Isolation, Structure Elucidation, and Standardization of Suberosin and Glycyrrhizin from *Ferulago trifida* L. and *Glycyrrhiza glabra* L.

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Abstract: *Natural products currently often considered as a starting point in drug discovery, followed by synthetic reforms to increase bioavailability and alleviate side effects. Approximately, fifty percent of the approved drugs by the U.S. Food and Drug Administration (FDA) are inspired by natural products. Glycyrrhizin is a well-known anti-inflammatory component that has been shown to prolong the coagulation time of thrombin and fibrinogen and to increase the duration of plasma recalcification. Suberosin, also known as 7-methoxy-6-prenylcoumarin, belongs to the class of coumarin compounds. The purpose of this article is to purify, identify, and standardize glycyrrhizin and suberosin, from Glycyrrhiza glabra L. and Ferulago trifida L. First, two plants were pulverized and then extracted using a percolator. The extracts were then divided into different fractions by column chromatography and mixed with thin-layer chromatography. Finally, we obtained pure compounds after using column chromatography and thin-layer chromatography. ^{1}H-NMR, ^{13}C-NMR have been used for the purified compounds and the HPLC method was used to standardize these compounds. Glycyrrhizin and Suberosin were extracted from Glycyrrhiza glabra L. and Ferulago trifida L respectively and ^{1}H-NMR ^{13}C-NMR techniques approved the structure of these two compounds, also, standardization of these compounds was correctly done by HPLC.*

KEYWORDS: *Structure elucidation; Glycyrrhizin; Suberosin; Standarztition; Glycyrrhiza glabra L.; Ferulago trifida L.*

INTRODUCTION

In both modern and traditional medicine natural products have been used. Natural products currently often consider as a starting point in drug discovery, followed by synthetic reforms to increase bioavailability and alleviate

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side effects. Natural compounds are the vision for fifty percent of the U.S. Food and Drug Administration (FDA) approved drugs. In addition to medicine, natural compounds are commonly used as food additives as a spice. Some herbs are also used as antibacterial agents and antioxidants to protect the freshness of food [1].

The roots of *Glycyrrhiza glabra* L. flavonoids active compounds, such as coumarin-GU-12, glycyrrhizin, glucoliquiritin apioside, isoflavone, liquirtin, , liquiritigenin, prenyllico flavone A, licopyrano coumarin, licoaryl coumarin, and metho-xyphaseolin, rhamnoliquirilin, shinpterocarpin, shinflavanone, and saponins [2]. Glycyrrhizin inclusive glycyrrhizic acid and triterpenoid aglycone, related to glucuronic acid disaccharide, and it can be naturally in licorice root as calcium potassium salts [3, 4]. Glycyrrhizin metabolized in the human body and become glycyrrhetinic acid. The pharmacological activities of glycyrrhetinic acid and Glycyrrhizin are similar [5].

glycyrrhetinic acid with its ability to inhibit Helicobacter pylori is effective in treating gastric ulcers, gastric mucosal problems and reducing gastric acid [6]. *G. glabra* also affects the body's endocrine system and its consumption may reduce the amount of testosterone in the blood. *G. glabra* or dried *G. glabra* root has also been shown to increase the secretion of serotonin and prostaglandins in the stomach and exert anti-inflammatory effects in this way [7-9]. Complete German Commission E use this plant in congestion of the upper respiratory tract and wounds the stomach and duodenum are confirmed [10-12].

Suberosin, also known as 7-methoxy-6-prenylcoumarin, is acoumarin and Suberosin is actually insoluble in water and based on its pKa an extremely weak basic nearly neutral compound. Suberosin also can be found in lemon, mandarin orange and sweet orange, which makes suberosin a potential biomarker for the utilization of these food products [13]. Several studies have reported different coumarins isolated from *Ferulago* species (Apiaceae), *Ferulago aucheri* Boiss., *Ferulago asparagifolia* Boiss., Ferulago *Bernardii* Tom. & M. Pimen., *Ferulago brachyloba* Boiss., *Ferulago capillaris* Cout., *Ferulago granatensis* Boiss., *Ferulago meoides* (L.) Boiss., *Ferulago nodosa* (L.) Boiss., *Ferulago sylvatica* Rchb., and *Ferulago turcomanica* Schisch. [14]. Moreover, in previous studies, the acetylcholinesterase inhibitory [15] and cytotoxic activities were evaluated [14]. Coumarins with medical activity several times are have been isolated from *Ferulago* species [16].

