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Chromatographic finger print analysis and antimicrobial activity of *Cassia Fistula* L. bark by HPTLC technique

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ABSTRACT

To evaluate the phytochemistry, HPTLC fingerprint profile and antimicrobial activity of *Cassia fistula* L. bark, preliminary phytochemical screening was done by the methods of Treas and Evans and Sofowora. The HPTLC analysis were carried out as Harbone and Wagner et al. described. The in vitro antimicrobial activity were performed by agar well diffusion method of Perez et al. The phytochemical screening showed the presence of alkaloids, glycosides, fats, tannins, flavonoids in methanol extract of bark, where as alkaloids, glycosides, saponins, tannins, flavonoids, photobatalines and anthraquinone were found in the aqueous extract. The HPTLC finger printing of the methanolic extract of bark showed 8 compounds with different R_f values. The highest values are 0.86 (79.20%), 0.27 (5.14%) and 0.92 (4.23%) respectively. The compound with 79.20% area appeared to be the major compound. The HPTLC results confirmed that the alkaloids are the major constituents in bark of *C. fistula* L. This was followed by the glycosides, tannins and flavonoids in trace amount. The purity of sample was confirmed by comparing the absorption spectra at start, middle and end position of the band. It can be concluded that HPTLC finger printing of *Cassia fistula* L. bark may be useful in differentiating the species from the adulterant and act as a biochemical marker for this medicinally important plant in the pharmaceutical industry. Among the different extracts methanol and aqueous extracts showed significant antimicrobial activity against test microbes and gram negative bacteria *P.aeruginosa* and *S.aureus* also.

Key words: Phytochemistry, HPTLC Finger Print, antimicrobial activity, bark, *Cassia fistula* L.

INTRODUCTION

Herbal remedies are found to be safe and effective. Many plants species have been used in folkloric medicine to treat various ailments. Even today compounds from plants continue to play a major role in primary healthcare as therapeutic remedies in many developing countries [1]. The prevailing threat on existing plant wealth compels us for an immediate scientific evaluation of the medicinal properties of these plants and globally there has been an increased interest to identify compounds that are pharmacologically potent, that have low or no side effects for use in therapeutic purpose [2]. Standardization of plant materials is the need of the day. The modern methods describing the identification and quantification of active constituents in the plant material may be useful for proper standardization of herbals and its formulations. The WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled technique and applying suitable standard methods[3]. HPTLC is a valuable tool for reliable identification and analyzing plant materials. It can be visualized and stored as electronic images[4]. High performance thin layer chromatography (HPTLC) is emerging as a versatile, high throughput and cost effective technology that is uniquely suited to assessing the identity and quality of botanical materials[5,6].

Cassia fistula L. (Caesalpiniaceae) commonly known as Amaltas and in English popularly called Indian labrum has been extensively used in Ayurvedic system of medicine for various ailments. It is native to India, Amazon, Sri Lanka

and is now cultivated worldwide. It is laxative, used in liver disorder, Jaundice, antidiabetic and lowers cholesterol activity. The ripe pods and leaves contain epiafzelechin, epiafzelechin-3-O- glucoside, sennoside A and B, chrysophanol [7,8] . So the objective of the present study is to develop phytochemical screening and HPTLC finger printing of *Cassia fistula* L. which may be used as a marker for quality evaluation and standardization of the drug.

MATERIALS AND METHODS

Plant Material

The fresh bark of *C. fistula* L. was collected from different regions of Parbhani district of Maharashtra, India. The taxonomic identification of the plant was confirmed from Herbarium, Dept. of Botany at Dr. B.A.M. University Aurangabad and Voucher specimen was deposited in the Department of Botany, D.S.M. College, Jintur, District-Parbhani (M.S.) India. The collected bark was cleaned and shade dried at room temperature. The dried bark was powdered and stored in air tight bottles for further use.

Preparation and Extraction of Plant Material

About 20gm of the Shade dried powder of bark were extracted with 150ml of petroleum ether, methanol, ethanol, aqueous and acetone respectively for 8-12 hours by using the soxhlet apparatus [9]. Collected extracts were concentrated by evaporation under room temperature and used for the study.

Phytochemical Screening

All the extracts of bark were individually analyzed for the various classes of phytoconstituents such as alkaloids, carbohydrates, glycosides, saponins, tannins, flavanoids, photobatalins and anthraquinonines by following the chemical tests and methods[10,11].

HPTLC Profile

HPTLC studies were carried out following the method[12,13].

Sample Preparation

The methanolic bark extract was dissolved in HPTLC grade methanol which was used for sample application on precoated silica gel 60 GF254 aluminium sheets.

