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Research Article

Phytochemical evaluation and HPTLC fingerprint profile of *Cassia fistula*

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ABSTRACT

The present study was aimed to develop the high performance thin layer chromatography (HPTLC) finger print profile of methanol and ethyl acetate extracts of leaves of *Cassia fistula*. Chromatographic technique was used for separation of components from different extracts of leaves. This study was planned to develop a HPTLC fingerprint profile of extracts in different solvents such as petroleum ether, toluene, ethyl acetate, chloroform, acetone and formic acid. A HPTLC method for the separation of the active constituents in extracts has been developed and TLC of these extracts on silica gel pre-coated aluminum plates of Merck by automatic TLC applicator and using solvent system toluene: ethyl acetate: formic acid::5:4:1 was performed. HPTLC profiling of the extract confirm about the presence of various phytochemicals. HPTLC finger print scanned at 400 nm for methanol and ethyl acetate leaf extracts revealed 15 and 16 peaks with Rf values in the range of 0.06 to 0.99 and 0.02 to 0.98 respectively. The bands revealed presence of greenish, purple, pink and light yellowish orange bands showing the presence of steroids, terpenoids and saponins after spraying with anisaldehyde sulphuric acid reagent. The HPTLC method for routine quality control of present species can be carried out using this method for extracts of plant and serve in qualitative, quantitative and was appropriate for standardization of the extract.

Keywords: Authentication, *Cassia fistula*, Fingerprint, HPTLC, Profile, Standardization.

1. INTRODUCTION

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. This knowledge is accessible from thousands of medical texts and manuscripts. The substances having medical value have been extensively used for treating various disease conditions. Herbs being easily available to human beings have been explored to the maximum for their medicinal properties. Products of primary metabolism such as aminoacids, carbohydrates and proteins are vital for the maintenance of life processes, while others like alkaloids, phenolics, steroids, terpenoids are products of secondary metabolism and have toxicological, pharmacological and ecological importance[1]. Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some need to be explored[2]. The phytochemical evaluations of plants which have a suitable history of use in folklore have often resulted in the isolation of principles with remarkable bio-activities[3]. Identification and quality evaluation of crude herbal extracts is a fundamental requirement. It is an accepted fact that the qualitative analysis of crude herbal extracts constitutes an important and reliable part of quality control protocol

as any change in the quality of extract directly affects the constituents.

Standardization and quality control of herbal drugs is very complicated because herbal products contain a group of phytoconstituents and are very capable of variation. There is the variability within the same plant material or between the different parts of the same plant. The variability may be from grower to grower, crop to crop and also depends on the harvest and post harvest handling. On the other hand herbal drugs have multiple phytoconstituents including active, inactive, unknown which are dietary rather than therapeutic[4].

Hence, methodologies that can generate a fingerprint of each extract in large collections would be useful to detect stability of the same extract over time. Preferably, the method should be based on electronic storage, retrieval and analysis of the data[5]. Various extraction methods and analytical methods as spectrophotometry, high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC–MS) and Fluorescence Transmission-Infrared Spectroscopy (FT-IR) are developed for the study about plant active compounds[6]. High-performance thin layer chromatography (HPTLC) based methods could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. This reduces time and cost of analysis. In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters[7,8,9]. *Cassia fistula* (Amaltas) belongs to family Leguminosae. It is a semi-wild Indian Labernum. It is distributed in various countries including Asia, South Africa, Mexico, China, West Indies, East Africa and Brazil. This plant is widely used by tribal people to treat various ailments including ringworm and other fungal skin infections and it is widely used in traditional medicinal system of India. It has been reported to possess hepatoprotective, anti-inflammatory, antifungal and antibacterial properties[10]. The present research deals with the phytochemical investigation and development of HPTLC fingerprints of methanol and ethyl acetate extracts of *C. fistula* leaves which can be used for identification, authentication and characterization.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material:

Cassia fistula belonging to family Leguminosae was selected on the basis of traditional applications and pharmacological reports. The plant material (leaves) was collected from Botanical Garden Kurukshetra University. Authentication of plant material was done from Wild Life Institute of India, Dehradun with specimen number GS-414.

