M., Bertsch, C., and Szakiel, A., "Changes in the Triterpenoid Content of Cuticular Waxes during Fruit Ripening of Eight Grape (Vitis vinifera) Cultivars Grown in the Upper Rhine Valley." *Journal of agricultural and food chemistry*, *62* (32). 7998-8007. Jul. 2014

[6] Wang, X., Ye, X. L., Liu, R., Chen, H. L., Bai, H., Liang, X., and Hai, C. X., "Antioxidant activities of oleanolic acid in vitro: possible role of Nrf2 and MAP kinases," *Chemico-biological interactions*, *184* (3). 328-337. Mar. 2010.

[7] Lee, W., Yang, E. J., Ku, S. K., Song, K. S., and Bae, J. S., "Anti-inflammatory effects of oleanolic acid on LPS-induced inflammation in vitro and in vivo," *Inflammation*, *36* (1). 94-102. Aug. 2013.

[8] Ghosh, S., Bishayee, K., and Khuda-Bukhsh, A. R., "Oleanolic acid isolated from ethanolic extract of Phytolacca decandra induces apoptosis in A375 skin melanoma cells: drug-DNA interaction and signaling cascade," *Journal of integrative medicine*, *12* (2). 102-114. Mar. 2014.

[9] Mahapatra, A., Chauhan, N., Patel, D. R., Kalia, N. P., Rajput, V. S., and Khan, I. A., "Synthesis and Antitubercular Activity of Oleanolic Acid Analogs", *Pharmaceutical Chemistry Journal*, 48 (1). 39-43. May. 2014.

[10] Fu, Q., Zhang, L., Cheng, N., Jia, M., and Zhang, Y., "Extraction optimization of oleanolic and ursolic acids from pomegranate (*Punica granatum* L.) flowers," *Food and Bioproducts Processing*. 92 (3). 321-327. Jul. 2014.

[11] Yang, Y. C., Wei, M. C., Hong, S. J., Huang, T. C., and Lee, S. Z., "Development/optimization of a green procedure with ultrasound-assisted improved supercritical carbon dioxide to produce extracts enriched in oleanolic acid and ursolic acid from *Scutellaria barbata* D. Don," *Industrial Crops and Products*, *49*, 542-553. Aug. 2013.

[12] Pai, S. R., Upadhya, V., Hegde, H. V., Joshi, R. K., and Kholkute, S. D, "New Report of Triterpenoid Betulinic Acid along with Oleanolic Acid from Achyranthes aspera by Reversed-Phase-Ultra Flow Liquid Chromatographic Analysis and Confirmation Using High-Performance Thin-Layer Chromatographic and Fourier Transform-Infrared Spectroscopic Techniques," *JPC-Journal of Planar Chromatography-Modern TLC*, *27* (1), 38-41. Feb. 2014

[13] Ahmad, M. M., and Przybycien, T. M., "Towards optimal aqueous two‐phase extraction system flowsheets for protein purification," *Journal of Chemical Technology and Biotechnology*, *88* (1), 62-71. Jan. 2013.

[14] Wu, X., Liang, L., Zou, Y., Zhao, T., Zhao, J., Li, F., and Yang, L., "Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (*Morus atropurpurea* Roxb.),"*Food Chemistry*, *129* (2), 443-453. Nov. 2011.

[15] Yang, X., Zhang, S., Yu, W., Liu, Z., Lei, L., Li, N., and Yu, Y., Ionic liquid-anionic surfactant based aqueous two-phase extraction for determination of antibiotics in honey by high-performance liquid chromatography. *Talanta*, *124*, 1-6. Jun. 2014.

[16] Liu, X., Mu, T., Sun, H., Zhang, M., and Chen, J., "Optimisation of aqueous two-phase extraction of anthocyanins from purple sweet potatoes by response surface methodology," *Food chemistry*, *141* (3), 3034-3041. Dec. 2013.

[17] Chen, F., Sun, Y., Zhao, G., Liao, X., Hu, X., Wu, J., and Wang, Z., "Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography–mass spectrometry," *Ultrasonics Sonochemistry*, *14* (6), 767-778. Sep. 2007.

[18] Wang, B. Z., and XIE H.X., "Determination of concentration of total saponins and oleanolic acid of Rubus sachalinensis at different collecting time," Chinese Journal of experiment medical formulae, 20 (12), 77-79. Jun. 2014.

[19] Liu, S. C., Lin, J. T., Wang, C. K., Chen, H. Y., and Yang, D. J., "Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers," Food Chemistry, 114 (2). 577-581. May. 2009

[20] Wang, Y., Han, J., Xu, X. H., Hu, S. P., and Yan, Y. S., "Partition behavior and partition mechanism of antibiotics in ethanol/2-propanol-ammonium sulfate aqueous two-phase systems," *Separation and Purifition Technology*, 75 (3). 352-357. Nov. 2010

[21] Yan, J. K., Ma, H. L., Pei, J. J., Wang, Z. B., and Wu, J. Y., "Facile and effective separation of polysaccharides and proteins from *Cordyceps sinensis* mycelia by ionic liquid aqueous two-phase system," *Separation and Purification technology*. 135. 278-284. Oct. 2014.

[22] Zafarani-Moattar, M. T., & Hamzehzadeh, S., "Phase diagrams for the aqueous two-phase ternary system containing the ionic liquid 1-butyl-3-methylimidazolium bromide and tri-potassium citrate at T=(278.15, 298.15, and 318.15) K," *Journal of Chemical & Engineering Data*, *54* (3), 833-841. Dec. 2008.