Developing Solvent System

A number of solvent systems were tried for extract but the satisfactory resolution was obtained in the solvent toluene-ethyl acetate- formic acid (7:3:0.1).

Sample Application

The 5 μ l sample were spotted in the form of bands of width 6 mm with 100 μ L sample using a Hamilton syringe on silica gel which was precoated on aluminum plate GF-254 plates (20 \times 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

Development of Chromatogram

The mobile phase consisted of toluene-ethyl acetate- formic acid (7:3:0.1) and 15 μ l of mobile phase was used per chromatography run. The linear ascending development was carried out in a (20 \times 10 cm) twin through glass chamber saturated with the mobile phase.

Detection of Spots

The developed plate was dried by hot air to evaporate solvents from the plate. The developed plate was sprayed with anisaldehyde sulphuric acid reagent as spray reagent and dried at 100 ⁰C in hot air oven for 3 minutes. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images under UV light at 254 and 366 nm respectively. The R_f values and finger print data were recorded by WIN CATS software.

Test Microorganisms

Authentic pure cultures of human pathogenic bacteria like gram positive *Staphylococcus aureus* (SRTCC 1073), *Bacillus subtilis* (SRTCC 1091), and gram negative *Pseudomonas aeruginosa* (SRTCC 708) and *Escherichia coli* (SRTCC 3260) and two species of fungi viz. *Aspergillus niger* (SRTCC1073) and *Candida albicans* (SRTCC 3971) are taken for this study. These microorganisms were obtained from the School of Life Sciences, S.R.T. M. University, Nanded (M.S.).

Preparation of Test Organisms Suspension

The test organisms were maintained on slants of medium containing nutrient agar (2.5 gm/10 ml) and sub cultured once a week. The slants were incubated at 37°C for 24 hrs and stored under refrigeration. The inoculum was 1×10^8 cells/ml in each case [14].

Antimicrobial Activity Assay

The *In vitro* antimicrobial activity of bark extracts of *Cassia fistula* L. was determined by the agar well diffusion method [15]. The plant extracts were dissolved in distilled water at concentration 2 mg/ml. The standard antimicrobial solution containing 50µl streptomycin was inoculated with 20µl microbial suspension having concentration 1×10^8 cells/ml. 0.1 ml extract was added to each well. The plates containing bacteria were incubated at 37°C for 24 hrs and those containing fungi were incubated 25°C for 7 days. Positive antimicrobial activity was based on growth inhibition zone and compared with standard drug [16]. The diameter of zone of inhibition surroundings each of the well was recorded.

RESULTS

The result of the phytochemical analysis of the different extracts of bark of *C. fistula* L. are presented in Table 1. The different extracts showed the presence of secondary metabolites such as alkaloids, glycosides, saponins, fats and oils, tannins, flavonoids, photobatalines and anthraquinonines, whereas the carbohydrates and triterpenes were found to be absent in all the extracts of bark. Among the different extract methanol extract showed the presence of alkaloids, glycosides, fats and oils, tannins and flavonoids, whereas the aqueous extract of bark showed the presence of alkaloids, glycosides, saponins, tannins, flavonoids, photobatalin and anthraquinonines. Acetone extract shows the presence of alkaloids and flavonoids only. These phytoconstituents were found to be absent in petroleum ether extract. Among the different extracts, aqueous and methanol extracts shows better solubility of the active compounds.

Table 1: Phytochemical analysis of bark of *C. fistula* L.

Sr. no.	Phyto Constituents	Chemical tests	<i>C. fistula</i> bark extract				
			PE	Meth	Eth	Aq	Ac
1	Alkaloids	1. Mayer's test	-	+	+	+	+
		2. Dragendroff's test	-	+	+	+	+
		3. Wagner's test	-	+	+	+	+
		4. Hagers test	-	+	+	+	+
2	Carbohydrates	1. Molisch's test	-	-	-	-	-
		2. Benedict's test	-	-	-	-	-
		3. Fehling's test	-	-	-	-	-
3.	Glycosides	1. Modified Borntragers	-	+	-	+	-
		2. Legal test	-	+	-	+	-
4	Saponins	1. Foam test	-	-	-	+	-
		2. Froth test	-	-	-	+	-
5	Triterpenes	1. Salkowski test	-	-	-	-	-
		2. Libermann Burchard	-	-	-	-	-
		3. Tschugajew test	-	-	-	-	-
6.	Fats & Oil	1. Stain test	-	+	+	-	-
7	Tannins	1. Gelatin test	-	+	-	+	+
8	Flavonoids	1. Alkaline Reagent test	-	+	-	+	-
		2. Lead acetate test	-	+	-	+	+
		3. Shinoda test	-	+	-	+	+
		4. Zn-HCl reduction test	-	+	-	+	+
9	Photobatalins		-	-	-	+	-
10	Anthraquinonines		-	-	-	+	-

(- : absent, + : present; P.E.- Petroleum ether, Meth.- Methanol, Eth.- Ethanol, Aq.- Aqueous, Ac- Acetone.)

