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ISOLATION AND ESTIMATION OF GLYCYRRHETINIC ACID FROM MARKETED ATHIMADHURAM CHURNA BY UV SPECTROPHOTOMETRY

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ABSTRACT

Athimadhuram or scientifically called as Glycyrrhiza Glabra. Most liquorice is used as a flavoring agent for tobacco. Liquorice has been a part of Chinese traditional medicine for the past hundreds of years. It also occupies an important place in the Indian Ayurvedic system of medicine. It was mentioned by Susruta in the ancient text books on Ayurveda.It is also known as "sweet root" as it contains a compound which is almost 50 times sweeter than sugar. Liquorice is grown in Syria, Turkey, Russia, Greece and southern Europe. In India it is grown in the northwestern parts.Liquorice is a soothing moist herb which has expectorant, anti-inflammatory properties. It relieves coughs effectively and exhibits laxative and hormonal effects. It has been used to relieve adreno-corticoid insufficiency and tuberculosis.Hence we aimed to study the physicochemical and preliminaryphytochemical relevance. Separation of Glycyrrhetinic acid was attempted. This study paved the presence of Glycyrrhetinic acid in ethanol Athimadhuram Churna.

Keywords: Ayurvedic churnas,Physicochemical, Glycyrrhetinic acid, UV-Visible spectrophotometry.

Introduction

Ayurveda is considered by many scholars to be the oldest healing science. In Sanskrit, Ayurveda means "The science of life". Ayurvedic knowledge originated in India more than 5000 years ago and is often called the "Mother of All Healing".[1-2] It stems from the ancient Vedic culture and was taught for many thousands of years in an oral traditional from accomplished masters to their disciples. The principles of many of the natural healing systems now familiar in the west have their roots in Ayurveda, including homeopathy and polarity therapy.

Ayurveda places great emphasis on prevention and encourages the maintenance of health through close attention to balance in one's life, right thinking, diet, life style and the use of herbs.

Just as everyone has unique fingerprint, each person has a particular pattern of energy- an individual combination of physical, emotional and mental characteristics which comprises their own constitution.[3-4]

Athimadhuram or scientifically called as Glycyrrhiza Glabra or Glyceria Glabra is a cultivated liquorice plant hailed for its medicinal properties. It is commonly called as sweetwood. Glycyrrhiza contains triterpene glycoside called Glycyrrhizin, Glycyrrhizic acid, or Glycyrrhizinic acid. This is converted to glycyrrhetinic acid (the aglycone) and two moles of gucuronic acid (the glycone) on hydrolysis. Glycyrrhizinic acid is potentially 50 times sweeter than sucrose. Liquorice is the alteration of Glycyrrhiza glabra, a Mediterranean perennial plant with blue pealike blossoms.[5-6] Glycyrrhiza is the active principle for sweetening, flavoring and pharmaceutical applications. It is effective in treatment of peptic ulcer. It is used in brewing and for confectionery and tobacco flavorings. It is frequently used in medicines to mask the unpleasant flavors. It has been used medicinally for highly effective coughs and as a mild laxative. It promotes the ejection of mucus or exudate from the lungs, bronchi, and trachea; sometimes extended to antitussives. Glycyrrhetinic acid has corticosteroid-like structure, thus is useful as an anti-inflammatory and co-emulsifier to treat skin disorders and in cosmetics.

This research provides the information regarding comparison of extraction techniques, pre-formulation studies of glycyrrhetinic acid like physical characterization, organoleptic properties, loss on drying, extractive values, UV, IR.

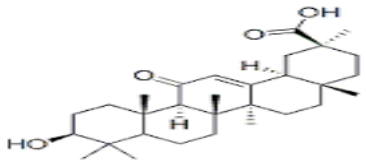


Fig No: 1 Glycyrrhetinic acid

Glycyrrhetinic acid is chemically 3b-Hydroxy-11-oxo-18b, 20b-olean-12-en-29-oic acid.

Materials and Methods

Apparatus: Heating mantle, vacuum filtration assembly, sieves, Sonicator, hot plate etc.

Instruments

Double beam UV-Visible Spectrophotometer (Systronics model 2203) with matched cuvettes was used in this study. In addition, an electronic balance (Shimadzu TX223L), digital ph meter (Systronics model 802), and an ultrasonic bath Sonicator (spectra lab, model UCB 40).

Chemicals and Reagents

The Athimadhuram Churna was procured from different markets. Methanol, anhydrous sodium sulphate, calcium chloride etc., were purchased from Merck Pvt. Ltd. Mumbai, India. All the other chemicals used including the solvents were of analytical grade. Glacial acetic acid (Rankem Ltd., Mumbai, India,).

Thin Layer Chromatography

Preparation of mobile phase and Standards [7-8]

Preparation of sample (test) solution

Add 10 ml of 70% methanol to 1 gr of dried athimadhuram powder, heat by shaking on water bath for 5 min, cool and filter.

Preparation of reference solution

Add 1 ml of 70% methanol solution to 1 mg of standard Glycyrrhetinic acid solution.

Mobile phase (Toluene: Ethyl acetate: Glacial acetic acid 12.5:7.5:0.2 v/v)

To 12.5 parts of Toluene, 7.5 parts of Ethyl acetate, 0.2 parts of Glacial acetic acid was mixed to get one liter of the mobile phase. The mobile phase was then filtered through 0.22 µm nylon membrane vacuum filtration and degassed by sonication.

