

Effect of Solvent Polarity on Extraction Yield of Total Flavonoids with Special Emphasis to Glabridin from *Glycyrrhiza glabra* Roots

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Effect of Solvent Polarity on Extraction Yield of Total Flavonoids with Special Emphasis to Glabridin from Glycyrrhiza glabra Roots

Çözücü Polaritesinin Özellikle Glabridin Olmak Üzere Glycyrrhiza glabra Köklerindeki Toplam Flavonoidlerin Ekstraksiyon Verimine Etkisi

SUMMARY

Different organic solvents (ethanol, dichloromethane, ethyl acetate and acetone) were studied for their effects on the extraction efficiency of glabridin and total flavonoids (TF) from *Glycyrrhiza glabra* roots. The extract yield of *Glycyrrhiza glabra* roots was in the range of 3% to 6% following the extraction efficiency in the order ethanol>acetone>ethyl acetate>dichloromethane. A higher extraction yield of TF and glabridin was obtained with dichloromethane, followed by ethyl acetate, acetone and ethanol, indicating that the non-polar solvents help in optimal extraction of TF and glabridin. We also demonstrate for the first time, that the extraction efficiency of the flavonoids is not significantly affected by the use of the recovered solvents except in case of ethanol which reflects that the moisture-absorbing capacity of the solvent dictates the extraction efficiency of such compounds. The glycyrrhizin content in all the extract types was rather low (0.1 % to 1%) except for extract prepared with water, where the glycyrrhizin content was ~10% as expected since glycyrrhizin is a polar compound. Interestingly, we observed that ethyl acetate selectively isolated only glabridin with no traces of glycyrrhizin, which is a finding reported for the first time.

Key Words: Licorice, Glabridin, Flavanoids, Glycyrrhizin, Extraction, Solvent polarity

ÖZ

Farklı organik çözücülerin (etanol, diklorometan, etil asetat ve aseton) *Glycyrrhiza glabra* köklerinden glabridin ve toplam flavonoid (TF) ekstraksiyon verimliliğine etkileri çalışıldı. *Glycyrrhiza glabra* köklerinden ekstre verimi % 3 ile % 6 aralığında, ekstraksiyon etkinliği etanol>aseton>etil asetat>diklorometan sırası ile bulunmuştur. Diklorometan, ardından etil asetat, aseton ve etanol ile daha yüksek bir TF ve glabridin ekstraksiyon verimi elde edilmiştir, bu da TF ve glabridinin en uygun şekilde ekstraksiyonunda polar olmayan çözücülerin etkili olduğunu göstermektedir. Ayrıca flavonoidlerin ekstraksiyon verimliliğinin, çözücünün nem absorplama kapasitesinin bu tür bileşiklerin ekstre edilmesinde rol oynadığını yansıtan etanol haricinde, geri kazanılan çözücülerin kullanımından büyük ölçüde etkilenmediği de ilk kez gösterilmiştir. Su ile hazırlanan ekstre haricinde tüm ekstre türlerinde glisirizin içeriği düşük olup % 0,1 ile % 1 aralığındadır, bileşik polar bir bileşik olduğu için su ekstresinde içerik beklendiği gibi ~% 10'dur. İlginç bir şekilde, etil asetatın glisirizin izleri olmadan sadece glabridini seçici olarak izole ettiği ilk kez bildirilen bir bulgu olarak gözlenmiştir.

Anahtar Kelimeler: Meyankökü, Glabridin, Flavonoidler, Glisirizin, Ekstraksiyon, Çözücü polaritesi

Received: XX.XX.XXXX

Revised: XX.XX.XXXX

Accepted: XX.XX.XXXX

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INTRODUCTION

Glycyrrhiza glabra Linn, commonly known as 'licorice' and 'sweet wood' belongs to Leguminosae family and is cultivated in Italy, Russia, France, UK, USA, Germany, Spain, China and Northern India (Bhan et al., 2017).

Licorice is widely used from the ancient medical history of Ayurveda, both as a medicine and as a flavouring agent. Its use as an anti-inflammatory agent during allergenic reactions, as a contraceptive, as a laxative and as an anti-asthmatic agent is reported (Ammosov et al., 2003). The use of licorice in the treatment of gastric ulcers, hepatitis C and pulmonary and skin diseases is known and, its use in treating cough because of its demulcent and expectorant property, also exists (Damle, 2014). Licorice extract is also incorporated in medicinal oils to treat rheumatism, haemorrhagic diseases, epilepsy, paralysis etc. (Kaur et al., 2013).

