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Quantitative estimation of (+)-Catechin in stem bark of *Saraca asoka* Linn using HPTLC

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Abstract

Saraca asoka is an important plant of Indian system of medicine for its chemical constituents and its well known pharmacological activities. Catechin is a well-known flavonoid found in this plant and also in many foods plants and often utilized by naturopaths for the symptomatic treatment of several gastrointestinal, respiratory and vascular diseases. Our aim was to quantify this flavonoid by using HPTLC from *Saraca asoca* stem bark. In the present paper we report our work on quantification of this marker compound by thin layer chromatography densitometric methods using high performance thin layer chromatography. To the best of our knowledge, this is the first report of quantification of this compound using HPTLC from this plant. The thin layer chromatography densitometric methods were found to be precise with RSD for intra-day in the range of 0.35-2.5 and for inter-day in the range of 0.85-3.05 for different concentrations of Catechin. Instrumental precision was 3.36 (% RSD) for Catechin. Accuracy of the method was checked by conducting recovery studies at three different levels for the compound and the average percentage recoveries obtained was 101.45%. *Saraca asoca* sample was found to contain 0.048% w/w of Catechin.

Keywords: High Performance Thin layer chromatography, (+)-Catechin, *Saraca asoka*, TLC densitometry.

Introduction

For past few decades compounds from natural sources have been gaining importance because of the vast chemical diversity that they offer. This has led to phenomenal increase in the demand for herbal medicines in the last two decades and a need has been felt for ensuring the quality, safety and efficacy of herbal drugs. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening; chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades High Performance Thin Layer Chromatography (HPTLC) has emerged as an important tool for the qualitative semi-quantitative and quantitative phytochemical analysis of herbal drugs

and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers. HPTLC provides an efficient, fast and reliable alternative for quantitative determination of natural products. The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase [1].
The use of HPTLC for fingerprinting of herbal extracts, characterization and quantification of secondary metabolites isolated in small quantities from a large number of plants affords a number of advantages over conventional methods in use for this purpose [2]. Determination and use of TLC/HPTLC profiles with other physiological parameters can be good tools for standardization and validation of plants and constituents. TLC/HPTLC profiling are simple and effective methods for determination of the phytochemicals. Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity [3-10].

Saraca asoca Roxb.. de Wilde, syn. *S. indica* auct non L. (Ashoka) is an evergreen tree belonging to the Caesalpiniaceae subfamily of the legume family [11]. It occurs almost throughout India up to an altitude of 750 m, in the central and the eastern Himalayas and in the Khasi, Garo and Lushai hills [12]. It is an important tree in the cultural traditions of the Indian Subcontinent and adjacent areas and considered sacred throughout the Indian subcontinent, especially in India and Sri Lanka.

Ashoka is wonderful herb that claims to cure several diseases. According to Ayurvedic medicine, it is the one herb that stands out as especially useful for treating excessive uterine bleeding. It is extensively used in the Ayurvedic system of medicine for a variety of ailments [13, 14] as a blood purifier, in stomach ache [15], and as a hypothermic and diuretic [16]. Ayurvedic texts describe more than 50 preparations of Ashoka for the treatment of a variety of ailments in which its stem bark is used as one of the main ingredients. Ashoka is an excellent herb for gynecological problems. It is useful in stimulating the uterus, the endometrium and the ovarian tissues, uterine bleeding associated with fibroids, leucorrhoea, menstrual disturbances without producing any side effects. Apart from this it is also useful for other ailments such as internal piles, diabetes, dyspepsia, indigestion, burning sensation, blood disorders, fractures, tumors, bites, ulcerations, and skin discoloration. The plant is found to have spasmogenic, oxytocic, uterotonic, antibacterial, anti-implantation, antitumour, antiprogestational, antioestrogenic activity [17].

