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IDENTIFICATION AND QUANTIFICATION OF LUPEOL IN FORMULATION CONTAINING FICUS SPECIES USING HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

There is a growing demand for biocompatible natural products and formulations for the treatment of various new era diseases like Cancer, Parkinson's, Alzheimer's, and Diabetes. Plants have many phytochemicals with different bioactivities like antioxidant, anti-inflammatory, and anticancer activities. In the present study, we report the identification and quantification of Lupeol, an anticancer agent, from an Ayurvedic formulation 'Nalpamaradi choorna' and its four Ficus species constituents using High-Performance Thin Layer Chromatography (HPTLC). The chromatographic separation of the methanol extract of the samples was done on TLC alumina plates coated with silica gel 60 F_{254} as the stationary phase with Toluene: Ethyl acetate: Formic acid (5:5:0.5 v/v/v) as the mobile phase. The plates were scanned at reflectance absorbance mode at 540 nm after derivatizing with Anisaldehyde- Sulphuric acid reagent. The system was found to give compact spots with a R_f value of 0.72±0.02. The method was found to be linear in a range of 50-250 ng with a correlation coefficient r = 0.9982. The results showed the presence of 0.021%, 0.024%, 0.019%, 0.0.027% and 0.029% of Lupeol in F. benghalensis, F. racemosa, F.religiosa, F. microcarpa and the formulation respectively.

KEYWORDS: HPTLC, *Nalpamaradi Choorna*, Ficus species, Lupeol, Anticancer activity.

INTRODUCTION

Plant-based medicines have a broad spectrum of activities from ancient times. The plant cells contain many compounds produced by the basic metabolism, which includes all processes

necessary for its survival. Secondary plant metabolites are usually synthesized by special, differentiated cells, to function primarily in defense against predators and pathogens, and attract pollinators and seed dispersers to help reproductive activities. Many of them are highly toxic too.[1] The drug activity of a plant is due to these compounds. Plants have many phytochemicals with various bioactivities like antioxidant, anti-inflammatory and anticancer activities. Extracts from natural products like fruits, vegetables, and medicinal plants have positive effects against cancer compared with chemotherapy or hormonal treatments.[2] Naturally occurring phytochemicals of dietary and non-dietary origin have gained interest in the recent years due to their capability to modulate degenerative diseases like cancer, cardiovascular diseases (CVD), diabetes, arthritis, cataract, aging,and so on. This resurgence of interest in the bioactive phytochemicals could mainly be attributed to the large body of scientific evidence gathered from well-designed epidemiological and experimental studies conducted during the last two decades.[3]

Ficus species are reported as a rich source of naturally occurring antioxidants of which phenolic compounds and flavanoids play a vital role in preventing numerous health disorders related to oxidative stress including cardiovascular diseases, neurodegenerative diseases, and cancer.[4] Among the many Ficus species, the most important are the four trees with milky latex, namely *Ficus racemosa* L., *Ficus microcarpa*, *Ficus religiosa* L., and *Ficus benghalensis* L. that constitute the group "Nalpamara" in Ayurveda. All parts- root, bark, leaf and fruit of these trees have extensive applications in medicine. The bark of these species forms an essential ingredient in many Ayurvedic formulations, like Nalpamaradi choorna, *Nalpamaradi tailam,Chandanasavam, Saribadyasavam,*etc.[5] The bark of these four lactiferous tree species is reported to contain tannin, wax, saponin, steroid, terpenoid, flavonoid, and alkaloid, as the major class of phytochemicals.[6] Various phytochemicals have been found to possess a broad range of activities, which may help in protection against chronic diseases. For example, the terpenoids have been shown to decrease blood sugar level in animal.[7] Steroids, triterpenoids, and Saponins showed the analgesic properties and central nervous system activities.[8-9]

Lupeol, a triterpene had been reported to have anti-arthritic and anti-inflammatory activities.[10] Investigations showed the anti-inflammatory activity of Lupeol and Lupeol acetate to be better than the commonly used non-steroidal drug Indomethacin.[11] Lupeol is reported to have antinociceptive properties during inflammatory pain other than post

operative pain.[12] Lupeol acts as a therapeutic and chemopreventive agent for the treatment of inflammation and cancer. It is also noteworthy that Lupeol at its effective therapeutic doses exhibits no toxicity to normal cells and tissues,[13] and protective against glutamate toxicity.[14] In another study, Lupeol was demonstrated as an antitumor-promoting agent.[15] Waxes of some common fruits, like apple, grape, berry, olive, and tomato,were demonstrated as rich sources of Lupeol.[16] Another study demonstrated that lupeol/mango pulp extract are effective in combating oxidative stress-induced cellular injury of mouse prostate; so could be a potential chemopreventive agent against prostate cancer.[17]

Lupeol was extracted from the stem bark powder of *Crataeva nurvala* and Antitrypsin (Anti-protease) activity was determined by using Folin Lowery method.[18] Even though few research groups have developed HPTLC method for fingerprinting and quantification of bioactive compounds in various formulations and food products,[19-23] the constituents and formulations of Nalpamara are not investigated thoroughly. So we have developed an HPTLC densitometric method for the standardization of *Nalpamaradi choorna*, and the bark powders of *F.benghalensis, F. racemosa, F. religiosa,* and *F. microcarpa* with Lupeol as marker compound.