The chemical constituents of the *G. glabra* roots include several bioactive compounds, such as glycyrrhizin (~16%), flavonoids (1.5%), coumarin, alkaloids, polysaccharides, sitosterol, amino acids, gums and essential oils (Li et al., 2012). The hydrophilic fraction of licorice extracts which includes glycyrrhizin and glycyrrhetic acid, are known to suppresses the replication of numerous viruses such as HIV, HCV, influenza HSV, rotavirus, coxsackievirus, HRSV, HBV (Wang et al., 2015), while the hydrophobic fraction of licorice extracts containing flavonoids and glabridin (Yokota et al., 1998) have pharmacological activities of wound-healing, (Yip et al., 2016), anti-microbial (Alwan et al., 2015), anti-ulcer agent (Alilzadeh-Amin et al., 2015) etc.

The conventional methods for extraction of glabridin from licorice utilize solvents that need to be free of moisture to have an optimal yield of glabridin, thereby leading to massive amount of solvent waste. Some of the solvents used for glabridin extraction include ethanol (Tian et al., 2008), acetone (Ao et al., 2010), ethyl acetate (Xu et al., 2009), supercritical fluid CO₂ (Cho et al., 2004), methanol (Bhan et al.,

2017) and imidazolium-based ionic liquids (Li et al., 2012). Also, since glabridin is sensitive to temperature (Ao et al., 2010), the high energy consumption due to sample extraction time and high extraction temperature affects the final yield of the product. Since the licorice extracts are used in the cosmetic and food industries, having these extracts without residues of toxic solvents would be beneficial. In the light of these issues, an alternate, safe and cost-effective process for extraction of glabridin from licorice assumes critical importance.

The requirement of pure glabridin as a chemical reference standard for quality control studies and identification of *G. glabra* roots and for biological/pharmacological investigations (Vishwanathan et al., 2019), tempted us to reinvestigate the method of extraction of glabridin from licorice so that the processes are cost-effective and reproducible without impacting their quality.

The present study was designed to study the impact of different extraction solvents and its moisture content on the yield of glabridin and total flavonoids (TF) from *Glycyrrhiza glabra* roots, with particular emphasis also on effects of recovered solvents on the extraction efficiency of TF and glabridin, under similar extraction conditions.

MATERIALS AND METHODS

Reagents and Chemicals

The HPLC grade solvents like acetonitrile and methanol, and potassium dihydrogen phosphate) were purchased from Rankem (Bangalore, India). Glabridin and glycyrrhizin reference standards were purchased from Natural Remedies Pvt. Ltd, Bangalore, India.

Collection of *Glycyrrhiza glabra* roots

Roots of *Glycyrrhiza glabra* were collected from a commercial source from the Northern part of India. The identity of the roots was confirmed and documented by a taxonomist at Durva Herbal, Tamil Nadu, India. The freshly collected samples of licorice roots were washed, air-dried, and stored at 4°C, protected from light and humidity before analysis.

Preparation of extracts

100 g licorice roots raw material was processed for extraction with four volumes of fresh acetone followed by three volumes of the acetone two times (each extraction for three hours) at 45-50°C. After extractions, all acetone extractions were pooled and concentrated on a rotary evaporator under vacuum at 45-50°C to get a dry powdered acetone extract of *Glycyrrhiza glabra*. A similar protocol was followed to obtain powdered licorice extracts with ethyl acetate and ethanol. For extraction with dichloromethane, the extraction temperature was maintained between 30-35°C, below the boiling point of the dichloromethane solvent, keeping all other extraction parameters constant.

Preparation of licorice extracts using fresh, primary and secondary recovered solvents

After completing the first round of extraction with ethyl acetate (EA), the used EA was recovered and its volume measured and its moisture content estimated. The primary recovered and distilled EA was later used to extract the glabridin and TF from a fresh aliquot of the *Glycyrrhiza glabra* roots. Similarly, the volume and the moisture of the secondary recovered EA generated from this extraction run were used for the third extraction of glabridin and TF from a fresh batch of *Glycyrrhiza glabra* roots powder. A similar protocol was followed for all the other three solvents used, unless mentioned otherwise.

Estimation of moisture content in solvents

Karl Fisher Titration is a technique for the determination of moisture content. KF Titrand, Metrohm was used, for this purpose. The sensitivity of the method is an accurate determination of water content from 0.001 to 100%.

