EXTRACTION AND PURIFICATION OF ANDROGRAPHOLIDE FROM ANDROGRAPHIS PANICULATA

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ABSTRACT

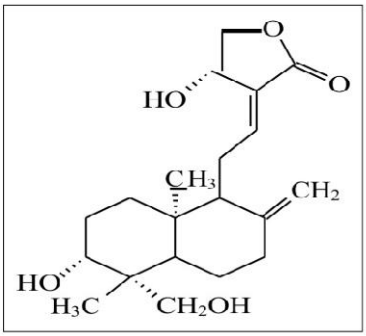
In the study, the purification of andrographolide from Andrographis paniculata was carried out using various physical separation methods such as extraction and crystallization followed by drying. Extraction of andrographolide was carried out using various solvent. The ratio of the solvents was checked for extraction efficiency. The ratio of andrographolide / solvent was found to be 1: 3. 5 weight / volume offers a higher degree of purity of andrographolide. A solubility study of andrographolide was investigated. The extract obtained after the extraction was then treated with activated charcoal in order to remove undesired impurities that could disrupt the crystallization process. The extract was concentrated by evaporation after clarification. The cooling crystallization process was effectively used to further purify andrographolide to obtain 95% high purity andrographolide. The crystallization process was examined from the point of view of supersaturation (more purified output). Andrographolide was confirmed by Thin Layer Chromatography, Melting point, Infra-Red, NMR and Mass Spectra. To look for the morphology of purified andrographolide inverted microscopy and SEM was used. It has been observed that Andrographolide gives different sizes of whitish cuboid crystals of the order of 30µ m- 40 µm.

Keywords: *Crystallization, herbal products, medicinal plant, anti-pyretic.*

Introduction

Andrographis paniculata belongs to the own circle of relatives Acanthaceae, is a medicinal herb with an incredibly bitter taste generally used to treat liver disorders, bowel problems of children, colicache, cold, and higher respiratory tract infection (Negi A.S. et.al, 2008; Roxas M. and Jurenka J., 2007; Kligler B., 2006). The herb is local of Taiwan, Mainland China, and India. The shoot a part of A. paniculata is normally utilized in the drugs of China. In the Ayurveda, A. paniculata is used to relieves our bodies inner heat, inflammation, and ache and extensively utilized for detoxication of body (Huang C.J. and Wu M.C., 2008; Chao, W. et.al, 2009; Mandela, S.C et.al, 2001). Inside*A. paniculata* leaves andrographolide, neo-andrographolide, and deoxyandrographolide had been recognized because the three principal diterpenoid lactones (Choudhury B.R. et.al, 1987; Rajani M. et.al, 2000). Andrographolide, that's grouped as an unsaturated trihydroxy lactone has the molecular formulation of $C_{20}H_{30}O_5$. In Fig.1, the molecular shape of andrographolide is shown.

Figure 1: Molecular structure of Andrographolide



Andrographolide, the principle thing withinside the leaves of A. paniculata may be effortlessly dissolved in methanol, ethanol, pyridine, acetic acid, and acetone, however has restrained solubility in ether and water. Its bodily houses are melting factor at 228-230oC, and the ultraviolet spectrum in ethanol λmax 223nm (Rajani M. et.al, 2000). Various techniques of extraction of andrographolide had been recorded, consisting of hydrotropic, microwave-assisted, and Soxhlet extraction, etc. (Mishra S.P. and Gaikar V.G., 2006; Wongkittipong R. et.al, 2004; Xu H.N. and He C.H., 2007; Patil V.V. et.al, 2010). Followed through extraction, to obtain higher enrichment of andrographolide, numerous column chromatographic strategies for andrographolide purification from the crude drug of

