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Short report
Analytical Section

Rapid High Performance Thin Layer Chromatographic Method for Quantitation of Catechin from Extracts of Cashew Leaves – a Short Report

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Cashew is a tropical tree with immense commercial value and the use of its various parts in the confectionary, food, herbal and tanning industry has gained tremendous importance. Thus, the development of quality control method for routine analysis of various products of this widely used plant would be a great help to analysts. The phytochemistry of this plant has not been explored in detail. *Anacardium occidentale* Linn. (cashew) is reported to contain many antioxidant and polyphenolic compounds. However, there have been no reports published for the HPTLC analysis of the extracts of cashew leaves. Hence, an attempt has been made to develop a HPTLC method for estimation of catechin from leaf extracts. The present paper reveals a rapid high performance thin layer chromatographic method developed for quantitation of catechin from extracts of cashew leaves. The chromatographic parameters such as solvent system, development time, saturation time, detection wavelength were optimized. The mobile phase toluene: ethyl acetate: methanol: formic acid (6:6:1:0.1v/v/v/v) gave the best resolution for various components. The separation of various components and quantitation of amount of catechin was successfully carried out from extracts of cashew leaves. The aqueous extract of leaves contained a higher amount of catechin as compared to ethanol extract. The presence of tannins and phenolics was visualised as bluish-black bands with 5% alcoholic $FeCl_3$ as visualising agent. The method can prove to be a rapid, sensitive and economic alternative as compared to other chromatographic methods especially HPLC for detection of catechin in various food products and herbal formulations.

INTRODUCTION

With the increasing global popularity of herbal remedies, the need of assuring safety and efficacy of these herbal products has increased as well. Herbal products comprise of many constituents and have complex matrices. They are susceptible to variation due to various manufacturing and storage conditions. Moreover, many traditional preparations are composed of several herbs. Thus only highly selective, sensitive and versatile analytical techniques will be appropriate for quality control of herbal products. Recently, chromatographic fingerprint technique has been accepted by WHO as an approach for the evaluation of the quality of herbal medicines [WHO, 2005]. Chromatographic analysis techniques such as high performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) have recently gained growing importance due to their utility in the characterisation of the entire sample composition [Liang *et al.*, 2004]. The quality of herbal products can be enhanced by application of these chromatographic techniques for the estimation of bioactive phytoconstituents [Mahady *et al.*, 2001]. The analysis of phytoconstituents is a difficult task because of their complex nature, usually low availability and variability even within the same plant species. Sensitivity is the major problem for the detection of phytoconstituents when various analytical techniques are used. Thus, most frequently chromatographic techniques in combination with different detections are the preferred techniques for this purpose. Due to extremely small sample volumes and the aspects mentioned above, HPLC and HPTLC are preferred for the analysis of herbal products [Liang *et al.*, 2004].

The technique of HPTLC has the advantage that several samples can be analysed at the same time. The established importance of HPTLC fingerprints is attributed to the visual impression, which can be further explored by multiple detection (*i.e.* pre and post chromatographic visualisation). A wide spectrum of constituents can be detected at the same time in a single run in an experiment. Moreover performing HPTLC helps in identifying and quantifying various phytoconstituents present in the extracts.

The phytochemistry of the leaves of this plant has not been explored in detail. There have been no reports published for the HPTLC of the extracts of cashew leaves. *Anacardium occidentale* Linn. (cashew) is reported to contain many antioxidant and polyphenolic compounds [Kubo *et al.*, 2006; Mathew & Parpia, 1970; Jaiswal *et al.*, 2010]. Thus, an HPTLC method was developed and optimised for quantify-

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ing catechin and obtaining a fingerprint of various extracts of leaves by this planar chromatographic method. This method can serve as a rapid, economic and specific alternative to other detection techniques for quantitation of catechin in cashew samples. Cashew is used in a number of beverages in various forms. The leaves are consumed as food in various forms in tropical countries like Malyasia and are reported to have a number of pharmacological effects [Abas *et al*., 2006; Kudi *et al*., 1999; Goncalves *et al*., 2005; Trevisan *et al*., 2006; Schmourlo *et al*., 2005]. Thus development of an economic and rapid analysis method would help in quality control of various formulations and food products of cashew available commercially.

