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ISOLATION, CHARACTERISATION AND QUANTIFICATION OF EXTRACTED D-PINITOL FROM *BOUGAINVILLEA SPECTABILIS* STEM BARK

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ABSTRACT

Bougainvillea spectabilis is a most abundantly found plant having an insulin mimicking agent D-Pinitol (3-o-methyl-chiroinositol). The aim of the present study was to extract, isolate and characterize D-Pinitol from *Bougainvillea spectabilis* stem bark. D-Pinitol extraction from *Bougainvillea spectabilis* was processed with ethanolic extraction followed by isolation and separation using silica gel column chromatography. This was followed by recrystallisation using methanol. D-Pinitol isolated from the plant was identified on the basis of Infrared Spectroscopy (IR), Nuclear Magnetic Resonance Spectroscopy (NMR), Differential scanning calorimetry (DSC) and Thin Layer Chromatography (TLC) as 1-methoxy-2,3,4,5,6-pentahydroxy cyclohexane. For the purpose of quantifying Ultraviolet Spectroscopy (UV) and High performance liquid chromatography (HPLC) methods were developed and validated.

KEYWORDS: *Bougainvillea spectabilis*, recrystallization, liquid chromatography.

1. INTRODUCTION

Bougainvillea Spectabilis commonly known as bougainvillea, great bougainvillea belonging to the family *Nyctaginaceae* contain many biologically active phytochemicals such as flavonoids, phenolic compounds, amylase inhibitors, oxides and pinitol.[1] *Bougainvillea Spectabilis* is medicinally used as antibacterial, antidiabetic, antilipidemic, antioxidant, antiviral, anti-inflammatory and antifertility agent.[2,3,4,5,6,7]

Diabetes is one of the major causes of premature illness and death worldwide. Currently available treatment for diabetes are costly therefore WHO suggests the use of antidiabetic agents from plants and other natural origin.[8]

D-Pinitol (Fig.1) chemically 3-o-methyl-chiroinositol exerts insulin like effect to improve glycemic control in normal and alloxan-induced albino mice without acute toxicity.[9,10] D-pinitol also has antioxidant and antilipidemic activity which improves diabetic control and reduce associated risk factors.[11,12] In developing countries the availability of allopathic medicine is difficult and is expensive.[13] Phytoconstituents isolated from various herbs can be used to a greater extent due to its fewer side effects[14]. An antidiabetic principle, pinitol was successfully isolated by column chromatography from *Bougainvillea spectabilis* and identified by different analytical techniques.

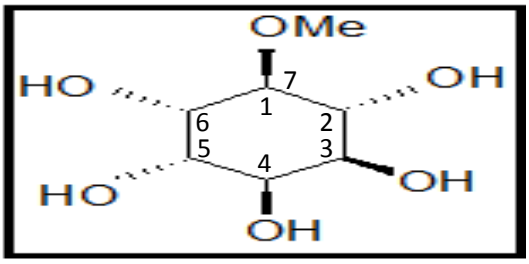

Fig.1 Chemical structure of D-Pinitol

2. MATERIAL AND METHOD

2.1 Materials

The *Bougainvillea spectabilis* stem bark was collected from the botanical garden of Sinhgad Technical Education Society, Vadgaon (Bk.), Pune, Maharashtra, India. All other solutes and reagents used in this study were of analytical grade.

2.2 Extraction Method

Stem bark of *Bougainvillea spectabilis* was dried in sunlight for 7 days and powdered. Powdered material was kept for soaking in 90% ethanol with continuous shaking for 12 hours and filtered. The process was repeated four times for residue. All filtered portions were combined and evaporated at 45-50ºC to yield a crude extract residue. The crude extract residue was suspended in hexane and ethyl acetate (50:50 v/v) and subjected to column chromatography on silica gel. Elution of D-pinitol was carried by gradual addition of methanol to enhance polarity of mobile phase. The eluted portion was evaporated to get solid residue. The residue was subjected to recrystallisation using methanol.[3,9] The yield of D-pinitol was calculated with respect to the weight of the powdered stem bark.

2.3 Phytochemical studies of extract

Ethanol Extract of *Bougainvillea spectabilis* stems bark was investigated for flavonoids by reacting with metallic magnesium and Hydrochloric acid, saponins using the foam test, for alkaloids dragendroffs reagent, for steroids by sulfuric acid and for tannins with ferric chloride reagent.[9,15,16] The isolated pinitol was further identified by different analytical technique.

