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Semi-preparative HPLC preparation and HPTLC quantification of tetrahydroamentoflavone as marker in *Semecarpus anacardium* and its polyherbal formulations

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ABSTRACT

Application of modern scientific knowledge coupled with sensitive analytical technique is important for the quality evaluation and standardization of polyherbal formulations. *Semecarpus anacardium*, an important medicinal plant with wide medicinal properties, is frequently used in a large number of traditional herbal preparations. Tetrahydroamentoflavone (THA), a major bioactive biflavonoid was selected as a chemical marker of *S. anacardium* and RP-semi-preparative HPLC conditions were optimized for the isolation of tetrahydroamentoflavone. HPTLC analytical method was developed for the fingerprinting of *S. anacardium* flavonoids and quantification of tetrahydroamentoflavone. The method was validated in terms of their linearity, LOD, LOQ, precision and accuracy and compared with RP-HPLC-DAD method. The methods were demonstrated for the chemical fingerprinting of *S. anacardium* plant parts and some commercial polyherbal formulations and the amount of tetrahydroamentoflavone was quantified. HPTLC analysis showed that *S. anacardium* seed contained approximately 10 g kg^{-1} of tetrahydroamentoflavone. The methods were able to identify and quantify tetrahydroamentoflavone from complex mixtures of phytochemicals and could be extended to the marker-based standardization of polyherbal formulations, containing *S. anacardium*.

1. Introduction

In the recent past there has been resurgence in interest in natural products consequent to the emerging evidence to associate consumption of dietary and non-dietary phytochemicals with modulation of degenerative diseases. Herbal products supported by scientific evidence have been gaining consumer confidence as complimentary and alternative medicine (CAM) for preventive health and disease management even in developed countries. Though traditional medicines specific to each country and culture have been in vague for centuries they have not been standardized and validated for preventive or therapeutic efficacy. For instance, Indian traditional medicine known as 'Ayurveda' (Knowledge of life) has been practiced during last 2500 years and 70% of Indian population is still dependent on this system for primary healthcare. This is true for many developing countries comprising large majority of world population. The world trade in herbal products is growing rapidly as a result of popularization of CAM, but the information on the products suffers from lack of the knowledge on active principles, validation, etc. Plants, and plant products are subject to wide variation in their phytochemical profile due to their variety, climatic conditions, maturity, post-harvest processing, storage, stability, etc. It is extremely important to standardize the ingredients and formulations based on marker compounds specific to each plant followed by validation for their efficacy.

Seeds of *Semecarpus anacardium* (Marking nut) are used in Indian traditional medicines (Ayurveda and Sidha) either alone or as an ingredient of many polyherbal formulation for treating various ailments. *S. ancardium* of Anacardiaceae family is a medium sized tree grown in arid parts of tropical and subtropical regions. Ayurveda describes it as a potent drug for neuritis, arthritis, leprosy, helmintic infection and venereal disorders [1–4]. But supporting data are lacking. Recently antioxidant [5,6], anti-inflammatory [7], anti-cancer [8,9], antibacterial [10], anti-rheumatic [11] and anti-helminthic [4] activities of its seeds have been reported. A variety of flavonoids such as tetrahydroamentoflavone (THA) [12], jeediflavanone [13,14], semicarpouflavonone [15], galluflavonone [16], nallaflavonone [17], semecarpetin [18] and anacardioflavonone [19] along with other phenolic compounds such as bhilawanols and anacardic acids [20] have been reported. However standardized and validated methods for their quantification are not available. Among the flavonoids reported tetrahydroamentoflavone is a major

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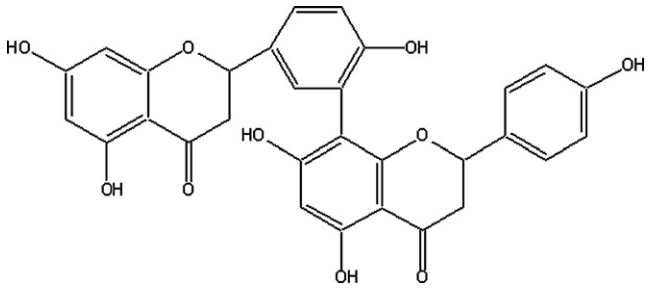

Fig. 1. Tetrahydroamentoflavone.

biflavonoid to which the therapeutic effects are attributed. The main objective of the present study was to isolate pure THA (Fig. 1) and develop a sensitive, reproducible and fast method for quantification of THA as marker compound using HPTLC that enables high throughput analysis. The seeds of *S. anacardium* are used in several commercial polyherbal formulations and the method developed should be able to track THA in trace quantity in such complex systems to validate the method. Separate method using RP-HPLC-DAD was also used for comparison of the results to further validate the HPTLC method.