The bark is bitter, astringent and sweet in taste. It has stimulating effect on endometrial and the ovarian tissue. It is useful in internal bleeding, hemorrhoids, ulcers, uterine affections, menorrhagia especially due to uterine fibroids, meno-metrorrhagia, leucorrhoea and pimples.
The fresh flowers are an excellent uterine tonic and are used in cervical adenitis, biliousness, syphilis, hyperdipsia, burning sensation, hemorrhagic dysentery, piles, scabies in children and inflammation. Dried flowers are used in diabetes. The seeds are used in treating bone fractures, strangury and vesicle calculi. The bark of the tree is also useful in treating scorpion-sting [18].

The bark is also effective for internal piles. A decoction prepared in the same manner as in case of uterine disorders is taken in this condition. The bark of the Ashoka tree is used to make a drug, which is reported to possess a stimulating effect on the endometrium and ovarian tissue [19].

Chemical Constituents:
It mainly contains glycosidic principles, non-phenolic, sapogenetic glycoside, sterols and aliphatic alcohols. catechol, (-) epicatechol and leucocyanidin has been isolated from pods and wood contains quercetin. In the powdered bark ash of Ashoka minerals like silica, sodium, potassium, phosphate, magnesium, iron, calcium, strontium and aluminium have been found [20-23]. The flowers contain fatty acids and gallic acid; apigenin-7-O-beta-D-glucoside, cyanidin-3, 5-diglucoside, kaempferol 3-O-beta-D-glucoside, pelargonidin-3, 5-diglucoside, quercetin and its 3-O-beta-D-glucoside and sitosterol. The bark yields alkanes, esters and primary alcohols. It gave n-octacosanol, tannin, catechin, (+)-catechol, (−)-epicatechin, (−)-epicatechol, leucocyanidin, leucopelargonidin, procyanidin derivatives, methyl-and ethylcholesterol derivatives. Quercetin and its 3-O-rhamnoside, kaempferol-3-O-α-L-rhamnoside, amyrin, ceryl alcohol and β-sitosterol have been isolated from leaves and stems [24]. It also contains flavonoids and sterols [25, 26].

Catechin is a polyphenolic antioxidant plant metabolite. The term catechin is also commonly used to refer to the related family of flavonoids and the subgroup flavan-3-ols (or simply flavanols). The health benefits of catechins have been studied extensively in humans and also in various animal models. Reduction in atherosclerotic plaques was seen in animal models [27] and reduction in carcinogenesis was seen *in vitro* [28]. Many studies on health benefits have been linked to the catechin content. Various studies proved that epicatechin can reduce the risk of four of the major health problems: stroke, heart failure, cancer and diabetes and epicatechin should be considered essential to the diet and thus classed as a vitamin [29, 30]. According to one researcher [31] epigallocatechin-3-gallate is an antioxidant that helps protect the skin from UV radiation-induced damage and tumor formation.

(+)-Catechin shown to possess antibiotic properties due to their role in disrupting a specific stage of the bacterial DNA replication process [32], it also helps in DNA protection [33], and possesses anti-carcinogenic effects [34]. Catechin and epicatechin are also selective monoamine oxidase inhibitors (MAOIs) of type MAO-B [35]. (+)-Catechin has been shown to have antioxidant [36], glucosidase-II [37] and matrix metalloproteinase inhibitory activity [38] and renoprotective effect [39]. It has also been reported to induce cancer preventive activity mediated through a chaperone like property [40].

In this present study we developed a simple TLC densitometric method for the quantification of (+)-Catechin in marketed formulation. However, various analytical methods have so far been reported for its determination from other plants [41, 42]. But till date no analytical method is reported for quantification of (+)-Catechin from *Saraca asoka* stem bark. Hence this paper reports a simple, precise, rapid and cost effective HPTLC method for the estimation of (+)-Catechin.

Results and Discussion

Saraca asoka is an important plant of Indian System of Medicine and there is no report of TLC densitometric quantification of (+)-Catechin from this plant. Hence we developed a simple and precise method for quantification of this marker compound.

