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SIMULTANEOUS QUANTIFICATION OF LUPEOL, B-SITOSTEROL AND OLEANOLIC ACID USING VALIDATED HPTLC METHOD FROM *NYCTANTHES ARBOR-TRISTIS* AND ITS MARKETED FORMULATION

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ABSTRACT

A simple, accurate and reproducible HPTLC densitometric method has been developed for the simultaneous quantification of Lupeol, β-sitosterol and Oleanolic acid from the methanolic extract of leaves of plant *Nyctanthes arbor-tristis* and its marketed formulation. The mobile phase n-Hexane: Ethylacetate: Formic acid (8: 2 : 0.5 v/v/v) gave good separation and resolution for the mentioned compounds. The CAMAG HPTLC system was used for the analysis. The separated compounds were visualized at 540 nm after derivatization using 10% Methanolic sulphuric acid reagent. The quantity of Lupeol was found to be 0.03124 % and 0.003054 %, β-sitosterol was 0.0777 % and 0.008369 % and Oleanolic acid was 0.1062 % and 0.008128 % in plant and formulation respectively. The developed method was then validated in accordance with the ICH guidelines in terms of specificity, linearity, LOD, LOQ, precision and recovery. The validated method was successfully applied for quantification of three components in a formulation containing *Nyctanthes arbor-tristis* extract. This method can also be used as a quality control tool for other formulations or dietary supplements containing the extract of *Nyctanthes arbor-tristis*.

KEYWORDS: Nyctanthes arbor-tristis, Lupeol, β-sitosterol, Oleanolic acid, simultaneous quantification.

INTRODUCTION

Herbal drugs have been used since ancient times as medicines for the treatment of wide range of diseases. Medicinal plants have played a key role in world health especially in rural areas. The recent global resurgence of interest in herbal medicines has led to an increase in the demand for them. Quality certification of products of botanical origin has been in the news for several reasons. It is a complicated job to standardize thousands of plant extracts with respect to their medicinal value and constituents.[1] Quality control is a term that refers to processes involved in maintaining the quality and validity of manufactured product. Currently, there is no Government body that certifies the label claim of herbal preparations. To maintain the quality of traditional medicines, WHO has issued guidelines for quality control methods of medicinal plant materials. These guidelines facilitate the work carried out by scientific bodies, regulatory authorities, and industries, involved in production of medicinal preparations. WHO has also emphasized the need to ensure quality control of herbs and herbal formulations by using modern analytical techniques.

Chromatographic and spectroscopic techniques are the most commonly used methods in standardization of herbal medicines, but the herbal system is not easy to analyze because of their complexity and variations of chemical composition. Many cutting-edge analytical technologies have been introduced to evaluate the quality of medicinal plants and significant amount of measurement data has been produced. Extremely valuable are techniques like high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), mass spectrometry (MS), high-performance liquid chromatography (HPLC), LC-MS, and GC-MS.
The Parijata is regarded in Hindu mythology as one of the five wish-granting trees of Devaloka. Different parts of *Nyctanthes arbor-tristis* Linn are known to possess various ailments by tribal people of Indian subcontinent with its use in Ayurveda, Sidha and Unani systems of medicines. The flowers are used as stomachic, carminative, astringent to bowel, antibilious, expectorant, hair tonic and in the treatment of piles and various skin diseases and in the treatment of ophthalmic purposes. The powdered stem bark is given in rheumatic joint pain, in treatment of malaria and also used as an expectorant. The bark is used for the treatment of snakebite and bronchitis. 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. The seeds are used as anthelmintics and in alopecia. It is an expectorant and is also

useful in bilious fevers. The powdered seeds are used to cure scurfy affections of scalp, piles and skin diseases.

Many forms of raw plant material and herbal drugs derived from *Nyctanthes arbor-tristis* are distributed in herbal market; however, the content of bioactive components in these products have 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 method has been reported for simultaneous quantitation of Lupeol, Oleanolic acid and β-sitosterol from methanolic extract of leaves of *Nyctanthes arbor-tristis*. Therefore, the aim of the study was to develop a rapid, precise and reproducible HPTLC method for quantification of Lupeol, Oleanolic acid and β-sitosterol from *Nyctanthes arbor-tristis* plant materials that can be used to determine their content in commercial herbal drugs. The proposed method was validated in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

MATERIALS AND METHOD

COLLECTION OF PLANT

Leaves of *Nyctanthes arbor-tristis* were collected from Kankeshwar, 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 grinder and sieved through an ASTM 80 mesh. It was then homogenized to fine powder and stored in an air-tight container for further analysis.