Analytical TLC for identification: [9]

Analytical TLC was carried out on preparative TLC plates (5 × 5 cm with 0.2mm thickness, silica gel GF_{254}, Merck, Darmstadt, Germany) cut from the commercially available sheets. An aliquot of standard solution of Glycyrrhetinic acid and a sample solution of crude extract was spotted onto the silica gel plate and allowed to dry for a few minutes. Afterwards, the chromatoplate was developed withToluene: Ethyl acetate: Glacial acetic acid (12.5:7.5:0.2 v/v) as mobile phase in a previously saturated glass chamber with eluting solvents for some time at room temperature. The developed plate was dried under normal air and the spots were visualized or examine under U.V light at 254 & 365 nm and by spraying Anasaldehyde. The (retention factor) values of isolated compounds and standard were calculated and compared.

UV spectrophotometric method

Preparation of Standard Solutions

The standard stock solutions of glycyrrhetinic acid was prepared by dissolving 10 mg of glycyrrhetinic acid in ethanol and final volume was adjusted with same solvent in 10mL of volumetric flask to get a solution containing 1000 µg/mL of glycyrrhetinic acid. Aliquots of working stock solutions of glycyrrhetinic acid was prepared with in the same solvent to get concentration in range of 2-10 µg/ml of glycyrrhetinic acid. The absorbance of resulting solutions was measured at 254nm. A calibration curve as concentration vs. absorbance was constructed to study the Beer-Lambert's Law and regression equation.

Preparation of Plant Extract:[13-14]

Whole plant *Glycyrrhiza Glabra* was used in this study. The material is cleaned and set free from moulds, insects, animal faecal matter and other contaminations such as earth, stones and extraneous materials. The specimen was shade dried and protected from sun light for several days not less than one month. It was ground to a fine powder using mortar and pestle without any loss of powdered drug. Then it was passed through a sieve of 40 mesh and the material passed by the sieve was collected and stored in a well tight amber coloured container and it was used for further study.

A coarsely powdered aerial part of the plant (about 5gr) was taken into a neat conical flask. To that 100ml of ethanol is added and heat on the hot plat for 1hr. keep the conical flask aside for 24 hr. The homogenate was filtered using Whatman's filter paper and the volume of the filtrate was recorded. About 25ml of filtrate is taken into a clean china dish.

Place the china dish on the hot plate to evaporate the solvent totally and collect the extract at the last.

Preparation of extract solutions

Accurately weighed 100 mg of herbal alcoholic extract of Licorice or Athimadhuram was transferred to 100mL volumetric flask and dissolved with ethanol and final volume was adjusted with same solvent in 100mL volumetric flask to get the solution containing 100µg/mL. The sample solution was then filtered through Whatman's filter paper No.41. Aliquots of working stock solutions of glycyrrhetinic acid was prepared with in the same solvent to get concentration in range of 2-10 µg/ml of glycyrrhetinic acid.

Assay

Amount = concentration * dilution factor

Determinationof λmax

Selection of analytical wavelength

Athimadhuram ofdifferent extracts andscanned for absorption maxima, the observed wavelengthused for analysis. Glycyrrhetinic acid standard absorbs at 254 nm, and ethanol extract absorb at250.2 nm.

Selection of analytical concentration range

From the above solution take10ml and scanned by UV –VIS spectrometer in the range 200-400nm, using respective solvent as a blank. The wavelength corresponding to the maximum absorbance λmax was found.

METHOD VALIDATION

Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific activity in consistently desired result or a product meeting is predetermined specifications and quality characteristic. The method was validated for different parameters like linearity, accuracy, precision, specificity, and ruggedness, limit of detection and limit of quantification.[10-12]

Linearity

For each solvent various aliquots were prepared from the stock solution ranging from 2-10 µg/ml. the sample analyzed with the help of UV-VISIBLE spectrophotometer using a respective solvent as a blank.

Accuracy

The accuracy of the method was determined by preparing solutions of different concentrations that is 80%, 100%, 120% in which the amount of drug was kept constant i.e., 50µg and amount of pure drug was varied., that is 40µg, 50µg, 60µg for 80%, 100%, 120% respectively. The solutions were prepared in triplicate and the accuracy was indicated by % recovery.

Precision

The precision of method was demonstrated by Interday and intraday variations study, the solutions of same concentration were prepared and analyzed thrice for 3 consecutive day, and the absorbance were recorded. In the intraday variations study, 9 different solutions of the same concentration were prepared and analyzed thrice a day that is morning, afternoon and evening. The result was indicated by % RSD.

Limit of detection:

The detection limit is determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

LOD=3.3(SD/S)

Where SD= the standard deviation of the response

S= the slope of the calibration curve

Limit quantification

The quantification limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precise.

LOQ=10 (SD/S)