In this study, we purified and identified the structure of two compounds of Glycyrrhizin and Suberosin from two plants *Glycyrrhiza glabra* L. and *Ferulago trifida* L. and also standardized these two compounds for the first time in Iran in the laboratory.

EXPERIMENTAL SECTION

General instrumental procedures

^{1}H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) spectra were measured on a Varian – INOVA spectrometer (USA), using $CDCl_3$ as solvent and TMS as internal standard (Merck, Germany). Mass spectra were obtained using an Agilent, 6410 QQQ mass spectrometer (USA). Open column chromatography was performed using silica gel (70–230 mesh), Sephadex LH-20 (Sigma-Aldrich) and analytical grade solvents (Merck, Germany). Separations were monitored by thin layer chromatography plates and were visualized by UV inspection and/or staining with Cerium Sulphate/molybdate (Merck, Germany) proceeding by heating.

Plant material

To isolate glycyrrhizin, first, prepared the root of the licorice plant with the scientific name of *Glycyrrhiza glabra* L. from the pharmaceutical market. Its scientific name was then approved by the Herbarium of the Medicinal Plants Research Institute and registered in the Herbarium of the Medicinal Plants Research Institute under IMPH-614.

Extraction

Glycyrrhiza glabra L.

100 g of plant roots were ground entirely by the mill. Poured the plant into a 2-liter Erlenmeyer flask and 1 liter of distilled water was added. Also, 1 ml of concentrated ammonia was poured into the Erlenmeyer flask. The Erlenmeyer was placed on a magnetic stirrer and 3 cm magnet was inserted into it. The contents of the Erlenmeyer flask stirred for 100 hours at 100 ° C at 500 rpm. The resulting extract was smoothed with filter paper. Transfer 50 ml of pre-prepared licorice extract to 100 ml beaker and 8 ml of 25% sulfuric acid solution was added drop by drop. The resulting mixture was centrifuged at 3000 rpm for 5 minutes and then the top solution was separated. Distilled water and the remaining residue were mixed and centrifuged again to remove the top solvent. The residue is dissolved in 4 ml of ammonia and

transferred to a petri dish. For 24 hours the petri dish was settled in the room air to dry its contents. Sediments were stored in the freezer until the compounds are separated.

Ferulago trifida L.

First, 110 g of chloroform root extract of *Ferulago trifida*, was divided into several fractions with a smaller number of compounds to facilitate the separation of chloroform extract. The mixture was poured into a rotary balloon so that the extract adhered entirely to the silica gel and dried thoroughly.

Isolation

Glycyrrhizin

The residue obtained in the previous step was first completely dissolved in concentrated ammonia to isolate the glycyrrhizin composition. Then added 96% ethanol dropwise to the resulting mixture. Ethanol was caused a white residue in the solution. The resulting solution was washed with strainer paper and the residue on the strainer with ethanol and thoroughly dried.

Sephadex LH20 column removed this residue. A column with a 1 cm diameter and a height of 100 cm have been used for glycyrrhizin's further purification. The mobile phase of this column is 80% methanol—separate 22 ml fractions from this column. Glycyrrhizin is present in fractions 26 to 30.

Chloroform-methanol-water (65: 10: 50) was used as a mobile phase to identify glycyrrhizin in thin layer chromatography. The product was sprayed with a benzaldehyde-sulfuric acid detector and placed in an oven at 100 °C for 5-10 minutes.

Suberosine

A chromatographic column with a 10 cm diameter and 50 cm height was selected and packed up to 30 cm with delicate mesh silica gel (230-400 mesh).

Silica gel was poured of the above extract and make a mixture. The height of the silica gel inside the column at this stage was 35 cm. The fractionation of column chloroform extract with hexane-ethyl acetate solvent system started with hexane alone and then increasing by 5% -5% ethyl acetate at each stage, increasing the polarity to 50% with 50% hexane-ethyl acetate. The column ended. The above solvents were also added to the column until the exhaust solvent of the column contained no compound.

Table 1: HPLC condition

HPLC device model	Knauer
Column type	Eclipse –XBD-C18
Column specifications	15 cm × 4.6 cm × 5 µm
pump	Knauer- K1001
Detector	Knauer- UV K2501: 254 nm
Flow rate	1.5 ml/min
Mobile phase	glacial acetic acid R, acetonitrile R, water R (6:30:64 V/V/V)
Temperature	20 ± 1 °C.