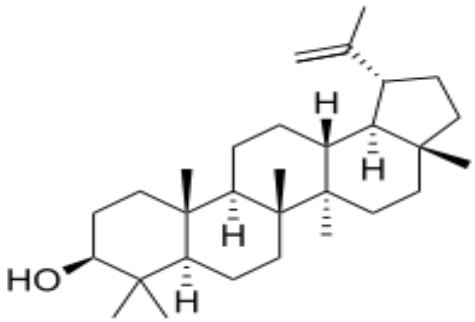

Figure 1: Chemical Structure of Lupeol.

MATERIAL AND METHOD

Chemicals and reagents

Lupeol was purchased from Sigma-Aldrich, Mumbai, India. Toluene, Ethyl acetate, Formic acid, and methanol used were of HPLC grade and were procured from Merck Chemicals, Mumbai. All the other chemicals used were of analytical grade. Silica gel 60 F_{254} TLC plates (20×10cm and 10×10 cm, layer thickness 0.2 mm, Merck, Germany) were used as stationary phase.

Sample preparation

Two samples of Nalpamaradi Choorna were purchased from a local Ayurvedic Pharmacy. Fresh Bark of F. benghalensis, F. racemosa, F. religiosa, and F. microcarpa were collected from Somaiya, Vidyavihar campus, Mumbai, and authenticated by Blatter Herbarium, St. Xaviers College, Mumbai, India. They were dried in the shade, powdered and stored at 25^0C. 2 g each of the powdered drug and the formulations [procured (A &B) and prepared in the laboratory by mixing equal amounts of the four constituents(C)] were extracted with methanol (10 ml × 4) under reflux on a water bath. It was filtered through Whatman I filter paper, filtrates were combined, concentrated under vacuum and the volume was made up to 10 ml in a volumetric flask.

Preparationof standard solution.

Lupeol (5.0mg) was weighed accurately and dissolved in 5.0 ml methanol in a standard measuring flask. This stock solution was further diluted to a concentration of 0.05 µg/µl and used as the standard solution for the HPTLC analysis.

Instrumentation and Chromatographic conditions

The standard and sample were spotted as bands of width 6.0 mm using a CAMAG 100.0 µl sample syringe on silica gel pre-coated Aluminium plate 60 F_{254} TLC plates (20×10 cm) with 0.2 mm thickness, using a CAMAG Linomat V [Switzerland] sample applicator. The plates were activated at 110.0^0C for 5 minutes prior to chromatography. The distance from lower edge was 8.0 mm and from the side 15.0 mm. The distance between the tracks was 10.0 mm. The slit dimensions were 5 mm×0.45 mm, micro and scanning speed 20 mm/s with data resolution 100 µm/step. The mobile phase consisted of Toluene: Ethyl acetate: Formic acid 5:5:0.5 (v/v/v) and 20.0 ml of it was used per chromatographic run. Linear ascending development was done in a 20×10 cm twin trough glass chamber [CAMAG, Switzerland] saturated with the mobile phase for 15 minutes at room temperature [25^0C±2]. Each chromatogram was developed over a distance of 8.0cm followed by drying in a stream of air with the help of hair drier. The plate was dipped in to Anisaldehyde-Sulphuric acid reagent (1.0ml Anisaldehyde reagent added to a mixture of 10.0ml Sulphuric acid and an ice-cold solution of 10.0 ml Acetic acid in 170.0 ml methanol) using the TLC immersion device (CAMAG). The immersion time was 3 s. The plate was heated on a TLC Plate Heater at 110^0C for 5 min to get violet coloured bands. Densitometric scanning was done using a

CAMAG TLC scanner III and scanned at 540 nm in the absorbance mode using Tungsten lamp. Data processing was done with the software platform winCATS (CAMAG).

Linearity and calibration curve

Linearity of the method was evaluated by constructing calibration curve at eight concentrations. Aliquots of standard working solutions of Lupeol [0.5µl to 4.0µl] were applied to the plate to obtain concentrations in the range 50 to 200 ng/spot. The calibration curve was developed by plotting peak area verses concentration with the help of winCATS software.

Sample analysis

10µl of each of the extracts of the three formulations and the four ingredients were spotted on the pre-coated TLC plate in duplicate. The parameter specifications were maintained as stated above. The samples were applied under continuous drying stream of nitrogen gas at a constant application rate. The linearity of the detector response for the prepared standards was assessed by means of linear regression regarding the amounts of each reference standard, measured in ng, and area of the corresponding peak on the chromatogram. After development and scanning of the plate densitometric estimation was carried out based on the peak areas. The peak areas were plotted against the corresponding concentration and least square regression analysis was performed to generate the calibration equation.

RESULT AND DISCUSSION

TLC densitometric quantification of Lupeol using HPTLC.

