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Introduction to Modules

Module	Details	Hours
1	**Ensuring quality of the herb as the starting material :** Overview of the herb, review of the literature, Report writing, presentation, Aims and objectives of the work, authentication and standardization of the herb, pharmacopeial specifications and compliance.	**50**
2	**Development of extraction schemes and optimization :** Review of various extraction methods, designing extraction technique based on the nature of phytoconstituent to be extracted, percent yield and native extract ratio	50
3	**Chromatographic techniques in evaluation of natural products :** Overview of Chromatographic techniques, analytical method development, qualitative and quantitative analysis - TLC, HPTLC, HPLC, application of the Chromatographic techniques in authentication and standardization of extracts.	50

Literature of Work undertaken under Practice School

Glycyrrhiza glabra, commonly known as liquorice, is a flowering herb belonging to the Fabaceae family (also known as Leguminosae). The genus *Glycyrrhiza* is derived from the Greek words glykos (sweet) and rhiza (root). It is also called, glycyrrhiza, sweet wood, licorice. The genus Fabaceae consists about 30 species such as *G. glabra, G. uralensis,b G. aspera, G. korshinskyi, or G. eurycarpa.* This species is a native of Mediterranean areas, but it is now also present in India, China, and Russia. The various reported pharmacological activit of liquorce are neuroprotective , antidepressive, anti-inflammatory, antiviral, antimicrobial, anticarcinogenic, hepatoprotective [1].

A large number of phytoconstituents have been isolated from liquorice including glycyrrhizic acid, glycyrrhizin, glycyrrhetinic acid, glabridin, triterpene saponins,glabrene, coumarin, flavonoids, isoflavonoids and chalcones.[2] Glabridin a natural occurring prenylated isoflavan, accounts for 0.08–0.35% of the roots dry weight in *Glycyrrhiza glabra* which has a wide range of biological activities including anti-inflammatory as in Atopic (dermatitis, Psoriasis) anti-oxidation, anti tumor, cardiovascular protection, hepatoprotection, anti-microorganism, bone protection neuroprotection, anti-obesity, and anti-diabetes. Glabridin is also found to be effective against P. aeruginosa-caused lung infection,growth inhibition of S. aureus and considered to mitigate COVID-19. [3]

The stolon of liquorice consists of a yellowish brown or dark brown longitudinally wrinkled outer layer. The cut surface shows a cambium ring and a central pith. According to Indian Pharmacopoeia 2022, the transverse section of liquorice stolon shows cork, secondary cortex of parenchymatous cells containing calcium oxalate crystals, secondary phloem, cambium, secondary xylem, xylem vessels, medullary rays, lignified fibers with crystal sheaths similar to those of phloem, xylem parenchyma of two kinds, those between the vessels having thick pitted walls without intercellular spaces, the remaining with thin walls and pith. [4] Total ash value, acid insoluble value , water extractive value and alcohol extractive value evaluation was done as per Indian Pharmacopoeia 2022 and British Pharmacopoeia 2022.[4,5,6] Glabridin is among the main fat-soluble flavonoid components of Glycyrrhiza glabra . Many studies have been conducted on the extraction of the crude extract from Glycyrrhiza glabra, including organic solvent extraction, ultrasonic-assisted extraction, microwave-assisted extraction, carbon dioxide

supercritical fluid extraction [7]. The majority of documented liquorice extraction techniques include the use of hydroalcoholic solvents.. The method for alcoholic extraction of liquorice reported by Lim et al., 2010 [8] discusses the alcoholic extraction was done using a mechanical shaker for 3h and extract was further concentrated using rotary vaccum evaporator.

The method for alcoholic extraction of liquorice reported by Vivek K Gupta et al., 2008 [9] discusses the alcoholic extraction by using soxhlet apparatus for 10h and then the extract was concentrated by rotary vacuum evaporator. The method for alcoholic extraction of liquorice reported by Fei Liu et al., 2022 [10] discusses the alcoholic extraction was performed by constant stirring for 3h at 25°C and filtered. The filterate was extracted twice with ethanol in the same manner and concentrated using rotary vacuum evaporator.

Various analytical methods for qualitative and quantitative analysis of glabridin have been published which include TLC, reversed phase HPLC, HPTLC, capillary zone electrophoresis and preparative chromatography. [2,11,12] TLC is the simplest and easiest method used for qualitative analysis of glabridin in extracts of liquorice. The TLC performed by using silica as stationary phase and mobile phase hexane:ethyl acetate (4:1) proportion showed expected RF at 0.55 for glabridin [13]
HPTLC carried out using mobile phase methanol:water(7:3) and silica plate as stationary phase and samples were applied as 6 mm bands and were allowed to run upto 80mm the RF was at 0.28 detected at 233nm wavelength.[14]. The drawback of this method was extreme polar nature of mobile phase which was ineffective for the seperation of glabridin. For HPLC ,The restricted linearity range, poor analyte recovery, and ineffective separation of glabridin from other extract constituents are the drawbacks of the HPLC approach described by Shanker et al. [15].
The development and validation of a high performance liquid chromatography method for measurement of glabridin, which can be used for liquorice quality control and standardization, is covered in the HPLC method reported by Y T Kamal et al., 2012 [16]. The method's good sensitivity is indicated by the low LOD and LOQ readings
The HPLC method for glabridin analysis in liquorice is discussed in the report by Vivek Viswanathan et al., 2016 [2]. It covers the development and validation of HPLC and HPTLC methods. Glabridin can be effectively separated from other extract constituents using the method outlined.