2. Experimental

2.1. Materials

The seeds of the plant *S. anacardium* were collected from authorized herbal suppliers at Trivandrum, India in December 2006 and authenticated. The plant materials were dried, powdered and stored in airtight containers at 4 °C.

2.2. Semi-preparative HPLC isolation of THA from S. anacardium seeds

S. anacardium seed powder (200 g) was defatted with hexane (1l) in a Soxhlet apparatus. The powder thus obtained was homogenized at 200 r.p.m. with methanol (5 × 500 ml) at room temperature. The extracts were pooled, centrifuged at 2000 × *g* and evaporated at ≤45 °C under vacuum. The methanol extract thus prepared was redissolved in methanol, filtered through 0.45 μm PTFE filter and subjected to semi-preparative HPLC, under the conditions given below.

HPLC System	Shimadzu (Japan) LC-8A system equipped with a binary pump, a Rheodyne injector (Rheodyne, California, USA)
Detector	Diode array detector (Shimadzu, SPD-M10A)
Injection volume	5 ml
Column	Reverse phase C-18 Phenomenex (15 μm, 250 mm × 21.20 mm) column

Time (min)	Water A (%)	Methanol B (%)
Mobile phase (gradient), flow rate 25 ml min^{-1}		
0	70	30
5	70	30
20	50	50
30	0	100
55	0	100
60	70	30

Chromatogram was monitored at 290 nm and UV–vis spectra of major peaks were recorded. The peak at 12 min with biflavonoid type UV-spectra was separated and identified as THA by ^{1}H-and ^{13}C NMR (Avance DPX 300, Bruker, UK), MS (The MS Route JMS 600H, JEOL, Japan) and FT-IR (PerkinElmer 100 series). It was further purified (95%) by crystallization from methanol and used as the reference compound.

2.3. Preparation of reference THA solutions

A reference stock solution with a concentration of 1 mg ml^{-1} of THA was prepared in methanol. Various concentrations of the standard solutions (1–300 μg ml^{-1}) were prepared with appropriate dilution of the stock solution in initial mobile phase for HPLC. For HPTLC analysis 50–500 μg ml^{-1} solutions of THA in methanol were prepared. The solutions were filtered through 0.45 μm PTFE filter prior to analysis.

2.4. HPTLC instrumentation

Silica gel HPTLC plates (Kieselgel 60F$_{254}$, 20 cm × 20 cm, 0.2 mm thickness, Merck, Darmstadt, Germany) prewashed with methanol and kept at 60 °C for 30 min were used for the analysis. The samples were spotted in the form of bands of width 6 mm with a Hamilton micro liter syringe using a Camag Linomat V (Switzerland). A constant application rate of 0.1 μl s^{-1} was employed and space between two bands was maintained as 5 mm. The plates were developed in an ascending manner with a solvent system consisting of toluene–acetone-acetic acid (7.5:2:0.5, v/v) in a development chamber presaturated with the mobile phase. The developed plates were air dried and scanned at 290 nm (TLC Scanner 3, Camag, Switzerland). The slit dimension was kept at 5 mm × 0.45 mm and 10 mm s^{-1} scanning speed was employed. The analysis was performed in an air-conditioned room maintained at 22 °C and 65% relative humidity. A minimum of three spottings were done for each reference and unknown sample. Data processing was performed using the software 'win CATS planar chromatography manager' (version 1.4.3). In order to establish the purity of resolved HPTLC bands, UV–vis spectra of the THA bands were obtained using Camag TLC Scanner 3. THA bands in the plates were located by comparing them with reference compound in a UV chamber and marked. The bands corresponding to THA were scrapped off from plates, eluted with methanol and spectral analyses were conducted.

