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Research Article

GAS CHROMATOGRAPHY – MASS SPECTROMETRY METHOD FOR SEPARATION, IDENTIFICATION AND QUANTIFICATION OF B-SITOSTEROL, LUPEOL AND FRIEDELIN FROM *NYCTANTHES ARBOR-TRISTIS* AND ITS MARKETED FORMULATION

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ABSTRACT

In the present work a simple, precise, accurate and reproducible Gas Chromatography-Mass Spectrometry method is developed and validated for separation, identification and quantification of three pharmacologically active compounds, β-sitosterol, Lupeol and Friedelin simultaneously in the methanolic leaf extract of *Nyctanthes arbor-tristis* and from its marketed formulation. These compounds were extracted from the *Nyctanthes arbor-tristis* leaves and from its formulation using Soxhlet extraction followed by chromatographic separation using gas chromatography instrument coupled with mass spectrometer for online identification. The method was developed using Restek RTX-5MS column which gave good resolution for the components with retention time of 14.540 min, 16.257 min and 19.357 min for β-sitosterol, Lupeol and Friedelin respectively. The total run time for the programme was 21.67 min. The quantity of β-sitosterol, Lupeol and Friedelin in leaves of *Nyctanthes arbor-tristis* was found to be 0.074377 %, 0.03295 % and 0.010902 % respectively, while in the formulation it was found to be 0.0085548 %, 0.003384 % and 0.0028246 % respectively. Friedelin was linear between 1 – 30 µg/mL, Lupeol was linear between 1 – 50 µg/mL range & β-sitosterol was found to be between 10 - 120 µg/mL. The method showed recovery within the acceptable range for all the three standards. Recovery for β-sitosterol, Lupeol and Friedelin was found to be 93.12 %, 98.78 % and 105.34 % respectively in the plant, whereas in the case of formulation it was found to be 98.24%, 102.93 % and 107.56 % respectively. The RSD for precision and reproducibility calculated were below 2%. This method can be used as a quality control tool for standardization of plant material and formulation containing its extract.

Keywords: *Nyctanthes arbor-tristis*, Gas chromatography, β-sitosterol, Lupeol, Friedelin.

INTRODUCTION

Nyctanthes arbor-tristis belongs to the family Oleaceae and is known as Paarijat in Sanskrit and as Night flowering jasmine in English[1]. *Nyctanthes arbor-tristis* Linn is native to India, distributed widely in sub-Himalayan regions and southwards to the Godavari.[2] Different parts of *Nyctanthes arbor-tristis* Linn are known to possess various ailments by tribal people of the Indian subcontinent with its use in Ayurveda, Sidha and Unani systems of medicines.[7] The leaves of *Nyctanthes arbor-tristis* Linn are used extensively in Ayurvedic medicine for the treatment of various diseases such as sciatica, chronic fever, rheumatism, and internal worm infections and as a laxative, diaphoretic and diuretic[8].

A variety of constituents belonging to different chemical classes such as terpenes, steroids, glycosides, flavonoids, alkaloids and aliphatic compounds have been isolated and characterized from different parts of *Nyctanthes arbor-tristis*. The bark contains a glycoside and two alkaloids, one soluble in water and the other soluble in chloroform[3]. Its roots are composed of alkaloids, tannins and glucosides.[4] The chemicals constituents present in Leaves of *Nyctanthes arbor-tristis*

are D-mannitol, β-sitosterol, flavanol glycosides, Astragaline, Nicotiflorin, Friedelin, Nyctanthic acid, tannic acid, ascorbic acid, methylsalicylate, carotene, Friedelin, lupeol, mannitol, Glucose and fructose, iridoid glycoside, benzoic acid.[9] Bark contains glycosides and alkaloids.[1]

Nyctanthes arbor-tristis plant parts possess various biological activities such as Anti-oxidant activity, Anti-inflammatory, Immunostimulant, Anti-microbial, Anti-viral, Anti-plasmodial, Hepatoprotective, Anti-allergy etc.

Many forms of raw plant material and herbal drugs derived from *Nyctanthes arbor-tristis* are distributed in the herbal market; however, the content of bioactive components in these products has not necessarily been quality-controlled. Therefore, a simple, low-cost, and rapid method for screening and quantitating bioactive components is strongly desired. Literature survey revealed that no GC-MS method has been reported for simultaneous quantitation of B-sitosterol, Lupeol and Friedelin from the methanolic extract of leaves of *Nyctanthes arbor-tristis* using Gas Chromatography – Mass Spectrometry.