The HPLC method reported by Vivek Viswanathan et al., 2016 [2] discusses the development and validation of HPLC and HPTLC methods for analysis of glabridin in licorice. The method

described provides excellent separation of glabridin from other constituent of the extract. The current study concerns the development of a method for extraction of Glabridin with Soxhlet using alcohol as solvent. Followed by qualitative and quantitative analysis of glabridin in the alcoholic extract of liquorice by TLC, HPTLC and HPLC.

Aims and objectives of work undertaken under Practice School

The aim is to develop and evaluate alcoholic extract of *Glycyrrhiza glabra.*

Objectives:

1.To verify authenticity, look at the microscopic and macroscopic properties of licorice powder and stolon.

2. To ascertain the liquorice powder's percentage of ash content in order to confirm that it complies with BP, and IP standards.

3. To determine the liquorice powder's percentage of water and alcohol-soluble extractives and to confirm that it complies with pharmacopoeial requirements.

4. To assemble soxhlet apparatus to carry out an extraction of liquorce using alcohol

5. To carry out TLC for identification of glabridin present in liquorce alcoholic extract.

6.To prepare the samples used for determination of glabridin present in the alcoholic extract of liquorice using high-performance thin-layer chromatography.

7. To detect and measure the amount of glabridin present in the alcoholic extract of liquorice using high performance liquid chromatography.

EVALUATION OF LIQUORCE AS PER PHARMACOPEIAL SPECIFICATIONS

Reagents and apparatus :
Stolon of liquorice, microscope,Anhydrous calcium chloride, Dessicator, microscope slides.
Reagents : Concentrated Hydrochloric acid
Phloroglucinol,
Bleach
Water
Sulphuric acid
Ethanol,

Projection microscope- (Make and model:Lawrence and Mayo- LYNX, N- 126),
Water bath- (Make and Model: BioTechnics India- AI-7981),
Weighing balance -(Make and Model:Wesnar- AND, GR-200)

MACROSCOPY AND MICROSCOPIC CHARACTERISTICS

Method of preparation of sample- Root of *Glycyrrhiza glabra* L. was washed with potable water to remove the adhering dirt and then with distilled water. After that the roots were dried in shade under normal room temperature. Before using the sample for the study it was assured that the drug sample is not infected with fungal or bacterial contaminants.

Macroscopic characteristics :
The color, texture, the stolon surface's appearance, and organoleptic properties were studied

Microscopic characteristics :
Liquorice stolon was sectioned manually. TS and RLS were collected as possible to as thin. The sections were cleaned with water then bleached using concentrated sulphuric acid by allowing it to stand in it for 1 min then the sections were washed multiple times with water to remove the

bleaching agent and stained using phloroglucinol and mounted with glycerin on microscope slides

Powder characteristics : The stolon powder samples were crushed into powdery mass, passed through a sieve to obtain a fine powder, and used for microscopic characterization. The powder was bleached using concentrated sulphuric acid by allowing it to stand in it for 1 min then the sections were washed multiple times with water to remove the bleaching agent and stained using phloroglucinol and mounted with glycerin on microscope slide and were examined under a compound microscope to study the various cell types and cell contents.

ASH VALUES

Total ash value :
Weigh accurately 2-3 g of drug in a weighing balance. Incinerate the drug in a crucible until it is free of carbon making sure the temperature does not exceed 450 degrees celsius. Cool in a desiccator and weigh. Calculate the percentage ash with respect to air dried drug.

Acid insoluble ash value :
Boil the total ash with 25 ml of 2N HCl for 1 minute. Filter the solution with an ashless filter paper. Wash the residue with hot water. Incinerate the residue along with the ashless filter paper. Cool in the desiccator and weigh it.

EXTRACTIVE VALUES

Water soluble extractive value :
Macerate 5 gm of the drug coarsely powdered with 100ml water for 24 hours. Shake frequently for 6 hours and remaining 18 hours allow it to stand. Filter rapidly and prepare aliquot of 20 ml. Transfer that 20 ml to an evaporating dish and use direct flame till it dries completely. Upon complete drying allow it cool down in a desiccator. Weight and calculate the % content of water soluble extractive value.

Alcohol soluble extractive value :
Macerate 5 gm of the air dried drug to coarsely powdered form with 100ml ethanol at the specified strength in a closed flask for 24 hours. Shake frequently for first 6 hours and allow it to stand for 18 hours.Filter rapidly and take precautions to prevent any loss of ethanol. Take 25 ml of the filtrate in an evaporating dish. Use water bath to completely dry the filtrate at 105°C. Let

the evaporating dish cool in the desiccator. Calculate percentage of ethanol soluble extractive value with reference to air dried sample.

DESIGN AND OPTIMIZATION OF EXTRACTION PROTOCOL

Preparation of crude drug extract :

Apparatus :

Heating Mantle (Remi 2RML), Soxhlet Apparatus (Round Bottom Flask, Soxhlet Chamber, Condenser), Weighing balance (Make and Model:AND, GR-200), Thermometer, Rotary evaporator (Model and Make: BUCHI Rotavapor model - R210/v), Vacuum pump (V-700), Vacuum controller - V-850, Heating bath - B-491 and Distillation chiller F-105

Method : Dried roots of liquorice (40 g) were extracted with ethanol (400ml) in a Soxhlet apparatus for 10 hours until the siphoning solvent turns almost colourless. The solvent was filtered and evaporated under vacuum (50 °C) to afford a crude extract.

