Research Article

Development and Validation of a Densitometric HPTLC Method for Estimation of Resveratrol *in Vitis vinifera* Linn. and in a Polyherbal Formulation

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Abstract

Pancharishta is an Ayurvedic polyherbal formulation contains Draksha (*Vitis vinifera* L., Vitaceae) as one of the key ingredients prescribed for digestive impairment, respiratory disorders and weakness. Draksha contains resveratrol as biologically active compounds. Therefore, it is necessary to carry out the standardisation of bioactive marker compounds present in the polyherbal ayurvedic formulation like Pancharishta. The aim of the present work was to develop and validate a HPTLC method for determination of resveratrol in both commercially available marketed formulation and the herbs used in the formulation. Quantification of bioactive marker resveratrol using HPTLC in Pancharishta had never been reported previously. The method employed silica gel precoated thin layer chromatography plates with $60F_{254}$ as the stationary phase. The respective mobile phases were used to develop the plates which separated bands according to the marker compound. Camag scanner IV was used for densitometric scanning. Further, the method was validated according to the International Conference of Harmonization (ICH) guidelines. Correlation coefficients were calculated from the standard graph of linearity. Accuracy, precision and recovery were all within the required limits. The developed HPTLC methods for bioactive marker compounds present in marketed formulations and herbs were found to be simple, accurate, precise and robust

Keywords: HPTLC, Poly herbal formulation, Resveratrol, Quantification

1. Introduction

Herbal medicine is the fulcrum of complementary and alternative medicine. In spite of the enormous improvements in modern medicine, a large number of populations in the developing as well as developed countries still have faith on herbal medicines for primary health care. According to the World Health Organization (WHO) report, it is estimated that about 80% of the population living in developing countries use extensively traditional and alternative medicines for their primary healthcare. The high cost and the side effects of modern medicines attract traditional and alternative medicine systems in our societies [1]. In contrast to the modern medicine which often consist primarily of single chemical entity (pure compounds), herbal formulations are typically made up of several compounds usually in the crude state. Many finished herbal formulations are made from folk recipes often containing more than one herbal material as the active entity in the form of generic and ethical range for the treatment of different ailments [2].

The fruits of the *Vitis vinifera* L. (Family: Vitaceae), are commonly known as Draksha (raisins) in the Indian sub-continent are used in traditional Ayurvedic formulation to treat respiratory disorders, digestive disorders and general weakness. Pancharishata is an Ayurvedic polyherbal alcoholic formulation included in the Ayurvedic Pharmacopoeia of India in which dried fruits of *V. vinifera* are used as one of the key ingredients. Resveratrol (Figure 1), the main biomarker of *V. vinifera* has become one of the most studied natural polyphenolic compound belongs to stilbenoid class [3]. It is a phytoalexin, which is a group of low molecular weight secondary

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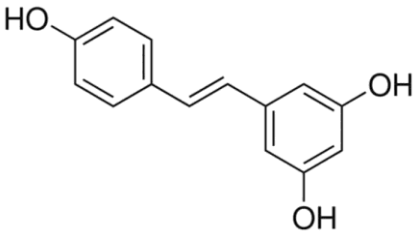

Figure 1: The Chemical Structure of Resveratrol

metabolites of plants, biosynthesized naturally by several plants in response to pathogen infection, traumatic damage, ultraviolet (UV) irradiation, and other stresses [4,5,6]. It has gained significant global attention due to its promising biological properties including antioxidant, anticancer, cardio protective, anti-inflammatory, antiplatelet, antiviral action and life span extension in diverse organisms, from yeast to vertebrates [7 - 15]. Moreover, it is observed that it has the synergistic anti-HIV with Didanosine [16]. In addition, ethanol extract of black grape showed significant activity against *E. coli*, *S. aureus*, *P. aeroginosa* and *H. pylori* [17].

Enormous effort is required to maintain the consistent quality and therapeutic efficacy of Ayurvedic polyherbal formulations. However, most of the conclusions drawn in the Ayurvedic texts are based on the ancient knowledge and clinical observations; they lack the modern observations of analytical methods during preparation of a drug [18].
Therefore, standardization plays a key role to prove the uniform composition of the formulation as well as to get efficacious result. It is important to ensure the presence of an individual component in the finished formulation which could be introduced as a rapid tool during in-process quality evaluation especially for formulations having multiple herbs.