2.2 Preparation of Plant Extract:

The leaves were carefully washed under running tap water followed by sterile water and shade dried for 4-5 days. The dried leaves were ground to powder and stored in airtight containers. Plants secondary metabolites possess various biological activities and methanol is capable of extracting a wide range of polar and rather non-polar compounds such as alkaloids, sterols, flavonoids and carbohydrates due to its high polarity therefore it was used for extraction. 10g of powdered leaves were soaked in conical flask containing 100ml of methanol for 24 hrs. Conical flask was allowed to stand for 30 mins in a water bath (at 100°C) with occasional shaking followed by keeping all the flasks on rotary shaker at 200 rpm for 24h[11]. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rotary evaporator. The dried extract, thus, obtained was sterilized by overnight UV-irradiation and stored at 4°C in refrigerator for further use[12].

2.2.1 Preparation of sub fractions:

Sub fraction of the methanol extract of *C. fistula* was prepared in ethyl acetate[13]. To prepare the sub fraction the methanol extract of the plant was dissolved in hot water. The aqueous solution of methanol extract was transferred into a separating funnel for partitioning with ethyl acetate. The sub fraction was dried in rotary evaporator and stored in refrigerator for further use. Phytochemical investigation and HPTLC fingerprints of methanol and ethyl acetate extracts of *C. fistula* leaves were determined.

2.3 Qualitative Phytochemical Analysis

The extracts were tested for the presence of bioactive compounds by using standard methods[14,15].

2.3.1 Flavonoids:

Extract mixed with few fragments of magnesium turnings. Concentrated HCl was added drop wise. Appearnce of pink scarlet colour after few minutes indicates the presence of flavonoids.

2.3.2 Phenols and Tannins:
The sample mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicates the presence of phenols and tannins.

2.3.3 Saponins:
5ml of distilled water mixed with extract in a test tube shaken vigorously. The formation of stable foam is taken as an indication for the presence of saponins.

2.3.4 Alkaloids:
2ml of 1% HCl mixed with crude extract and heated gently. Mayer's and Wagner's reagent was added to the mixture. Turbidity of the resulting precipitate is taken as evidence for the presence of alkaloids.

2.4 High Pressure Thin Layer Chromatography (HPTLC) Profile
The standardization of a crude drug is an integral part of establishing its correct identity. The results of this investigation could therefore, serve as a basis for proper identification of the plant. Chromatographic fingerprint profile of methanol and ethyl acetate extracts of *C. fistula* were studied by HPTLC.

2.4.1 Sample Preparation and Application:
5 mg/ml concentration of extracts were prepared in respective solvents of chromatographic grade and then filtered by whatman filter paper No. 1. Prepared samples of different extracts were applied on TLC aluminium sheets silica gel 60 F 254 (Merck) 07 µl each with band length of 5 mm using Linomat 5 sample applicator set at a speed of 150 nl/sec.

2.4.2 Developing Solvent System:
A number of solvent systems were tried, for each extract for better resolution and maximum number of spots, but the satisfactory resolution was obtained in the solvent Toluene: Ethyl acetate: Formic acid:: 5:4:1.

2.4.3 Development of Chromatogram:
The chromatograms were developed in twin trough glass chamber saturated with solvent Toluene: Ethyl acetate: Formic acid::5:4:1 for 20 minutes up to the distance of 80 mm.

2.4.4 Scanning and Detection of spots:
The air dried plates were viewed in ultraviolet radiation to mid-day light (Figure 1). Spots were visible without derivatization at 254 and 366 nm wavelengths but best results were shown when TLC plates were sprayed with detection reagent (Anisaldehyde sulfuric acid reagent and plate was heated at 110°C for 5 minutes) and then visualized in visible light range 400-600nm. Scanning was performed by CAMAG HPTLC Densitometer (Scanner 3) in absorbance mode at both 254 and 366 nm, the extracts were also scanned at 350-600 nm using deuterium and tungsten lamp with slit dimension 6.0 X 0.45 macro. The R_f values and colour of the resolved bands were noted.