MATERIALS AND METHODS

Collection of Plant

Leaves of *Nyctanthes arbor-tristis* were collected from Kanakeshwar, around 10 km away from Alibaug, Maharashtra, India in the month of June. The plant was authenticated and voucher specimen no. PMP-1 was deposited in The Botanical Survey of India, Pune, India.

Preparation of Plant Material

The leaves were washed thoroughly with tap water. The leaves were dried initially using paper to remove excess of water and later were air dried thoroughly under shade at room temperature to avoid direct loss of phytoconstituents from sunlight. The shade dried material was powdered using a grinder and sieved through an ASTM 80 mesh. It was then homogenized to a consistency of a fine powder and stored in an air-tight container for further analysis.

Preparation of the Extracts

Nyctanthes arbor-tristis leaf powder was extracted using Soxhlet extraction. One grams of plant powder was weighed and packed in a Whatman paper thimble. This weighed powder sample was damped with 5 mL of concentrated HCl. It was then extracted with 200 mL methanol for 12 hours using Soxhlet extractor. The solvent after extraction was evaporated to reduce its volume. The final volume was made to 10 mL using methanol. Formulation extract was prepared in similar manner with five grams as sample size.

Reagents and standards

All chemicals and solvents used were of HPLC grade and purchased from Merck (Darmstadt, Germany). Analytical standards B-sitosterol, Lupeol and Friedelin were procured from Sigma-Aldrich (Bengaluru, India).

Preparation of standard solutions

Stock solutions of standards were prepared in methanol just before use. 5 mg each of B-sitosterol, Lupeol and Friedelin was dissolved in 5 mL of methanol to make a concentration of 1000 µg/1000 µL. All three standards were further diluted using methanol to make solutions of appropriate concentrations.

CHROMATOGRAPHIC CONDITIONS

Table 1: Optimized Chromatographic Conditions

Parameter	Description		
Instrument	Shimadzu QP 2010 Ultra GCMS		
Column	Restek RTX-5MS (5% Diphenyl ; 95% Dimethylpolysiloxane) L- 30 m, Id- 0.25 mm, d_f -0.1 µm		
Injector Temperature	280 °C		
Flow Rate	1.00 mL/min		
Split Ratio	1 : 5		
Column Oven Program	Ramp Rise (º C / min)	Temperature (º C)	Hold Time (min)
	-	200	0
	15	285	1
	0.5	290	5
Injection volume	1 µL		
Carrier Gas	Helium		
Flow Control Mode	Linear Velocity (35.0 cm/sec)		
Ion Source Temperature	280 °C		
Interface Temperature	280 °C		
m/z Range	35 – 450		

METHOD VALIDATION

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.[4] Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.[10]

System Suitability and Specificity

Suitability of the system was checked by injecting six replicates of 10 µg/mL solutions of each standard. The solution was injected under the optimized conditions. Parameters like retention time and peak area were evaluated for the system suitability. Specificity test was carried out by injecting 1 µl of the diluent and individual standards.

Precision

The variability of the method was studied by carrying out repeatability, inter-day and intra-day precision. Repeatability was carried out in same laboratory, on same day as well as on two consecutive days, by analyzing standard solutions of β-sitosterol, Lupeol and Friedelin using optimized chromatographic conditions.

Linearity

The Linearity of a method is the measure of how well a calibration plot of detector response against concentration approximates to a straight line. Concentrations of Friedelin were 1 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL and 30 µg/mL were selected for linear dynamic range experiment. For Lupeol 1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL were selected and for β-sitosterol, concentrations of 10 µg/mL, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL and 120 µg/mL were selected for linear dynamic range experiment. The chromatograms were recorded and the peak areas of β-sitosterol, Lupeol and Friedelin for each applied concentration of these standards were noted. The response factors were calculated for each concentration of β-sitosterol, Lupeol and Friedelin by dividing each peak area by concentration of β-sitosterol, Lupeol and Friedelin at that level.

Limit of Detection and Limit of Quantification

ICH defines the limit of detection (LOD) is the lowest concentration of an analyte that can be detected under the operational conditions of the method but not necessarily quantitated as an exact value. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy, under the operational conditions of the method.