ANALYTICAL METHOD FOR QUANTIFICATION OF ACTIVE CONSTITUENTS

Instruments : Precoated silica gel aluminum plate 60 F254 (250 µm thickness) (Merck Ltd, Darmstadt, Germany), UV spectrometer (CAMAG UV CABINET), CAMAG HPTLC SYSTEM WITH LINOMAT V APPLICATOR (Make & model: Camag Scanner 3) winCATS - Planar Chromatography Version: 1.1.3.0, CAMAG twin trough chamber, HPLC System (Make and Model : Agilent 1260 Infinity Quaternary LC VL), Diode Array Detector, Software: Agilent OpenLab CDS (EZChrom edition) Version : A.04.06, Phenomenex Luna C-8 column (5 µm silica particle size, 15 cm length)

TLC :

Chromatographic reactions were monitored on analytical TLC (MERCK TLC Silica gel 60 F254) precoated plates using hexane:ethyl acetate:chloroform (5:4:3 v/v/v) as the mobile phase. TLC plates were developed in TLC chamber and visualized by UV spectrometer at 254 nm.

HPTLC :

The samples were applied on a precoated silica gel aluminum plate 60 F254 (10 × 10 cm with 250 µm thickness) (Merck Ltd, Darmstadt, Germany). Samples were applied as 6 mm bands with a 100 µL syringe (Hamilton, Switzerland) using a CAMAG Linomat 5 sample applicator (Muttenz, Switzerland). A constant application rate of 0.15 µL/s for all applications was followed. A linear ascending development was done in a 10 × 10 cm CAMAG twin trough chamber using a mobile phase of hexane–ethyl acetate–chloroform (5 + 4 + 3, v/v/v). The chamber saturation time was 15 min at room temperature (25 ± 2°C) at a relative 8humidity of 60 ± 5%. The chromatogram run length was 70 mm and the approximate time taken for development was 15 min. After development, the plates were dried at room temperature

protected from heat and light. The developed plates were scanned at 285 nm using CAMAG Scanner 3 operated by WinCATS software version 1.1.3.0 (CAMAG). The slit width for scanning was 5.00 × 0.45 mm and scanning speed was 20 mm/s for all analyses. The source of radiation used was a deuterium lamp, which continuously emits a UV spectrum between 200 nm and 400 nm.

HPLC :

The LC separations were performed using a Agilent HPLC system equipped with a binary pump (model–P2000), autosampler (model–AS3000), and UV detector (model–UV1000). The data acquisition was done using ChromQuest software version 4.1. The stationary phase was Phenomenex Luna C-8 column (5 µm silica particle size, 15 cm length). The mobile phase consisted of a (0.2%) Acetic acid : Acetonitrile (45 + 55 v/v) HPLC grade. Sample injection volume was 20 µL for all the analyses. The flow rate was kept at 1 mL/min throughout the analysis and the acquisition was performed at ambient temperature. The eluent from the column was analyzed using a Diode Array Detector.

Preparation of standard solution of glabridin :

A stock solution containing 500 µg/mL of glabridin was prepared in methanol. This solution was further diluted with methanol to obtain solutions containing 5–50 µg/mL of glabridin for HPLC and HPTLC. These solutions were filtered through 0.45 µm, 47mm polyfluorotetraethylene (PTFE) membrane syringe filters (Agilent Technologies Pvt. Ltd, Bangalore, India) before injection.

Preparation of sample solution of glabridin

RESULTS AND DISCUSSIONS

Macroscopy : Stolon consists of a yellowish brown or dark brown outer layer, externally longitudinally wrinkled, with occasional small buds,the cut surface shows a cambium ring about one-third of radius from outer surface and a small central pith.

Microscopy :
The transverse section of liquorice stolon shows cork (10-20 or more layers of tabular cells) secondary cortex of radially parenchymatous cells containing calcium oxalate crystals, secondary phloem, cambium, xylem vessels, medullary rays, secondary xylem vessels distinctly radiate with medullary rays, lignified fibers with crystal sheaths similar to those of phloem, xylem parenchyma of two kinds, those between the vessels having thick pitted walls without intercellular spaces, the remaining with thin walls and pith

Figure 1 and 2 : Transverse section of stolon of liquorice

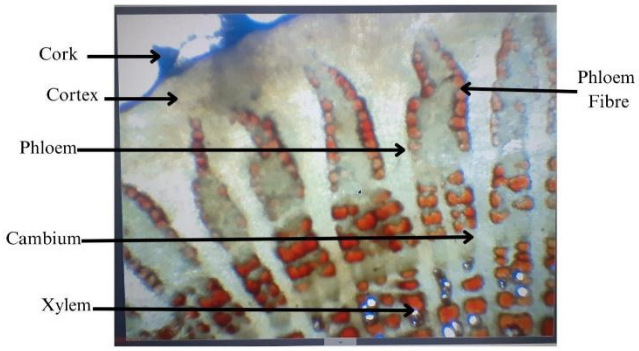


Figure 1.