Preparation of the plant extracts

Nyctanthes arbor-tristis leaf powder was extracted using Soxhlet extraction. One gram of plant powder was weighed and packed in a Whatman paper thimble. This weighed powder sample was wetted 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 the volume, filtered through 0.45 µ filter and finally made upto 10 ml volume using methanol.

Preparation of the formulation extracts

Five grams of formulation powder was weighed and packed in a Whatman paper thimble. This weighed powder sample was wetted 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 the volume, filtered through 0.45 µ filter and finally made upto 20 ml volume using methanol.

Reagents and standards

All chemicals and solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). Analytical standards Lupeol, Oleanolic acid and β-sitosterol were procured from Sigma-Aldrich (Bengaluru, India). TLC aluminium plates precoated with silica gel 60 F_{254} were purchased from Merck (Darmstadt, Germany).

Preparation of standard solutions

Stock solutions of standards were prepared in methanol just before use, with a concentration of 1000 µg/ml. All three standards were further diluted using methanol to make a stock solution of 100 µg/ml.

Chromatographic conditions

High Performance Thin Layer Chromatography was performed on a 20 X 10 cm HPTLC Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Three µl of plant extract and twenty µl of formulation extract were separately applied on the plate as 8 mm wide and 10.6 mm apart bands, 8 mm from the bottom with a semi-automatic TLC Sampler Linomat 5 (CAMAG, Switzerland). Different volumes of standard solutions; Lupeol, Oleanolic acid and β-sitosterol were also applied as bands. The TLC plate was dried for 5 minutes after application for spots to dry thoroughly. CAMAG Twin Trough Chamber (20 X 10) was saturated without saturation pad for 10 minutes with mobile phase consisting of n-Hexane : Ethyl acetate : Formic acid (8 : 2 : 0.5 v/v/v). Plate was developed till 8 cm, dried with hair dryer for 5 minutes. Developed plate was derivatized using 10 % Methanolic sulphuric acid, heated on TLC plate heater for110^0 C for 5 minutes and then scanned at 540 nm using TLC Plate Scanner 3 (CAMAG, Switzerland) for densitometric analysis. The plate was photo documented using TLC visualizer (CAMAG, Switzerland).

Method Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.[3] 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.

Specificity

Specificity test was carried out by applying 3µl of *Nyctanthes arbor-tristis* leaf methanolic extract and 20 µl formulation; and 2 µl of each standard solution (100 µl/ml of Lupeol, Oleanolic acid and β-sitosterol), diluent and mobile phase.

Precision

The variability of the method was studied by carrying out repeatability and intermediate precision. Repeatability was carried out in same laboratory, on same day, by analyzing quality control samples containing the mixture of Lupeol, Oleanolic acid and β-sitosterol using optimized chromatographic conditions. The experiment for inter-day precision was carried out using quality control samples of Lupeol, Oleanolic acid and β-sitosterol on different days.

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. For Lupeol, concentrations of 50 ng, 100 ng, 150 ng, 200 ng, 250 ng, 300 ng, 350 ng and 400 ng were selected for linear dynamic range experiment. Concentrations of Oleanolic acid were 100 ng, 200 ng, 300 ng, 400 ng, 500 ng, 600 ng, 700 ng and 800 ng. For β-sitosterol, concentrations of 50 ng, 100 ng, 150 ng, 200 ng, 250 ng300 ng, 350 ng and 400 ng were selected for linear dynamic range experiment. The densitograms were recorded and the peak areas of Lupeol, Oleanolic acid and β-sitosterol for each applied concentration of these standards were noted. The response factors were calculated for each concentration of Lupeol, Oleanolic acid and β-sitosterol by dividing each peak area by concentration of Lupeol, Oleanolic acid and β-sitosterol at that level.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

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.

LOD and LOQ were calculated using the formula;

Assay

Three microliters of sample solution i.e. *Nyctanthes arbor-tristis* leaf extract and 20 microliters of formulation extract were applied separately on the same chromatographic plate along with different concentrations of Lupeol, Oleanolic acid and β-sitosterol and the plate was developed using optimized chromatographic conditions. The peak corresponding to Lupeol, Oleanolic acid and β-sitosterol in the sample solution was identified by comparing the R_f values of the sample, with that of standards. The amount of Lupeol, Oleanolic acid and β-sitosterol present in sample solution was determined from the calibration curve by using the peak area of Lupeol, Oleanolic acid and β-sitosterol generated by the densitogram.