3. Results
3.1 Phytochemical Screening
The Phytochemical tests on methanol and ethyl acetate extracts of *C. fistula* leaves showed the presence of various phytoconstituents like alkaloids, saponins, flavonoids, phenols and tannins (Table 1).

3.2 HPTLC Profile
The study revealed that *C. fistula* showed best results in Toluene:Ethyl Acetate: Formic acid::5:4:1 solvent system for both extracts. After scanning and visualizing the plates in absorbance mode at both 254nm, 366 nm and visible light range (400-600nm after spraying with anisaldehyde sulphuric acid reagent) best results were shown at 400nm. The HPTLC images shown in Figure 1 indicate that all sample constituents were clearly separated without any tailing and diffuseness.
The results from HPTLC finger print scanned at wavelength 400 nm for methanol extract of *Cassia fistula* leaf revealed the presence of fifteen polyvalent phytoconstituents (Table 2). The R_f values ranged from 0.06 to 0.99. It is also clear from Table 2 and the chromatogram as shown in Figure 2 and 3 that out of 15 components, the component with *R*f values 0.95, 0.82 were found to be more predominant as the percentage area is more with 28.85% and 12.91% respectively. TLC plate showed different colour phytoconstituents of *C. fistula* methanol extract (Track 1-2). The bands revealed presence of one greenish, three purple, one pink and two light yellowish orange bands showing the presence of steroids, terpenoids and saponins (Figure 1) after spraying with anisaldehyde sulphuric acid reagent.
The HPTLC finger print scanned at wavelength 400 nm for ethyl acetate extract of *C. fistula* leaf showed sixteen polyvalent phytoconstituents and corresponding ascending order of R_f values ranged from 0.02 to 0.98 in which highest concentration of the phytoconstituents was found to be 30.26 % and its corresponding R_f value was found to be 0.95 respectively and was recorded in Table 2 and Figure 4. TLC plate showed different colour phytoconstituents of *C. fistula* ethyl acetate extract (Track 3-4) by presence of one greenish, three purple, two yellow, one pink and three merged yellowish orange bands showing the presence of terpenoids,

steroids and saponins (Figure 1) after spraying with anisaldehyde sulphuric acid reagent.

Thus the developed chromatogram is specific with selected solvent system, *R*f value and serve the better tool for standardization of the extract. HPTLC fingerprint of a plant species helps in the proper identification and quality control of a particular plant species and also provides basic information regarding isolation, purification, characterization and identification of marker chemical compounds of the species. Thus, the present study provides sufficient information about phytoconstituents present in the ethyl acetate and methanol extracts of *C. fisula* and also in the identification, standardization and quality control of this medicinal plant.

4. DISCUSSION

Phytochemicals are chemical compounds synthesized during the various metabolic processes. Various phytochemicals possess a variety of pharmacological activities. These chemicals are often called secondary metabolites. Some of these are found to have antimicrobial activity and serve as plant defense mechanisms against pathogenic organisms. These compounds are classified as phenols, quinines, flavonoids, tannins, alkaloids, glycosides and polysaccharides. Phytochemical analysis revealed the presence of alkaloids, saponins, flavonoids, phenols and tannins in *C. fistula* methanolic and ethyl acetate leaf extracts. HPTLC fingerprint studies confirmed the results of phytochemical screening by the presence of various coloured bands at different wavelengths with specific solvent systems, symbolizing the presence of particular phytocompounds.

Sujogya *et al.* in 2011 reported the presence of alkaloids, flavonoids, triterpenoids, carbohydrates, glycosides, saponins, protein and amino acid in methanolic leaf extracts of *C. fistula*[16]. Our results are in accordance by showing the presence of alkaloids, flavonoids and saponins in the methanolic extract of leaves of *C. fistula*.

Jyothi *et a.l* reported quercitin in *Cassia auriculata* L. using HPTLC fingerprint profile[17]. The present study is first to report the HPTLC fingerprint of ethyl acetate and methanol extracts of *C. fistula* leaves showing maximum number of components 16 and 15 respectively at 400nm with solvent system Toluene: Ethyl acetate: Formic acid:: 5:4:1.