HPLC conditions

The glabridin content in the powdered extracts recovered from the process was analyzed using an HPLC system (Shimadzu, 2010CHT) consisting of a quaternary pump with a vacuum degasser, thermostatted column compartment, autosampler,

and UV detector. A reverse-phase column (Inertsil ODS, C18, 3V, 5 µm, 250 X 4.6 mm; GL Sciences, Japan) was used with a column temperature of 40 °C. The HPLC mobile phase- Solution A: Potassium dihydrogen phosphate (1.36 g) was dissolved in 1000 mL of HPLC grade water. The solution was filtered through a 0.45 µm membrane filter and degassed in a sonicator for 3 min; Solution B: Acetonitrile (100%). The mobile phase was run using gradient elution. The gradient program for separation of glabridin and other flavonoids by HPLC was set as (time/% B) 0/5, 25/40, 40/42, 45/60, 50/60, 55/80, 60/80, 61/5, 65/5, that pumped the phases at a rate of 1 mL/min. The detection wavelength for glabridin was chosen as 280 nm, based on the UV spectra of pure glabridin solution (Shrikant et al., 2020). The flow rate was kept as 1.0 ml/minute and injection volume was 20 µL. The run time for detection of glabridin by HPLC was 65 min.

The estimation of glycyrrhizinic acid was carried out by HPLC under conditions as follows: Hypersil BDS column (3 V, 250 mm × 4.6 mm, 5 µm) using an isocratic mobile phase consisting of 2% formic acid and acetonitrile in the ratio 65:35 v/v. The flow rate was 1.0 mL/minute, injection volume was 20 µL, the column oven temperature was 30°C and the run time was 20 minutes. The peak of glycyrrhizinic acid was observed at 254 nm.

Sample preparation

50 mg of *Glycyrrhiza glabra* extract was precisely weighed, and transferred to a 50 mL volumetric flask. About 30 mL methanol was added and the contents were sonicated for 20 min. The volume was then made up to 50 mL with methanol, filtered through a 0.45µm filter and the resulting filtrate was used as a test solution. A stock solution of glabridin standard was prepared at a concentration of 5.0 mg/mL in absolute methanol. The calibration curves were prepared using solutions of different concentration levels from 10 – 550 µg/mL. 5 mg of glycyrrhizin in 50 mL methanol was used as the reference glycyrrhizin standard solution.

RESULTS

HPLC method

Standard glabridin peak was obtained at the

retention time of 50.707 min (Figure 1) while the glabridin peak was seen at 50.56 min with the glabridin extracted using ethyl acetate (Figure 2).

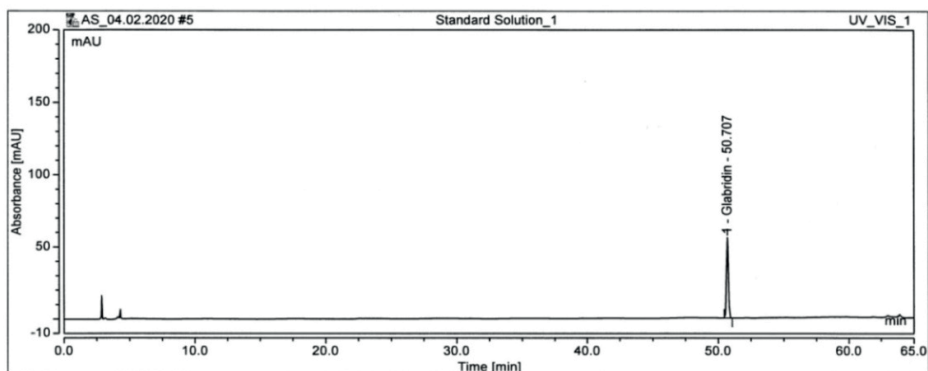


Figure 1. Chromatogram of standard Glabridin. Mobile phase A: Phosphate buffer; Mobile phase B- 100% acetonitrile, flow rate: 1 mL/min; detection: 280 nm. The retention time of glabridin was ~51 min.