Assay

One microliter of sample solution i.e. *Nyctanthes arbor-tristis* leaf extract and of formulation extract were injected. Also different concentrations of β-sitosterol, Lupeol and Friedelin were injected under optimized chromatographic conditions. The peak corresponding to β-sitosterol, Lupeol and Friedelin in the sample solution was identified using Mass Spectrometer as the detector. The amount of β-sitosterol, Lupeol and Friedelin present in sample solution was determined from the calibration curve by using the peak area of standards generated by the chromatogram.

Recovery

The recovery experiment was carried out to check if there is any interference of other constituents with the peaks of β-sitosterol, Lupeol and Friedelin present in leaves of *Nyctanthes arbor-tristis* and formulation Arthrum Capsule containing extract of *Nyctanthes arbor-tristis*. Accuracy of the method was established by carrying out recovery experiment at three different levels, using standard addition method. Each sample was analyzed in triplicates and the amounts of β-sitosterol, Lupeol and Friedelin recovered for each level, were determined. The value of percentage recovery for the three components was then calculated.

Ruggedness

Ruggedness of the method was checked under following conditions

Parameters	Optimized conditions	Experimental conditions
Injection temperature (° C)	280 ° C	± 5 ° C
Ion source temperature (° C)	280 ° C	± 5 ° C
Interface temperature (° C)	280 ° C	± 5 ° C
Flow rate (mL/min)	1.00 mL/min	± 0.2 mL/min
Split ratio	1 : 5	1 : 5 ± 0.5

Stability Studies

The stability of stock solutions for all three standards were studied by storing them at room temperature and under refrigeration between 0 - 4° C. Triplicate injections of the standard solutions were done at regular intervals of 12 hours.

RESULTS AND DISCUSSION

A Gas Chromatography Mass Spectrometry method for the simultaneous quantification of β-sitosterol, Lupeol and Friedelin from *Nyctanthes arbor-tristis* leaves methanolic extract and from a formulation containing extract of *Nyctanthes arbor-tristis* was developed and validated in the present research work.

Retention time values of β-sitosterol, Lupeol and Friedelin was found to be 14.540 min, 16.257 min and 19.357 min respectively.