Nevertheless, no ready reference is available to confirm the presence of each and every individual component except marker-based standardization which needs both sophisticated instruments along-with reference standard of each herb. TLC can play an important role to ensure the presence of an individual ingredient in the formulation. Hence, the present study is aimed to provide a simple method to identify the presence of an individual component in the finished formulation by comparing both the retention factor (RF) and reflectance spectrum of each ingredient with those of the finished formulation to ensure the presence of the same in the finished formulation.

2. Materials and Methods

2.1. Apparatus

Soxhlet apparatus (Borosil, Mumbai, India), rotary vacuum evaporator (R-300, Flavil, Switzerland), water bath, sonicator (Trans-O-Sonic, Mumbai, India), and weighing balance (Sartorius, Göttlingen, Germany) were used for general experiments. CAMAG (Muttenz, Switzerland) Linomat 5 sample applicator, CAMAG TLC Scanner 4, and CAMAG Photo document chamber were used for HPTLC analysis.

2.2. Chemicals, reagents and raw materials

Solvents namely Toluene, ethyl acetate, formic acid, methanol and pre-coated aluminum silica gel 60F254 TLC plates were procured from Merck Specialties Private Limited (Karnataka, India).Resveratrol (≥99% purity) was purchased from Sigma Aldrich. Anisaldehyde reagent was procured from Sigma-Aldrich (St. Louis, MO, USA). *Vitis vinifera* L. fruit was obtained from Kolkata, West Bengal, India. The plant species were authenticated by a botanist from Corporate Analytical Design Excellence, Emami Limited, Kolkata and the crude drug repository number is CADE/COG/PRS/067.

2.3. Preparation of test sample

The three batches of commercial formulations of 100 ml each were fractionated with 100 ml ethyl acetate separately. The process was repeated thrice for each batch. Combined the ethyl acetate fractions of each batch separately and concentrated by rotary vacuum evaporator. Reconstitute the ethyl acetate fractions with 10 ml methanol. The concentrations of three in-house formulations obtained were 62, 67 and 71 mg/ml respectively.

Accurately weighted 1.0 g of crushed fruit was taken into a 50 ml conical flask. 25 ml methanol was added and sonicated in a sonicator for 15 minutes, followed by warming on water bath for another 15 minutes. The solution was allowed to cool, filtered through Whatman No 1 filter paper into 25 ml volumetric flask, and made up the volume with methanol.

2.4. Preparation of standard solution

A common stock solution (1 mg/ml) of resveratrol was prepared by dissolving 10 mg in methanol and making the volume of solution up to 10 ml. The working standard solution of 0.1 mg/ml was prepared by diluting 10 times the stock solution with methanol.

2.5. HPTLC conditions

The chromatographic estimation was performed by spotting marker compound, reference crude herb and three batches