Where SD= the standard deviation of the response

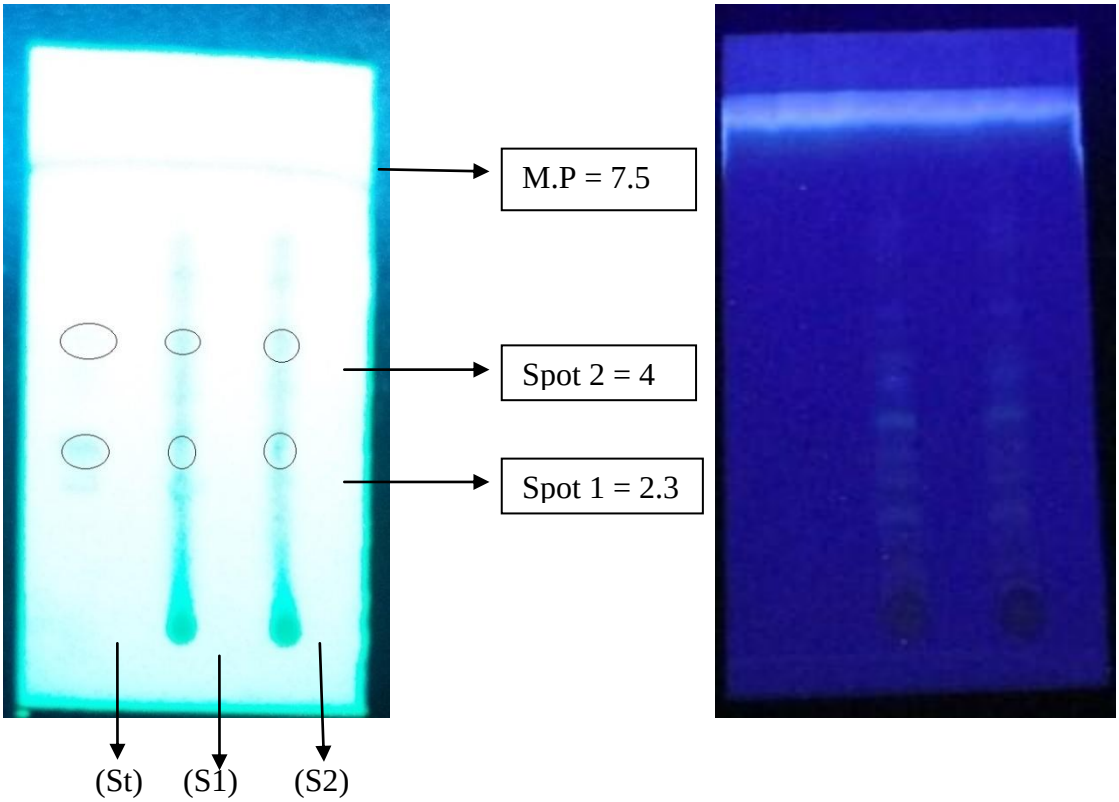
S= the slope of the calibration curve

Validation of the developed method.

The effect of wide range of other constituents and other additives usually present in the extract was investigated to know the specificity of the method. It shows no interference from other compounds. For linearity**,** Aliquots of primary working standard solutions containing Glycyrrhetinic acid were diluted such a way that the final concentrations of Glycyrrhetinic acid are in the range of 2-10 µg/ml. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. Precision was determined by intra-day and inter-day study. Precision of the method was evaluated by carrying out the assay and analyzing corresponding responses 6 times on the same day and on different days for the sample solution. The percent relative standard deviation (% RSD) was calculated. Accuracy studies were performed for Glycyrrhetinic acid at three different levels (80%, 100% and 120%) and the mixtures were analyzed in triplicate by the proposed method. Known amount of standard Glycyrrhetinic acid at 80%, 100% and 120% of sample (which was previously analysed) was added and it was reanalyzed by the proposed method. And the percentage recovery was evaluated. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3(σ)/S and LOQ= 10 (σ)/S, Where (σ) = standard deviation of response (peak area) and S= slope of the calibration curve.

Results and Discussion

Complete extraction of Glycyrrhetinic acid was achieved by successive solvent extraction with ethanol.Several mobile phase combinations were tried and Toluene: Ethyl acetate: Glacial acetic acid (12.5:7.5:0.2 v/v) was found optimum for separation of Glycyrrhetinic acid from Ethanolic extract of*Athimadhuram Churna*. The $R_f$values of standard and sample compound matches each other and the R_f value was found as 0.5. TLC profile of compound was represented in Figure no. 2.



a. Short wavelength (254nm) b. Under long wavelength (365nm)

Figure No:2 TLC showing the equal R_f (0.306, 0.53) for standard, sample 1, sample 2.

Note: R_f value of about 0.3 (β-glycyrrhetinic acid) and smaller spot with an R_f value of about 0.5 (α-glycyrrhetinic acid).

