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HRLC-MS analysis of isoliquiritigenin from the root extract of *Glycyrrhiza glabra* for developing a novel depigmenting agent

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ABSTRACT

In the present study, a sensitive, reliable and rapid ultra performance liquid chromatography method, coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) was established for determination of isoliquiritigenin (ISL) from the root extract of *Glycyrrhiza glabra*. Chromatographic separation was accomplished on a C18 column with a multiple-step gradient elution using acetonitrile and water as mobile phase. The accuracy of the method was checked by conducting recovery studies for two different levels of ISL; the average recovery was found to be 99.49%. The average ISL content, as estimated by use of the proposed method, was 106.25 µg mg-1. Chemical structure of the purified ISL from root extract of *G. glabra* was identified by electrospray ionization–HRMS analysis using positive ion mode. The validated method can be successfully applied for the accurate analysis of ISL from licorice extract, which can be a possible depigmenting agent for treatment of hyperpigmentation of skin.

KEY WORDS: UPLC-QTOF-MS, *GLYCYRRHIZA GLABRA*, LIQUID CHROMATOGRAPHY, ISOLIQUIRITIGENIN.

INTRODUCTION

Glycyrrhiza glabra Linn. of the family Leguminosae, is a genus of perennial herbs and under shrubs is distributed in the subtropical and warm temperate regions of the world, chiefly in the Mediterranean and certain areas of Asia. Licorice, the dried root of *G. glabra*, is widely used as flavoring and sweetening agent, but has also been proposed for various clinical applications, (Fiore *et al.*, 2005). Traditions from ancient Assyrian, Egyptian, Chinese and Indian cultures have documented its extensive medicinal use as demulcent, expectorant and in ulcer

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healing (Armanini *et al.,* 2002). Pharmacological effects of licorice including inhibition of gastric acid secretion, anti-inflammatory, antiviral and anti-atherogenic properties have been well verified, (Fiore *et al.,* 2008; Tanaka *et al.,* 2008).

Pharmacological investigations have concluded that, flavonoides are the main bioactive components of licorice and have antioxidant and antibacterial bioactivities, (Vaya *et al.,* 1997; Sharma *et al.,* 2013). Among them, the remarkable one such as Isoliquiritigenin (ISL) has been reported to carry strong biological activity. ISL is a flavonoid contained in licorice with a simple chalcone structure (4,2',4'-trihydroxychalcone) (Fig. 1). ISL has been shown to exert various actions including vasorelaxant, antioxidant, neuroprotective, anti-platelet, anti-tumor, angiogenic, anti-allergic, and antispasmodic (on intestine) effects, and has estrogenic properties (Ii *et al.,* 2004; Chin *et al.,* 2007; Ye *et al.,* 2009; Kang *et al.,* 2010; Lee *et al.,* 2012).

In addition, it has been reported that ISL can inhibit the activity of tyrosinase, a key enzyme of melanin biosynthesis (Nerya *et al.* 2003). However, there has been no comprehensive study on its melanolytic activity on mammalian melanocytes .With the growing of its potential pharmaceutical role, there is an increasing demand for analyzing ISL in plant extract and evaluating its melanolytic behaviour for a better understanding of the mechanism of its action and facilitating further research.

The separation and purification of ISL from licorice by conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) is tedious, time consuming and usually requires multiple chromatography steps (Cheng *et al.,* 2005). Over the past few years, the applications of liquid chromatography coupled, with mass spectrometry (LC–MS) in natural product analysis have been dramatically growing because of the increasingly improved separation and detection capabilities of HRLC–MS instruments.

Compared to other conventional detection methods, HRLC-MS can not only allow accurate determination of chemical structure of natural compounds with known and unknown structures, but also offers excellent sensitivity and attain high-quality data within minimum acquisition time. In particular, these analytical techniques greatly aid unequivocal identification and highly sensitive quantification of natural products at trace concentrations in complex matrices (Ho *et al.,* 2003; Sauvage *et al.,* 2006).