Andrographis Paniculata had been reported. However, those strategies had been observed to be time-consuming, expensive, and tedious, leaving impure andrographolide. By preserving this stuff in mind, the goal of this studies paintings become to broaden a solvent-primarily based totally extraction of andrographolide from *Andrographis paniculata*. It became apparent that crystallization ought to be a powerful approach implemented to split herbal merchandise from natural extract (Nie Q, et.al, 2006; Xu W.L. et.al, 2005). Crystallization is truly rank because the oldest unit operation with inside the chemical engineering sense. Apart from being one of the satisfactory and most inexpensive techniques to be had for the manufacturing of natural solids from impure solutions, crystallization has the extra benefit of giving a cease product that had many suited houses. There are a huge variety of solvents and solvent combos appropriate for the crystallization purification method. However, while aiming at a easy purification method it's miles useful to apply handiest one solvent rather than a solvent combination. The solubility traits of a solute in a given solvent have a sizable impact on the selection of a technique of crystallization. Both the solubility electricity and the solubility electricity with temperature ought to be taken into consideration while selecting a solvent for a crystallization method; the previous amount inspired the quantity of the crystallizer, and the latter decided the crystalsynthesis. Thus, the selection of the best solvent and right running situations have become in particular essential for the duration of the method of separation. The choice of the 'satisfactory' solvent for a given crystallization operation become now no longer usually a clean matter. There are a few reviews at the solubilities of andrographolide in ethanol, methanol, and dichloromethane (Chen M. et.al, 2010). Hence, comparable to be had solubility records become used to perform crystallization and to examine the consequences of solvents at the polymorphs formation. Moreover, the improvement of medication from herbal plant life typically calls for the isolation and purification of the goal compound from a complicated multi-thing combination to provide a excessive purity. Therefore, the goal of the prevailing paintings is to analyze the opportunity of mixing the benefits of crystallization to generate a hybrid method for the isolation and purification of andrographolide from the crude extract of Andrographis Paniculata.

Materials and Methods

Chemicals:

The fine powder of leaves of *A. paniculata* was collected from a local herbal supplier, Mumbai, India. The pure (approx. 98%) andrographolide was obtained from Sigma Aldrich, Mumbai. Silica gels and Thin layer chromatographic plates were purchased from S.D.Chemicals, India. Solvents such as methanol, ethanol, ethyl acetate, acetone, petroleum ether, dichloromethane, chloroform were obtained from S.D. Chemicals,India.

Solid-Liquid Extraction:

The extraction was performed using different solventsin the first set of experiment. To get higher andrographolide enrichment, the suitable solvent in the extracted phase was selected for the further experiment. The leaves were ground to a powder (80 mesh size) and extracted at reflux temperature for 3 h. The extraction of andrographolide from the ground powder was carried by mixing powder and solvent using the Soxhlet extraction technique. The powder to the different solvent ratio (w/v) used for all the studies. The solvent was removed and the process was repeated one more time to remove the final traces of andrographolide from the ground powder of leaves. The extracts were then combined and concentrated by recovering the solvent using Buchirota vapor. The brownish extract enriched with andrographolide was used for further studies. The effect of andrographolide on the solvent ratio on the extraction efficiency was also studied by varying the andrographolide to solventratio.

Enrichment of Andrographolide Extract:

The most important operation in the phytochemical separation process is the extract clarification because it results in the better visual quality of the final product. Since the leaf of *A. Paniculata* contains the coloring matters such as chlorophyll which gets sticky

extract after extraction and makes andrographolide purification difficult. To get rid of this difficulty, the crude green and dark brown extract was treated with different percentages of activated charcoal and reflux for 20 min. The andrographolide extract was filtered and the residual charcoal was once again mixed with methanol and reflux one more time for 10 min. The filtrates in this way obtained were then combined and concentrated. The amount of chlorophyll was checked to find the level of andrographolide in the extract and it was done by taking the absorbance at 646 and 662 nm (Iain, L. *et.al,* 1995; Sukran, D. *et.al,* 1998). The obtained yellow-colored extract was used for further study.

Determination of Solubility of Andrographolide in Menthol:

The solubility of andrographolide in methanol was measured at different temperatures. To the 10 mL of solvent, the excess quantity of andrographolide was added. Subsequently, the liquid-solid suspension was constantly agitated at 120 rpm at 30°C for 2h in REMI Shaker to achieve uniform mixing. The clear solution was then removed using a syringe filter and dried in the vacuum oven at 50ºC. The solid thus obtained wasmeasured and the solubility was tested as milligram of andrographolide per ml of the solvent. The same procedure was repeated at different temperatures to get a solubility curve.