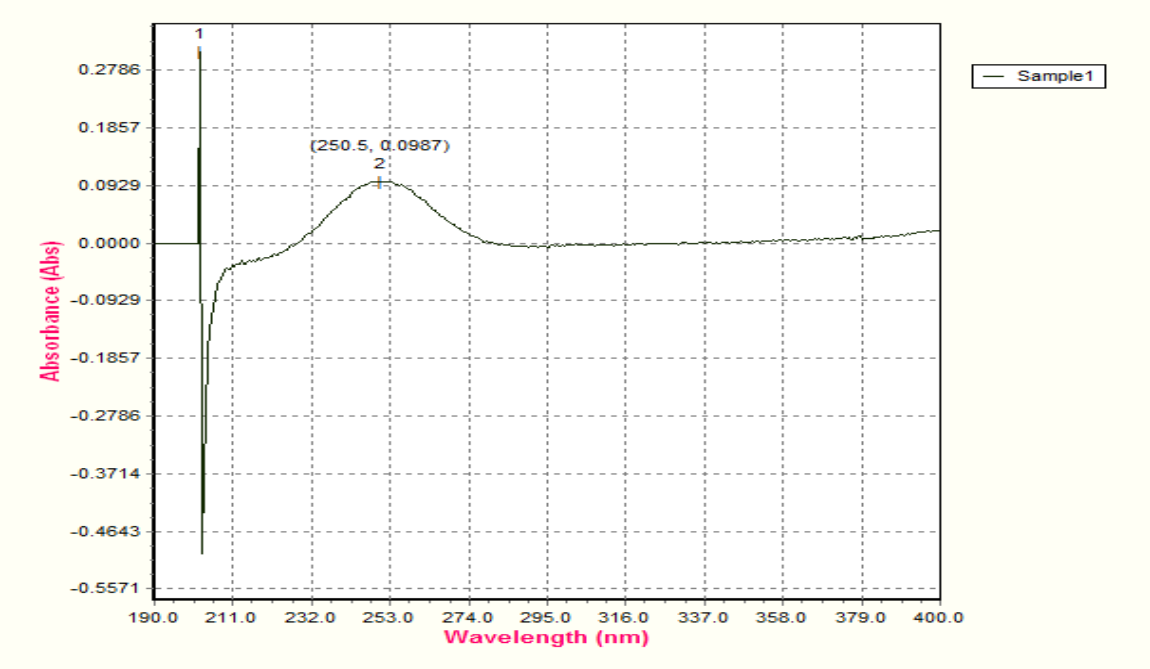


Figure no: 3 Calibration curve of standard Glycyrrhetinic acid

Table No: 1Linearity result of Glycyrrhetinic acid

S. No.	Concentration of standard Glycyrrhetinic acid (µg/ml)	Absorbance at 250.2 nm
1	0	0
2	2	0.0987
3	4	0.1949
4	6	0.3001
5	8	0.4232
6	10	0.5251