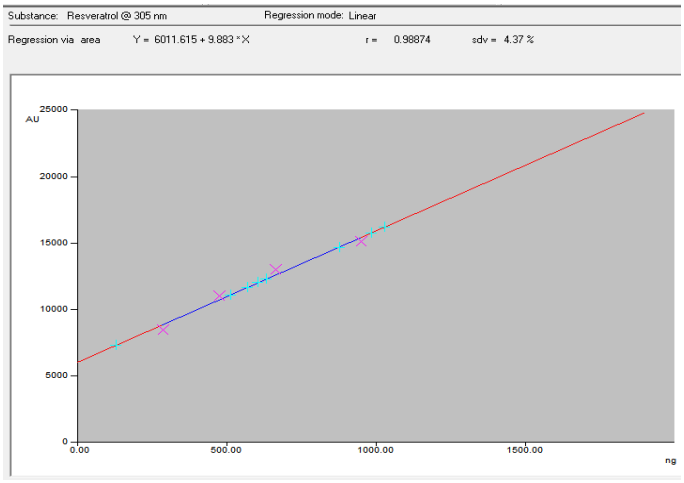

Figure 3: Calibration curve of Resveratrol

Table 1: Results of sensitivity study

Linearity range	r	Slope	Intercept
0.3 µg to 1.0 µg	0.989	9.883	6011.61

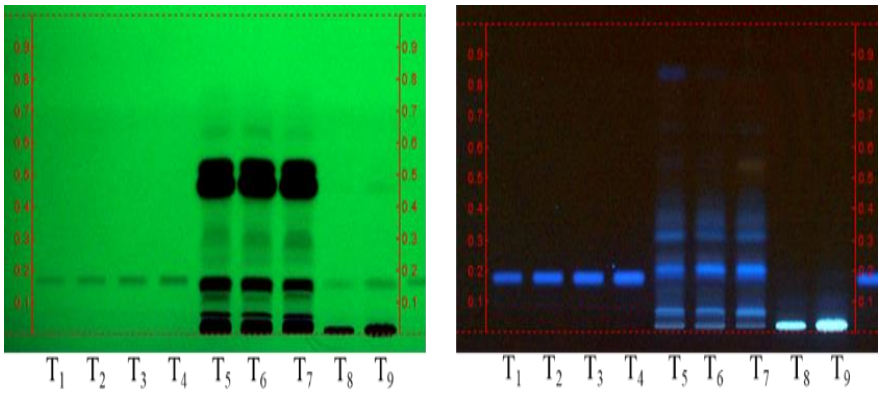

Figure 2: HPTLC photo document (visible in 254 nm & 366 nm) of resveratrol, polyherbal formulation (Pancharishta) and *Vitis vinifera* L. T1-T4: Resveratrol standard; T5-T7: Pancharishta; T8-T9: *Vitis vinifera* L.

of formulation samples on pre-coated aluminum TLC plates of silica gel $60F_{254}$ (20 cm x 10 cm, E. Merck) using a CAMAG Linomat V sample applicator and a 100 mL syringe. The samples, in the form of band length 8 mm, were spotted at a constant application rate of 80 nL/s using nitrogen aspirator. The plates were developed using the mobile phase toluene: ethyl acetate: formic acid (2.5:1:0.1, v/v/v). Linear ascending development was carried out in 20 cm x 10 cm twin-trough glass chamber (CAMAG) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 15 min at room temperature. The length of the mobile phase run was 70 mm. Approximately 20 mL of the mobile phase was used for development. After the development, TLC plates were dried with an air-dryer for 5 min. Then, the spots were visualized at 254 and 366 nm (Figure 2). Camag Scanner controlled by winCATS Planar Chromatography manager software version 1.4.9 was used as a densitometric scanner. The slit dimensions were 6 × 0.45 mm and the scanning speed 20 mm/s. The radiation source used was a deuterium lamp at a wavelength of 305 nm for resveratrol.

2.6. Statistical analysis

All measured data was expressed as: mean ± standard deviation (S.D.), n = 3. The calculation was carried out using MedCalc software.

3. Method validation

3.1. Calibration curve

The calibration curve of resveratrol was obtained from standard concentrations of 0.3 µg to 1.0 µg, which were prepared from the stock solution of 0.1 mg/mL. An amount of 10 µL of each solution was spotted on the TLC plate to obtain a linear range of 300–1000 ng/spot. The data of peak area versus sample concentration were treated by linear regression analysis (Figure 3).

3.2. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on three and ten times of the noise levels, respectively.

3.3. Specificity

The specificity of the method was established by analysing resveratrol and test samples.

Table 2: Results of recovery study

V. Vinifera [mg]	Resveratrol added [mg]	Amount spotted [mg]	Amount of Resveratrol detected [µg] (mean ± SD, n = 3)	%RSD	Recovery (%)
1000	1	800	0.94±0.02	2.25	98.92

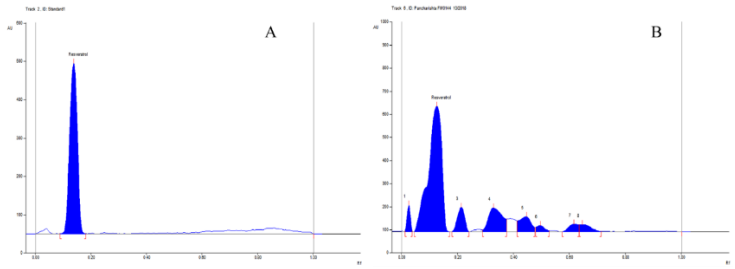

Figure 4: HPTLC chromatogram of (A) Resveratrol and (B) Pancharishta.