The process was monitored using TLC paper. This process caused the extract to be converted to 25 fractions with fewer compounds.

Standardization

After separation and purification of glycyrrhizin and Suberosine compounds, the validated method in the instruction number PSA.AA.BB.CCC.332 was used to determine the amount of these substances.

Repeated the test to determine the amount of glycyrrhizin and Suberosine compounds on three different days by three experts and three times, and finally, picked the RSD and the accuracy of the analysis of the percentage of glycyrrhizin composition.

High-performance liquid chromatography

Specifications and conditions for the quantitative analysis of glycyrrhizin are shown in Table 1.

RESULTS AND DISCUSSION

Isolation and Characterization

Glycyrrhizin

In the end, a colorless amorphous powder was obtained, which was glycyrrhizin. Analysis of Spectroscopic (^{1}H and ^{13}C NMR, and mass spectroscopy) confirmed its structure as glycyrrhizin. Positive mode of ESI Time of Flight (TOF) mass spectrum indicated an [M+H] + ion at *m/z* 823.4138 together with [M+Na] + adduct ion at *m/z* 849.3954, respectively; which were confirmed the molecular formula $C_{42}H_{62}O_{16}$. The ^{13}C NMR spectral further data supported the chemical composition of glycyrrhizin. The ^{1}H NMR (Fig. 1) spectra of glycyrrhizin showed 0.79, 1.05, 1.21, 1.27, 1.37, 1.42 and 1.44 chemical shift that they confirmed seven methyl singlets. The terpenoid skeleton of glycyrrhizin is

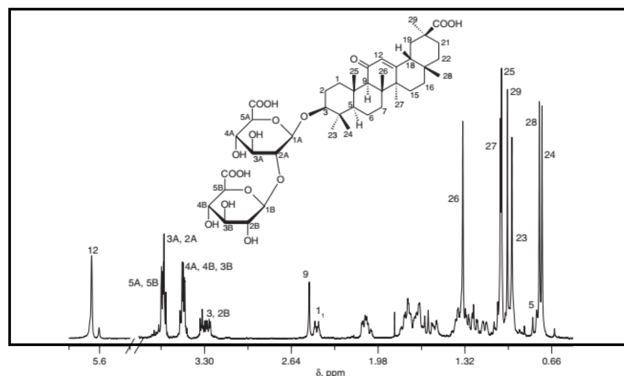

Fig. 1: ***^{1}HNMR Spectrum of the isolated compound-glycyrrhizin***

confirmed by the Liebermann-Burchard reaction. A doublet of doublets at δ 3.37 confirmed the signal corresponding to the H-3 of the oxymetholone proton in glycyrrhizin's terpene. A trisubstituted olefinic bond at δ 5.98 also shown in glycyrrhizin.

Further, a carbonyl group at the C-11 position with downfield shift of the trisubstituted olefinic proton, which was supported by the carbonyl group resonating at δ 200.1. The mentioned spectral data have supported the attendance of an oleanane triterpene skeleton having a hydroxyl group at C-3 position with a double bond at C-12/C-13 with seven methyl groups. Tthe ^{1}H NMR spectrum of glycyrrhizin was supported the attendance of two sugar units in its structure, which showed the anomeric protons doublets at δ 5.08, and 5.47. To identify the stereochemistry of sugar in glycyrrhizin, as stated in the literature, by preparing thiocarbamoyl thiazolidine carboxylate derivatives with L-cysteine methyl ester O-tolyl isothiocyanate, and comparing their retention times with standard sugars, could Identified sugar's stereochemistry is D-glucuronic acid [16]. At δ 5.08 (d, J=8.1 Hz) the two anomeric protons of the glucose moieties shown the large coupling constants, and suggested their β- orientation5.47 (d, J=8.4 Hz), as reported earlier. All values for the ^{1}H and ^{13}C NMR is in Table 2.

Glycyrrhizin and its corresponding aglycone 18β-glycyrrhetinic acid weren't veritably surface-active [17]. Nonetheless, the slightly higher affinity of glycyrrhizin compared to aglycone can be attributed to the higher polarity of the glycyrrhizin molecule than aglycone. At an oil-water interface, 18β-glycyrrhetinic acid showed no surface activity at all, whereas glycyrrhizin was appeared to gently reduce the interfacial traction [18]. The relatively rigid structure of glycyrrhizin is due to its low surface activity [19]. Moreover, Hydrophobic interactions contribute to the stabilizing forces of similar relationships, as evidenced by the dominance of hydrophobic surfaces over polar surfaces in both glycyrrhizin and 18β-glycyrrhizinic acid molecules [18].