2.4 ^{1}H Nuclear Magnetic Resonance study

NMR spectra were recorded in dimethyl sulfoxide at room temperature using Brucker Advance (500 MHz) Fourier Transform. The solution of 1 mg/mL of pinitol was prepared in dimethyl sulfoxide to record the proton NMR spectrum. Spectra were recorded with a spectral width of 12019 Hz with 64 k data points.

2.5 Fourier Transform Infrared Spectroscopic (FTIR) study

The dry sample of isolated pinitol was mixed with IR grade KBr with the drug: KBr ratio 1:100. This mixture was compressed to form pellets by applying 10 tons of pressure in a hydraulic press. The pellet was scanned over a wave number range of 4000 to 400 cm^{-1} on the FTIR instrument (Perkin Elmer, Spectrum Bx) and spectral analysis was done.

2.6 Differential Scanning Calorimetry (DSC) study

The DSC thermogram of isolated pinitol was recorded by using a Perkin Elmer 4000 system equipped with a computerized data station. All samples were weighed and heated at a scanning rate of 10°C/min between 30 and 300°C and 60 ml/min of nitrogen flow.

2.9 Thin Layer Chromatography (TLC) Study[18]

TLC of isolated D-pinitol was run on a pre-coated silica gel plate. The plate was developed with mobile phase ethyl acetate: methanol (3:2). After complete development the plate was dried in the oven at 100°C for 15 minutes. The dried plate was kept in an iodine chamber for development of the spots. The R_f value of the spots was calculated.

R_f = Distance travelled by substance from origin/distance travelled by solvent from origin.

2.7 UV-Visible Spectrophotometric study[17,19]

2.7.1 Determination wavelength of maximum absorption

Considering the drug solubility, stability of various solvents, solvents like methanol were tried for analytical purpose. To determine λ_{max} (wavelength maximum) for isolated pinitol,

the solution (10µg/ml) of the drug was scanned in the spectrum mode from 400nm to 200nm using UV visible spectrophotometer Shimadzu, V-1800, Japan.

2.7.2 Linearity and Range

A stock solution of concentration 1000 µg/mL of the drug in methanol was prepared by dissolving accurately weighed 10 mg of isolated pinitol in 10 ml of methanol. Appropriate volumes of this solution were diluted to 10.0 mL with methanol to get the concentration in the range of 10 to 60 µg/ml. Limit of detection (LOD) and limit of quantification (LOQ) also calculated from the following formula.

LOD = 3.3 × (SD/S)

LOQ = 10 × (SD/S)

Where, S is the mean slope of the regression equation of six calibration curves and SD is the standard deviation of y-intercept of the calibration curves.

2.7.3.1 Repeatability

Six aliquots of 35 µg/mL were prepared from stock solution and absorbance was measured at 229nm. From the absorbance reading % RSD was calculated.

2.7.3.2 Reproducibility

For interday precision the six aliquots of 30, 35, 40 µg/mL each was prepared from the stock solution and absorbance was measured at 229nm. The same procedure was repeated for intraday precision, from the observed readings % RSD was calculated.

2.7.4 Accuracy

The accuracy of the method was determined by calculating recoveries of D-pinitol by the method of standard additions. The accuracy of the analytical procedure was determined at 80 %, 100 % and 120 % levels of standard solution. All solutions were analyzed at 229nm and % recovery was calculated.

2.7.5 Robustness

Small but deliberate change in analytical method was carried out for determination of robustness. For this, concentration of 35 µg/mL was analysed at 229±1nm and %RSD was calculated.

2.7.6. Assay

For assay of formulated transdermal formulation of D-pinitol, concentration containing 35µg/mL of D-pinitol from prepared transdermal patch was analysed in triplicate and drug content was determined.

2.8 High performance liquid chromatography

HPLC system consisting Spectra system 2010, Autosampler, a Spectro system with UV detector, Thermostat column compartment connected with LC2010 software.

2.8.1 Chromatography Condition

Column: C_{18}, 250mm X 4.6mm, 5µ

Flow rate: 1 ml /min

Mobile phase: acetonitrile: 20mM Potassium dihydrogen orthophosphate pH 3.5 (70:30)

Wavelength: 230nm

Column temperature: 30°C

Injection volume: 20 µ L

Run time: 10 minutes

2.8.2 Preparation of pinitol Standard Stock solution

Weigh and transfer 10mg of pinitol powder into the 10ml volumetric flask, add 30ml of mobile phase and sonicate and further filter the solution through 0.45µ filter paper and make up with same. Appropriate volumes of this solution were diluted to 10.0 mL with mobile phase to get the concentration in the range of 10 to 100 µg/ml.