Crystallization: Technique for Purification of Andrographolide:

The isolation of andrographolide from diterpene lactones mixture of A. *Paniculata* was carried out by using evaporation followed by a slow cooling crystallization technique. For the crystal growth and nucleation, supersaturation of the solution is act as an important step. To achieve supersaturation, the methanol was recovered using the evaporation process from the final extract which was obtained after the clarification step. This leads to an increase in the solid concentration of andrographolide. Andrographolide extract which was obtained after the clarification process was concentrated by recovering the methanol by evaporation at 65-70ºC till the volume of extract reduced to the volume of starting extract. A yellowish clear solution was then filtered and the filtrate was allowed to cool slowly at room temperature to attain the supersaturation level. During cooling, after attaining the supersaturation level after over some time expels the yellowish solids from the solution. The formation of yellowish solid can be referred to as the appearance of super saturation and thereafter, crystal formation. The crystals were collected by decanting mother liquid and dried in a vacuum dryer at 50^{o}C for 3-4 hrs. By carrying out crystallization repeatedly a couple of times, more refined, whitish, high purity andrographolide could be obtained.

Furthermore, for crystallization to take place a solution must be "supersaturated". The supersaturation is measure of the concentration variation between supersaturated solution in which the crystals growsas that of solution in equilibriumofcrystal.

HPLC Analysis of Andrographolide:

The reputed Germany Agilent HPLC system, consisting of a model G1329A standard auto-sampler, model G1316A thermostat column, model G1322A vacuum degasser, quaternary pump, model G1314B variable wavelength detector, was used. The separation was achieved on a stainless-steel silica-based Zorbax Eclipse XDB–C18 column (ϕ4.6 mm×150 mm, 5 µm). The column temperature at 30ºC was maintained. Andrographolide was eluted using a mobile phase consisting of methanol and 0.1% v/v H_3PO_4 (70:30) at the flow rate of 1 ml/min. At 223 nm, the eluent was monitored. The standard curve was obtained by analyzing the known concentration of Andrographolide. The concentration of andrographolide and the area under the curve is used to plot the standard curve. This plot was used for the determination of the concentration of the andrographolide in the unknown solution. In the methanol of 10 mg/l concentration, all the samples were prepared and filtered through 0.22µm filter to remove any suspended particles. The amount of sample injected in the column was kept constant at 10 µl. To remove any dissolved gases, all solvents used in the HPLC analysis were filtered through

0.22 µm filter and then 10 min are given for sonication.

Scanning Electron Microscopy:

To know more about the morphology and size of andrographolide crystals, scanning electron microscope was used to get electron micrographs of the crystals (Leica Cambridge S360, UK) operating at 5 kV. The specimens were mounted on plasma coated with JEOL-JFC-1600 AUTO FINE COATER.

Result and Discussion

Extraction:

The **Fig.2** shows, the % yield of andrographolide obtained by the use of various solvents such as ethanol, methanol, and DCM was studied. It shows that methanol extraction gives a higher amount compared to other solvent. **Fig.2.** displayed HPLC chromatograms of the extracts, where a represent main andrographolide.

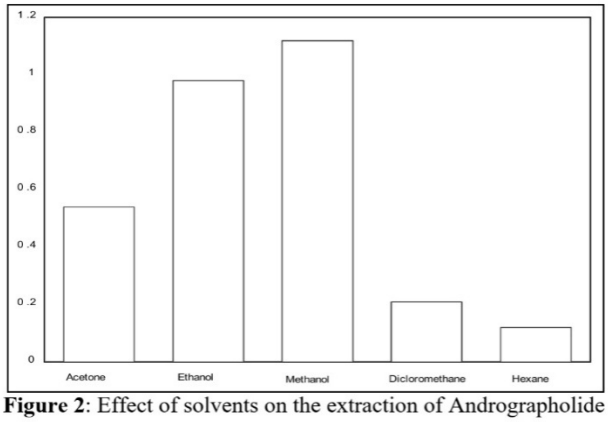


Figure 2: Effect of solvents on the extraction of Andrographolide

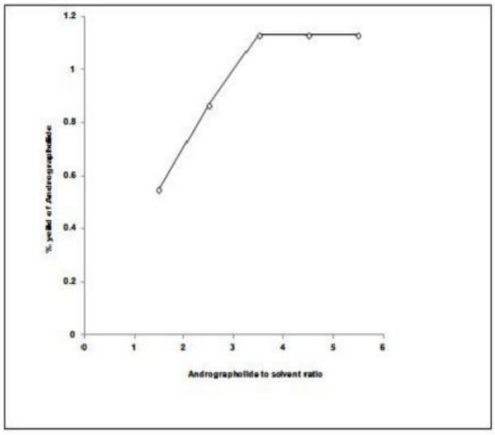


Figure 3: Effect of dry feed to solvent ratio on the percent yield of andrographolide