2.5. HPLC instrumentation

Analytical HPLC was done using a Shimadzu (Japan) LC-8A system equipped with a binary pump (LC-8A), a Rheodyne injector (California, USA) provided with 20 μl loop, a column temperature controller (CTO 10 AV), diode array detector (SPD-M10A) and a C18 reverse phase column (Phenomenex (Torrance, USA) ODS-2.5 μm, 250 mm × 4.6 mm) and the system was controlled by Class-VP software. The reference THA solutions and methanol extract of *S. anacardium* seed were injected into the HPLC and the mobile phase was optimized as gradient of acetonitrile (A) and 1% acetic acid in water (B). The optimized gradient elution was as follows; 0–8 min 20% B, 15 min 40% B, 40 min 100% B; 60 min 100% B and 65 min 20% B. The temperature of the column was maintained at 35° C. A minimum of three injections were done for each reference and unknown sample.

2.6. Method validation

The specificity of the methods was ascertained by comparing the retention data and spectra of the peaks with those of reference THA. The peak purity was assessed by comparing the spectra at three different levels, i.e. start, apex and end positions of the chromatogram corresponding to THA. Linearity of HPLC and HPTLC methods were obtained by determining the detector

responses against a series of varying concentrations of reference THA. 5 analyses per concentration were conducted and calibration plots were constructed. Limits of detection and quantification of the methods were calculated using the equations LOD = 3.3 σ/S and LOQ = 10 σ/S where, σ is standard deviation of response and S is the slope of calibration curve. The precision of the methods was validated in terms of repeatability and intermediate precision. Repeatability was studied by analyzing 3 different methanol extracts of seeds each extract 3 times on the same day. Intermediate precision included the analysis of the same three extracts and each of them analyzed three times a day over three days by different analyst. The results of repeatability and intermediate precision are expressed as %R.S.D. The concentrations of methanol extracts were selected so that their THA contents were in experimental range. Accuracy of the methods was determined by standard addition techniques. Known amounts of reference THA in a range of low, medium and high concentrations were added to preanalyzed samples of seed methanol extracts and analyzed under the optimized conditions. Addition experiments for each concentration were performed in triplicate and the accuracy was calculated as the % of analyte recovered. Three analyses per concentration were performed and mean ± S.D. was determined. Robustness of the methods was determined by introducing small changes in certain chromatographic parameters and expressed in terms of %R.S.D.

2.7. Analysis of polyherbal formulations containing S. anacardium

Samples of polyherbal formulations (50 g) were extracted with methanol (5 × 500 ml) at room temperature. The extracts were centrifuged at 3000 × g for 15 min and supernatants were filtered, evaporated at ≤45 °C and made up to 50 ml with methanol. The extracts were filtered through 0.45 μm filters and analyzed by HPTLC and HPLC. The analysis was repeated five times and mean ± S.D. values were determined.

2.8. Statistical analysis

Statistical analysis was performed with Microsoft Excel 2003 and Microcal origin 6.0.

3. Results and discussion

3.1. Isolation of THA

Biflavanoids are polyphenolic molecules comprised of two identical or non-identical flavonoid units conjoined in a symmetrical or unsymmetrical manner through an alkyl or an alkoxy-based links of varying length. These possibilities introduce significant structural variation in biflavanoids, which is further amplified by the positions of functional groups – hydroxy, methoxy, keto, or double bond – and stereogenic centers on the flavanoid scaffold. In combination, the class of biflavanoids represents a library of structurally diverse molecules, which remains to be fully exploited. Seeds of *S. anacardium* plants contain an array of bioactive biflavonoids. Biflavonoids are moderately polar in nature, hence a reverse phase column was selected as stationary phase for the profiling and various proportions of methanol and water were tried as mobile phase to optimize. UV–vis spectra of the major peaks in the chromatogram, were recorded using DAD and used as a primary identification tool. The methanol extract of the seed was used to isolate THA from other biflavonoids on preparative scale using HPLC as described under Section 2.2. The peaks with characteristic biflavonoid spectra were selected and separated. Among the selected peaks, the peak at 12 min was identified as THA by spectral studies. The purity and the identity of isolated THA were established by various spectral data as presented below.