From the HPTLC studies, it has been found that ethyl acetate and methanol extracts contain a mixture of compounds. This densitometric HPTLC fingerprint profile may be used as marker for quality evaluation and standardization of the drug. Thus, HPTLC fingerprint profile along with their R_f values were recorded, which would serve as a reference standard for the scientist engaged in research on the medicinal properties of plant.

5. CONCLUSION

HPTLC fingerprint analysis not only gives the idea for the authentication of the plant extracts and its constituents but also provides the parameters for quality of herbal formulations. In HPTLC technique, as the sample is applied as a rectangular band it provides more resolution and better separation of spots as compared to the TLC technique because of the shape of the area in which the compounds are present on the plate. The chromatographic fingerprint, therefore is suitable for monitoring the identity and purity profile of a plant extract. In addition to qualitative detection, HPTLC technique also provides semiquantitative information about the major active phytoconstituents present in a plant extract, thus enabling an assessment of plant extract quality.

HPTLC fingerprint analysis can be used as a diagnostic tool for the correct identification of the plant. Though further work to characterize the other chemical constituents and perform quantitative estimation with marker compounds is also necessary these data can also be considered along with the other values for fixing standards to this plant. In conclusion, the results obtained from qualitative evaluation of HPTLC fingerprint images will be helpful in the identification and quality control of the drug and ensure therapeutic efficacy. It can be concluded that HPTLC fingerprint analysis of leaf extract of *C. fistula* can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also a good estimator of genetic variability in plant population.

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CONFLICT OF INTEREST

There is no conflict of interest.

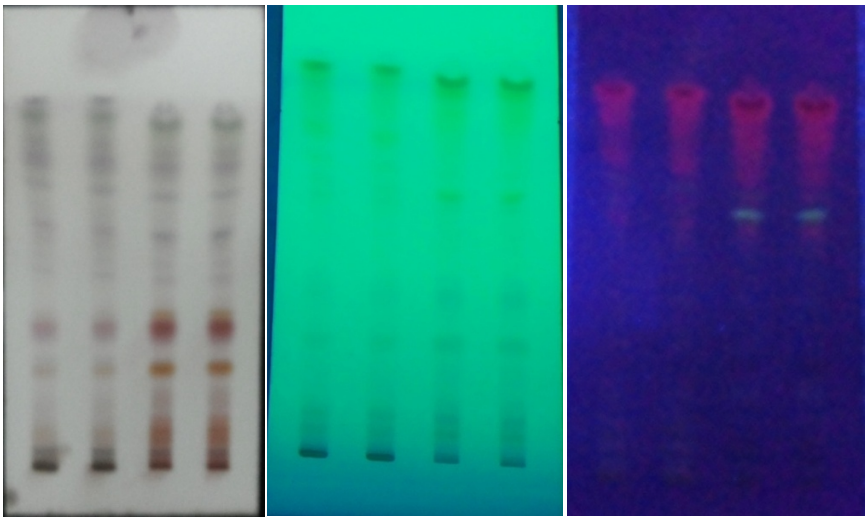
Table 1
Preliminary phytochemical screening of different extracts of *Cassia fistula* leaf.

Phytoconstituents	M	EA
Flavonoids	+	+
Phenols &Tannins	+	+
Saponins	+	+
Alkaloids	+	+

M: Methanol extract, EA: Ethyl acetate extract, "+" Present," – "Absent.

Table 2
Data pertaining to HPTLC fingerprint of different extracts of *Cassia fistula* at 400 nm.