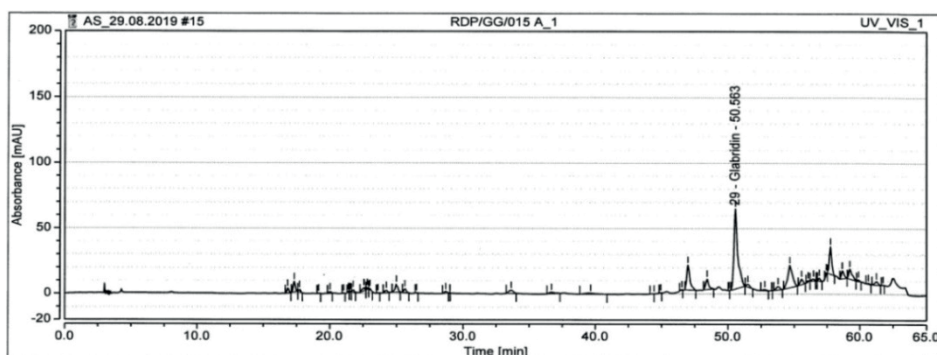


Figure 2. HPLC chromatogram of ethyl acetate extract of licorice roots. Mobile phase A: Phosphate buffer; Mobile phase B- 100% acetonitrile, flow rate: 1 mL/min; detection: 280 nm.

Several peaks of the flavonoids eluted at the retention times between 5 min to 63 minutes in the sample extract and these were integrated for enumeration of total flavonoid (TF) content. Figures 3,

4 and 5 represent the HPLC chromatograms of licorice extracts of acetone, dichloromethane and ethanol, respectively, wherein the retention time of glabridin was seen at 51.098, 50.29 and 50.71 min, respectively.

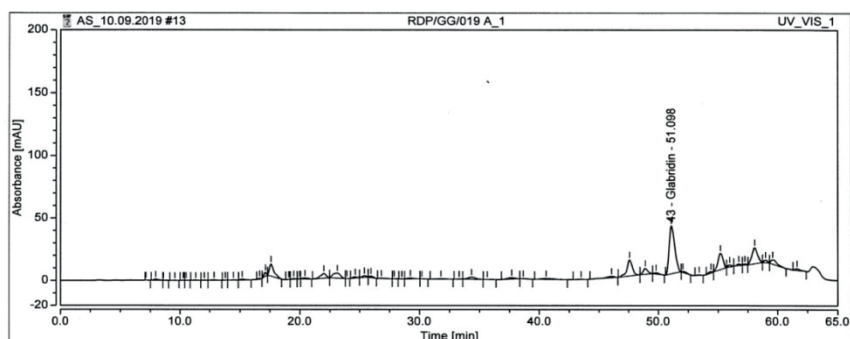


Figure 3. HPLC chromatogram of acetone extract of licorice roots. Mobile phase A: Phosphate buffer; Mobile phase B- 100% acetonitrile, flow rate: 1 mL/min; detection: 280 nm.

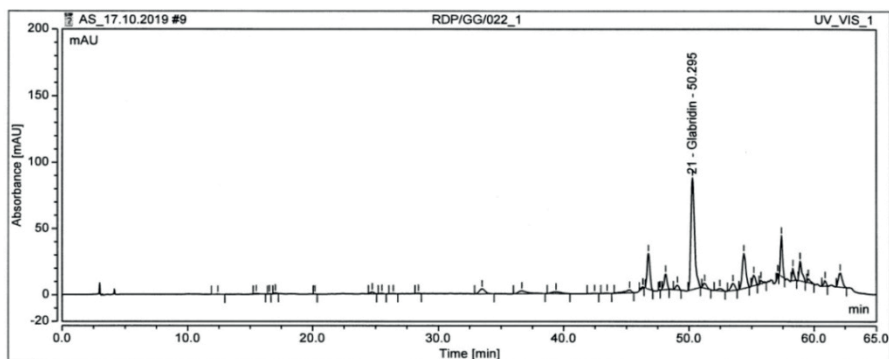


Figure 4. HPLC chromatogram of dichloromethane extract of licorice roots.

Mobile phase A: Phosphate buffer; Mobile phase B- 100% acetonitrile, flow rate: 1 mL/min; detection: 280 nm.

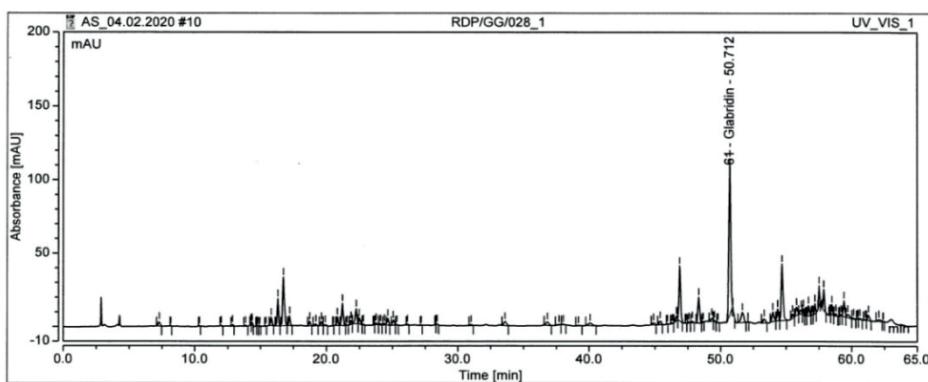


Figure 5. HPLC chromatogram of ethanol extract of licorice roots. Mobile phase A: Phosphate buffer; Mobile phase B- 100% acetonitrile, flow rate: 1 mL/min; detection: 280 nm.