2.8.3 Method Validation[19]

Method validation for pinitol was carried out as per procedures given in the 2.7.2-2.7.6.

2.8.4 Forced degradation study

Forced Degradation Studies of the drug substance can help to identify the likely degradation products which can assist to establish the degradation pathways and the stability of the molecule. D-Pinitol standard samples were degraded under different stress conditions like acidic, alkali and oxidative hydrolysis. For acid and base degradation, samples were refluxed with 0.1M HCl and 0.1M NaOH at 70^0C for 5hrs. For oxidation, 3% v/v H_2O_2 was refluxed at 70^0C for 5hrs. Acid and base treated samples were neutralized and all the samples were

then diluted to a concentration of 50µg/ml with the methanol. The samples were withdrawn and subjected to HPLC analysis under optimized conditions.

3. RESULT AND DISCUSSION

The yield of D-Pinitol from *Bougainvillea spectabilis* powdered stem bark was found to be 0.1128%. This low yield is responsible for the higher prices of D-pinitol in the market. The extraction of D-pinitol from *Bougainvillea spectabilis* can be an alternative to overcome the problem of higher prices.

3.1 Phytochemistry of extract

The ethanol extract of *Bougainvillea Spectabilis* stem bark showed the presence of flavonoids, saponins, alkaloids, steroids and tannins. The observations of phytochemical constituents in ethanolic extract were shown in the Table.1.

Table.1 Phytochemical study of ethanolic extract of *Bougainvillea spectabilis*

Phytoconstituents	Reagents	Observation	Inference
Flavonoids	Metallic magnesium and hydrochloric acid	Green colour	Presence of Flavonoids was confirmed
Saponins	(Foam test) Check for appearance of foam	Appearance of foam was seen	Presence of Saponins was confirmed
Alkaloids	Dragendroff's reagent (potassium bismuth iodide)	Reddish brown precipitate	Presence of Alkaloids was confirmed.
Steroids	Acetic anhydride and Conc. H_2So_4	Brown ring formed at upper layer which turned green.	Presence of steroids was confirmed.
Tannins	Ferric chloride	Blue colour	Presence of tannins was confirmed.

3.2 ^{1}H NMR Spectroscopy Study

The ^{1}H NMR spectroscopy is one of the methods for structural elucidation of the compounds. An NMR spectrum of isolated D-pinitol (Fig.2) shows all peaks corresponding to the structure that are interpreted in the Table.2. The ^{1}H NMR spectrum has three doublet at δ value 4.520-4.510, 4.472-4.459, 4.341-4.337 for OH attach to carbon 6, 2 and 4 respectively. Broad singlet at 3.446 due to the hydrogen attach to the methoxy group. Two singlets at δ value 4.720 and 4.629 due to the OH attached to C-5 and C-3 respectively. One triplate and one multiplate at δ value 3.016-2.978 and 3.35-3.31 of H attach to C-1 and C-6. Hydrogen

attach to the C-3 and C-5 gives broad singlet at δ 3.50 and 3.622. The spectral data obtained from the ^{1}H NMR were identified structure of the D-pinitol.