Terpenoids are biosynthesized from isoprene molecules (CH_2=C(CH_3)-CH=CH_2), significant in plant growth, metabolism, and growth. Terpenoids have cyclic structures with one or more functional groups like hydroxyl, carbonyl, etc. They are found in resins, barks, and latex of trees.[24] They can be extracted with hot methanol. The detection is slightly difficult as they are colourless compounds and not sensitive to universal chromogenic compounds. However, can be detected on TLC plate with H_2SO_4 and heating.

In the present study, a slightly modified method was developed. Initial fingerprinting studies were performed on the pure compound and the conditions were optimized. HPTLC chromatograms of methanol extracts of all the test samples were developed under identical conditions. The visualization of Lupeol bands were made possible with the derivatization using Anisaldehyde- Sulphuric acid reagent and heating at 110^0 C for five minutes. The well

resolved TLC profile of the marker compound corresponding to the individual constituents and the formulation (violet coloured bands) authenticate the presence of the marker in all the samples (Figure 3). The correlation coefficient was found to be 0.9982, which is indicative of peak purity. In the linearity study, response was found to be a linear function of the amount applied on the range 25-250 ng/spot (Table 1). The detectability, ie, LOD and LOQ were determined to be 18.0 and 61.25 ng/spot respectively.

The amount (%) of the bio-active marker in different samples was determined on the basis of the calibration curve as given in Table 2.

Table 1 Linearity study results (n=3)

Parameters	Lupeol
Solvent system	Touene:ethyl acetate: Formic acid (5:5:0.5)
Saturation time	15 minutes
Rf value R_f Value	0.72±0.02
Derivatizing Agent	Anisaldehyde sulphuric acid
Linearity (r)	o.9982
Range	25-250 ng.
LOD	18.00 ng
LOQ	61.25 ng

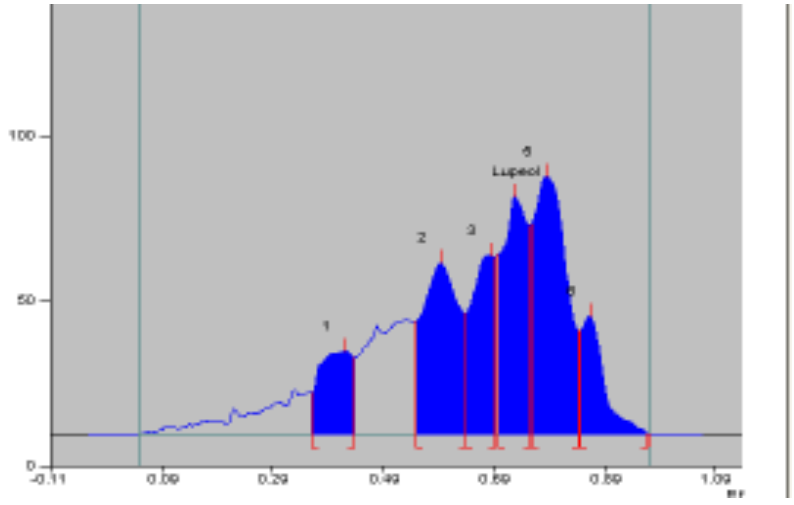

Figure 2: A Representative HPTLC Chromatogram of Formulation C.

Figure 3: HPTLC Fingerprint profile of Standard and samples at 540 nm after derivatization.

Table 2: Analysis of sample.

No.	Sample	Amount of Lupeol [% w/w] (*n=3)
1	F. benghalensis [I]	0.021 ± 0.006
2	F. racemosa [II]	0.024 ± 0.005
3	F. relegiosa [III]	0.019 ±0.007
4	F.microcarpa [IV]	0.027 ± 0.004
5	Formulation (A)	0.0147 ± 0.005
6	Formulation (B)	0.0130 ±0.006
7	Formulation (C)	0.029 ±0.005

***n= no. of times procedure repeated.**

Among the four Ficus species, F. microcarpa contain the highest amount (0.027%) and F. religiosa the least quantity of Lupeol (0.019%). But this is comparable to that reported for F. religiosa by another group (0.020).[25] The Lupeol content in the formulations A & B were found to be lesser than the individual constituents. But C, the one prepared in the laboratory using fresh bark from Somaiya, Vidyavihar Campus demonstrated to be much better (0.029 %) than the marketed samples. It contains a better percentage of Lupeol than the constituents even indicating a strong synergistic effect.

CONCLUSION

A simple HPTLC method was developed for the quantification of Lupeol, a triterpenoid from Nalpamaradi choorna, an Ayurvedic formulation and its Ficus constituents. Lupeol, has been reported to have a broad range of medicinal properties that include strong antioxidant, anti-mutagenic, anti-inflammatory and anti-arthritic effects. The present study confirms the

presence of Lupeol in the tested samples. The medicinal properties of these Ficus species plants may be related to their bioactive compounds. Lupeol being one of the bioactive compounds, could be responsible for the biological activities of these plants. This feature makes these common plants promising candidates for further studies. Application of this developed method on isolation and pharmacological studies of this compound could lead to the discovery of modern medicine with less side effects for the treatment of inflammation and cancer as it is a chemopreventive and therapeutic agent.

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