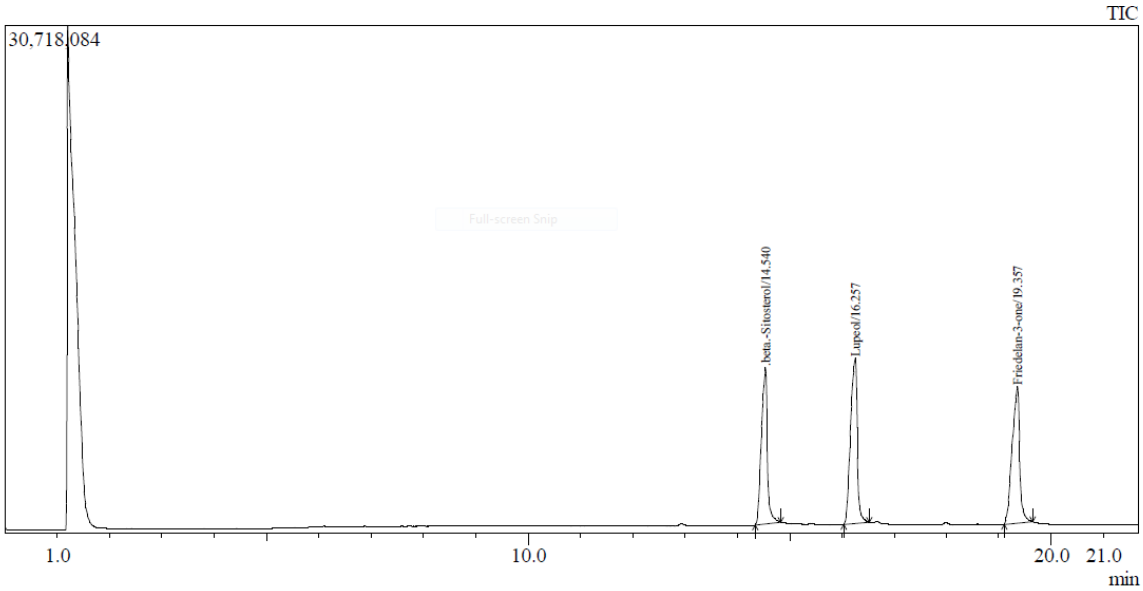

Fig. 1: Chromatogram of Standard Mixture of β-sitosterol, Lupeol and Friedelin

The method was validated for specificity, linearity, LOD, LOQ, intra- day and inter-day precision, recovery, ruggedness and stock solution stability. The method was found to be linear between 1 – 30 µg/mL for Friedelin, Lupeol was linear between 1 – 50 µg/mL range & β-sitosterol was found to be linear between 10 - 120 µg/mL. The limits of detection (LOD) for β-sitosterol, Lupeol and Friedelin was found to be 3.333 µg/mL, 0.333 µg/mL and 0.33 µg/mL respectively and the limit of quantification (LOQ) was found to be 10 µg/mL, 1 µg/mL and 1 µg/mL respectively.

The relative standard deviation for inter-day and intra-day precision was <2%. The correlation coefficient was found to be ≥0.99 for the components under evaluation. The precision (% RSD) of the method was found to be < 2%, indicating that the proposed method is precise.

The recovery values for all the standards were within acceptable limits. Stock solution stability was checked by monitoring the peak area response over a given time range. Standard solutions were analyzed right after its preparation and at regular intervals till 72 hrs. There was no significant change (% RSD ≤ 2%) in the retention time and area values of standard peak.

The quantity of β-sitosterol, Lupeol and Friedelin in leaves of *Nyctanthes arbor-tristis* was found to be 0.074377 %, 0.03295 % and 0.010902 %respectively. While the quantity of β-sitosterol, Lupeol and Friedelin in formulation was found to be 0.0085548 %, 0.003384 %and 0.0028246 % respectively. The method is specific for all the three components because it resolved all the standards well in the presence of other phytochemicals in *Nyctanthes arbor-tristis*. The method was found to be suitable for qualitative and simultaneous quantitative analysis of β-sitosterol, Lupeol and Friedelin in the methanolic extract of *Nyctanthes arbor-tristis*.