Recovery

The recovery experiment was carried out to check if there is any interference of other constituents with the peaks of Lupeol, Oleanolic acid and β-sitosterol 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. To 3 µl leaf extract and 20 µl of formulation, known amounts of pure standards of Lupeol, Oleanolic acid and β-sitosterol were added at different levels. The sample was then analysed by HPTLC method using the developed optimized chromatographic conditions. Each sample was analyzed in three replicates and the amounts of Lupeol, Oleanolic acid and β-sitosterol recovered for each level, were determined. The value of percentage recovery for the three components was then calculated.

RESULTS AND DISCUSSION

A normal phase high performance thin layer chromatographic (HPTLC) method for the simultaneous quantification of Lupeol, Oleanolic acid and β-sitosterol 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.

R_f value of Oleanolic acid, β-sitosterol and Lupeol was found to be 0.40, 0.49 and 0.574 respectively.

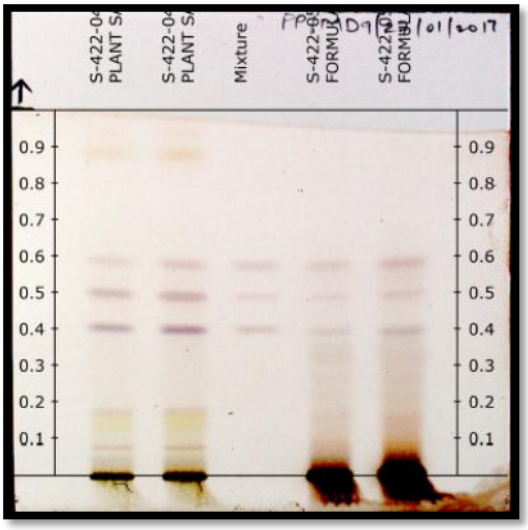

Fig.1: HPTLC plate for Method Development of Oleanolic acid, β-sitosterol and Lupeol. (Track 1,2: Plant extract; Track 3: Standard mixture; Track 4,5: Formulation extract).

The method was validated for specificity, linearity, LOD, LOQ, intra- day and inter-day precision, recovery and stock solution stability. The method was linear from 50 to 400 ng for Lupeol and β-sitosterol and 100 to 800 ng for Oleanolic acid. The limits of detection (LOD) and quantification (LOQ) for Lupeol and β-sitosterol was found to be 16.67 ng and 50 ng respectively and that for Oleanolic acid was found to be 33.33 ng and 100 ng respectively.

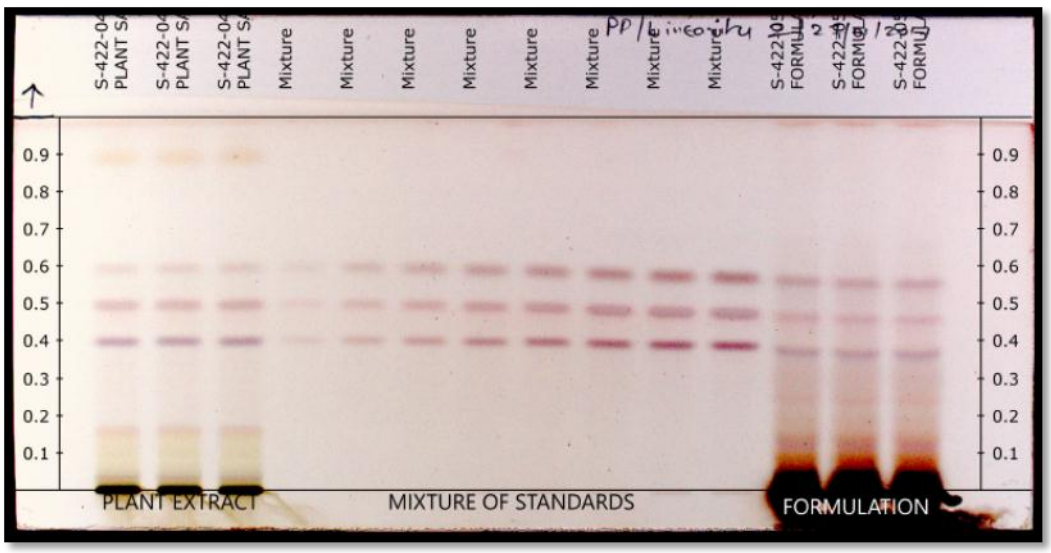

Figure 2: HPTLC plate for Quantitation of Oleanolic acid, β-sitosterol and Lupeol. (Track 1,2,3: Plant extract; Track 8-11: Standard mixture of different concentration; Track 12,13,14: Formulation extract).