The present report deals with the identification, quantification and structure analysis of ISL from the root extract of *G. glabra* for the first time, which is responsible for anti browning activities and also to seek new tyrosinase inhibitors for application as whitening agents in cosmetic industry.

MATERIAL AND METHODS

Analytical grade methanol and ethanol were obtained from Qualigens Fine Chemicals, Mumbai, India. Acetonitrile (HPLC grade) was purchased from Sigma Aldrich (St. 21 Louis, MO, USA). ISL standard was procured from Alfa Aesar (USA). Licorice, the root of *G. glabra*, was purchased from local market of Bhopal. The plant materials were identified and authenticated by Dr. S.S. Khan of Botany Department, Saifia College, Bhopal. A voucher specimen (287/Bot/Saifia/11) is deposited at herbarium of Department of Botany, Saifia College, Bhopal.

EXTRACTION PROCEDURE AND SAMPLE PREPARATION FOR LC–MS ANALYSES

Extract was prepared according to the method of Cheng *et al.,* (2005) with slight modification, where the roots of *G. glabra* were dried at 60°C and then pulverized. Five hundred grams of sample were put into a 3000 ml flask, to which 1200 ml 90% ethanol was added. After soaking extraction at room temperature for 24 h, the extraction procedure was repeated twice (1200 ml of 95% ethanol each time). All the filtrates were combined, filtered and evaporated to brown syrup ointment under reduced pressure. The ointment was then dissolved in 500 ml hot water, and extracted with ether (3×300 ml). The ether extracts were combined and evaporated to dryness under reduced pressure, which yielded 15 g of crude flavonoid extracts. Obtained dry residue was resuspended in 1 ml methanol. 2 µL were used for LC–MS analysis.

Standard stock solution of ISL was prepared in methanol. Appropriate amounts of standard were dissolved in volumetric flasks to obtain a stock solution with a concentration of 1 mg mL^{-1}. Working solution was prepared by diluting the stock solution with methanol to different concentration.

HRLC–MS INSTRUMENT AND CONDITIONS

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with a HSS T3 column18 (100 × 1.0 mm, particle size 1.8 µm; Waters); applying the following gradient at a flow rate of 0.5 mL min^{-1}; 0-0.5 min, 10% A (acetinitrile) and 90% B (water); 1-3 min linear from 10 to 90% A; 3.5-5.5 min, isocratic 10% A. The injection volume was 3.1 µL (full loop injection). The thermo stated auto sampler was kept at 4°C.

Eluted compound was detected from m/z 50 to 3000 using a Micro-TOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-Q-II Daltonics) equipped with an Apollo II electrospray ion source in positive ion modes using the following instrument settings: nebulizer gas: nitrogen; drying gas flow: 7.0 L/min;

nebulizer pressure: 1.2 bar; capillary voltage: 4500 V; end plate offset: -500 V; collision cell RF: 200 Vpp. drying gas temperature: 200 °C. System control and data acquisition were controlled by Bruker Compass Data Analysis 4.0

RESULTS AND DISCUSSION

High performance liquid chromatography (HPLC), High-speed counter-current chromatography (HSCCC), liquid chromatography-mass spectrometry (LC–MS) techniques were reported for qualitative and quantitative analyses of ISL in licorice and rat plasma (Zhang and Ye, 2009; Tanaka *et al.,* 2010; Montoro *et al.,* 2011). However there has been no report on accurate quantification of ISL from licorice extract using high resolution liquid chromatography - mass spectrometry (HRLC-MS). Licorice saponins and flavonoids are relatively polar compounds with carboxyl or phenol groups in the molecules, and thus could be readily ionized in the electrospray ionization (ESI) source.

The present method used slightly different mobile phases and a steeper gradient of water and acetonitrile. This allowed for the elution of all analytes within 6 min.