MATERIAL AND METHODS

Material

Cashew leaves were collected from Tungareshwar forests of Vasai Taluka, Dist. Thane in the state of Maharashtra, India. The plant specimen was authenticated and a herbarium of the plant specimen (voucher number no. YOGA1/No.BSI/WC/Tech/2008/69) was submitted at the Botany Department of Botanical Survey of India, Pune; (M.S), India.

Chemicals

Standard of catechin was purchased from Sigma–Aldrich (Germany). All the solvents were of analytical reagent grade and obtained from Merck (Mumbai, India).

Extraction of cashew leaves

Fully matured shade dried leaves of cashew were collected, cleansed and ground to coarse powder form. The samples were extracted by using Soxhlet extractor, with ethanol with a mass to volume ratio of 1:6. The ethanol extract was evaporated to dryness on the rotary evaporator. Aqueous extract was prepared by refluxing with water and mass to volume ratio of 1:6 (g/mL). The aqueous extract was freeze dried and used for analysis.

Preparation of solutions of extracts and catechin

Stock solutions (1 mg/mL) of reference catechin were prepared in methanol. Working solutions of catechin were prepared by appropriate dilutions of the stock solution with methanol. All solutions were prepared freshly prior to analysis. Working solutions of extracts (5 mg/mL) of cashew leaves were prepared with methanol.

Development and optimisation of HPTLC parameters

To make a choice of suitable solvent system, initially the elutropic series of different solvents was tried by running on the HPTLC plates. Neat solvents of varying polarity and solvents in different combination ratios were used to optimise elution of various components and a combination of solvents that gave better resolution of the maximum number of components in extracts was selected. Formic acid was used as a modifier to affect better resolution of bands. Various time periods from 10–25 min (10, 15, 20, 25 mins) were attempted to select the optimum saturation time, suitable for maximum resolution and faster development of the HPTLC plate. The samples were applied at 1 cm distance from the bottom on the HPTLC plates and the solvent front was marked at 8 cm distance from the application position. The plates were allowed to dry and then placed in chambers saturated with the solvent system (mobile phase) for a period of 20 min prior to placement of plates. The qualitative evaluation of the plate was done by determining the migration behaviour of the separated substances by calculating R_f value.

HPTLC analysis

Precoated HPTLC plates used for analysis were preconditioned by overnight washing with methanol in a twin-trough chamber. Preconditioning in methanol has been shown to be effective for layer cleaning. The prewashed plates were then heated at 105°C for 5 min before use. HPTLC was performed on precoated silica gel GF_{254} aluminum backed HPTLC plates (20 cm×20 cm, 0.2 mm thickness, 5–6 μm particle size, E-Merck, Germany). Five microlitres of the sample solutions were spotted as bands of 6 mm width by using a 100 μL Hamilton syringe. The plates were developed using toluene: ethyl acetate: methanol: formic acid (6:6:1:0.1, v/v/v/v) as the solvent system with saturation time of 15 min in a CAMAG twin-trough plate development chamber. The developed plates were air dried and scanned. A spectro-densitometer (Scanner 3, CAMAG) equipped with 'win CATS' planar chromatography manager (version 1.3.0) software was used for the densitometry measurements, spectra recording and data processing. Absorption/remission were done in the measurement mode at a scan speed of 20 mm/s. Densitograms were recorded at the wavelength of 254 nm for catechin and various components of extracts. The analysis was performed in air-conditioned room maintained at 22°C and 55% humidity.

Spot visualisation on the HTPLC plate

A visualising agent was selected based upon the class of phytoconstituents found in the preliminary phytochemical screening tests. The visualising reagent helps in visualisation as well as confirmation of the identity of the phytoconstituents. A 5% alcoholic $FeCl_3$ solution was used for visualisation of tannins and phenolic components in the extracts.

Quantitation of catechin in various extracts by HPTLC analysis

The extracts were dissolved in methanol and the solution of concentration 5 μg/μL was filtered through 0.45 μm PVDF filter and HPTLC was performed under the conditions optimised for the reference compound. The amount of catechin in the extracts was quantified by comparison with catechin bands from solutions of known concentration. The plates were then dipped in 5% alcoholic $FeCl_3$ solution for a few seconds and then kept at 100°C for 5 min for visualisation of bands of polyphenols.

Calibration curve of catechin

In order to establish a calibration curve for estimation of catechin, the limit of detection (LOD) and limit of quantitation (LOQ) were determined.