MS (FAB):	543 (M^+ + 1), 307.36, 176.21, 154.20
UV λ_{max}:	290 nm
^{1}H NMR (CD_3OD, 300 MHz):	δ 7.1(4H, m), 6.78 (1H, d), 6.6 (2H, d), 5.9 (1H, s), 5.7 (2H, s), 5.2 (2H, m), 2.9 (2H, m), 2.75 (2H, m)
FT-IR (cm^{-1}):	3436, 1639, 1518, 1163

THA was previously reported as a major bioactive compound in *S. anacardium* plant and not so common in other plants [12]. THA therefore could be considered as a chemical marker of *S. anacardium* plant. The THA thus isolated was used as reference compound for further studies. The method could be used for isolation of other biflavonoids in *S. anacardium* by slightly modifying the mobile phase which was not attempted here.

3.2. Optimization of HPTLC densitometric method

The reference THA and the seed methanol extracts were spotted on HPTLC plate and initially toluene–acetone–methanol in varying ratios was tried as the mobile phase. Replacing methanol with acetic acid improved the resolution between bands. Finally, the mobile phase consisting of toluene–acetone–acetic acid (7.5:2:0.5, v/v/v) gave a sharp, symmetric and well resolved peak for THA with an Rf value of 0.56 which was identical with that of reference THA (Fig. 2). In order to further establish the selectivity of the method, the band corresponding to THA in the methanol extract was separated and subjected to NMR and MS spectral analysis. The spectral data obtained were identical with those for the reference THA (Section 3.1). ^{1}H NMR and MS (FAB) signals obtained for the bands of THA were compared with those of the reference compound and previous report [12]. All signals were found to belong to THA which further confirms the purity and identity of the band.

3.3. RP-HPLC-DAD analysis of THA

RP-HPLC-DAD method was also developed to validate the HPTLC method. Acetonitrile and water mixtures with varying ratios were tried as mobile phases using reverse phase column for profiling of the methanol extract of *S. anacardium* seed. Substitution of water with 1% acetic acid resulted in improved resolution. A gradient mobile phase of acetonitrile with 1% acetic acid was optimized so as to obtain a complete biflavonoid profile. With this optimized gradient mobile phase THA was found to elute at 27 min as a symmetric and well-resolved peak, identical with that of reference THA as confirmed by spiking the sample with pure THA. Selectivity of the method was assessed by evaluating the similarity (≥95%) between UV spectra at start, middle and end of the THA peak.

3.4. Validation of methods

The developed HPTLC and HPLC methods were validated in terms of their linearity, limits of detection and quantification, precision, accuracy and robustness as detailed below.

3.4.1. Linearity

Linearity between the detector response and concentration of THA (50, 100, 200, 300, 400 and 500 μg ml^{-1}) in HPTLC analysis was evaluated. It was found to be linear over a range of 50–400 μg ml^{-1} with a correlation coefficient r = 0.9984. The LOD and LOQ of the