TLC fingerprint and co-chromatography
Quality control and quality assurance of herbal drugs remains a challenge as they contain a myriad of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy [43]. Hence a systematic consideration of all its

phytoconstituents is as important as the quantification of the active constituents present in it. TLC fingerprint profile of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality and ensuring the consistency and stability of herbal drugs and their products. In the present study, we developed TLC fingerprint profile for *Saraca asoka* and carried out co-chromatography with marker compound (+)-Catechin. (+)-Catechin resolved well at R_f 0.57, (Table 1, Fig.1) from sample solution when the plate was developed in optimized Solvent System. The identity of the band for (+)-Catechin in the sample extract was confirmed by overlaying their UV absorption spectra with those of respective reference standards using CAMAG TLC scanner 3 with WINCATS software (Fig. 2). The purity of each of these bands in the sample extract was confirmed by comparing the absorption spectra recorded at start, middle and end positions of the band.

Table 1: TLC Details of sample solution of *Saraca asoca* Stem Bark

S. No.	R_f value	Color of the band
1	0.24	Brown
2	0.32	Brown
3	0.40	Light brown
4	0.57	Brown ((+)- Catechin)
5	0.64	Violet
6	0.70	Light blue
7	0.76	Yellow
8	0.77	Light blue
9	0.82	Light purple
10	0.89	Light purple
11	0.92	Light purple

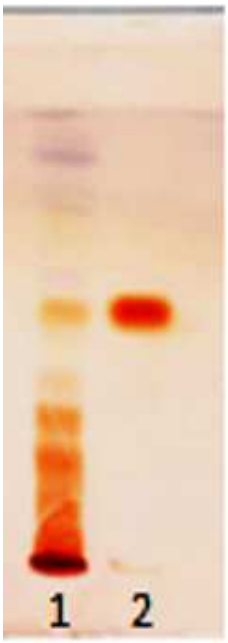

Fig.1. TLC profile of Sample solution of *Saraca asoca* stem bark
1: Sample solution; 2: (+)-Catechin standard

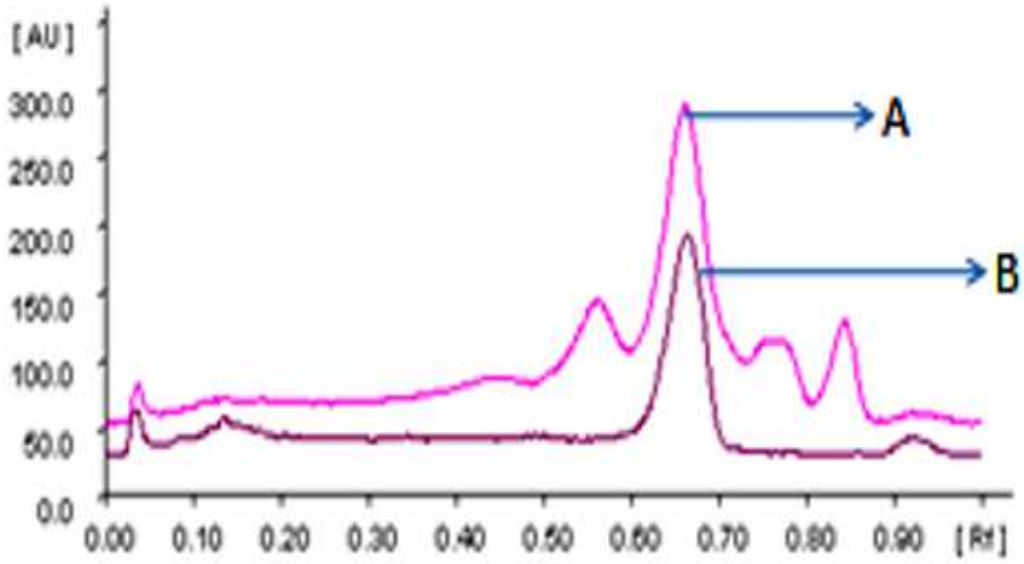

Fig.2. TLC densitometric scan at 280 nm of Sample solution of *Saraca asoca* stem bark
A: Sample solution; B: (+)-Catechin standard

TLC densitometric quantification of (+)-Catechin

The simplicity of the sample preparation, and the possibility of analyzing several sample of herbal products simultaneously in a short time, make HPTLC the method of choice. In the present work (+)-Catechin was quantified from *Saraca asoka* stem bark by TLC densitometric methods using HPTLC. For the quantification of (+)-Catechin TLC densitometric method was established and described.