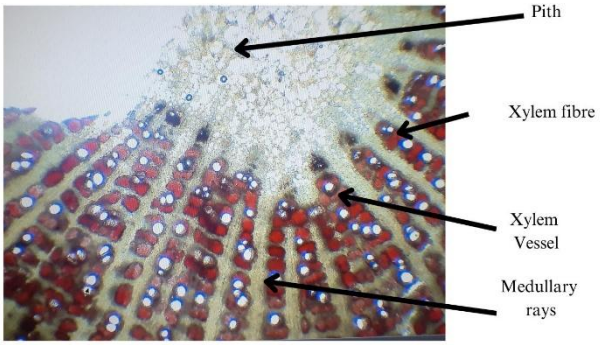


Figure 2.

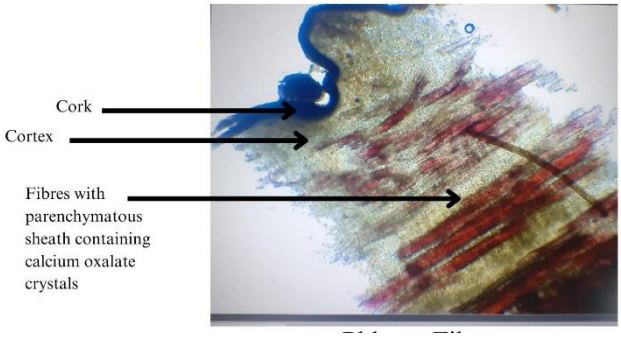


Figure 3. Phloem

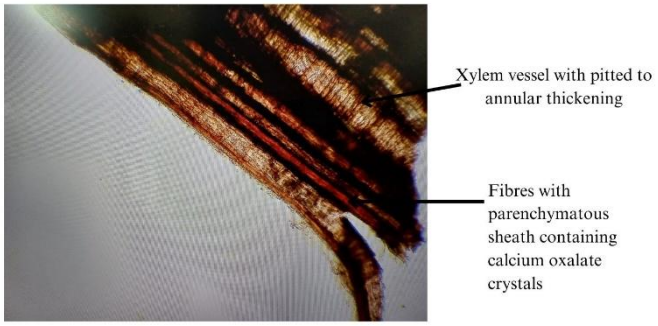
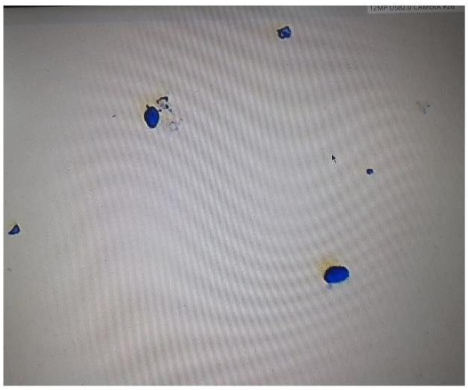
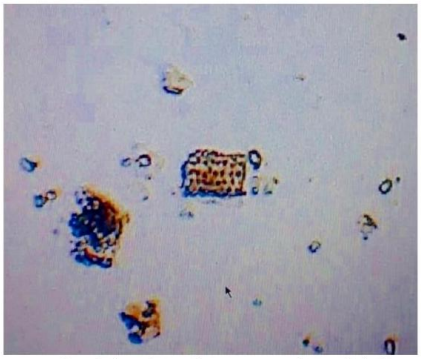


Figure 4. Xylem

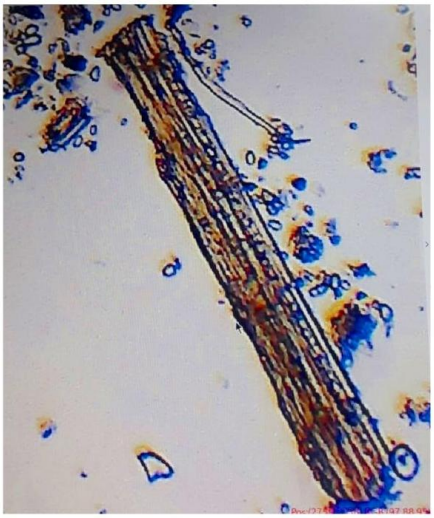
Powder Characteristics :



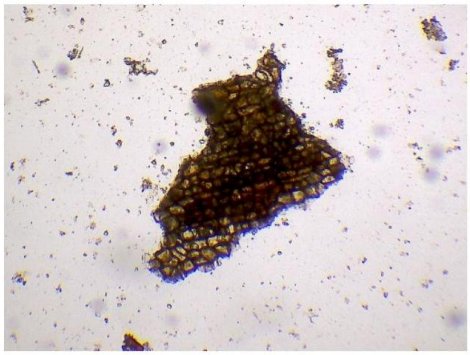
Iodine stained starch grain



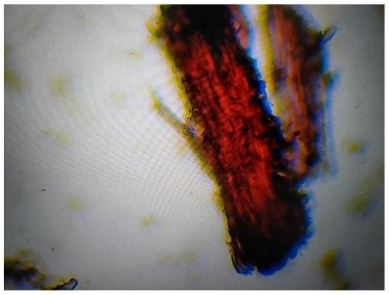
vessel with pitted to annular thickening



Fibres with parenchymatous sheath with calcium oxalate crystals



Cork



Lignified Fibres with parenchymatous sheath with calcium oxalate crystals

Total ash value :

The total ash value was found to be 5.66 % w/w. The Pharmacopoeial limits in British pharmacopeia 2022 and Ayurvedic pharmacopeia 2015 gives that the total ash value of liquorice should not be more than 10 % w/w. As the %w/w of total ash content is 5.66% w/w, the drug complies with the BP and Ayurvedic pharmacopeia standards for total ash content.