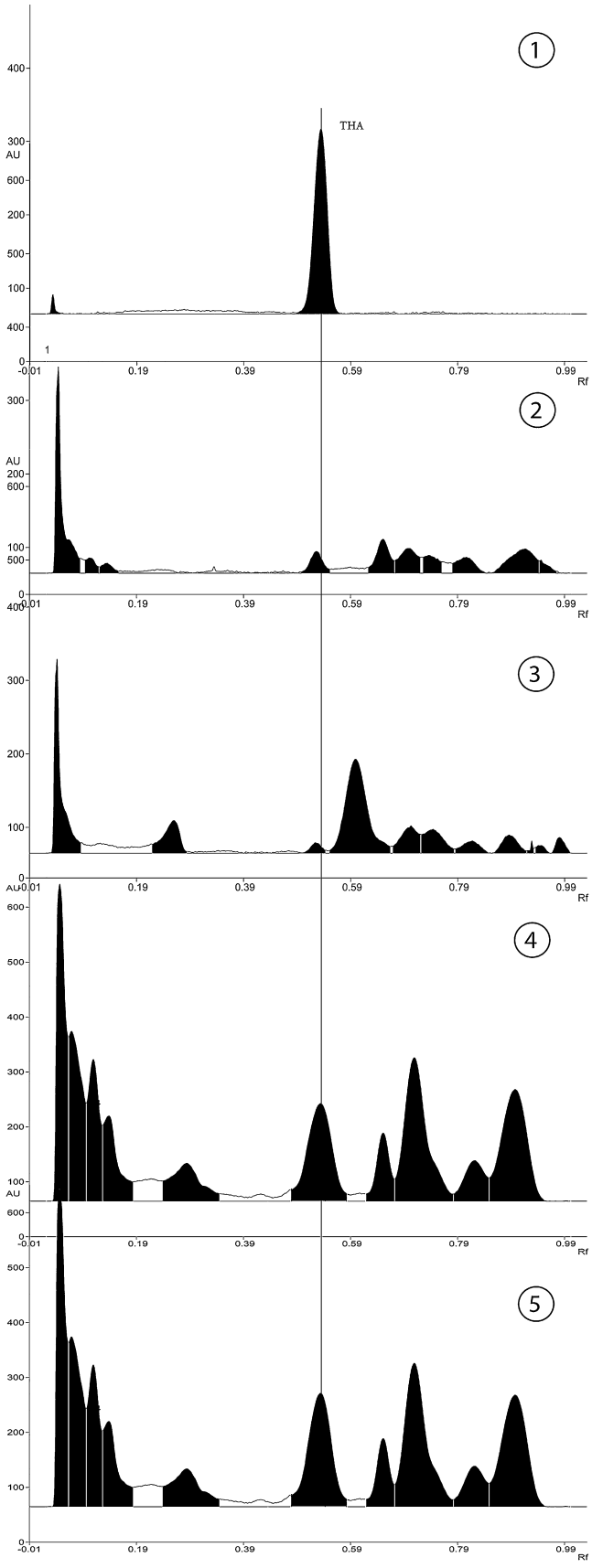

Fig. 2. HPTLC chromatograms of methanol extracts of polyherbal formulations and *S. anacardium* seed at 290 nm. (1) THA, (2) Gulugulu Thithakashayam, (3) Varanadhi Ghrudam, (4) *S. anacardium* seed and (5) seed methanol extract spiked with THA.

Table 1
Linearity and limits of detection and quantification of HPLC and HPTLC analysis of THA

Parameters	HPTLC	HPLC
Regression equation[a]	$y = 110x + 1547$	$y = 21692x - 39092$
Linear range ($\mu g\,ml^{-1}$)	50–400	5–300
Correlation coefficient (r)	0.9984	0.9941
LOD ($\mu g\,ml^{-1}$)	2.1	0.4
LOQ ($\mu g\,ml^{-1}$)	6.3	1.2

[a] y = peak area, x = concentration of THA in $\mu g\,ml^{-1}$.

method were respectively 2.1 and 6.93 $\mu g\,ml^{-1}$ (Table 1). For HPLC method the concentration of THA and peak area at 290 nm were linear in the range of 5–300 $\mu g\,ml^{-1}$, with a correlation coefficient of 0.9941, which is within the analytical limit. LOD and LOQ were 0.4 and 1.2 $\mu g\,ml^{-1}$ (Table 1), respectively.

3.4.2. Precision

Repeatability and intermediate precision of the methods are presented in Table 2. For HPTLC method repeatability and intermediate precision were respectively 1.32 and 1.65%. Repeatability and intermediate precision values obtained for the HPLC method were also in acceptable range.

3.4.3. Accuracy

The accuracy of the methods, assessed as % recovery for low, medium and high concentrations of THA (Table 3). For HPTLC, the recovery percentage of 100.6, 103.2 and 106.2% were obtained for low, medium and high concentrations of THA respectively (Table 3). HPTLC method showed % recoveries ranging from 99 to 101% (Table 3).

3.4.4. Robustness

For HPTLC method, robustness was validated by varying time from spotting to chromatography and from chromatography to scanning (0, 20, 40 and 60 min), composition and amount of mobile phase (±10%), temperature (±2 °C). dimension of HPTLC plate, development chamber (10 cm × 10 cm and 20 cm × 20 cm) and saturation time (±5 °C). The variations in HPTLC analysis due to these changes were ≤1.7 (%R.S.D.). For assessing the robustness of HPLC method, variations in flow rate of mobile phase (±10%), composition of acetic acid in mobile phase (±10%), column temperature (±2 °C) and detection wavelength (±2 nm) and their dependence on Tr and peak area were evaluated. The value obtained for the robustness was ≤1.4 (% R.S.D.) and it is within the limit required for HPLC analysis.