2 mcg

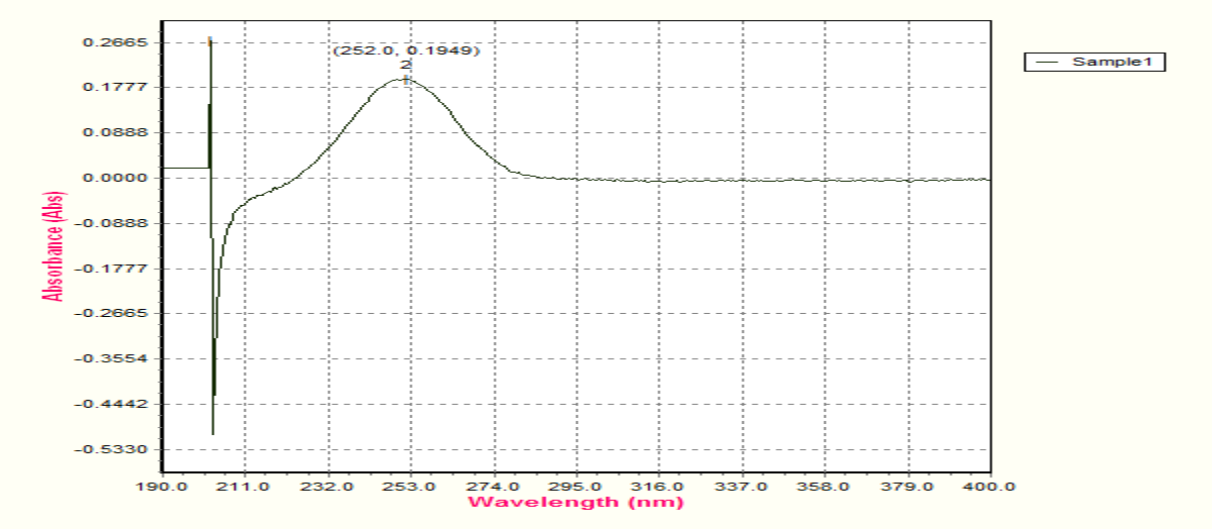


Figure no: 4Absorption curve of standard Glycyrrhetinic acid

4 mcg

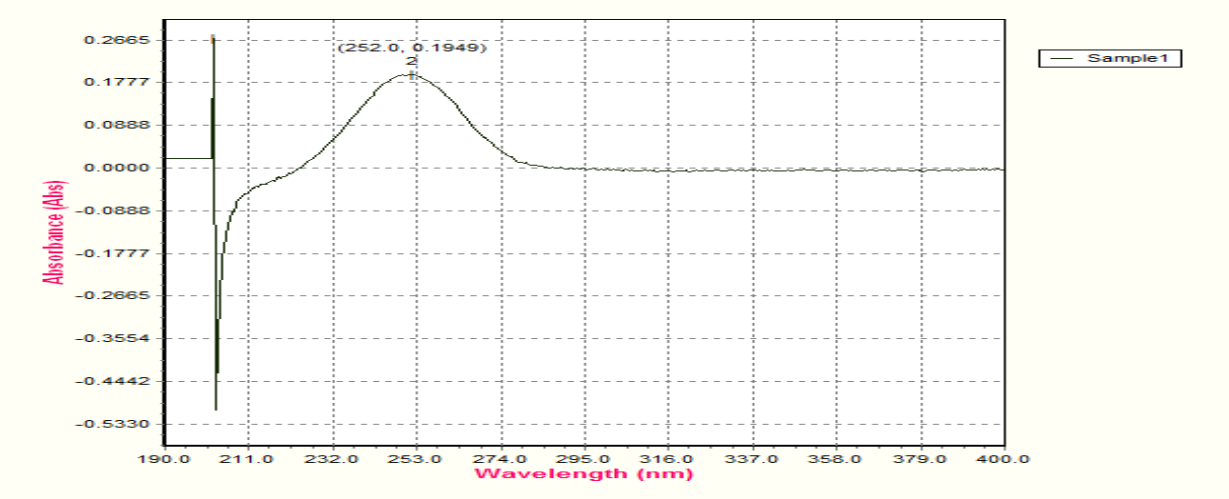


Figure no: 5Absorption curve of standard Glycyrrhetinic acid

6 mcg

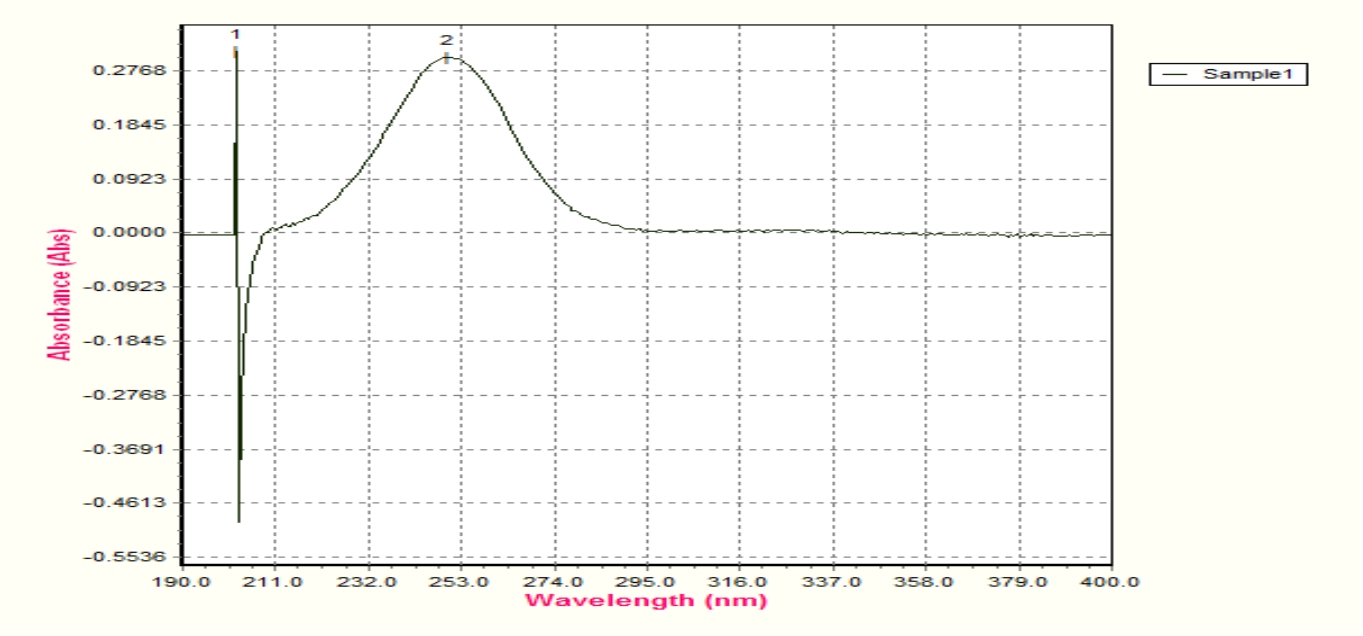


Figure no: 6Absorption curve of standard Glycyrrhetinic acid