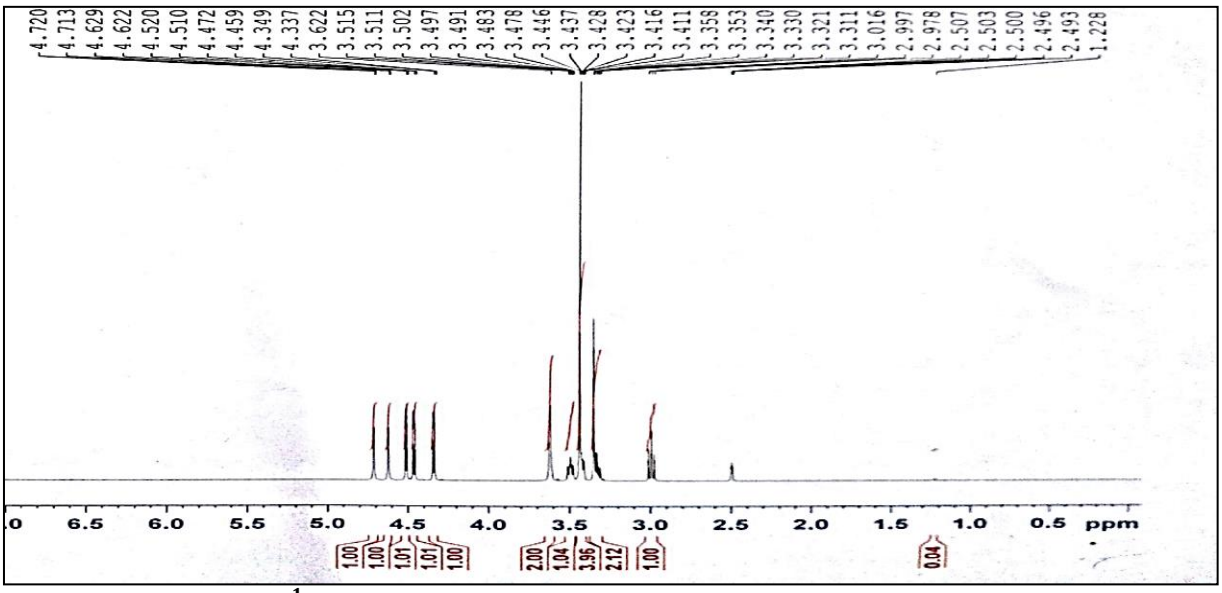

Fig.2 ^{1}H NMR Spectra of D-pinitol in DMSO at 500 MHz

Table.2 ^{1}H NMR spectra interpretation of isolated D-pinitol

δ value (ppm)	Splitting	Interpretation of protons
4.720	s	C_5-OH
4.629	s	C_3-OH
4.520-4.510	d	C_6-OH
4.472-4.559	d	C_2-OH
4.341-4-337	d	C_4-OH
3.446	br. s	7-OCH_3
3.016-2.978	t	C_1-H
3.50	br. s	C_3-H
3.622	br. s	C_5-H
3.35-3.31	m	C_6-H

s-singlet, d-doublet, t- triplet, m-multiplate, br.- broad

3.3 Infrared Spectroscopy study

FTIR spectrum of isolated pinitol showed all the peaks corresponding to the functional groups present in their respective structures. The FTIR spectrum for D-pinitol is shown in Fig.3 and interpretation of FTIR spectra is given in Table.3. The main functional group found in IR spectrum are O-H and C-H.

Table.3 IR spectrum interpretation of isolate D-pinitol

Functional group	Reported IR Frequency (cm^{-1})	Observed IR Frequency (cm^{-1})
OH strech	3400-3600	3402.33
C-H stretch of aliphatic	2900-300	2910.68
C-Hbend	1435, 1375	1455, 1380.08

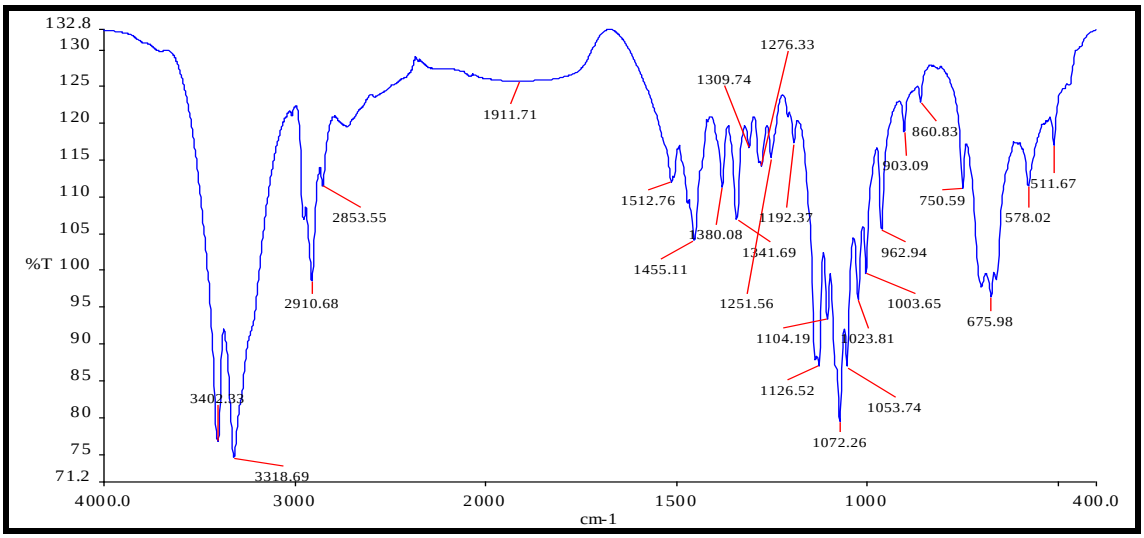

Fig.3 IR Spectra of D-pinitol with KBr

3.4 Differential Scanning Calorimetry (DSC) study

The differential scanning calorimetric analysis gives an idea about the purity of the compound. The DSC thermogram of isolated D-pinitol is shown in the Fig.4**.** The DSC thermogram of isolated pinitol indicated a sharp endothermic peak at the 175-198^{o}C range corresponding to melting of D-pinitol. The reported melting point of D-pinitol was 179-186^{o}C. The range of endothermic peak increases because small amounts of other phytochemicals remain in the isolated D-pinitol. However presence of single peak account for the acceptable purity of d-pinitol.