Acid insoluble ash value :

The acid insoluble ash value was found to be 1 % w/w. The Pharmacopoeial limits in Indian Pharmacopoeia 2022 and British Pharmacopoeia 2022 gives that the acid insoluble ash value of liquorice should not be more than 2 % w/w while Ayurvedic pharmacopoeia 2015 gives that the acid insoluble ash value of liquorice should not be more than 2.5 % w/w. As the %w/w of acid insoluble ash content is 1% w/w, the drug complies with the BP, IP and Ayurvedic pharmacopeia standards for acid insoluble ash content.

Water soluble extractive value :
The water extractive value was found to be 23 % w/w. The Pharmacopoeial limits in Ayurvedic pharmacopeia 2015 gives that the water soluble extractive value should not be less than 20% w/w. As the % w/w of water soluble extractive is 23 % w/w, it complies with Ayurvedic Pharmacopeia.

Alcohol soluble extractive value :
The alcohol extractive value was found to be 11.2 % w/w. The Pharmacopoeial limits in Ayurvedic pharmacopeia 2015 gives that the alcohol soluble extractive value should not be less than 10 % w/w. As the % w/w alcohol soluble extractive of the liquorice powder is 11.2 % w/w, it complies with standards mentioned in API.

Alcoholic extraction of liquorice :
Percent yield = 6.1 % w/w
Native extract ratio = 16.393:1

TLC :
Intially, TLC runs was performed using silica as stationary phase and mobile phase hexane:ethyl acetate (4:1) which is comparatively less polar keeping in mind the nature of glabridin . Different solvent combinations were tried (data not shown) Finally a good seperation of glabridin from other constituents was observed in the mobile phase hexane-ethyl acetate -chloroform in the ratio of 5+4+3v/v/v.
The retention factor was found to be 3.8

HPTLC :
Good separation of glabridin from other constituents of the extract was observed in the mobile phase composition ratio of hexane–ethyl acetate–chloroform in the ratio of 5 + 4 + 3 v/v/v. The R_f of glabridin obtained was 0.42 ± 0.02. The comparative densitogram of different concentrations of standard solution of glabridin, the chromatogram of alcoholic extract of liquorice solution and calibration curve of standard glabridin are given in the following text.