8 mcg

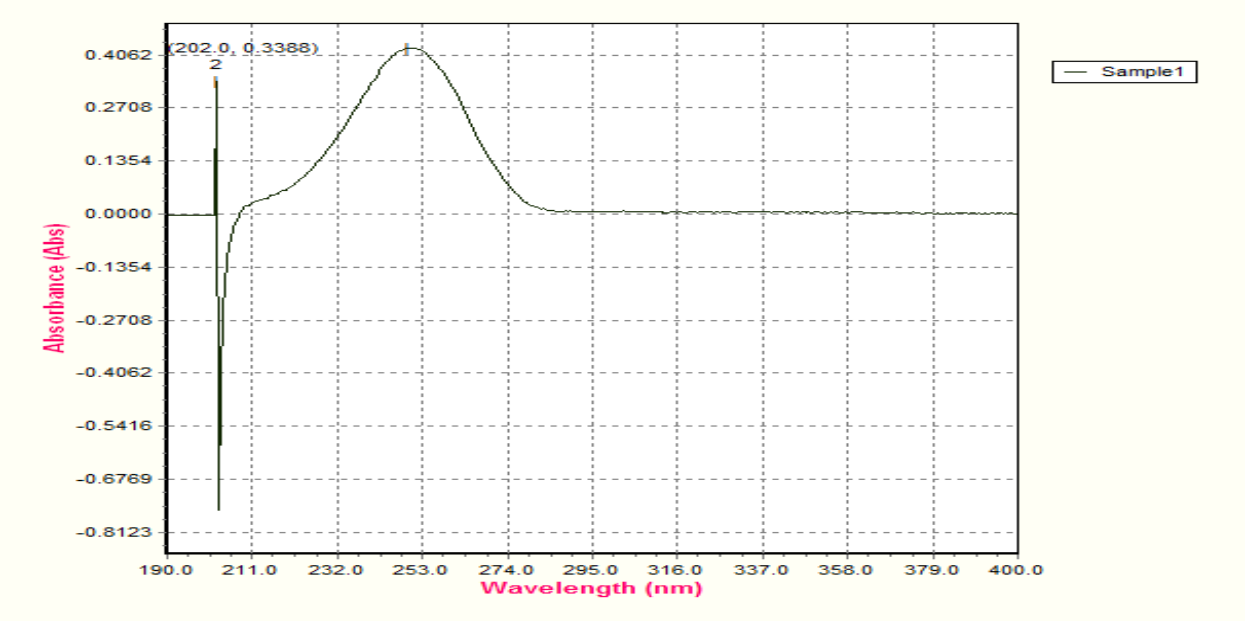


Figure no: 7Absorption curve of standard Glycyrrhetinic acid

10 mcg

Figure no: 8 Absorption curve of standard Glycyrrhetinic acid

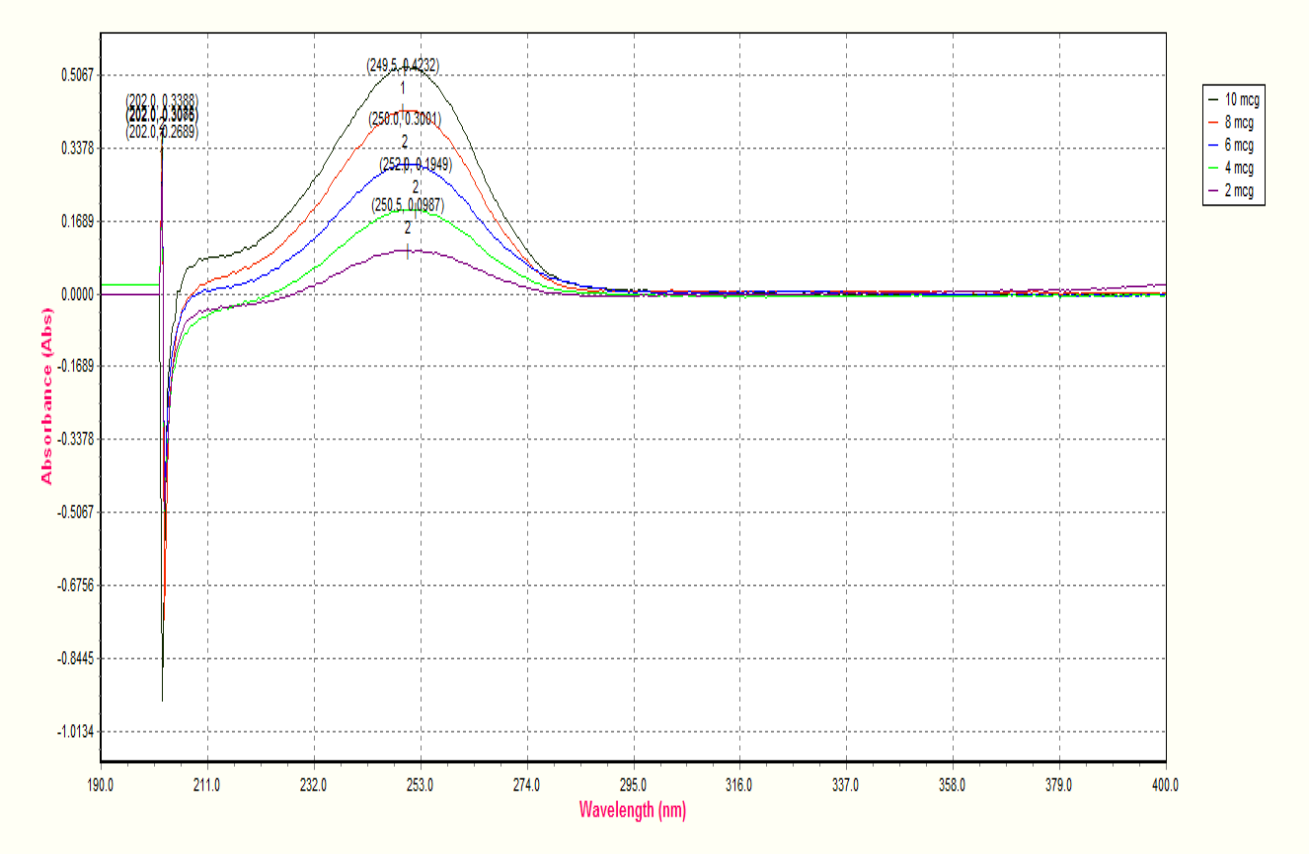


Figure No: 9 overlapping of absorbance

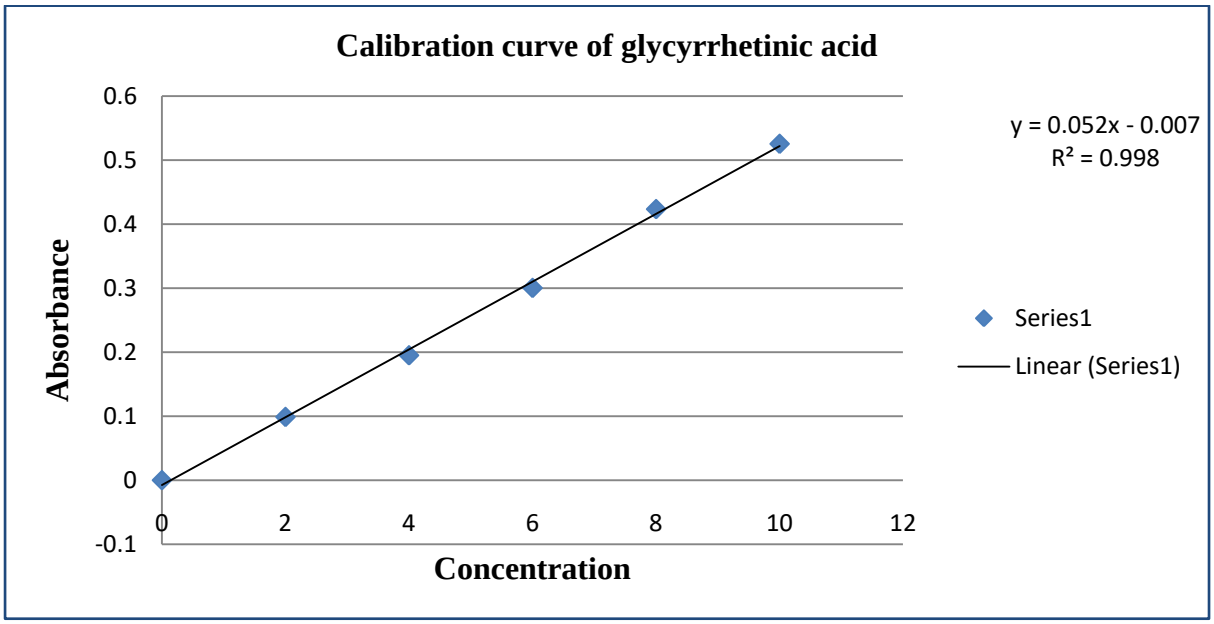


Figure No: 10 Linearity curve of standard Glycyrrhetinic acid

Table No: 2 Absorbance plant extract

S. No.	Concentration of standard Glycyrrhetinic acid (µg/ml)	Sample 1 Absorbance at 250nm	Sample 2 Absorbance at 250nm
1	0	0	0
2	2	0.2687	0.275
3	4	0.4972	0.5742
4	6	0.7266	0.8407
5	8	0.9808	1.0936
6	10	1.2281	1.3708