In evidence, **Fig.2 and Fig.3** implied that there were fewer desired components in the methanol extract than those obtained by them methanol or dichloromethane extraction. The purity of the product is the most important characteristic and quality parameters as per as the extract is concerned. It is easy to decide the extraction protocol if the target compound is single and well-defined. In comparison to non – polar solvents, polar solvents could extract andrographolide at a higher percentage except water, where hydrolysis and thermal degradation might occur. Methanol was found to be the best solvent for the extraction of andrographolide (Mishra, S.P.; Gaikar and V.G., 2006; Wongkittipong, R. *et.al,* 2004). The extraction of andrographolide in different solvents such as ethanol and aqueous acetone gives lower outcome although its solubility characters are closer of andrographolide. Methanol extracts much andrographolide as compare to other solvents. The study showed that the non-polar solvents did not extract andrographolide.

Isolation of Andrographolide:

The crude leaf extract was deep green in color due to the presence of pigments such as chlorophyll. Figure 4 is the chromatogram of the crude extract showing several peaks along with one major peaks of andrographolide at the retention time 4.1min **(Fig.4)** The chromatogram shows that the pigments such as chlorophyll present in the crude extract affects to the great extent the purification process and therefore the purity of the andrographolide. The crude green extract was treated with 5-20% of activated charcoal to remove the chlorophyll. The pigment content reduces by 90% in terms of chlorophyll as shown **Fig. 5**

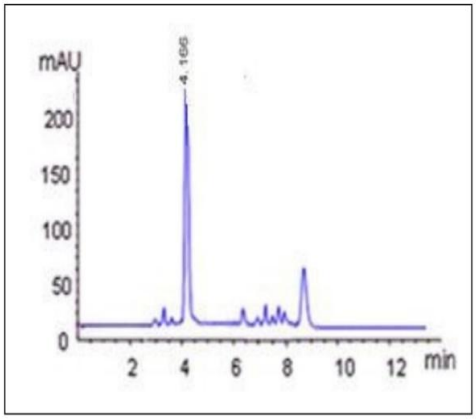


Figure 4: Chromatogram of initial extract of *Andrographis Paniculata* leaves

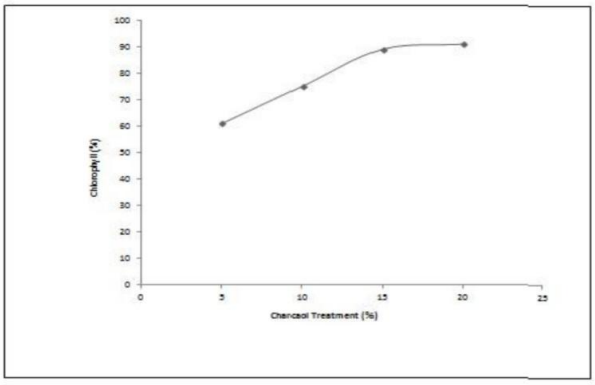


Figure 5: Total chlorophyll content after charcoal treatment

Crystallization:

The ICH parameters and solubility guidelines are considered as main aspect for selection of suitable solvent for crystallization process. As the andrographolide is highly soluble in methanol, the crystallization was carried out in the same medium. The solubility profile of andrographolide crystallization in methanol by considering the function of temperature is seen in **Figure 6**. The result showed that solubility tremendously increases from 15^0C to 65^0C, by almost double. The solubility change is seen less before 45^0C and post 65^0C,as compared to temperature range 15-65^0C. Equilibrium is reached when the solution is saturated and the equilibrium relationship indicates the significant crystallization point where maximum recovery of the crystallized product was obtained which was shown in Table 1. Such a type of curve is an ideal one for cooling crystallization, where supersaturation using cooling brings about these parathion of two phases rather easily. **Figure(7)** shows the plot of supersaturation as a function of the temperature variants and it is seen that approximately 0.003 mass fraction of solute was the degree of supersaturation and the corresponding, supersaturation (α) given by equation (2) was found to be in the range of 1.18. When the solubility of andrographolide increases appreciably with temperature, the supersaturation can be expressed as an equivalent temperature changes instead of a mass fraction difference. The relation between this driving potential is shown in **Figure (7)** which contains a small section of the solubility curve of andrographolide in mass fraction solute. The unsaturated solution is represented as a field above the line at 65^0C temperature and supersaturated solution represented below the line. Temperature T_c,is the temperature of saturated solution of the growing crystal represented by Point A, Temperature T represented point D of Supersaturated solution. Since, the heat is evolved by the crystal as it grows, T_c is slightly larger than T, providing the driving force of ΔT_h for heat transfer from crystal to the liquid.