Table 2: ^{1}H NMR, ^{13}CNMR Data of glycyrrhizin

Position	^{1}H NMR	^{13}C NMR
1	0.95 m, 3.05 dd (J = 8.1, 9.6)	39.9
2	1.75 m, 2.04 m	27.1
3	3.37 dd (J = 5.4, 11.6)	89.6
4	-	40.5
5	0.74 m	55.9
6	1.46 m, 1.68 m	18.1
7	1.48 m, 1.72 m	33.4
8	-	43.9
9	2.46 s	62.6
10	-	37.7
11	-	200.1
12	5.98 s	129.2
13	-	172.9
14	-	46.1
15	1.24 m, 2.12 m	27.2
16	1.08 m, 2.15 m	27.3
17	-	32.7
18	2.14 m	49.2
19	1.55 m, 2.34 m	42.2
20	-	44.6
21	1.53 m, 2.08 m	32.1
22	1.30 m, 1.73 m	38.9
23	1.21 s	28.6
24	0.79 s	17.2
25	1.27 s	17.3
26	1.37 s	19.3
27	1.42 s	24.1
28	1.05 s	29.2
29	179.7	-
30	1.44 s	29.3

Suberosine

To identify and determine the type of Suberosine hydrogens (Fig. 2), NMR spectra were taken in the deuterium chloroform solvent. According to the ^{1}H-NMR (Table 3, Fig. 3), ^{13}C-NMR (Table 4, Fig. 4), and Mass spectra (Fig. 3) presented below, we were able to achieve this combination's exact structure.

Table 3: ^{1}H NMR Data of suberosin

^{1}H NMR	Chemical Shift (δ)
1	1.70 (3H, s, CH3)
2	1.77 (3H, s, CH3)
3	3.31 (2H, d, J= 7.20 Hz, H-1')
4	3.89 (3H, s, OCH3)
5	5.28 (1H, t, J= 7.20 Hz, H-2')
6	6.23 (1H, d, J= 9.4 Hz, H-3)
7	6.76 (1H, s, H-8)
8	7.18 (1H, s, H-5)
9	7.62 (1H, d, J= 9.4 Hz, H-4)

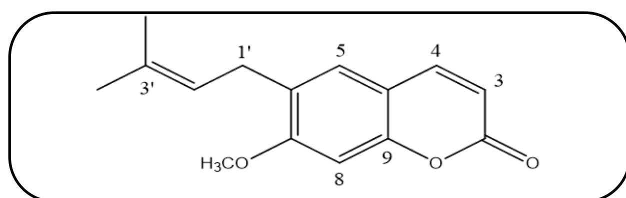

Fig. 2: Structure of suberosin

Table 4: ^{13}C NMR data of

^{13}C NMR	Chemical Shift (δ)
1	17.6 (C-5')
2	25.6 (C-4')
3	27.5 (C-1')
4	56.2 (OCH3)
5	98.7 (C-8)
6	111.7 (C-10)
7	112.3 (C-3)
8	121.7 (C-2')
9	126.5 (C-6)
10	127.0 (C-5)
11	132.5 (C-3')
12	144.4 (C-4)
13	154.1 (C-9)
14	160.2 (C-7)
15	160.5 (C-2)