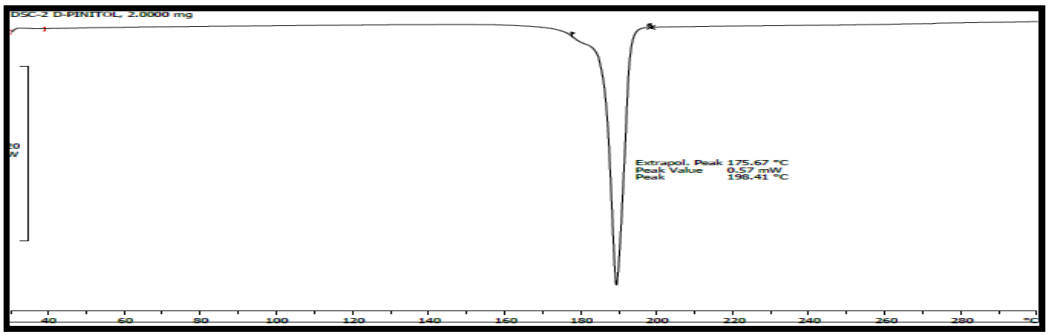

Fig.4 DSC thermogram of D-pinitol

3.5 Thin Layer Chromatography (TLC) Study

After the development of a TLC plate in iodine chamber two spots of yellow color were obtained with the different R_f value 0.75 and 0.59 respectively. As per the standard given in the literature the 0.75 R_f value is near to the D-pinitol R_f value[18]. The spot at 0.59 is due to the other phytochemical remaining in the isolated pinitol which is not identified in this work.

Fig. 5 Thin layer chromatography of D-pinitol

3.6 UV-Visible Spectroscopy study

Methanol was selected as the solvent for D-pinitol because provides good solubility and other characteristics for absorbance measurements. The UV spectra of isolated D-pinitol, it was found that it shows maximum absorbance at 229 NM in methanol. So absorbance at 229 was considered as λ_{max} for D-pinitol which is shown in Fig.6. The linearity range for D-pinitol was found to be 10-60 µg/mL with r^2=0.9994 and calibration curve shown in the Fig.7. The validation parameters of D-pinitol were calculated by the proposed method and are presented in Table 4. The results of precision for repeatability and reproducibility (Intraday and Interday precision) study given in Table.5 and 6 values obtained (≤2%) comply with the stated limits of the guidelines. The accuracy of D-pinitol was evaluated by % recovery studies at concentration levels of 80, 100, and 120% and the values obtained were found to be in the acceptable limits (≤2%) which are given in Table.7. Robustness were performed by three different wavelengths (229nm±1) and results were evaluated by calculating the %RSD value lying within the range which is shown in Table.8. The drug content in a transdermal formulation was found to be 98.76 % for an absorption spectrophotometric method which is given in Table.9.