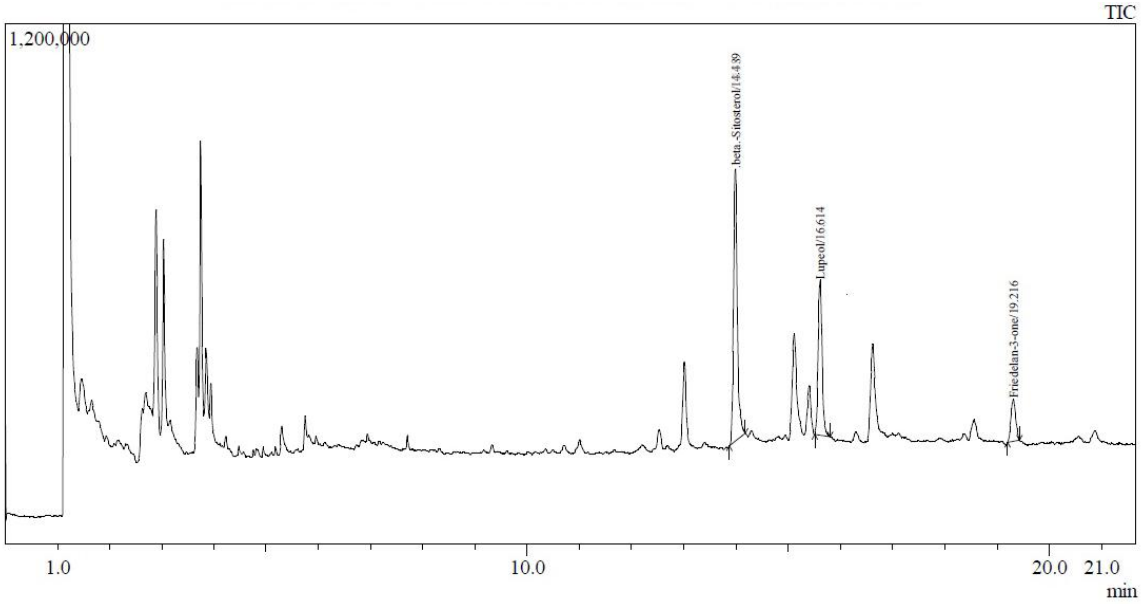

Fig. 2: Chromatogram of Nyctanthes arbor-tristis methanolic leaf extract

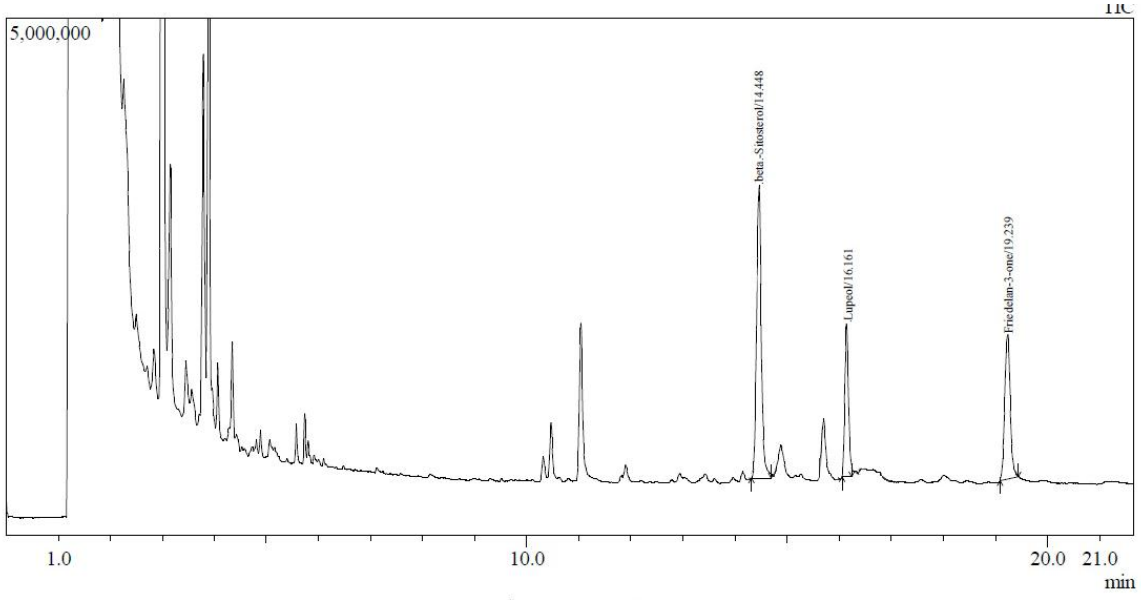

Fig. 3: Chromatogram of Formulation containing Nyctanthes arbor-tristis plant extract

Proposed method was not influenced by the factors considered for ruggedness study. Change in flow rate, temperature and split ratio affected the retention time and response area of the three analytes but the results were satisfactory since % CV was <2%.

Stock solution stability study of all the three standards stored for the period of 72 hours at 0-4°C showed % CV < 2%. During bench top stability study, the results vastly varied indicating lesser stability of the standards at room temperature. Stability studies showed that Friedelin, Lupeol and β- sitosterol were found stable for at least 12.0 h at room temperature and for 72 hours at 0-4°C of storage condition.

Table 2: Summary of method validation parameters

Parameter	β-sitosterol	Lupeol	Friedelin
Specificity	Specific	Specific	Specific
Precision	<2%	< 2%	<2%
LOD	3.333 (µg/mL)	0.333 (µg/mL)	0.33 (µg/mL)
LOQ	10 (µg/mL)	1 (µg/mL)	1 (µg/mL)
Linearity (µg/mL)	10-120	1-50	1-30
Quantity (Plant)	0.074377 %	0.03295 %	0.010902 %
Quantity (formulation)	0.0085548 %	0.003384 %	0.0028246 %
Stock solution stability	Stable for 72 hrs at 0 – 4 °C	Stable for 72 hrs at 0 – 4 °C	Stable for 72 hrs at 0 – 4 °C
Recovery (Plant)	93.12 %	98.78 %	105.34 %
Recovery (Formulation)	98.24%	102.93 %	107.56 %

CONCLUSION

The developed method in this research work is precise, accurate and reproducible. It is suitable for qualitative and quantitative analysis of β- sitosterol, Lupeol and Friedelin in the methanolic extracts of leaves of *Nyctanthes arbor-tristis*. Also it can be used as a quality control method for other market formulations or dietary supplements containing powder extract of *Nyctanthes arbor-tristis.*

CONFLICT OF INTEREST

The authors whose names are listed certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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