For LOD and LOQ calculations of glycyrrhizinic acid, the established linear equation was $y = 13,783.285x - 1,242.374$, the r^2 (coefficient of determination) was 0.998, and the calculated LOD and LOQ values were 0.31 mg/L and 0.95 mg/L, respectively while for glabridin, the established linear equation was $y = 52,367.4771x - 843.7015$, the r^2 (coefficient of determination) was 1.000, and the calculated LOD and LOQ values were 0.13 mg/L and 0.41 mg/L, respectively. Hence, the quantification range for glycyrrhizinic acid and glabridin by the method described in this paper is from 0.95 mg/L and 0.41 mg/L and above respectively.

Glabridin and water content in licorice root

extracts using fresh, primary and secondary recovered solvents

The polarity index of acetone, ethanol, ethyl acetate and dichloromethane is 5.1, 5.2, 4.4 and 3.1 respectively and we observed these solvents to have different hygroscopicity values (moisture-absorbing capacity) with acetone topping the list with 5.55% water content, followed by ethanol (3.4%), ethyl acetate (0.77%) and dichloromethane (0.07%). It is evident from Table 1 that different solvents yielded different extraction yields of licorice roots following the order acetone>ethanol>ethyl acetate>dichloromethane. The moisture content in the recovered solvents followed the order dichloromethane<ethyl acetate<

ethanol<acetone. The % glabridin content was maximum with dichloromethane (6.65%) extract followed by ethyl acetate (5.59%), acetone (5.09%) and ethanol (3.73%) extracts, with fresh solvents. The % glabridin and TF content remained constant in all the extracts when recovered solvents were employed for extraction except for ethanol which showed a

significant reduction in the yield of glabridin and TF in comparison to the yield achieved with fresh ethanol.

Glycyrrhizin content in licorice extracts using various solvents

The RT of the standard glycyrrhizin by the HPLC method employed was found to be nearly 12 min (Figure 6).

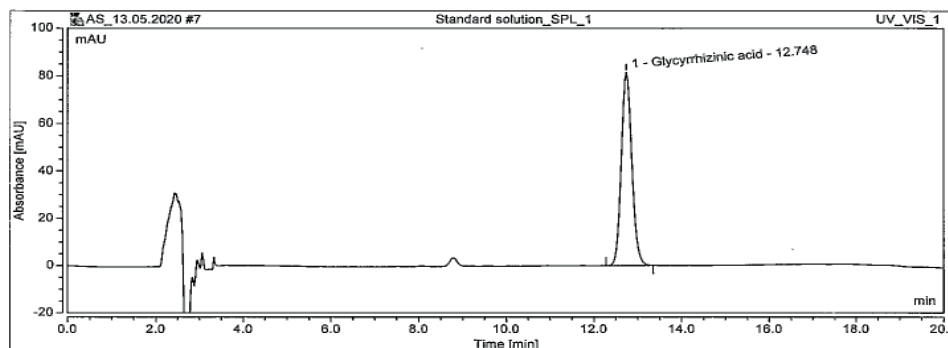


Figure 6. Chromatogram of standard Glycyrrhizic acid. The HPLC was done using an isocratic mobile phase consisting of 2% formic acid and acetonitrile in the ratio 65:35 v/v. flow rate: 1 mL/min; detection: 254 nm.

The retention time of glycyrrhizin was 12.7 min.