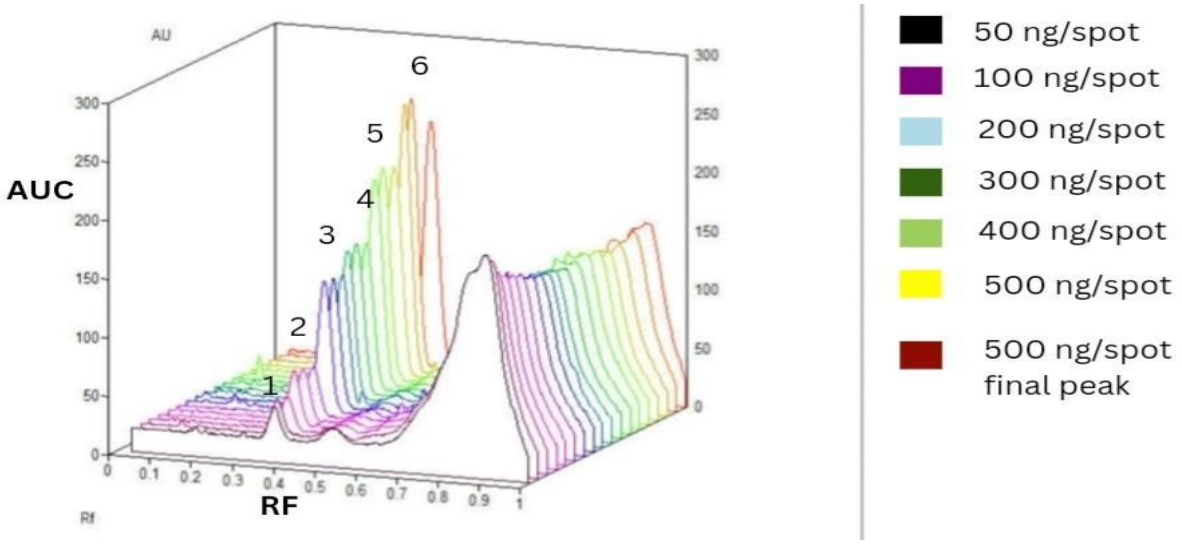


Figure : Densitogram of glabridin standard

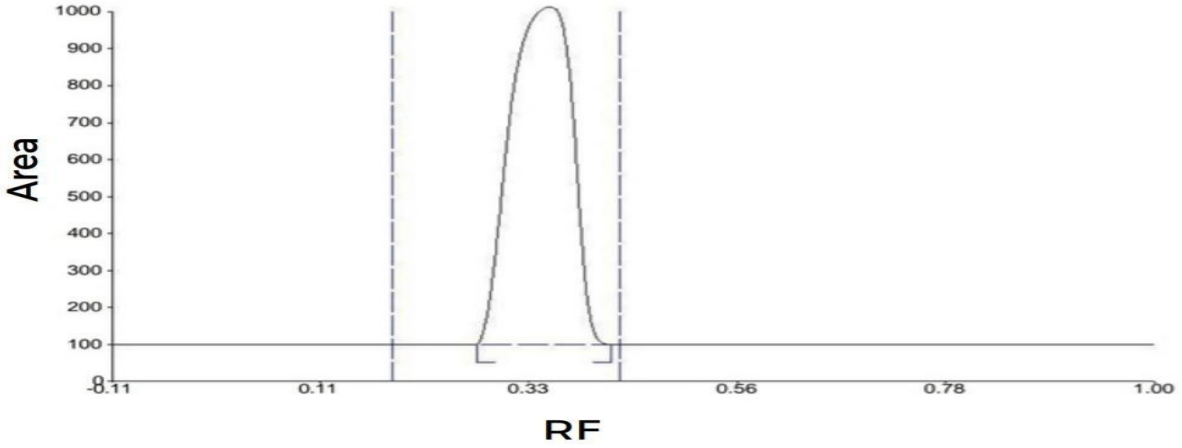
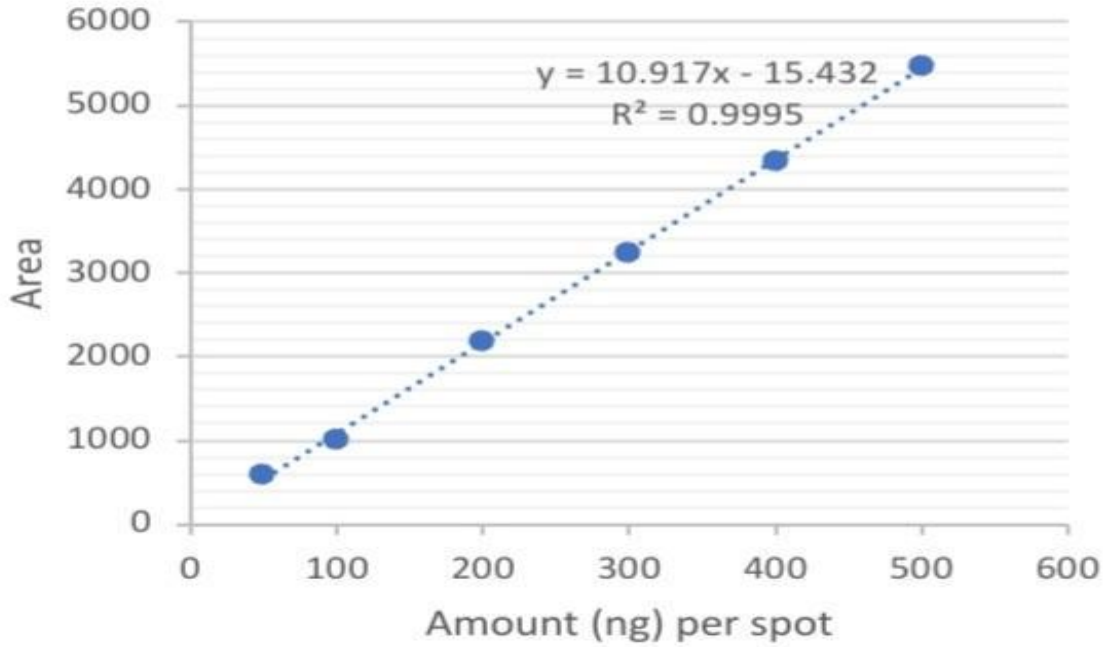


Figure : Chromatogram of alcoholic extract of liquorice



amount (nanogram per spot)	area
50	585.33333
100	1016.4333
200	2187.9667
300	3231.1333
400	4340.8667
500	5467.1667

amount (nanogram per spot)	area 1	area 2	area 3	average	sd	%rsd
50	571.1	588.6	596.3	585.3333333	12.91368783	2.206210905
100	1043.8	1005	1000.5	1016.433333	23.80679175	2.342189199
200	2193.5	2183	2187.4	2187.966667	5.272886622	0.240994833
300	3231.5	3280.4	3181.5	3231.133333	49.45101954	1.530454315
400	4322	4371.6	4329	4340.866667	26.84498712	0.618424595
500	5517.3	5510.8	5373.4	5467.166667	81.26932591	1.48649805