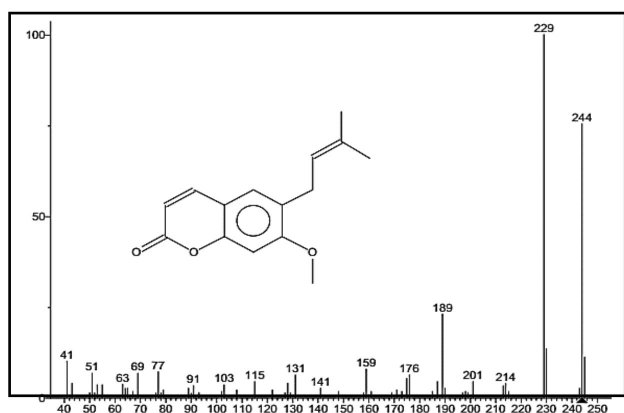

Fig. 3: LC-MS spectra of suberosin

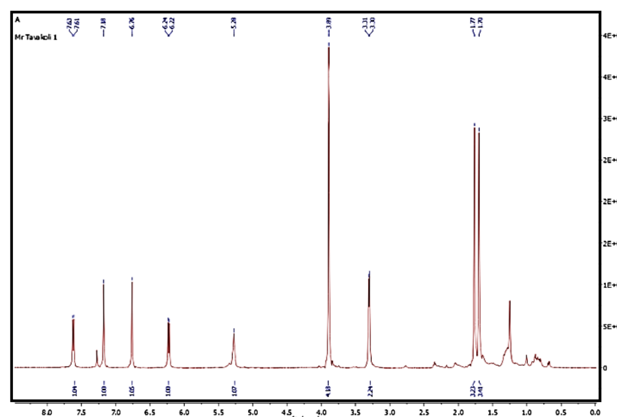

Fig. 4: ^{1}HNMR Spectrum of the isolated compound-suberosin

Study of this Spectrum indicates nine proton peaks, which they are interpreted in the following. The two-pronged peak with a chemical displacement of 7.62 ppm and a coupling constant of 9.4 Hz corresponds to proton number 4, paired with proton number 3. Single-branched peaks with chemical removals of 7.18 ppm and 6.76 ppm belong to protons 5 and 8, respectively. The three-pronged peak in chemical displacement is a 5.28 ppm constant of the 7/2 Hz coupling corresponding to proton number 2. The single-branch peak with the chemical displacement of 3.89 ppm and integral 3 belongs to 3 protons of the methoxy group.

A preliminary look at the ^{13}CNMR Spectrum shows this fifteen-carbon composition. The six peaks with shorter heights than the others belong to the six quaternary carbons. The peak with chemical displacements of 160/50 ppm corresponds to carbon No. 2 (carbonyl group). The peak with a chemical displacement of 160/21 ppm corresponds to carbon 7, which is uncoated due to its proximity to the oxygen-killing electron element. The remaining four quaternary peaks with chemical displacements are 154.09 ppm, 132.49 ppm, 126.46 ppm, and 111.69 ppm corresponding to 9, 3, 3, 6, and 10 carbon. The peak with the chemical displacement of 56.18 ppm belongs to the methoxy group.

Standardization

The glycyrrhizin and Suberisine compounds were weighed and poured into dark glass vials. The label of standard vials includes the name of the composition, the Jahad Medicinal

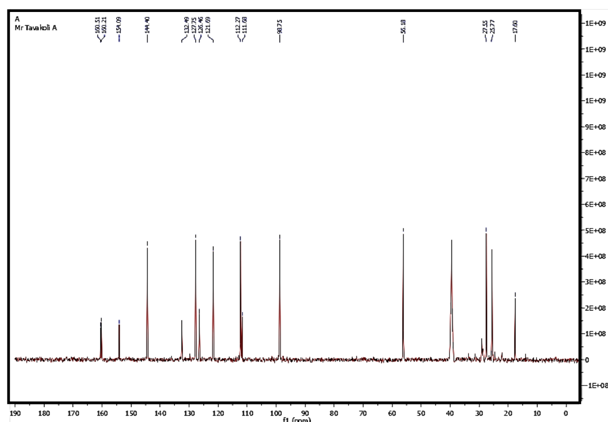

Fig. 5: 13C NMR Spectrum of the isolated compound-Suberosin

Plants Research Institute's name as the producer, the approximate amount of the substance in the container, and the batch number. The stability of vials containing standard (SOP number PSA.QA.IA.SOP-025) was insured.

CONCLUSIONS

Only a few papers regard methods to separate glycyrrhizine and Suberosine, using HPLC, GC or HPTLC, Thus, the object of this research is the development of a rapid and feasible open column chromatography method for the contemporary analysis of glycyrrhizine and Suberosine. Whereas, HPLC techniques entail chiral stationary phases for the separation of isomers that they are very expensive, while HPTLC requires high capability and it is not a wide-spreading technique. In this study, glycyrrhizine and Suberosine with high purity were obtained from *Glycyrrhiza glabra* L. and *Ferulago trifida* L. column chromatography and thin-layer chromatography, the purified compounds identified with ^{1}HNMR and ^{13}CNMR. Also, these compounds were measured and standardized by the HPLC method.

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