The variations in point E andD, givessupersaturation Δy which based on group temperature. Point B refers to a saturated solution of the same composition as the supersaturated solution in which the crystals are growing. It would be at a temperature T_s, where T_s> T. Point C indicates temperature $T_c$which is the concentration exactly similar to that of supersaturated solution.

Using Equation, the supersaturation potential can be represented by the line segment *A*. The similar temperature treating potential is shown by line segment *BC*. Segment *AB* of the solubility curve can be considered linear over the small concentration spanned by the line *AC* and the temperature potential defined by ($\Delta T_C = T_S - T_C$). T_s = Supersaturation Temperature, T_c = Saturation Temperature.

From the above equation, the temperature potential (ΔT_c) was found to be 20°C which was slightly smaller than the actual change in temperature, T of the solution, and the corresponding saturation temperatureT_s.

Table 1. Isolation of Andrographolide after Crystallization and Re-crystallization

Crystals produced from different operations	Melting point ^{0}C Andrographolide	Andrographolide %	Recovery or overall yield %
Crystallization by evaporation followed by cooling	223-230	93.67	94.1
Recrystallization	228-230	96	92.67

Andrographolides crystals as viewed via SEM at 1000x magnification and it was observed that andrographolide gives the different sizes of cube-shaped whitish crystals in the range to 30µm- 40 µ m which was having very good solubility in 20 % concentration of ethanol. This type of crystals can be used in a different type of herbal formulations such as antipyretic, cough, antiviralformulations**.**

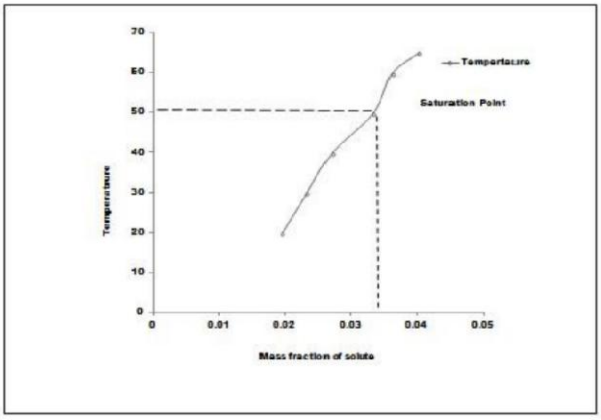
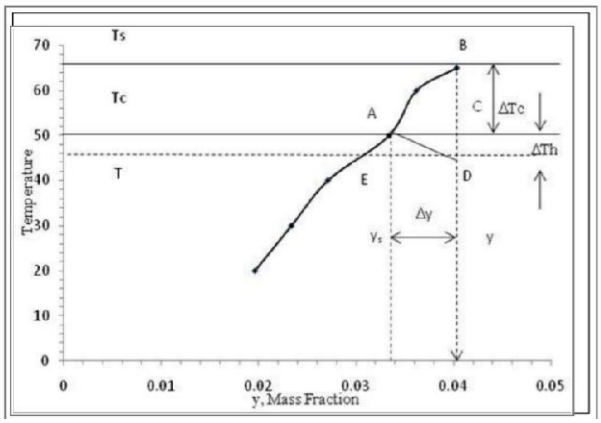


Figure 6: Equilibrium relationship for bulk andrographolide crystals
Figure 7. Supersaturation and Temperature potential



HPLC Analysis:

In the chromatogram of andrographolide, 96% of purity of crystals of andrographolide was found as compare to standard andrographolide. **Figure 8** shows SEM images of Andrographolide whereas, **Figure 9** and **Figure 10** show the chromatograms of standard andrographolide and crystals obtained in this study, respectively. The presence of andrographolide at 4.18 min retention time, clearly shows the intrinsic advantage of crystallization in attaining more bitter components as a substantial fraction in the extracted purified product.