RESULTS AND DISCUSSION

Ethanol and aqueous extract of cashew leaves were prepared and the extraction yields were calculated and listed in Table 1. In literature, reports indicate that Soxhlet extraction has been used as control for comparison with other extraction techniques [Ghosh *et al*., 2011; Ahmad *et al*., 2010; Chulet *et al*., 2010]. The extraction of cashew leaves with ethanol was carried out by Soxhlet extraction and with water by decoction process. Since cashew is reported to be a rich source of phenolic compounds, ethanol and water were selected as the solvents for extraction of leaves [Melo *et al*., 2006; Kamath *et al*., 2007; Rezali *et al*., 2008; Shobha *et al*., 1992; de Brito *et al*., 2007]. Such polar solvents would help in extraction of polar components like polyphenols. The extractive yield of ethanol extract was found to be higher than aqueous extract. This may be due to the ability of ethanol to extract polar as well as non-polar constituents and this is in agreement to the results of extraction yields obtained with ethanol in our previous work [Jaiswal *et al*., 2010, 2012].

An HPTLC method was optimised and developed for estimation of catechin from extracts of leaves of cashew. A 5% alcoholic $FeCl_3$ solution was used as a visualising agent to visualise the presence of polyphenols and bluish black coloured bands indicated their presence. Polyphenols are considered as one of the important secondary metabolites produced by plants, and hence our HPTLC studies focused on visualisation of polyphenols in the leaves of cashew [Mamyrbekova-Bekro *et al*., 2008]. Catechin is known to be a bioactive and potent polyphenolic compound, and hence we estimated and quantified its presence in the extracts prepared from cashew leaves. The peak areas obtained for standard catechin were used as a standard to quantify the amount of catechin present in ethanol and aqueous extracts of cashew leaves. The R_f value of catechin was found to be 0.4 by comparison with the reference catechin that showed same R_f value. Other 7 spots in addition to catechin were found to have R_f values of 0.3, 0.45, 0.52, 0.6, 0.74, 0.76 and 0.8 respectively. There were some spots which on spraying with ferric chloride showed clear patches and were not visualised. Spraying with the $FeCl_3$ solution on an HPTLC plate without any sample displays very faint brown or yellowish in colour after drying, due to deposition of ferric hydroxide on the plate. But in the HPTLC fingerprint of extracts of cashew leaves, number of clear patches (between R_f value 0.6 to 0.75) were observed where the plate retained its original white colour. The clear patches may thus be attributed to the presence of free acids which apparently stop the precipitation of ferric hydroxide [Roberts & Wood, 1951]. Identification of all the bands in those patches was not successful, but one of them corresponded to citric acid which is in agreement to our previous research work that explains the qualitative phytochemical tests of extracts of cashew leaves [Jaiswal *et al*., 2012].

With a decrease in the intensities of the individual spots up to R_f value 0.3, there was a marked increase in the intensity observed in the diffused area at and near the origin, after spraying with $FeCl_3$ solution. Such diffused area is possibly to be identified as the various condensation products that are formed from the preliminary oxidation products of the polyphenols [Harrison & Roberts, 1939; Roberts & Wood, 1951]. The presence of gallic acid was observed at R_f value 0.45. However the other bands at R_f value 0.52 and 0.6 were polyphenols which gave bluish colouration with ferric chloride solution and were present in trace amounts only. Only cat-

TABLE 1. Extractive yield of various extracts of cashew leaves.

Cashew leaf extracts	Extractive yields (%)*±SEM
Ethanol extract	25.94±0.70
Aqueous extract	8.64±0.50

* n=3 determinations for each of the values mentioned above.

TABLE 2. Catechin content in various extracts of cashew leaves.

Cashew leaf extracts	Content of catechin (%)*±SEM
Ethanol extract	4.75±0.7
Aqueous extract	5.70±0.9

* n=3 determinations for each of the values mentioned above.