Table 1. Effect of extraction solvent and water content of the used solvents on the yield of Glabridin, TF and Glycyrrhizin from licorice roots

Lab trial No.	Extraction solvent	Extract yield (g%)	% moisture content ^a	% Glabridin ^b	% Total Flavonoid content [#]	% Glycyrrhizin ^c
RDP/GG/015	ethyl acetate (fresh)	5.1	0.77	5.59	17.11	Not detectable
RDP/GG/016	ethyl acetate (recovered from RDP/GG/015)	5.0	1.71	5.90	18.08	Not detectable
RDP/GG/018	ethyl acetate (recovered from RDP/GG/016)	5.1	3.15	5.57	17.58	Not detectable
RDP/GG/019	acetone (fresh)	5.6	5.55	5.09	16.21	0.16
RDP/GG/020	acetone (recovered from RDP/GG/019)	6.8	8.49	4.06	16.09	0.21
RDP/GG/021	acetone (recovered from RDP/GG/020)	6.3	10.6	4.92	18.08	0.26
RDP/GG/022	dichloromethane (fresh)	3.5	0.07	6.65	20.04	0.12
RDP/GG/023	dichloromethane (recovered from RDP/GG/022)	3.0	0.21	8.24	23.48	0.05
RDP/GG/024	dichloromethane (recovered from RDP/GG/023)	3.0	0.28	7.37	22.06	Not detectable
RDP/GG/028	ethanol (100%- fresh)	6.7	3.40	3.73	15.08	0.68
RDP/GG/029	ethanol (recovered from RDP/GG/028)	7.1	5.74	2.04	12.13	0.94
RDP/GG/030	ethanol (recovered from RDP/GG/029)	6.0	7.15	1.89	11.87	1.57
RDP/GG/035	water	10.0	Not applicable	0.05	Not applicable	10.58

As expected, the maximum amount of glycyrrhizic acid was achieved in the water extract of *Glycyrrhiza glabra* roots (Table 1, Figure 7, panel

b), followed by ethanolic extract (Table 1, Figure 7, panel a), acetone extract and extract prepared with dichloromethane (Table 1, panel c and d respectively).