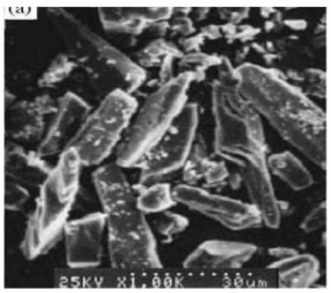
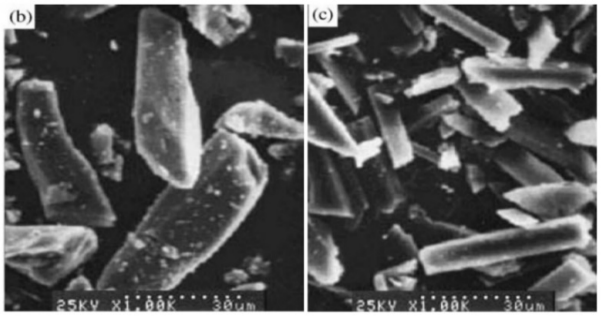


Figure 8: SEM images of Andrographolide (a,b,c)



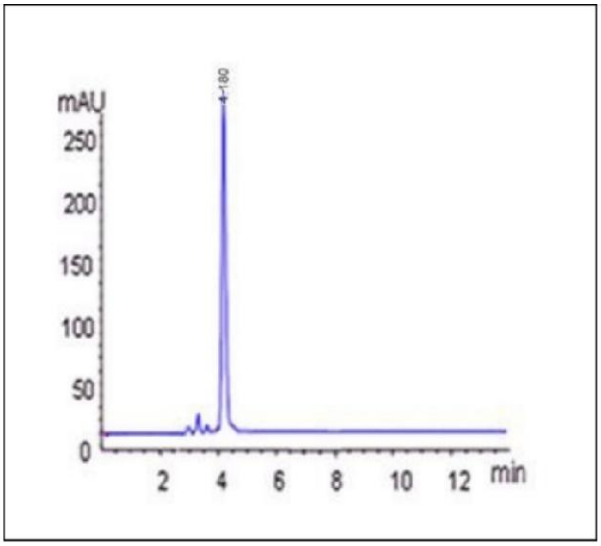


Figure 9: Chromatogram of Standard andrographolide

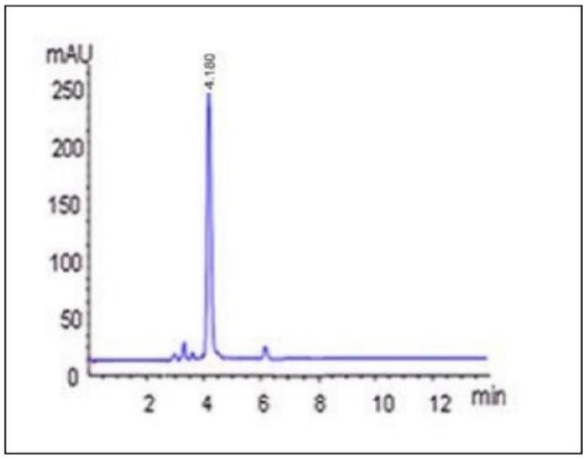


Figure 10: Chromatogram obtained crystals of andrographolide

Conclusion

By using methanol solvent, the extraction of andrographolide from the powder of *A.paniculata* was conducted. The optimum ratio of dried powder of *A. paniculata* to methanol was obtained as 1:3.5. Followed by conventional extraction, the extract clarification was successfully carried out using the charcoal treatment. Cooling crystallization and Evaporation was done for the recovery of andrographolide and it found to be in the range of 90-96% of recovery of andrographolide with 96% purity. Solubility study at the different temperatures of Andrographolide was carried out in methanol. The different process parameters such as supersaturation (Δy), supersaturation ratio (α), and temperature potential (ΔT_C) are studied for crystallization. To obtain a substantial yield of andrographolide, 20°C supercooling was found to be sufficient practically. This novel study approach for extraction followed by clarification and crystallization given in the present work might be one of the most promising techniques for this kind of natural bitter separation andpurification.

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