Table 3: Precision and accuracy

The actual amount of standard spotted (ng)	The amount detected (ng ± SD, n = 3)	% RSD
500	513.62 ± 4.30	0.84
700	713.82 ± 9.21	1.29
1000	946.61 ± 9.09	0.96

The band for resveratrol in the test samples was confirmed by comparing the RF and spectra of the spot with those of the reference standard (Figure. 4A and 4B). The peak purity of the marker compound was assessed by comparing the spectra at peak start, peak apex, and peak end of the band. HPTLC spectra and 3D overlay of standard and samples were the same as shown in Figure 5.

3.4. Precision

The repeatability of measurement (n = 3) of the peak area for Resveratrol was expressed in terms of the relative standard deviation (% RSD). The intra-day and inter-day variation studies were carried out at three different concentration levels.

3.5. Recovery

Recovery was determined by spiking Resveratrol (1.0 mg) to the herb powder (1.0 g) before extraction. The extract was evaporated and transferred to a 10 mL volumetric flask in methanol. The sample was applied (20 µL) in triplicates and analysed. The recovery was calculated by comparing the same samples without spiking. (Table 2)

4. Results and discussion

4.1. Sensitivity

The sensitivity of the method was determined concerning LOD, LOQ, linearity range, and correlation coefficient. Solutions containing 0.3–1.0 µg of the standard were spotted on a TLC plate. The LOD was calculated as 14.59 ng (3.3 times the noise level), and LOQ was calculated as 44.21 ng (10 times the noise level). The regression data for the three samples are given in Table 2, showing a good linear relationship in that range studied. The regression data of the samples are given in Table 1, showing a good linear relationship in that range studied.

4.2. Recovery study

Recovery of the standard was calculated by spiking 1.0 mg to 1000 mg. The recovery was found to be 98.92% w/w (Table 2).

4.3. Precision and accuracy

Different amounts of spiked samples were spotted on a TLC plate. These spots were analysed by using the above described HPTLC method (Table 3). Precision was expressed as the %

4.4. Reproducibility

The repeatability was evaluated by analysing the known amounts of spiked sample spotted on the TLC plate in replicates Reproducibility (n = 3). Inter-day and intra-day precisions were evaluated by analysing the same amount of

Table 4: Reproducibility

The actual amount of standard spotted (ng)	The amount detected (ng ± SD, n = 3)	% RSD
Inter day (n=3)		
500	513.62 ± 4.30	0.84
700	713.82 ± 9.21	1.29
1000	946.61 ± 9.09	0.96
Intraday		
500	510.82 ± 5.38	1.05
700	712.68 ± 9.27	1.3
1000	960.6 ± 12.44	1.29

analyte for 3 days (n = 3) and they were expressed in terms of % RSD (Table 4).RSD.

4.5. Sample analysis

The validated HPTLC method was applied for a quantitative evaluation of Resveratrol in the dried fruit of *V. vinifera* and Zandu Pancharishata as shown in Table 5. The Resveratrol was found to be present in the dried fruit of *V. vinifera* L. (0.02% w/w) and 0.28% w/w in the Zandu Pancharishta.

5. Conclusion

The identification and quantification of active ingredients in polyherbal Ayurvedic formulations like arishtas can be evaluated by use of validated analytical methods. A new HPTLC method has been developed for the identification and quantification of Resveratrol in herbs and marketed poly herbal formulations of Pancharishta. Low cost, faster speed, and satisfactory precision and accuracy are the main features of this method. The method was successfully validated as per ICH guidelines and statistical analysis proves that the method is sensitive, specific, repeatable and robust. This rapid and reproducible method can be successfully used for quality control and quality assurance of the herbs as well as different Ayurvedic formulations.

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Conflict of Interest;

No potential conflict of interest was reported by the authors.

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