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RESEARCH ARTICLE

A Selective High Performance Liquid Chromatographic Method for Estimation of Catechin in Ayurvedic Taila Preparations.

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ABSTRACT

A simple, sensitive, rapid and selective high-performance liquid chromatographic (HPLC) method has been developed and validated for the analysis of catechin in marketed Ayurvedic *Taila* (oil) formulations containing *Acacia catechu*. Chromatography of methanolic: 0.1% formic acid (7:3, v/v) extracts of these formulations was performed on C_{18} (5 µm x 25cm x 4.6mm i.d) column using isocratic mobile phase consisting of methanol: acetonitrile: water (40:15:45, v/v/v) containing 1.0% acetic acid at a flow rate of 0.5ml/min and SPD-10 A_{VP} photodiode array (PDA) UV-Visible detector. The analytical marker, catechin, was quantified at 279 nm. The retention time of catechin was about 3.27 min. The linear regression analysis data for the calibration plot showed a good linear relationship with correlation coefficient of 0.9988 in the concentration range of 15 to 90 µg/ml for catechin with respect to peak area. The limit of detection and limit of quantitation values were found to be 0.5µg/ml and 1.7µg/ml respectively. Repeatability of the method was found to be 0.62 RSD. Recovery values from 99.73 to 100.22 % indicate excellent accuracy of the method. The developed HPLC method is accurate, precise, and cost-effective, and it can be successfully applied for the determination of catechin in marketed ayurvedic oil formulations containing *Acacia catechu*.

KEY WORDS *Acacia catechu*; HPLC; oil; quantitation

INTRODUCTION:

'*khadir*' *(Acacia catechu* ,family: *Mimosaceae*), is considered as one of the most potent medicine used for various skin ailments in Ayurveda. It is widely used herb in Indian traditional system of medicine. The bark, heartwood and its decoction are used for medicinal purpose. It is useful in passive diarrhea, high blood pressure, dysentery, sore throat, colitis, gastric problems, bronchial asthma, cough, gingivitis, leucorrhoea, leprosy, dental and oral infections. The chief chemical constituents of *khadir* bark are catechin (2-12%), phlobatinin (25-33%), gummy matter, quercitrin and quercitin. There are number of ayurvedic *taila* (oil) formulations which contain '*khadir*' bark as one of the active ingredient [1-4]. Catechin is a free radical scavenger and largely responsible for the bio-potency of *acacia catechu.*

Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices, and composition is important to ensure quality, efficacy and optimum levels of active principles for their bio-potency.

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Recently, the concept of marker-based standardization of herbal drugs is gaining momentum. Identification of major and unique compounds in herbs as markers and development of analytical methodologies for monitoring them are the key steps involved in marker-based standardization.

Catechin being the major active principles largely responsible for the bio-potency of *Acacia catechu*, is recognized as analytical marker compounds for the quality control of ayurvedic oil preparation containing *Acacia catechu* [5, 6]. There are reports on the application of various analytical methods for isolation and quantitaion of catechin present in *Acacia catechu* and other botanical sources [7-10]. But no reported method deals with estimation of cathechin in complex matrix of ayurvedic *taila* formulations. In the last two decades high performance liquid chromatography has emerged as an efficient tool for the phytochemical evaluation of herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high throughput screening, etc. [11-16]. Hence it was thought worthwhile to develop a simple and rapid chromatographic method for determination of catechin in ayurvedic *taila* formulations. The method was validated and found to be sensitive and reproducible [17].

MATERIAL AND METHODS:

Reference compounds and reagents

Reference catechin (98%w/v), was purchased from Sigma-Aldrich (Germany).Analytical grade solvents were obtained from Merck (Mumbai, India).

Ayurvedic *taila* preparations

Commercial marketed *taila* preparations *'Tuvaraka taila'* and '*Khadiradi taila'* containing *khadir* bark as key component were selected for studies. Samples of the same formulations in triplicate, manufactured by three different reputed ayurvedic drug manufacturers were collected from retail pharmacies in Indore, India.

Preparation of standard solutions

The stock solution of 1 mg/ml was prepared after keeping the purity of reference cathechin into consideration. 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml and 1.2ml of aliquot of stock solutions were diluted to 5 ml using methanol: 0.1% formic acid (70:30%v/v) solvent system to obtain working standards.

Chromatographic conditions

The mobile phase consisted of methanol: acetonitrile: water (40:15:45, v/v/v) containing 1.0% acetic acid at a flow rate of 0.5ml/min.Before use ,the mobile phase was degassed by an ultrasonic bath and filtered using 0.4 µm membrane filter. Separation was performed at room temperature on HPLC system having a pump (Shimadzu LC 10AT$_{VP}$) with 20 µL Rheodyne injector ,Phenomenex Luna C_{18} (5 µm x 25cm x 4.6mm i.d) column and SPD-10 A$_{VP}$ photodiode array (PDA) UV-Visible detector set at 279nm and equipped with CLASS-VP software (Shimadzu. Kyoto, Japan).