The TLC densitometric method was validated in terms of precision, repeatability, and accuracy (Table 2). The linearity ranges for (+)-Catechin was found to be the same (160–720 ng/spot) with correlation coefficient (r-values) of 0.997 (Table 2). The TLC densitometric methods were found to be precise with RSDs for intraday in the range of 0.35–2.5 and for interday in the range of 0.85–3.05 for different concentrations of (+)-Catechin (Table 3). This indicates that the proposed method was precise and reproducible. The limit of detection (LOD) value for (+)-Catechin was found to be 60, and limit of quantification (LOQ) value was 160 (Table 2). The average percent recoveries at 3 different levels of (+)-Catechin were found to be 101.45 (Table 4). The amount of (+)-Catechin was found to be 0.0815 ± 0.0007 % w/w respectively in sample solution.

Table 2: Method validation parameters for the quantification of (+) - Catechin, by the proposed TLC densitometric methods

Sr. No.	Parameter	(+) -Catechin
1	Instrumental precision (% CV, n = 7)	3.36
2	Repeatability (% CV, n = 5)	1.83
4	Accuracy (average % recovery)	101.45
5	Limit of detection (ng)	60
6	Limit of quantification (ng)	160
7	Specificity	Specific
8	Linearity (Correlation coefficient)	0.997
9	Range (ng/ spot)	160-720

Table 3: Intra-day and Inter-day precision of (+)-Catechin

Marker	Concentration (ng/ spot)	Intra-day precision*	Inter-day precision*
(+)-Catechin	400	1.04	0.85
	480	2.5	3.05
	560	0.35	2.43

* % R.S.D.; Mean (n=3)

Table 4: Recovery studies of (+)-Catechin at 50 %, 100 % and 125 % addition by the proposed TLC densitometric method

Marker	Amount of marker present (µg)	Amount of marker added (µg)	Amount of marker found (µg)	Recovery* (%)	Average Recovery (%)
(+)-Catechin	60	30	92.37 ± 4.58	102.63 ± 0.96	101.45
	60	60	118.91 ± 7.25	99.09 ± 1.20	
	60	75	138.59 ± 2.17	102.65 ± 0.64	

*Mean ± SD (n=3)

Materials and Methods

Plant material: Stem bark of *Saraca asoka* was procured from the market in Sirsa Dist. Haryana, India. It was authenticated by our taxonomist and a voucher specimen (JCDP/P/02) was preserved in the Dept. of Pharmacognosy and Phytochemistry JCDM College of Pharmacy, Sirsa. The plant material was dried in a hot air oven at < 50°C, stored in airtight glass bottle at 30°C and powdered to 40 mesh whenever required.

Standard compound: (+)-Catechin (Purity: 98%w/w) was purchased from Natural Remedies Pvt. Ltd, Bangalore, India.
Chemicals: All chemicals used were of analytical grade.

Thin Layer Chromatography Conditions

The TLC plates were 20 × 10 cm, precoated with silica gel 60 F_{254} TLC plate (E. Merck) (0.2 mm thickness); spotting device was Camag Linomat V Automatic Sample Spotter, Camag (Muttenz, Switzerland); syringe was a 100 µL (Hamilton); developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); densitometer a Camag TLC Scanner 3 linked to winCATS software. Experimental conditions: Temperature 25 ± 2°C, relative humidity 40%.