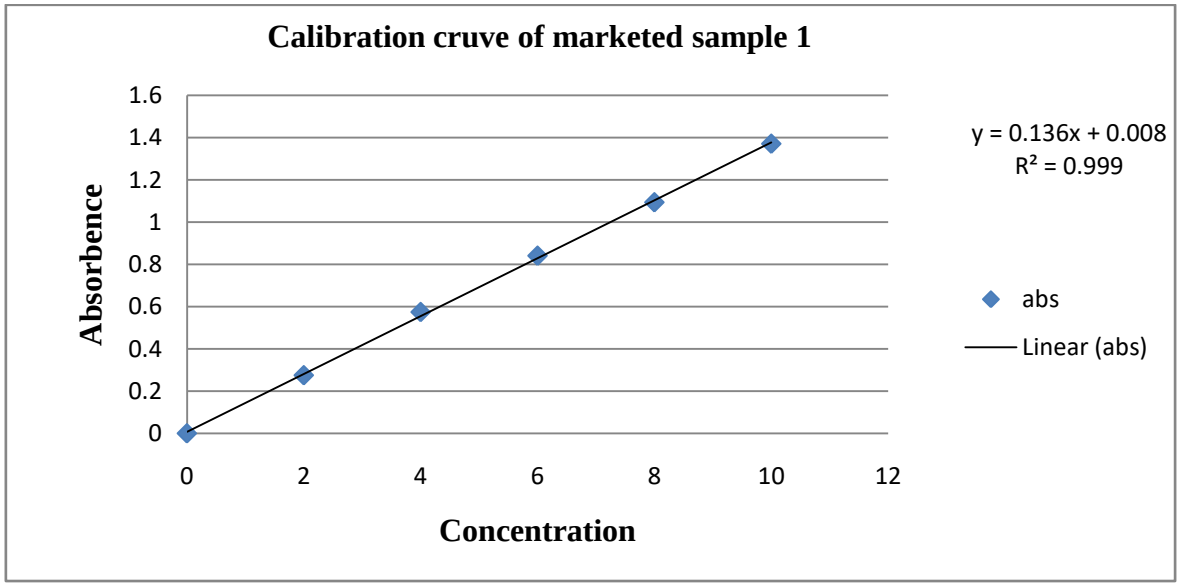


Figure No: 11 Linearity curve of sample 1 Glycyrrhetinic acid

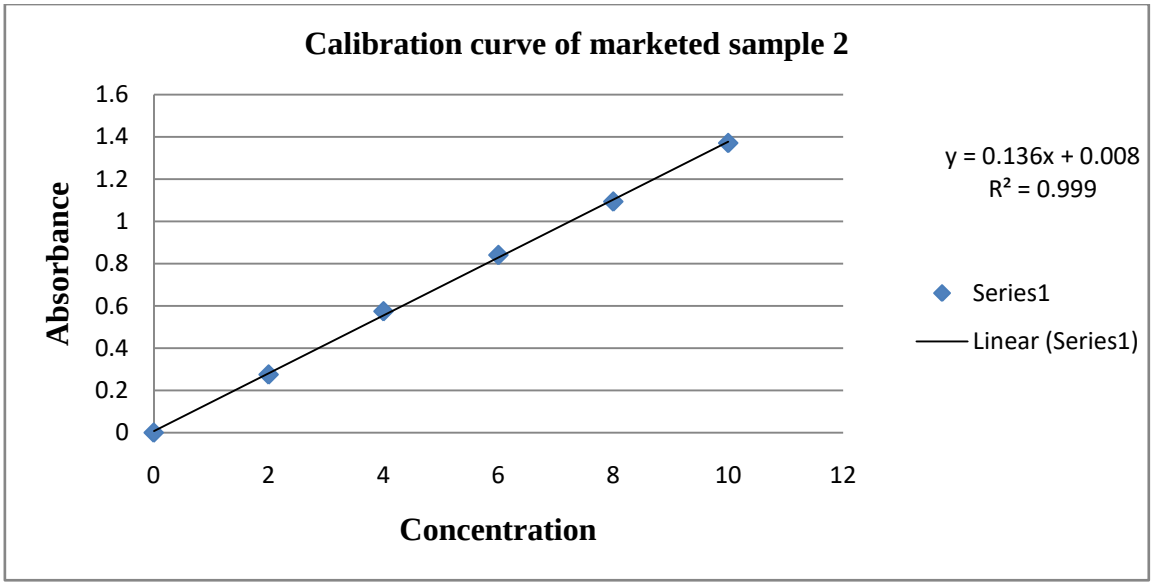


Figure No: 12Linearity curve of sample 2 Glycyrrhetinic acid

Table No: 3Result of validation parameters

S.No	Parameters	Results		
		Standard drug	**Marketed 1**	**Marketed 2**
1	Detection wavelength	250.2nm	250.2nm	250.2nm
2	Linearity range (µg/mL)	0.2-1.0 (µg/mL)	0.2-1.0 (µg/mL)	0.2-1.0 (µg/mL)
3	Regression equation (y = mx+c)	y = 0.0529x - 0.0076	y = 0.1215x +0.0093	y = 0.1368x+0.0084
4	Correlation Coefficient (R^2)	0.9984	0.9995	0.9995
5	Slope	0.052917	0.121517	0.136804
6	Intercept	-0.00759`	0.009314	0.008362
7	Limit of detection	0.49894	0.28533	0.33356
8	Limit of quantification	1.51194	0.86464	1.01080

Table no: 4 Result of precision

Parameters	**Intraday**			**Interday**		
	Standard drug	**Marketed 1**	**Marketed 2**	**Standard drug**	**Marketed 1**	**Marketed 2**
Mean	0.30422	0.7254	0.8435	0.30018	0.6319	0.9619
S.D	0.00587	0.0013	0.0031	0.00019	0.0050	0.0068
%RSD	1.9321	0.1807	0.3779	0.0640	0.8056	0.7130

Table no: 5 Result of Accuracy

	% mean recovery			Standard deviation (SD)			%RSD		
Accuracy (% mean recovery)	Standard drug	Marketed 1	Marketed 2	Standard drug	Marketed 1	Marketed 2	Standard drug	Marketed 1	Marketed 2
80 % level	98.6168	97.4772	97.406	0.610	0.586	0.5860	0.703	0.6778	0.6779
100 % level	98.8776	97.9351	101.40	0.589	0.568	0.6409	0.551	0.5326	0.6075
120 % level	99.0472	98.2656	100.24	0.554	0.566	0.6615	0.427	0.4671	0.5172

Table no: 6 Ruggedness studies

	Instrument-1(Systronics model 2203)		**Instrument-2** (Elico SL 159)	
parameter	Analyst 1	Analyst 2	Analyst -1	Analyst -2
Mean	0.1947	0.1949	0.30024	0.30012
SD*	0.000158	0.000158	0.000207	0.000148
% RSD	0.081209	0.081126	0.069066	0.049422

Table No: 7Assay values of Athimadhuram churnas.

Ethanolic extract of Athimadhuram churnas	**Drug present in 100mg**
Sample 1	0.104 mg
Sample 2	0.113 mg

UV Spectrophotometry

The main objective of the project work is to develop simple, objective, selective method for the determination of Glycyrrhetinic acid and to validate. The developed method according to the ICH guidelines and applying same for its estimation in crude drug of Athimadhuram churna. The absorption spectra were recorded in the wavelength range of 200-800 nm in UV spectrophotometer, the absorption maximum curve was observed at 250.2 nm. Beers law range was confirmed by the linearity of calibration curve of Glycyrrhetinic acid,

which has shown in figure no. 10. The linearity concentration range of 2-10 µg/ml and the results are given in table no. 1. Each concentration absorbance peaks are shown in figure no. 4-8. The overlay of these absorbances are shown in figure no. 9.

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, slope (b), intercept (c), correlation coefficient (R^2), obtained from different concentrations, percent Relative Standard Deviation (%RSD), LOD and LOQ values were given in table no. 3. The method validation parameters were established in this work, LOD and LOQ of the Glycyrrhetinic acid were found as 0.4989µg/ml and 1.5119µg/ml.

The accuracy of the methods was confirmed by the recovery studies, by adding known amount of the pure drug to the formulation and the percentage recovery was found to be between 98.61%, 98.87%, 99.04%w/w, indicating that the developed method is accurate which indicates a good accuracy of the method and it shows that the method was free from the interference of excepients used in the formulation and the results are given in table no. 5.

Precision of the method was reported in terms of relative standard deviation and it should be evaluated by using a maximum of 6 determinations over 100% concentration which shows RSD less than 2 indicates that the method was precise and the results shown in table no. 4. Also obtained in limits

Ruggedness of the method was reported and evaluated by using two different analysts and instruments. The results are shown in table no. 6. Where the mean, SD, %RSD are less than 2 indicates that the method was precise.

The assay percentage of Glycyrrhetinic acid present in the sample 1 and 2 were found to be`0.104 mg and 0.113 mg in 100 mg of ethanolic extract.

The developed UV spectrophotometer method was found to be rapid, simple, precise, accurate and economic for routine estimation of Glycyrrhetinic acid.

CONCLUSION:

Identification and UV spectrometry estimation of Glycyrrhetinic acid was achieved successfully which will be helpful for the standardization of herbal formulations containing this active constituent. The proposed UV method is linear, accurate and precise and can be adopted for the determination of concentration of Glycyrrhetinic acid in various samples from various herbs and formulations with shorter run time and good efficiency.

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