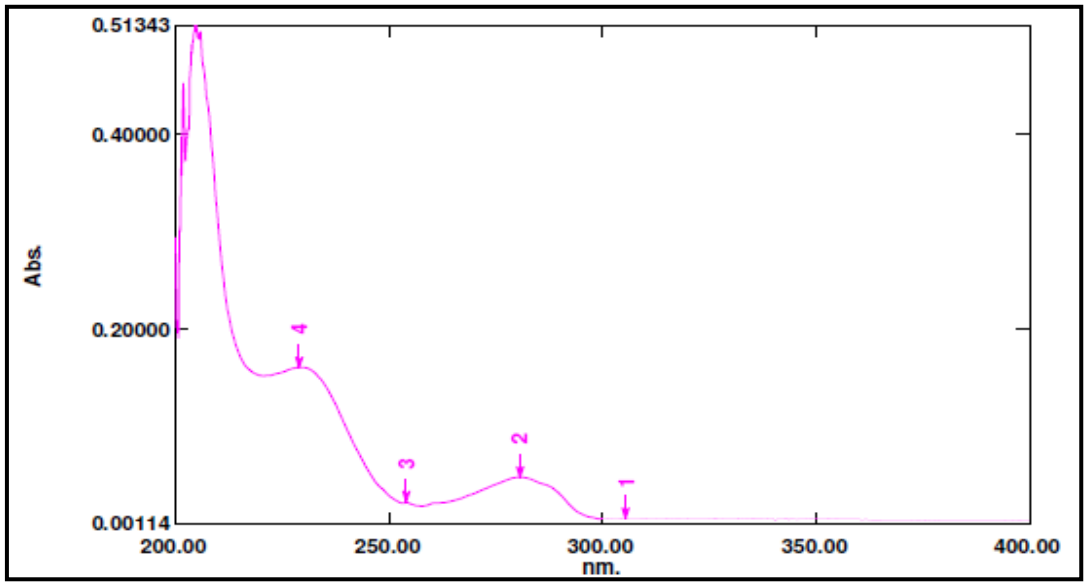

Fig.6 Graph of UV-Visible spectroscopy of D-pinitol in methanol

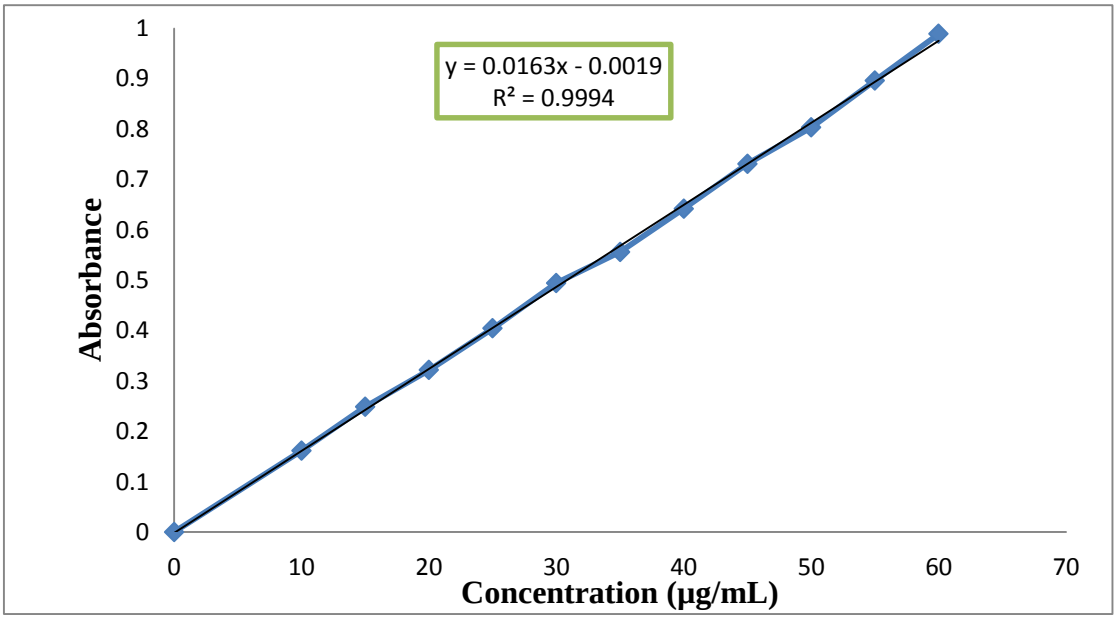

Fig.7 UV Calibration curve of D-pinitol by UV

3.7 High Performance Liquid Chromatography

The appropriate wavelength in UV region has been selected for the measurement of active ingredient in the proposed method. This method was validated by linear fit curve and all the other parameters were calculated. For Optimization of peak different mobile phase was tried with different combinations. The acetonitrile: 20mM Potassium dihydrogen orthophosphate pH 3.5 (70:30) mobile phase gives the optimized peak at 5.509 retention time which was shown in the Fig.8. Chromatogram of pinitol shows another small intensity peak at 3.14 which are small impurities remaining in the pinitol. All the values obtained from pinitol

validation were found to be in acceptable limit (≤2%). Validation parameters of validation is reported in the table. 4. The calibration curve was plotted concentration against an area of the curve, which is given in Fig.9. The result of forced degradation study is given in the table. 10 which interpret that pinitol get oxidized in presence of hydrogen peroxide, it shows that it works as an antioxidant by preferential oxidation mechanism. Pinitol do not degrade very fast in neutral condition, but it degrade slowly in acid and in alkali. The degradation peaks of pinitol after 5 hours is given in the Fig.10.