Extract	Solvent system	No. of peaks	R_f Value	Percent Area (%)
Methanol extract	Toluene:Ethyl Acetate: Formic acid 5:4:1	15	0.06, 0.11, 0.20, 0.31, 0.44, 0.53, 0.56, 0.61, 0.65, 0.70, 0.75, 0.82, 0.86, 0.95, 0.99	1.14, 1.80, 0.39, 3.86, 7.05, 3.37, 2.84, 5.60, 4.87, 6.18, 8.00, 12.91, 8.36, 28.85, 4.79
Ethyl acetate extract	Toluene:Ethyl Acetate: Formic acid 5:4:1	16	0.02, 0.05, 0.07, 0.11, 0.14, 0.22, 0.30, 0.44, 0.52, 0.56, 0.65, 0.69, 0.74, 0.80, 0.84, 0.98	0.31, 0.73, 0.37, 2.81, 0.54, 1.22, 8.14, 13.60,2.42, 3.46, 8.49, 3.52, 6.05, 9.83, 8.26, 30.26



1 2 3 4 1 2 3 4 1 2 3 4

HPTLC plate seen at Visible light after Spraying with anisaldehyde Sulphuric acid reagent

HPTLC plate seen at 254 nm

HPTLC plate seen at 366nm

Track 1 & 2: Methanol extract
Track 3 & 4: Ethyl acetate extract

Figure 1
HPTLC profile of leaf extracts of *C. fistula*

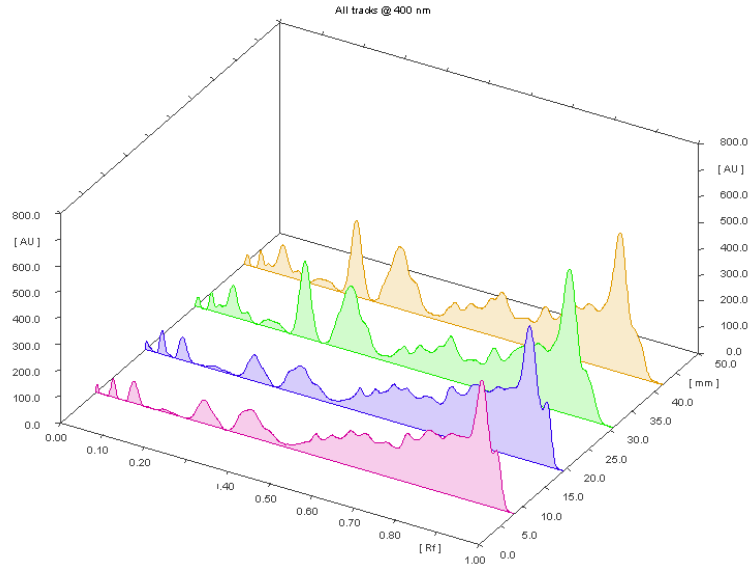


Figure 2
Three dimensional representation of HPTLC chromatogram of *Cassia fistula* methanol and ethyl acetate extract measured at 400 nm.