Figure : Calibration curve of standard glabridin

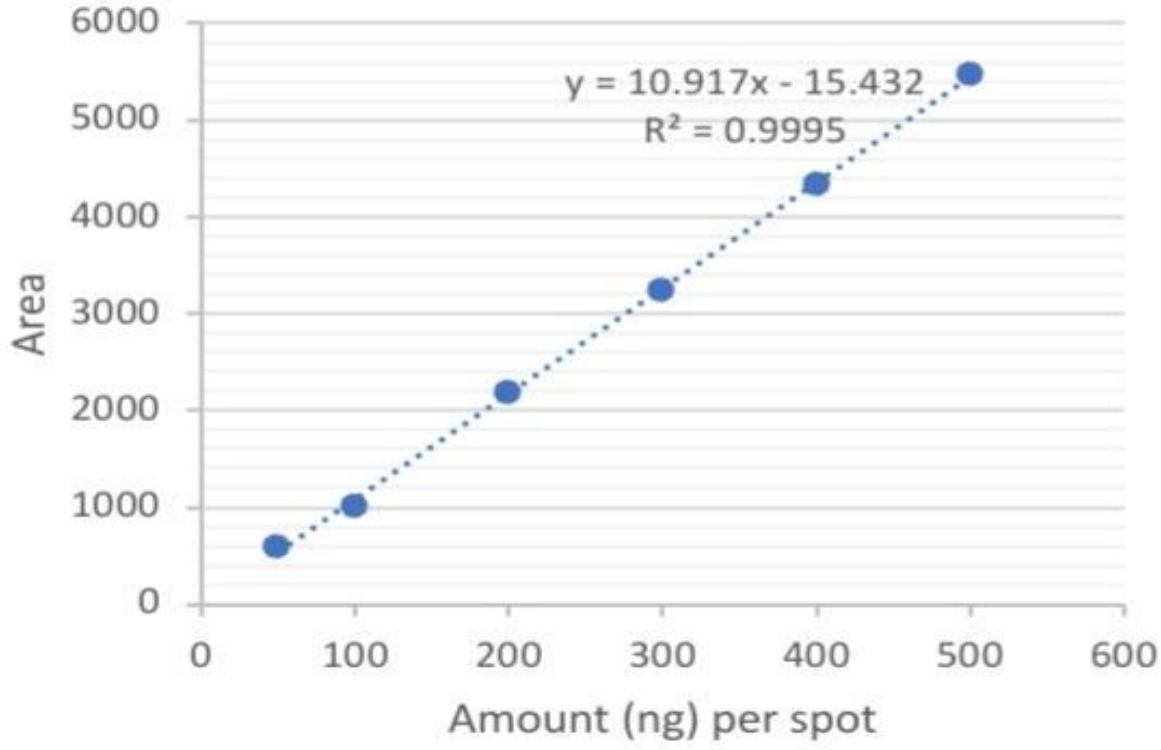


Table : Linear regression data for HPTLC method of analysis for glabridin in alcoholic licorice extract.

Parameters	Values
Concentration range	5–50 µg/mL
Slope	10.917
Intercept	15.432
Correlation	0.9995
Regression equation	y = 10.917x - 15.432

Calculations :
Average area of the sample spot = 3389.47
Standard Deviation-=4.239496825
% RSD =0.125067512
Equation: y=mx +C
y = 109.17x - 15.432
3389.47=109.17x - 15.432
So, amount per spot = 31.189 ng glabridin per spot

If 31.189 ng glabridin is present in 10µl sample solution (diluted),
Then 3118.9 ng glabridin is present in 1 ml (1000 µl) of sample solution (diluted).
Which is 3.1189 microgram glabridin is present in 1ml (1000 µl) of sample solution (diluted)
dilution factor = 66.67 ml.
Hence the amount of glabridin present in the sample stock solution = 3.1189 X 66.67 ml
= 0.20793 mg glabridin

If 0.20793 mg glabridin is present in 0.2 g of extract,
Then 2.5367 mg glabridin is present in 2.44g of extract which came from 40g liquorice powder.
If 0.0025367 g glabridin is present in 40g liquorice powder, then 0.006342 g glabridin is present in 100g liquorice powder.
Therefore, the content of glabridin in alcoholic extract of liquorice was found to be 0.006342 %.

HPLC :
The combination of mobile phase consisting 0.2% Acetic acid : Acetonitrile (45 : 55 v/v) HPLC grade yielded sharp peaks of glabridin and provided good separation of glabridin peak. The chromatogram of one of the concentration of standard glabridin, chromatogram of alcoholic extract of liquorice and the calibration curve of standard glabridin are given in the following text.

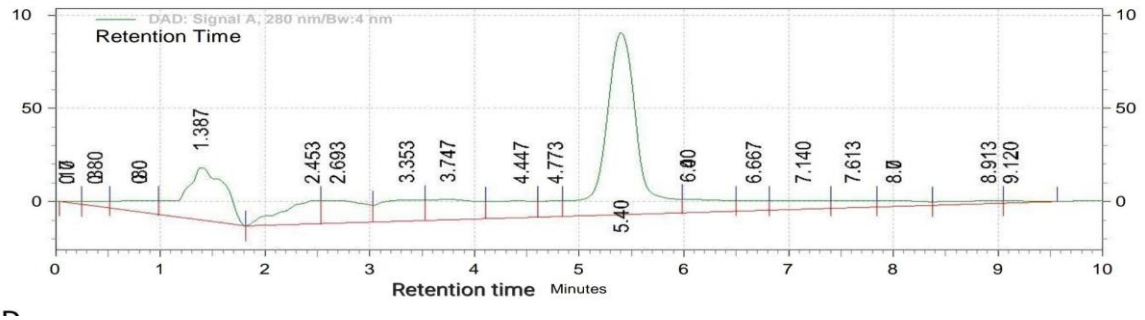


Figure : Chromatogram of standard glabridin

Figure : Chromatogram of alcoholic extract of liquorice

amount per injection (µg)	area 1	area 2	area 3	average	sd	%rsd
0.1	350822	350722	350850	350798	67.29041537	0.019182098
0.2	703088	677825	703807	694906.6667	14797.52487	2.129426235
0.4	1456260	1458563	1475570	1463464.333	10546.86334	0.720677853
0.6	2181699	2187315	2183684	2184232.667	2847.918597	0.130385313
0.8	2908932	2907262	2901582	2905925.333	3853.003158	0.132591265
1	3626977	3623497	3627134	3625869.333	2056.000081	0.056703645

amount per injection (µg)	area
0.1	350798
0.2	694906.67
0.4	1463464.3
0.6	2184232.7
0.8	2905925.3
1	3625869.3