The relative standard deviation for inter-day and intra-day precision was <3%. The correlation coefficient was found to be ≥0.99 for all the three components. The precision (% RSD) of the method was found to be < 3%, indicating that the proposed method is precise.

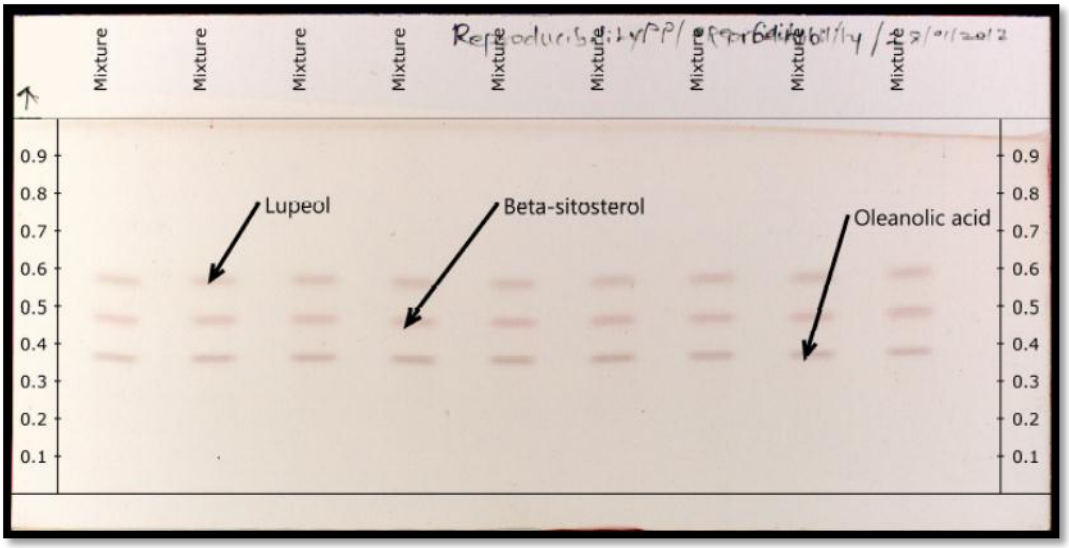

Figure 3: HPTLC plate for Reproducibility of Standards.

(Track 1-9: Standard mixture of different concentration).

The recovery values for all the three components were within acceptable limits (85.0 to 115.0%). Stock solution stability was evaluated by monitoring the peak area response. Standard solutions were analyzed right after its preparation and after 72 hrs. There was no significant change (% RSD ≤ 3%) in the R_f and area values of standard peak.

The quantity of Lupeol, β-sitosterol and Oleanolic acid in leaves of *Nyctanthes arbor-tristis* was found to be 0.03124 %, 0.0777% and 0.1062 % respectively. While the quantity of Lupeol, β-sitosterol and Oleanolic acid in formulation was found to be 0.003054 %, 0.008369 % and 0.008128 % 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 Lupeol, Oleanolic acid and β-sitosterol in the methanolic extract of *Nyctanthes arbor-tristis*.

Table 1: Summary of method validation parameters.

Parameter	Oleanolic Acid	β-sitosterol	Lupeol
Specificity	Specific	Specific	Specific
Precision	<2%	< 2%	<2%
LOD	33.33 ng	16.67 ng	16.67 ng
LOQ	100 ng	50 ng	50 ng
Linearity (ng)	100-800	50-400	50-400
Quantity (Plant)	0.1062 %	0.0777 %	0.03124 %
Quantity (formulation)	0.008128 %	0.008369 %	0.003054 %
Stocksolution stability	Stable till 72 hrs	Stable till 72 hrs	Stable till 72 hrs
Recovery (Plant)	100.08 %	103.02 %	101.05 %
Recovery (Formulation)	104.82 %	103.50 %	100.81 %

CONCLUSION

The developed method in this research work is precise, accurate and reproducible. It is suitable for qualitative and quantitative analysis of Lupeol, Oleanolic acid and β-sitosterol 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.*

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