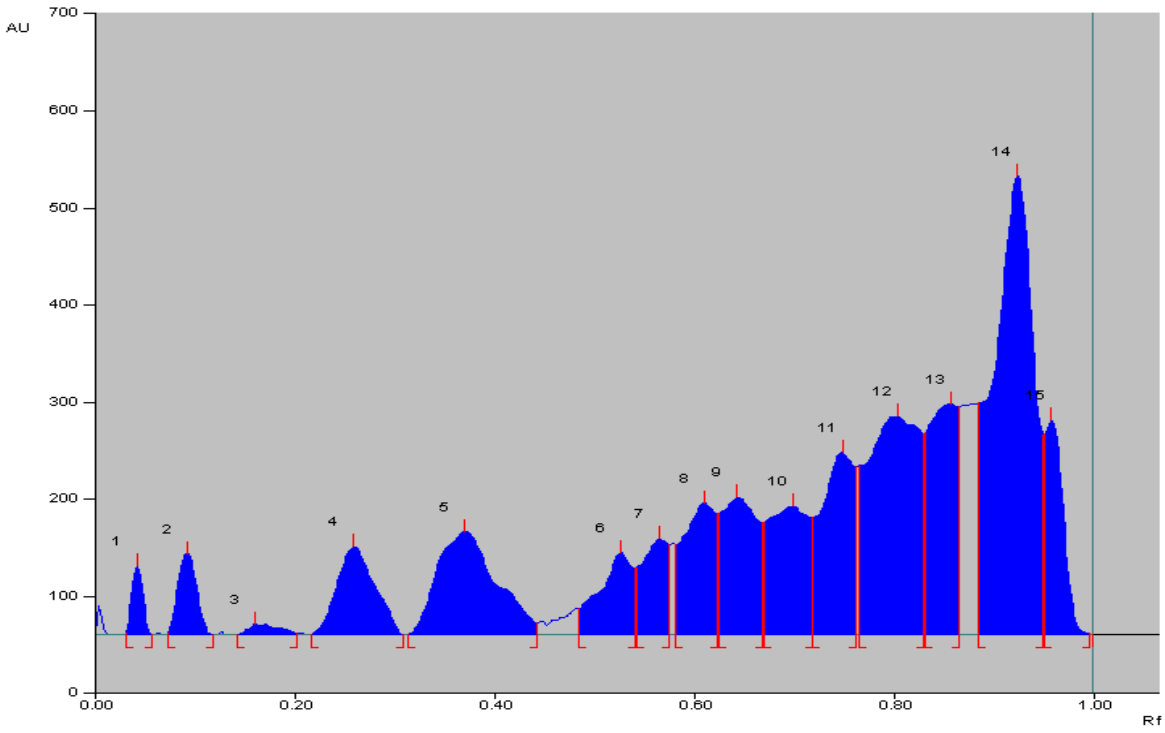


Figure 3
Chromatogram of methanol extract of *Cassia fistula* leaf at 400 nm.

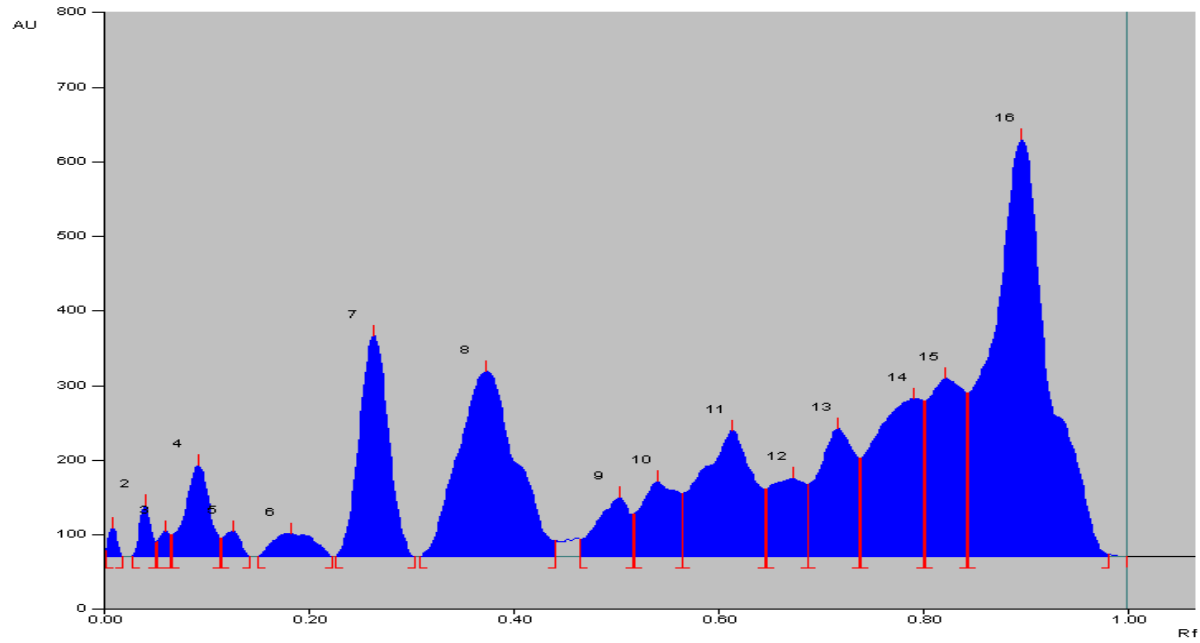


Figure 4
Chromatogram of ethyl acetate extract of *Cassia fistula* leaf at 400 nm.

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