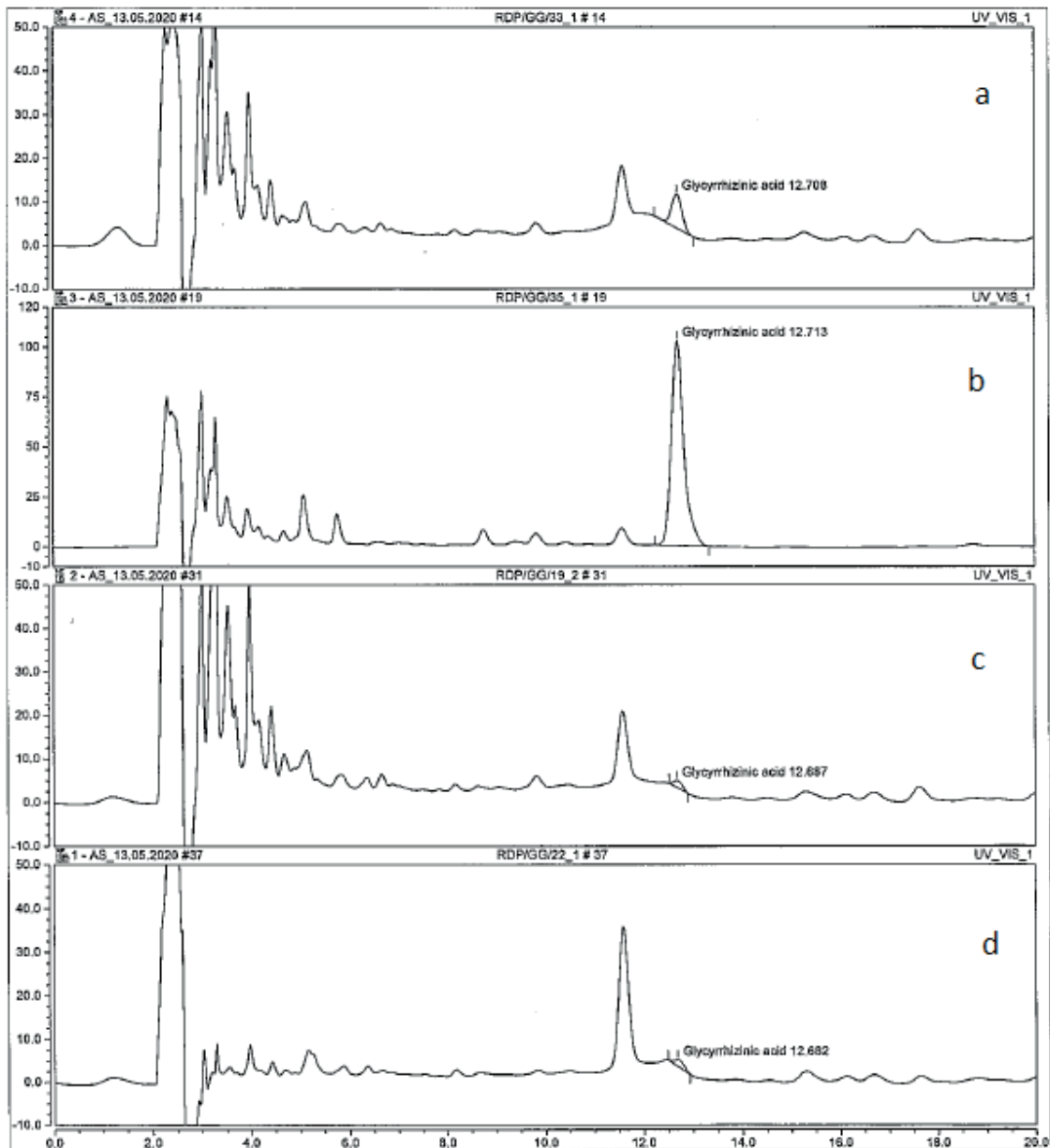


Figure 7. Chromatogram of *Glycyrrhiza glabra* extracts. Panel a: Ethanolic extract; panel b: water extract; panel c: acetone extract and panel d: dichloromethane extract. The HPLC was done using an isocratic mobile phase consisting of 2% formic acid and acetonitrile in the ratio 65:35 v/v. flow rate: 1 mL/min; detection: 254 nm. The retention time of glycyrrhizin was 12.7 min.

DISCUSSION

Medicinal herbs and herbal extracts have been in use for improving human health and building immunity in humans since decades. The first step in the preparation of herbal extracts is the extraction of the bioactive components, its (their) identification and characterization. The most crucial factor affecting the extraction efficiency of such bioactive is the type of extraction solvent employed in the extraction process. In the context of glabridin extraction from licorice, solvents that have been used to date include water, methanol, ethanol, acetonitrile and chloroform (Tian et al., 2008). Acetone (Ao et al., 2015), EA (Xu et al., 2009; Lv et al., 2010), supercritical carbon dioxide (SC-CO₂) with ethanol (Hong et al., 2019) and 95% ethanol (Nakagawa et al., 2002) are some of the solvents used for glabridin extraction.

Since Tian and co-workers reported an optimal extraction of glabridin at 50 and 60°C (Tian et al., 2008), we chose the extraction temperature as 50°C in our studies except for dichloromethane, which was operated at 30-35°C, below its boiling point. The highest yield of glabridin to date is 0.198 wt% with 95% ethanol, while it was 0.21% with acetone. Lv et al. describe the extraction of glabridin using ethyl acetate with a yield of 0.23% (Lv et al., 2010). Our present reporting ~0.29% yield of glabridin from licorice with EA is highest to date with the time of extraction to a maximum of 6 h as against 24 h and 48 h extraction times reported earlier (Tian et al., 2008; Hong et al., 2019). Since the solvent volumes used in this study is close to the volumes of solvents used by other workers, the higher yield of glabridin with EA seen is most probably due to the property of the EA solvent used for extraction.

From our studies, it is also clear that the yield of glabridin from *Glycyrrhiza glabra* roots was highest with dichloromethane, followed by EA, acetone and ethanol while the total licorice extract output per 100 g of the raw material (roots of *Glycyrrhiza glabra*) was maximal in acetone derived extract followed by

extract made using ethanol and EA. The extract output was least in extract made using dichloromethane.

The glabridin content per 100 g of raw material was least with ethanol, followed by dichloromethane, acetone and EA. The glabridin content obtained with dichloromethane was in the range of 7-8% irrespective of whether the solvent was fresh or recovered, which would make the large-scale production batches of glabridin using dichloromethane cost-effective. The next highest level of glabridin was achieved with EA as the extraction solvent which was consistent (5%) irrespective of the moisture status of the solvent. Our results of >7% extraction yield of glabridin from licorice roots using dichloromethane is the highest report to date. Also, our observation of using dichloromethane to extract glabridin and TF from licorice is reported for the first time. These findings suggest that non-polar solvents like dichloromethane and ethyl acetate are the ideal solvents for extracting bioactive compounds from *Glycyrrhiza glabra* roots.

Since ethyl acetate extract yield was almost double to dichloromethane, we recommend using ethyl acetate as a solvent for the extraction of glabridin and TF from licorice roots. Our suggestion of ethyl acetate as a preferred choice of solvent corroborates the observations of Ao and co-workers, who compared the stability of glabridin in seven different organic solvents and demonstrated maximum stability of glabridin in ethyl acetate followed by acetone (Ao et al., 2010).