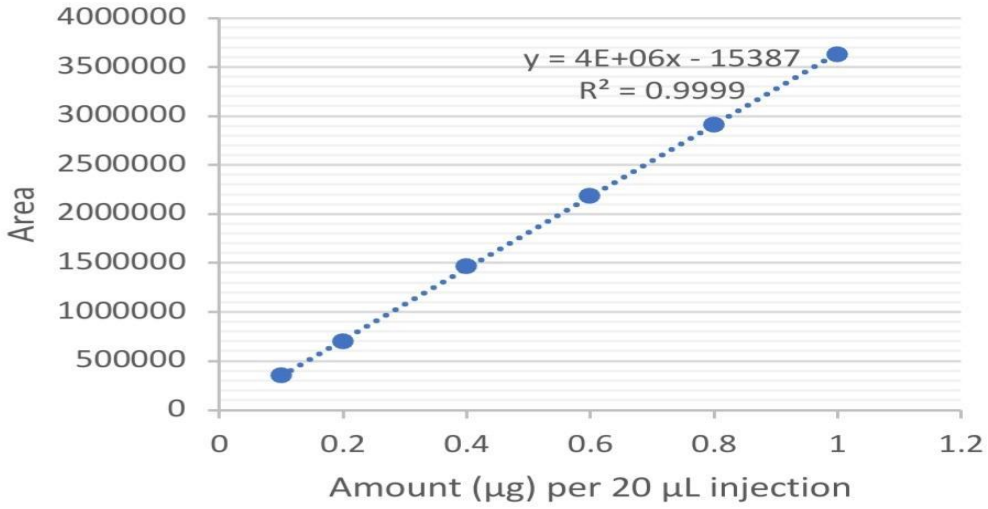


Figure : Calibration curve of glabridin standard

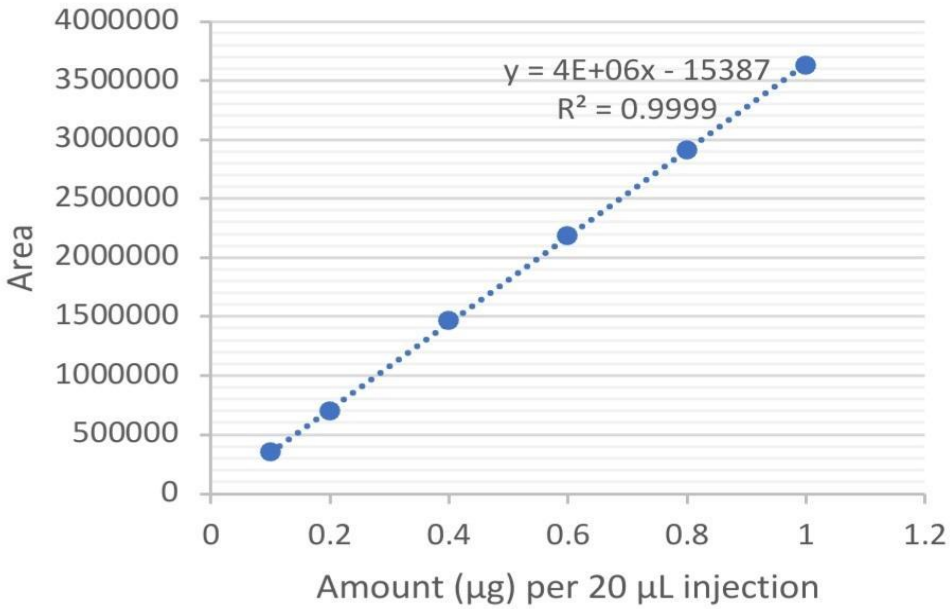


Figure : standard error bar graph

Table : Linear regression data for HPTLC method of analysis for glabridin in alcoholic licorice extract.

Parameters	Value
Concentration ramge	5–50 µg/mL
Slope	4000000
Intercept	15387
Correlation coefficient	0.9999
Regression line	y = 4E+06x - 15387

Calculation :
Avg Area of sample = 1,434,325
Equation of the linearity graph: y = 4000000x -15387
standard deviation= 4261.304
%rsd= 0.29.

Therefore, x = 0.3624 microgram of glabridin per injection

If 0.3624 microgram of glabridin is present in 20 µl of sample solution, then 18.1214 µg of glabridin is present in 1 ml of sample solution.

From the sample dilution steps, the dilution factor is 66.67 ml.
Hence the amount of glabridin present in the sample stock solution = 18.1214 X 66.67 ml
= 1.208 mg glabridin
Hence, 1.208 mg glabridin is present in 200 mg alcoholic extract of licorice.
If 1.208 mg glabridin is present in 0.2 g of extract,
Then 14.74 mg glabridin is present in 2.44g of extract which came from 40g liquorice powder.

If 0.01474 g glabridin is present in 40g liquorice powder,
then, 0.036844 g glabridin is present in 100g liquorice powder
Therefore the content of glabridin in alcoholic extract of liquorice was found to be 0.036844 %..

Conclusion :

The given liquorice sample complies with the Pharmacopoeial standards. The percent yield of extract was found to be 6.1 % w/w and the native extract ratio was found to be 16.393:1. The content of glabridin in the alcoholic extract of liquorice was found to be 0.006342 % by HPTLC and 0.036844 % by HPLC.

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