Table 2
Precision (R.S.D.%)

Precision	HPTLC	HPLC
Repeatability	1.32	0.96
Intermediate precision	1.65	1.49

Table 3
Accuracy

Excess of THA added (%)	% Recovery	
	HPTLC	HPLC
50	100.6	101.1
100	103.2	99.7
150	106.2	100.5

Table 4
Amount of THA in *S. anacardium* seeds and commercial polyhedral formulations (Ayurveda) (mean ± S.D.)

Material/formulations	THA (mg kg^{-1})	
	HPTLC	HPLC
Seeds	9870 ± 23	9986 ± 21
Gulugulu Thithakashayam-1	49.2 ± 1.2	51.8 ± 1.3
Gulugulu Thithakashayam-2	53.7 ± 0.9	54.6 ± 1.5
Gulugulu Thithakashayam-3	43.4 ± 0.6	45.3 ± 0.8
Varanadhi Ghrudam-1	8.4 ± 1.8	11.6 ± 1.2
Varanadhi Ghrudam-2	20.2 ± 1.6	21.6 ± 1.3

3.5. Analysis of *S. anacardium* seeds and its polyherbal formulations

Ayurvedic formulations are prepared primarily as water or alcohol extract following the traditional method. Obviously most of the phytochemical extracts would be predominantly polar in nature. Methanol extract of seeds of *S. anacardium* and formulations were profiled using HPTLC and HPLC following the methods developed here and amount of THA was estimated. Peak corresponding to THA was identified by comparing its retention data and UV spectra with those of reference THA and was confirmed by spiking studies. Purity of the HPTLC bands was further confirmed by NMR and MS analysis. The amounts of THA in the extracts were calculated by comparing the peak area with calibration curve. The seeds contained 9870 mg kg^{-1} of THA. The amount of THA was determined by HPTLC method was found to be matching with that from HPLC method within the error limit (Table 4). There are no reports on the quantification of THA in *S. anacardium* plant parts. The present results showed that *S. anacardium* seeds contained 1% THA. Being a major and unique biflavonoid with potential biological significance, THA could be considered as a chemical marker for the standardization of herbal formulations containing *S. anacardium* seeds.

Ayurvedic formulations often contain a large number of herbs with varying proportions and are prepared by different methods as described in classical texts. In this study, two frequently used Ayurvedic polyherbal formulations, namely 'Guluguluthikthakashayam' and 'Varanadhighrutham' containing *S. anacardium* seeds, were selected and the amount of THA was evaluated. The two formulations were of reputed brands with considerable difference between preparation and composition and are consumed in large volumes.

'Guluguluthikthakashayam' is a water decoction of 20 herbs with 0.5% *S. anacardium* seed powder and is recommended against arthritis, diabetics, skin diseases, cancer, etc. The amount of THA in the samples were quantified by analyzing their methanol extracts and given in Table 4. HPTLC and HPLC fingerprints of one sample are given respectively in Figs. 2 and 3. HPTLC analysis showed 43–54 mg kg^{-1} THA in the samples of 'Guluguluthikthakashayam'. 'Varanadhighrutham' is recommended for the treatment of arthritis. It is prepared by boiling 15 herbs with ghee and water and it contains 0.7 g of *S. anacardium* seed powder in 10 g of final product. Methanol extracts of the samples were profiled in HPTLC and HPLC (Figs. 2 and 3). Samples were found to contain 8–22 mg kg^{-1} of THA (Table 4).

3.6. HPTLC versus HPLC

In order to verify the reliability of the HPTLC densitometric analysis, five independently prepared methanol extracts of *S. anacardium* seed were analyzed simultaneously by HPTLC and HPLC methods. Each sample was analyzed in triplicate and mean values were compared by matched pair Student's *T*-test. The data were treated as paired data. The observed T (T_{obs}) was calculated using