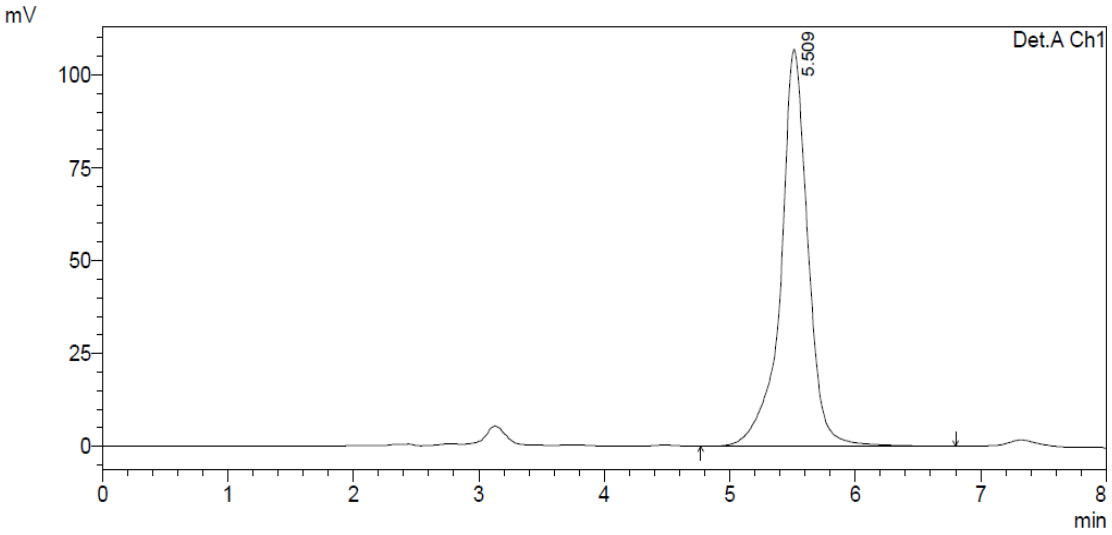

Fig.8 Chromatogram of Pinitol

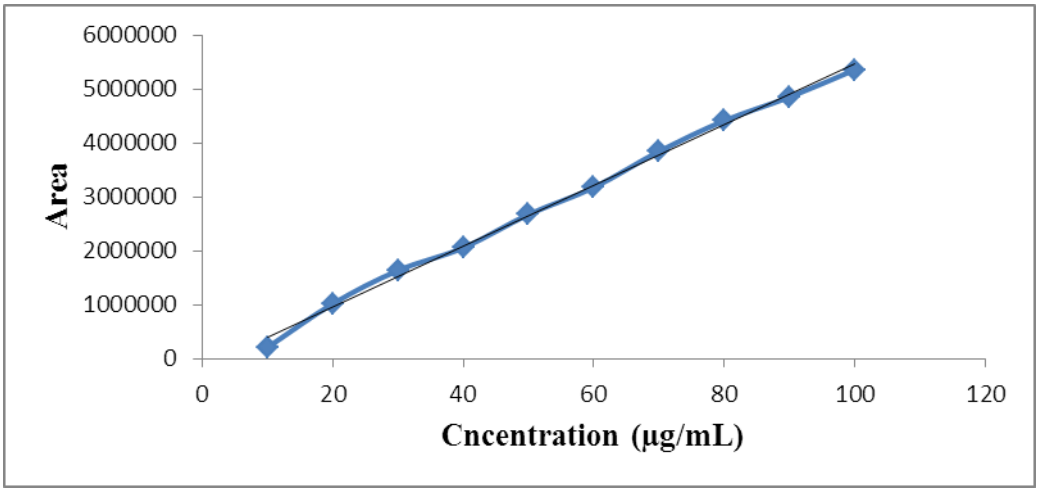

Fig.9 Calibration curve of pinitol by HPLC

Table.4 Validation Parameters

Parameters	UV	HPLC
λ_{max}	229nm	230nm
Linearity and range	10-60 µg/mL	10-100 µg/mL
Regression equation (y=mx+c)	y = 0.0163x - 0.0019	y = 56299x - 152243
Slope (m) ±SD	0.0163	56299
Co-relation coefficient (R^2)	0.9994	0.997
Precision (%RSD)	0.5527	1.456
Repeatability ntraday nterday	 0.5642 0.5740	 1.7797 1.5216
Accuracy (mean% Recovery)	99.04	99.97
LOD	54.58 µg/mL	99.69 µg/mL
LOQ	165.4 µg/mL	302.10 µg/mL
Assay (%)	98.76	99.97