HPLC analysis of Ayurvedic *taila* preparations

Oil formulations were homogenated using methanol: 0.1% formic acid (70:30%v/v) in proportion of 1:5, w/v at 50 ºC for 20 min. The mixture was centrifuged at 2000 rpm for 20 min at 4ºC and the supernatant was collected. The residue was resuspended in methanol: 0.1% formic acid (70:30%v/v) and the extraction was repeated five more times similarly. The supernatants were pooled and concentrated under vacuum at room temperature and made up to a known volume using methanol: 0.1%formic acid (70:30%v/v). The extracts were filtered through 0.45µm filter and HPLC was performed under the conditions optimized for the reference compound. The amount of catechin was quantified using calibration curves plotted with the reference compound.

Validation of Method

(a) Calibration graph (linearity of the HPLC method) -The calibration curve was obtained at 6 concentration levels of catechin standard solutions (15–90 µg/ml). The solutions (20 µl) were injected into the HPLC system (n = 6) with the chromatographic conditions previously given. The linearity was evaluated by the least-squares regression method.

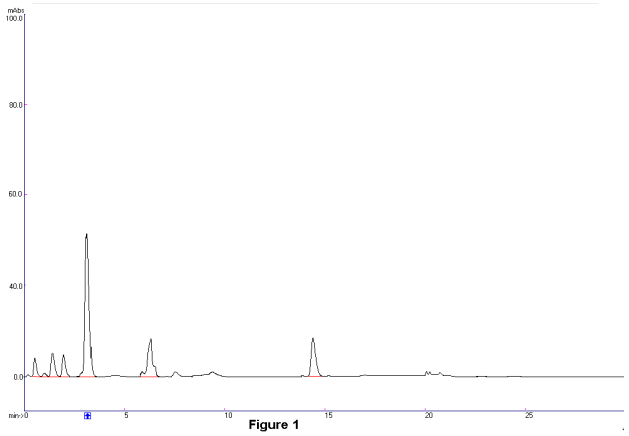

Figure 1

Figure 1. HPLC chromatogram at 279nm for taila preparations.

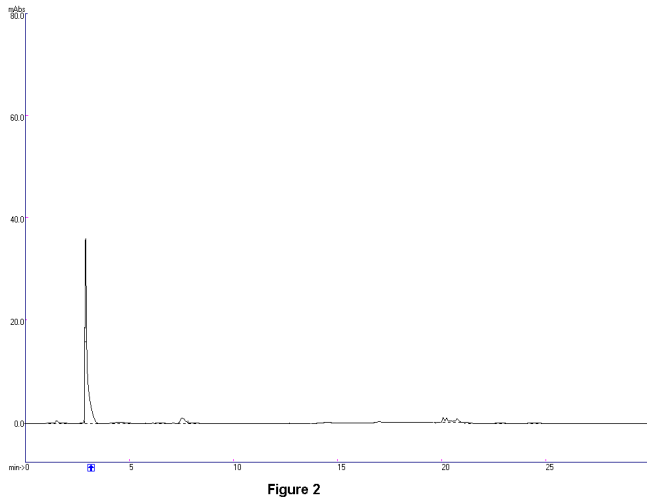

Figure 2

Figure 2. HPLC chromatogram at 279nm for reference catechin.

(b) Limits of detection and quantification -For determination of the limit of detection (LOD) and the limit of quantification (LOQ) different dilutions of the standard solution of catechin were analyzed using mobile phase as the blank. The LOD and LOQ were determined on the basis of signal-to-noise ratio until the average responses were approximately 3 and 10 times the responses of the blank respectively.

(c) *Accuracy (recovery)* -The accuracy of the methods was determined by calculating recovery of catechin by the standard addition method. Known amounts of standard solution of catechin (at three levels 50%, 100%, 150%) were added to pre quantitated sample solutions. The amount of catechin was estimated by applying values of peak area to the regression equations of the calibration graph. Five replicate samples of each concentration level were prepared.

(d) Method precision (repeatability) -The precision of the instruments was checked by repeatedly injecting and analyzing (n = 6) standard solutions of catechin (45

µg/ml). The results are reported in terms of relative standard deviation (RSD).

Table 1. Method validation parameters for estimation of catechin.

Parameters	
Linearity range (µg/ml)	15-90
Correlation coefficient (r^2)	0.9988
Regression equation	y = 15955x + 27426
Limit of detection (µg/ml)	0.5
Limit of quantification (µg/ml)	1.7
Repeatability (%RSD, n =6)	0.69

Table 2. Intermediate Precision studies.