TLC fingerprinting profile

Sample solution

Sample solution was optimized to achieve good fingerprinting and also to extract the marker compound efficiently. Different solvent systems were tried in order to resolve the marker compound. Accurately weighed 1.0 g quantity of powdered drug was extracted with methanol (25 mL × 4) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated under vacuum and the volume

was made upto 50 mL in a volumetric flask. This extract was used for TLC fingerprinting and co-chromatography with (+)-Catechin.

Standard solution of (+)-Catechin
2 mg of (+)-Catechin was dissolved in methanol and the volume was made upto 25 mL with methanol in volumetric flask.

Solvent system
Toluene : Ethyl acetate : Formic acid : Methanol (3 : 6 : 1.6 : 0.4 v/v/v/v)

Development of TLC Fingerprint profile of *Saraca asoka* and co-chromatography using (+)-Catechin

Procedure
10 µl each of sample solution along with (+)-Catechin standard was applied on a TLC plate and the plate was developed in solvent system to a distance of 8 cm. The plate was observed under UV 254 nm and UV 366 nm. The R_f value and the colour of the resolved bands were observed.

Quantification of (+)-Catechin
Followed by co-chromatography, the marker compound was quantified using HPTLC.

Preparation of standard solutions of (+)-Catechin
Stock solution of 80 µg/mL of (+)-Catechin by dissolving 4 mg of accurately weighed (+)-Catechin in methanol and making up the volume of the solutions to 50 mL with methanol in volumetric flasks. The aliquots (4 to 8 mL) of stock solutions were transferred to 10 mL volumetric flasks and the volume of each was adjusted to 10 mL with methanol, to obtain standard solutions containing 32 µg/mL, 40 µg/mL, 48 µg/mL, 56 µg/mL and 64 µg/mL of (+)-Catechin.

Preparation of calibration curve of (+)-Catechin
10 µL each of the standard solutions of (+)-Catechin (320-640 ng/spot) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20 × 10 cm) upto a distance of 8 cm using a solvent system of Toluene: Ethyl acetate: Formic acid: Methanol (3 : 6 : 1.6 : 0.4 v/v/v/v) for (+)-Catechin at 25 ± 2°C temperature and 40 % relative humidity. Visualization was done by spraying the plate with anisaldehyde-sulphuric acid reagent and heats the plate at 70°C till the coloured bands develop. Note the R_f and colours of the resolved bands (Table 1, Fig. 1). The plates were dried at room temperature and scanned at 260 nm in absorbance mode using deuterium lamp. The areas of the resolved peaks were recorded. Calibration curve of (+)-Catechin was obtained by plotting peak areas *vs* applied concentrations of (+)-Catechin.

Procedure
10 µL of suitably diluted sample solution (Methanolic Extract) along with the marker compound was applied in triplicate on a TLC plate. The plate was developed in the solvent system and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of (+)-Catechin was calculated using the calibration curve.

Validation of the Methods: ICH guidelines were followed for the validation of the analytical methods developed (CPMP/ICH/281/95 and CPMP/ICH/381/95) for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning (n = 7) of the same spot of (+)-Catechin (400 ng/spot) and expressed as relative standard deviation (% RSD). The repeatability of the method was affirmed by analyzing 400 ng/spot of (+)-Catechin individually on TLC plate (n = 5) and expressed as % RSD. Variability of the method was studied by analyzing aliquots of standard solution containing 400, 480, 560 ng/spot of (+)-Catechin, on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD.

For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of (+)-Catechin was applied along with methanol as blank and determined on the basis of signal to noise ratio.

The accuracy of the method was assessed by performing recovery study at three different levels 50 %, 100 % and 125 % addition of (+)-Catechin. The percent recoveries and the average percent recoveries were calculated for each.

Conclusion

We established TLC densitometric method for the quantification of bioactive compound (+)-Catechin from stem bark of *Saraca asoka* using HPTLC. The method was found to be simple, precise, specific, sensitive and accurate and can also be used for the quantification of (+)-Catechin in the herbal raw materials. It can also be used in routine quality control of herbal materials as well as formulations containing this compound.

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