The polarity index of the dichloromethane is lowest followed by EA, acetone and ethanol and this reason could be attributed for the observations of the highest glabridin and TF content in dichloromethane and ethyl acetate licorice extracts than extracts made with ethanol and acetone.

An HPLC method for simultaneous quantification of three flavonoids and four triterpenoids from licorice is reported (Wang and Yang, 2007). Our HPLC method also separates all flavonoids in a single run. The HPLC method presented, in this article,

appears to be better than the described method of Chandrasekaran et al., (2011) since in our gradient HPLC method, all the flavonoid peaks, including the peak of glabridin, are base to base resolved efficiently. Also, the proper resolution of all the flavonoid peaks has ensured the chromatographic purity of the individual peaks, thereby enhancing the specificity/selectivity of the developed method. It is noteworthy that the flavonoids, being comparatively less polar, were found mainly in the less polar media like dichloromethane and ethyl acetate. The extract made using dichloromethane is the optimum solvent of choice as it contains the maximum variety and number of flavonoids and glabridin.

Solvents used in herb extraction need to be volatile and leave no residue when dried. This solvent property will make downstream processing easier, including solvent exchange and bioassay. The used acetone produced during any processing contains varying quantities of water, and when recycled with most conventional recyclers, the distilled acetone has a purity of 96-97.5%. To remove the remaining water and increase the purity of recycled acetone to ~100%, molecular sieves are recommended (Baptista et al., 2013). All these additional steps would cause extra expenditure and cost to the herbal product. Hence, using a solvent that absorbs the least amount of moisture from plant extracts appears an attractive proposition. Hence, ethyl acetate appears as an attractive alternative to acetone for such bioactive extractions.

The relative rate of hygroscopicity (the rate of water absorption for a solvent with respect to chloroform) has been reported to be in the order ethanol, 2-propanol, acetonitrile, ethyl acetate, acetone, methanol, chloroform and the relative rate of evaporation was ether > acetone > chloroform > acetonitrile > methanol > ethyl acetate > ethanol > 1-propanol > water (Tan et al., 1982) with the rate of water absorption of acetone and ethyl acetate being close. Since the yield of glabridin and TF was

consistent with ethyl acetate, it appears tempting to suggest that ethyl acetate is a better solvent than acetone.

There are several known uses and advantages of ethyl acetate. Ethyl acetate is used not only in the pharmaceutical industry but also in the food and beverage industry (Lee et al., 2014). Ethyl acetate offers wines a fruity flavour and is used as an artificial flavour in ice creams and cakes. Its use as a solvent for varnishes, lacquers, dry cleaning, stains, for contact lenses, photographic films & plates, synthetic flavouring, chemical intermediate, perfumes, in the leather processing industry, printing industry, absorbents and adsorbents, adhesives and binding agents, cosmetics, colouring agents, etc. is reported. There are several reports on the use of ethyl acetate in the extraction of active constituents from medicinal plants where the extraction efficiency is dependent on the polarity of the isolated compound (Altemini et al., 2017) and our present article substantiates such reports.

Glabridin has many beneficial properties for use in cosmetics where its antioxidant, estrogenic, skin depigmentation, anti-inflammatory, and skin-whitening activities are utilized. In the light of these facts, it would benefit if the content of glabridin from licorice is optimal. Hence, although, dichloromethane showed significantly low moisture-absorption capacity, its low amount of glabridin limits the use in large-scale manufacturing.

A survey by the World Health Organization (WHO) has indicated the use of herbal medicines for primary healthcare since the herbal drugs are safe, natural, relatively accessible, and cheaper than the synthetic drugs (Yewale et al., 2020). Hence, our present article describing cost-effective methods to manufacture herbal extracts with active antiviral agents is attractive.

ACKNOWLEDGEMENTS

Thanks are due to Mr. Vinod Jadhav, Chairman, SAVA Healthcare Limited and Mr. Avinaash Mandale,

MD, SAVA Healthcare Limited for their constant source of support and encouragement. The authors would also like to thank Mr. Sachin Margaj for formatting the figures of the generated data as per the journal's format.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SP developed the concept and did a majority of the literature search while SB and SK designed the HPLC analyses methodology for estimation of the active constituents. ZF carried out some of the HPLC analysis while SY, SBP and NA carried out extraction of licorice roots using different solvents together with a few HPLC analyses. LS suggested the use of dichloromethane as one of the extraction solvents, tested in this study. The manuscript preparation, editing and review was done by SB and SP.

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