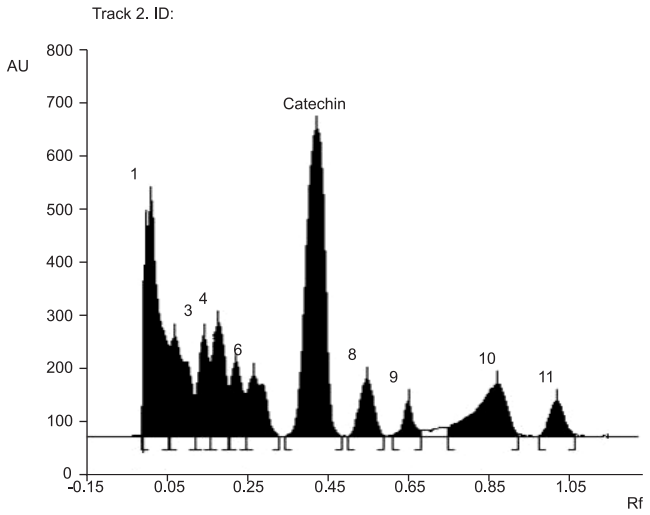

FIGURE 1. HPTLC chromatogram of an ethanol extract of leaves.

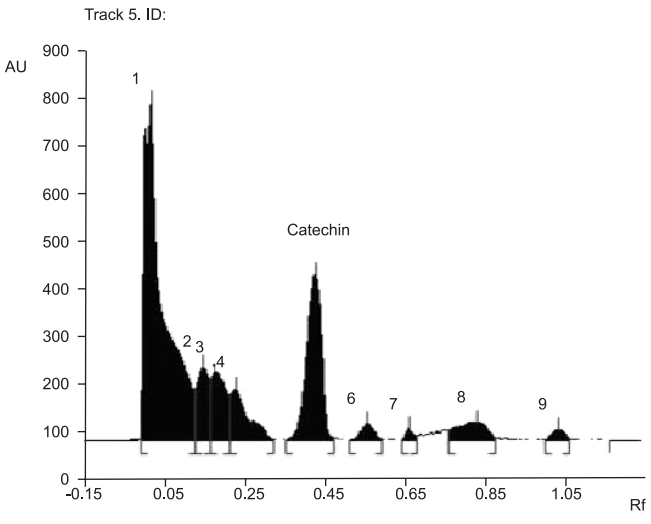

FIGURE 2. HPTLC chromatogram of an aqueous extract of leaves.

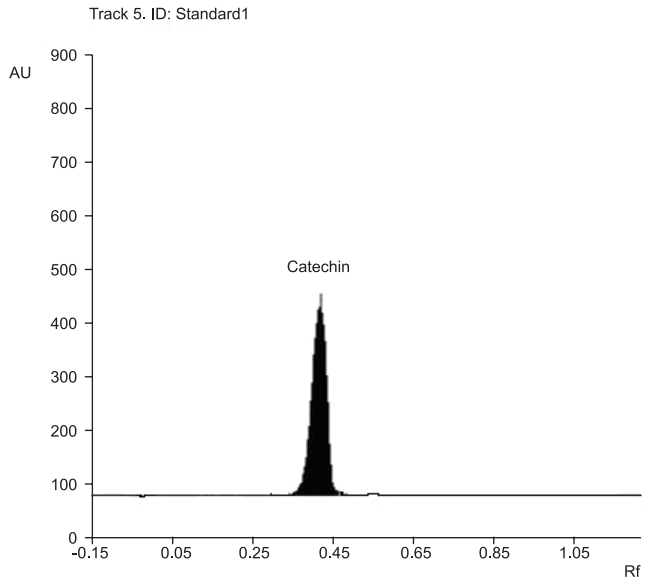

FIGURE 3. HPTLC chromatogram of standard catechins.

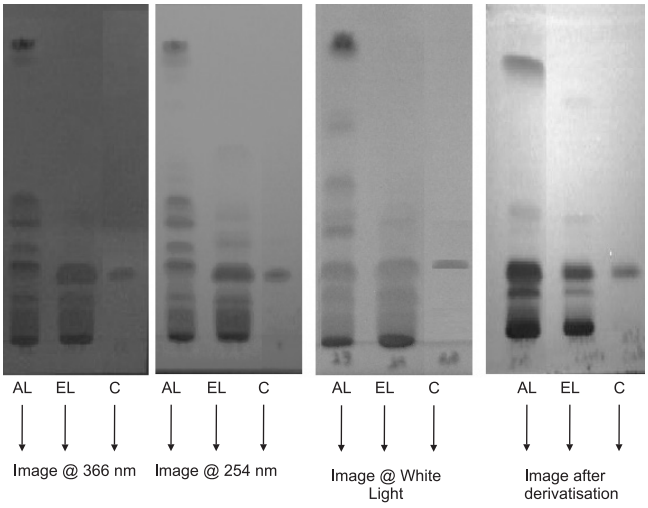

FIGURE 5. HPTLC Fingerprints of extracts of leaves and testa of cashew.