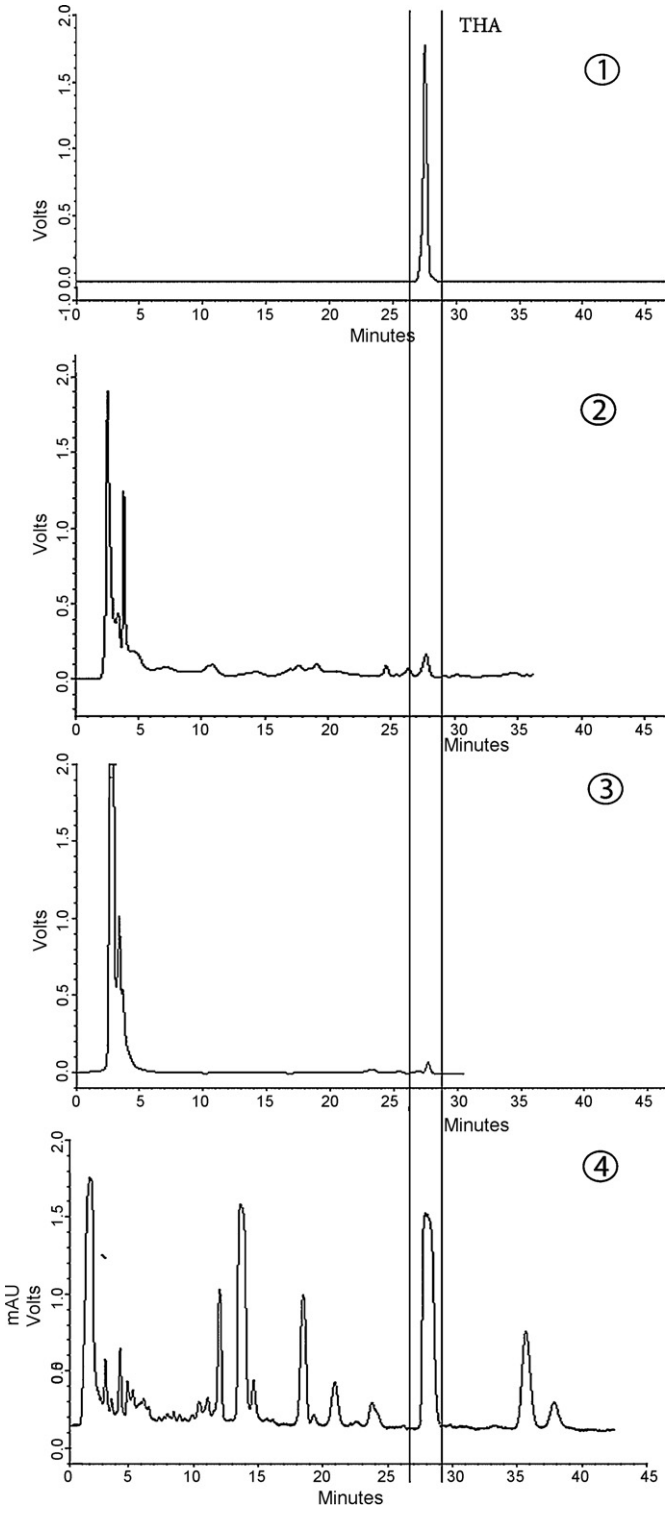

Fig. 3. HPLC chromatograms of methanol extracts of polyherbal formulations and *S. anacardium* seed at 290 nm. (1) THA, (2) Gulugulu Thithakashayam, (3) Varanadhi Ghrudam and (4) *S. anacardium* seeds.

the equation,

$$T_{(obs)} = \frac{|\bar{d}|}{\sqrt{\sum d_i^2 - 1/n(\sum d_i)^2/n(n-1)}}$$

where d_i is the difference between two pairs of measurements for the same observation i.

For five pairs of analysis the T_{obs} was 1.71 which was lower than the T value obtained from Student's distribution Table, T (95, 5, 4) = 2.78 for a risk factor of 5%, which showed that there was no statistically significant difference between HPLC and HPTLC analytical methods.

4. Conclusion

The HPTLC and HPLC techniques developed are precise, specific, accurate and robust for determination of THA. Statistical analysis proves that the methods are repeatable and selective for the analysis of one of the biologically active components, THA in plant and in pharmaceutical formulations. The method can be extended for the marker-based analysis of *S. anacardium* herbal formulations. HPTLC method is especially suitable for the fingerprinting and high throughput analysis of botanical samples and herbal formulations.

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