Concentration (µg/ml)	Intraday*	Interday *
15	1.19	1.13
45	0.43	0.66
75	0.38	0.64

*Relative standard deviation (%R.S.D, n = 3)

Table 3. Recovery studies.

Marketed Formulations	Amount added (µg/ml)	Recovered* (%)
Tuvaraka taila	15	98.36 ± 0.85
	30	97.41± 0.42
	45	99.19± 0.38
Khadiradi Taila	15	99.72±0.28
	30	101.29±0.39
	45	101.33±0.92

*Mean ± Relative standard deviation (%RSD, n=3)

Table 4. Catechin content found in various *taila* preparations.

Sample	Brand	Catechin Content in oil (%w/w)
Tuvaraka taila	1	0.765±0.46
	2	0.802±0.87
	3	0.628±0.52
Khadiradi Taila	1	1.74 ± 0.27
	2	1.85 ± 0.86
	3	1.16 ± 0.74

*Mean ± Relative standard deviation (RSD, n=3)

(*e*) *Intermediate precision (reproducibility)* -The intraday and interday precision of the proposed method were determined by analyzing standard solution of catechin at 3 different concentrations (15, 45, and 90µg/ml) three times on the same day and on three different days. The results are reported in terms of RSD.

(*f*) *Solution stability and mobile phase stability* -Solution stability in the assay method was evaluated by leaving test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature in the dark for 24 h. The same sample solutions were assayed every 6 h interval in the study period. Mobile phase stability was studied by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6 h intervals up to 24 h. Mobile phase was prepared and kept constant during the study period. The relative standard deviation (RSD) of the assay of catechin was calculated for the study period during mobile phase and solution stability experiments.

Statistical Analysis

The statistical analysis was performed using Microsoft Excel 2003.

RESULT AND DISCUSSION:

The literature revealed that methanol: 0.1% formic acid (70:30%v/v) is preferred for extraction of catechin from acacia catechu [8].The same was used for extraction of catechin from oil formulations. It is advantageous as the base oil in all the selected ayurvedic oil formulations is immiscible in this solvent. The immiscibility of oil in solvent will help in reducing number of interfering components in further chromatographic development. Multiple extractions were carried out to ensure complete extraction.

Development of the HPLC Method

The method development and selection of a suitable mobile phase involved several trials because of the complexity of the chemical composition of the herbals and the affinities of the components towards various solvents. The proportions of the organic and aqueous phases were adjusted to obtain a rapid and simple assay method with a reasonable run time, suitable retention time, and sharp peak. Under optimized conditions HPLC with C18 column and UV detector at 279nm using mobile phase (methanol: acetonitrile: water (40:15:45, v/v/v) containing 1.0% acetic acid) gave well resolved symmetric band for catechin from its oil formulation (Fig 1). Retention time was found to be around 3 minutes and catechin appeared on chromatogram at 3.27minutes. The retention time of the reference catechin was observed to be 3.269min (Fig 2). This indicates that the present HPLC method is rapid; which in turn shows that the method consumes less volume of HPLC solvents. When the same drug solution was injected 6 times, the retention time of the drug was found to be same.

Validation of method

The calibration curve was prepared by plotting the peak area against catechin concentration; it was found linear in the range of 15–90 µg/ml. The regression equation was found as y = 15955x + 27426 with r^2 of 0.9988, showing excellent linearity. The method was validated in terms of precision, repeatability, accuracy and other validation parameters (Table 1).The repeatability of the HPLC method and the intermediate precisions for intra-day and inter-day variations are given in Table 2. The LOD value was found to be 0.5µg/ml, which is the concentration that yields a signal-to-noise (S/N) ratio of 3/1. The LOQ value under the described conditions was 1.7µg/ml with an S/N ratio of 10:1. This confirmed the sensitivity for quantitaion of catechin in *taila* preparations. Recovery values from 99.73 to 100.22 % indicate excellent accuracy of the method (Table 3). The RSD values of

assay of catechin during solution stability and mobile phase stability experiments were within 2.0%. The data obtained in both experiments proves that the sample solutions and mobile phase used during assay were stable up to 24 h.

HPLC analysis of Ayurvedic taila preparations

Quantitative estimation of catechin in polyherbal oil formulations given in Table 4 revealed variation in its content in different brands, which indicates the need of standardization of raw material used and uniformity in method of manufacturing to be followed by different ayurvedic manufacturers . The method developed here does not require separation of unsaponifiable matter for quantification as reported for some active ingredients in oil formulation. Oil extract can be directly used for analysis. Avoidance of long and tedious step therefore makes this method more amendable to the high through put screening.

CONCLUSION:

A method for analysis of *Acacia catechu* using catechin as analytical marker in ayurvedic *taila* preparations was developed. The method was found to be simple, precise, specific, sensitive, and accurate. It can be used for routine quality control of ayurvedic *taila* preparations containing *Acacia catechu*.

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