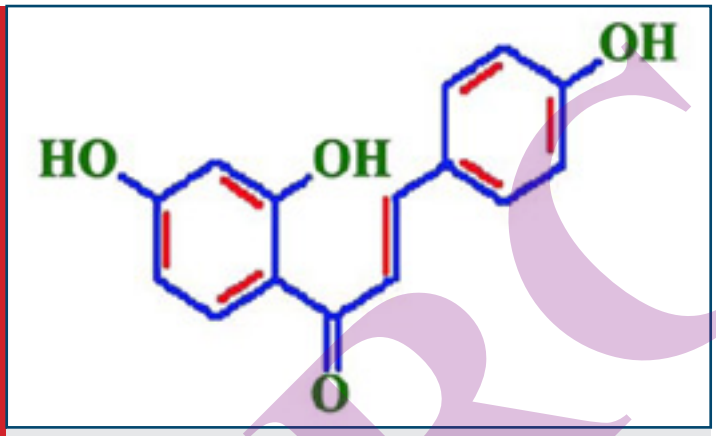

FIGURE 1: Molecular structure of Isoliquiritigenin (ISL).

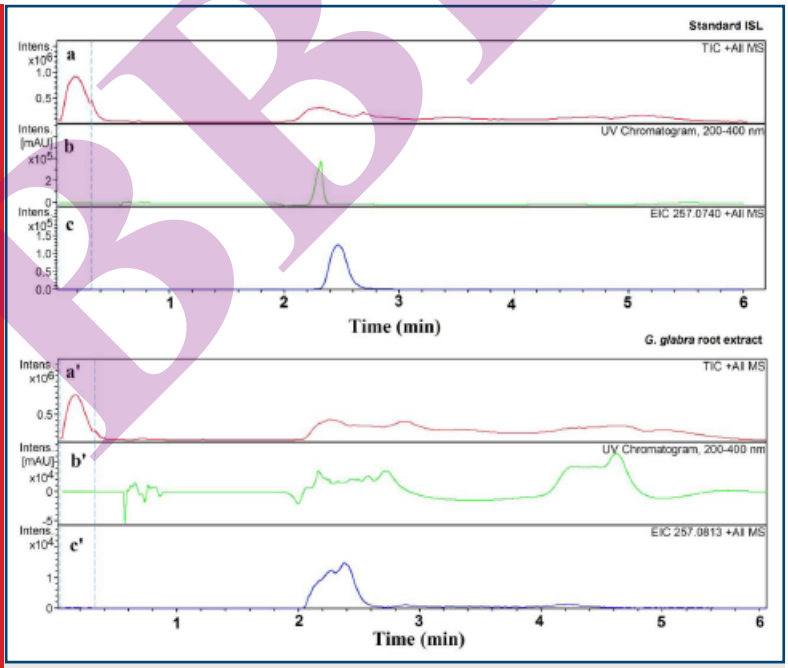

FIGURE 2: Comparative analyses of standard ISL and root extract from *G. glabra* using UPLC-HRLC–MS data (a-a') Total ion counts vs. Acquisition time (min) (b-b') UV chromatogram, (c-c') Extracted ion counts vs. Acquisition time (min)