echin and gallic acid were found to be present in appreciable amounts, and since gallic acid is a very common constituent, catechin was selected as a marker compound for quantitation. Catechin is a well known bioactive compound with significant antioxidant activity with a great commercial value. The bands with R_f value 0.74, 0.76 and 0.8 were chlorophyll pigments that did not respond to the ferric chloride solution after spraying.

The values obtained for LOD and LOQ were 0.1 and 0.3 μg/μL, respectively. The linear response was obtained between 0.4 and 2.0 μg/μL. The chromatograms for both the extracts of cashew leaves and for catechin are depicted in Figures 1, 2 and 3, respectively. The calibration curve is depicted in Figure 4 indicating excellent linear relationship with the correlation coefficient as 0.9990.

The amount of catechin in various extracts was estimated and listed in Table 2. It was found that an aqueous extract of leaves contained the maximum amount of catechin as compared to the ethanol extract. Similar results were obtained in our previous work, where HPLC method was used for quantitation of catechin in various extracts of cashew leaves and aqueous extracts were found to contain a higher content of catechin as compared to the ethanol extract [Jaiswal *et al*., 2010]. This may be due to the higher solubility of catechin in water as compared to ethanol. The video images of HPTLC fingerprints of the extracts and fractions at different wavelengths and after spraying with 5% ferric chloride are shown in Figure 5. The various components of the extracts were well resolved. A distinct band of catechin can be seen in both the extracts at R_f 0.4.

The developed method can be recommended for quality assurance of extracts and herbal formulations of cashew leaves using catechin as a marker. The method finds its merit in being an economic method of analysis with lower sample preparation and analysis time as compared to other chromatographic techniques like LC and GC [De Brito *et al*., 2007; Jaiswal *et al*., 2010].

CONCLUSION

The high performance thin layer chromatography with toluene: ethyl acetate: methanol: formic acid (6:6:1:0.1, v/v/v/v) as the mobile phase is a reliable method for fast and easy separation of components and quantitation of catechin from crude extracts of cashew leaves. The method can be used as a cost effective method with reduced analysis time as compared to other alternative methods of analysis.

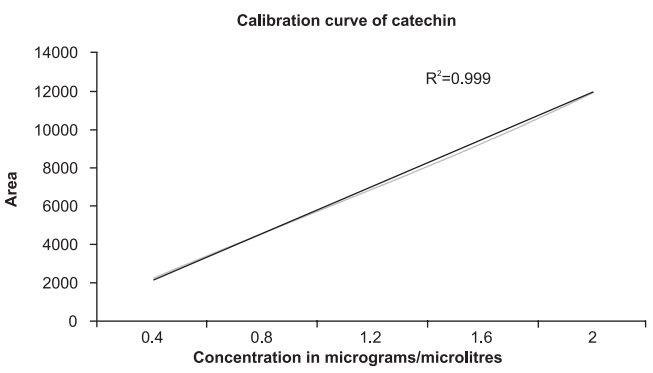

FIGURE 4. Calibration curve of standard catechins.

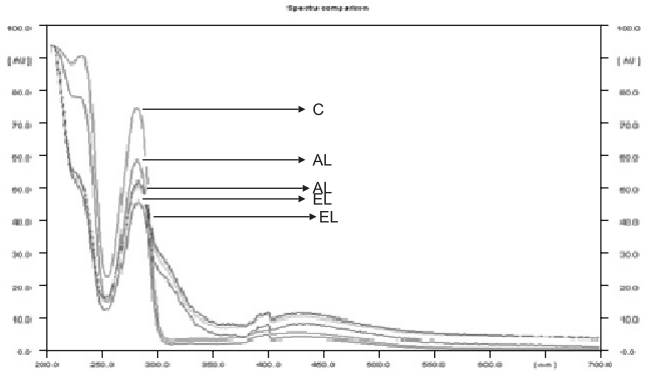

FIGURE 6. Spectra of catechin in various extracts.

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