Table.5 Repeatability results of precision

Drug	Concentration of drug (µg/mL) (n=6)	%RSD
D-pinitol	35	0.557

Table.6 Reproducibility results of precision

Drug	Concentration of drug (µg/mL) n=6	%RSD	
		Intraday	Interday
D-pinitol	30	0.4944	0.4942
	35	0.5562	0.5661
	40	0.6421	0.6618

Table. 7 Accuracy results

Accuracy levels	Amount added (µg/mL)	% recovery	% mean recovery	% RSD
80%	30	98.56±0.8945	99.04	0.7272
100%	35	99.31±0.9632		
120%	40	99.28±0.3241		

Table.8 Robustness results

Wavelengths (nm)	Concentration of drug (µg/mL)	%RSD
228	35	0.5445
229	35	0.5562
230	35	0.5439

Table.9 Assay of transdermal formulation

Sample Concentration (µg/mL)	% Amount Found	Mean amount	%RSD
35	98.12	98.76	0.56
35	98.54		
35	99.62		

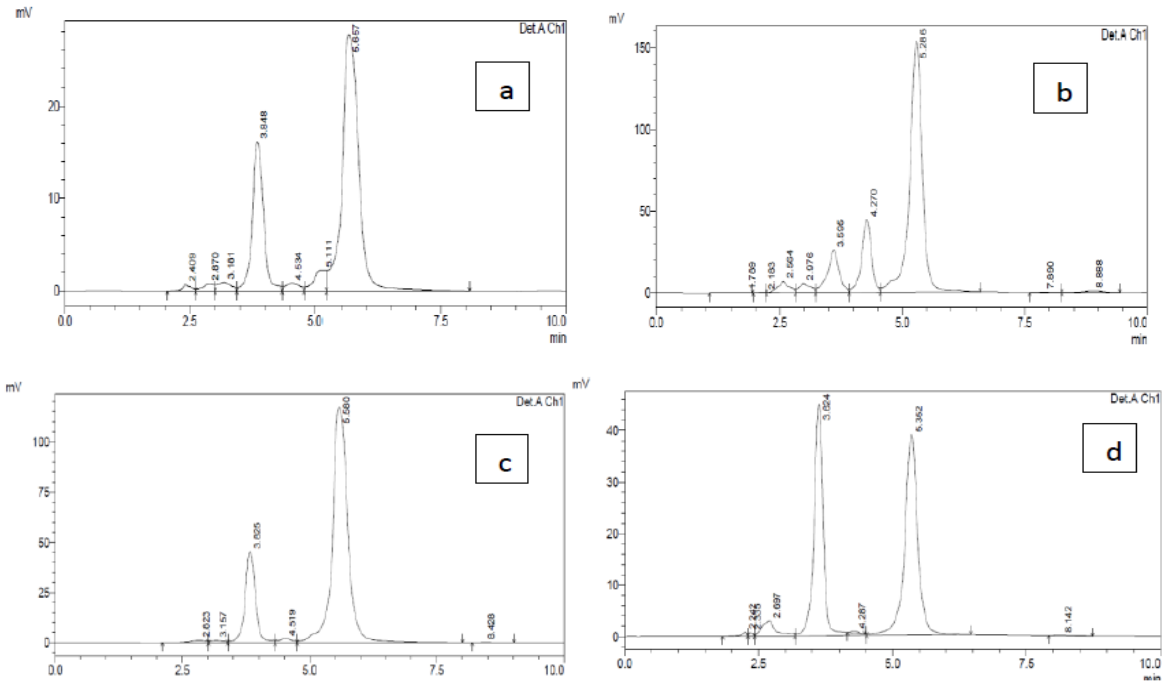

Fig. 10 Chromatogram of degradation of pinitol in a) 0.1M HCL b) 0.1 M NaOH c) Neutral d) H_2O_2

Table.10 Forced degradation study

Stress studies	% Amount of pinitol degraded (After 5 hours)	Retention time of degraded product
0.1M HCL (70°C)	39	3.8
0.1M NaOH (70°C)	37.97	3.5 and 4.2
Neutral (70°C)	25.96	3.8
H_2O_2 (70°C)	81.508	3.6

CONCLUSION

The present study showed that antidiabetic principle D-Pinitol can easily be extracted from *Bougainvillea spectabilis* stem bark with ethanol extraction followed by purification using silica gel chromatography and then successfully identified and quantified by standard analytical methods.

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