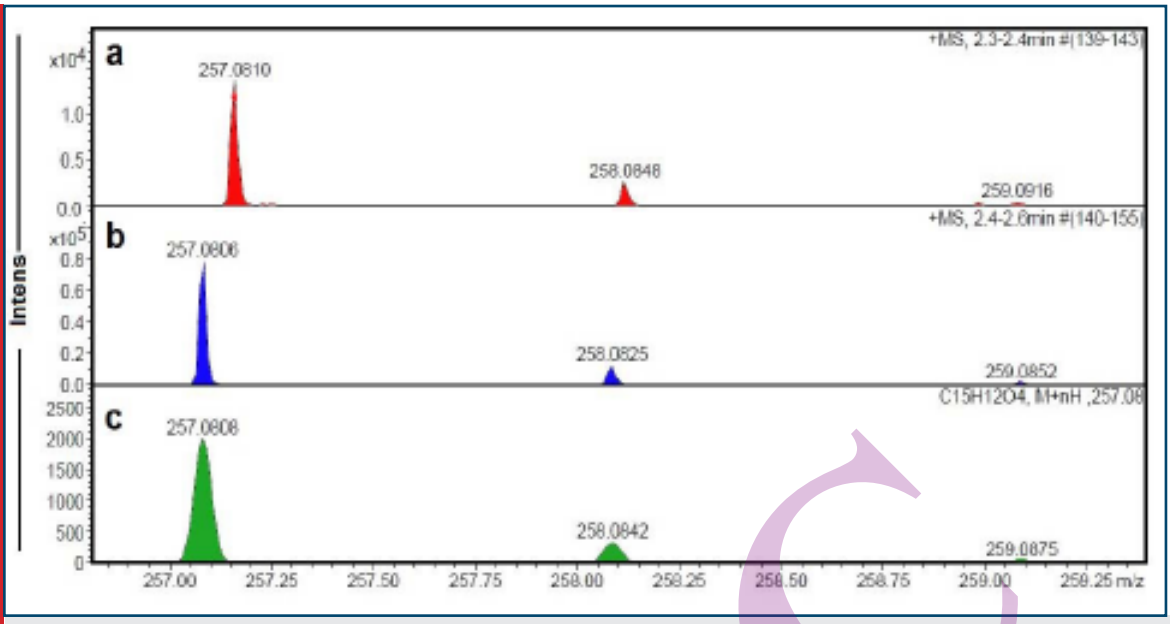

FIGURE 3: Comparison of LC–MS base peak intensity chromatograms derived from positive ionization mode (m/z 50–3000). Counts vs. Mass-to-charge (m/z), *G. glabra* extract (a) and standard Isoliquiritigenin (ISL) (b) theoretical composition (c).

simultaneously acquired LC–MS total ion chromatograms, UV characteristics and extracted ion chromatogram of *G. glabra* extract and standard ISL are presented in Fig. 2. The UPLC analyses of the root extract from *G. glabra* shows several compounds where the purity ISL was 42% based on UPLC peak area percentage. Based on the UPLC analysis, a total amount of 106.25 µg mg^{-1} of ISL was quantified.

The average recovery of ISL at two different levels was found to be 99.49%.This method was found superior in linearity, recovery, and sensitivity compared to other chromatographic methods (Zhang *et al.,* 2013; Wang and Yang, 2007) for quantitative estimation of ISL in the herbal extraction of *G. glabra*.

The identities, retention times, and observed molecular and protonated ions for individual components are presented in fig. 3. Structure and molecular formula were confirmed by comparing retention time, UV/Vis spectra and MS data (accurate mass, isotopic distribution in positive ion mode) of the compound detected. In the present study we found mass accuracy of our plant extract compared to standard ISL, matching with theoretical composition of $C_{15}H_{12}O_4$. The total ion chromatogram and computer-reconstructed selected ion chromatograms for the positive ion electrospray LC-MS was analysed. The exact mass of our plant extract ($[M-H]^+$ *m/z* 257.0810) was identical to that of ISL ($[M-H]^+$ *m/z* 257.0806), matching the theoretical composition of $C_{15}H_{12}O_4$ (($[M-H]^+$ *m/z* 257.0808). The positive ion electrospray product ion mass spectra of sample and ISL are shown in Fig. 3 and are also indistinguishable. The UV absorbance spectra of sample and ISL were recorded during LC-UV-MS and showed absorbance maxima of 272 nm. The overall results of the present study indicate that HRLC-MS is a powerful technique in separating and purifying ISL from herbal extracts of *G. glabra,* a possible candidate for treatment of skin hyper pigmentation.

CONCLUSION

Despite the widespread medicinal and culinary uses of *G. glabra*, the levels of most phytoconstituents in commercial extracts are not all standardized and several specific compounds responsible for its pharmacological properties are not yet fully elucidated. The present study provides an unbiased multiplex approach, combining UPLC and high resolution MS techniques to reveal the characterization and quantification with unbiased mass accuracy of ISL in root extract of *G. glabra* coupled with multivariate data analyses.

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