The results of HPTLC finger printing of methanol extract of *Cassia fistula* L. bark showed eight compounds (Table 2, Fig.1,2,3) with the different R_f values seen under UV 366 nm. The highest R_f values are 0.86(79.20%), 0.27(5.14%), 0.92(4.23%) respectively. The compound 7 appeared to be the major compound with 79.20% area. From the results it was confirmed that alkaloids were the major group of phytochemical in methanol extract of bark (Peak 7). This was followed by the glycosides, tannins and flavonoids respectively in trace amount. As the methanol and aqueous were having more activity and intensity when compared to other extracts these two extracts were taken for isolation of alkaloids, glycosides, tannins and flavonoids. Purity of the sample extracts compared by comparing the absorption spectra at start, middle and end position of the band [17]. The above R_f values of the *Cassia fistula* L. bark contain the tannin, anthraquinone and steroids.

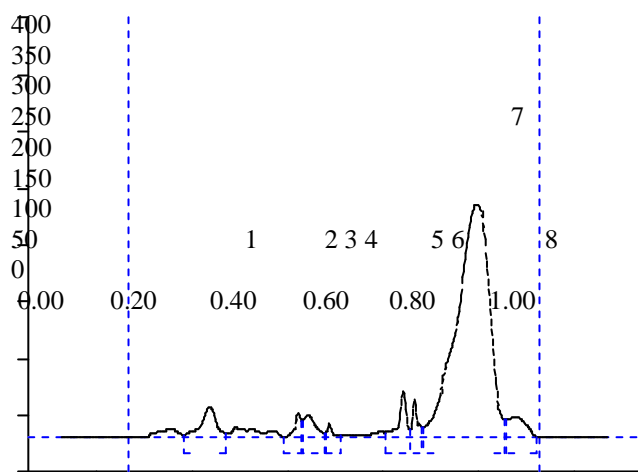
Fig.1.HPTLC analysis of bark of *Cassia fistula* L.

Fig.2. HPTLC Plate at 366 nm.



Fig.3. HPTLC Plate at 254 nm

Table 2. Rf values of the peak of *Cassia fistula* L. bark extract

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Assigned substances
1	0.18	2.7	0.24	27.8	7.26	0.27	3.8	744.4	5.14	unknown*
2	0.40	0.3	0.42	22.2	5.78	0.43	15.3	233.8	1.62	unknown*
3	0.43	15.7	0.45	20.6	5.37	0.48	4.5	441.0	3.05	unknown*
4	0.48	4.7	0.49	12.6	3.29	0.51	2.3	132.5	0.92	unknown*
5	0.61	5.1	0.64	41.5	10.82	0.66	8.2	544.5	3.76	unknown*
6	0.66	7.3	0.67	34.5	9.01	0.68	9.7	301.8	2.09	unknown*
7	0.69	9.7	0.80	206.1	53.78	0.86	16.4	11462.4	79.20	alkaloids*
8	0.86	16.4	0.88	18.0	4.69	0.92	1.7	611.8	4.23	unknown*

The results of the antimicrobial activities of the different extracts of bark are presented in Table 3. The results reported that all the solvent extracts was found to be effective against the tested microbes. Among the different extracts methanol extract showed significant antimicrobial activity against all the microorganisms as compared to standard reference antibiotic streptomycine. Aqueous extract of bark was found effective against *E. coli* and *S. aureus*. The highest antifungal activity was exhibited by methanol extracts of bark against *Aspergillus niger* and *Candida albicans*. The antibacterial activity of aqueous and alcoholic extract of stem and bark of *C. fistula*. Alcoholic extracts recorded greater inhibition against *S. aureus* compared with aqueous extract¹⁸.

Table 3. *In vitro* antimicrobial activity of bark of *Cassia fistula* L.

Microorganisms	Diameter of Zone of inhibition in mm of different extracts of leaves (2mg/ml) Mean± S.D.					Standards reference antibiotic (Streptomycine)
	Methanol	Ethanol	Aqueous	Pet. ether	Acetone	
Bacteria						
<i>Escherichia coli</i>	21±0.15	20±0.12	21±0.17	18±0.15	19±0.15	20±0.22
<i>Staphylococcus aureus</i>	24±0.12	17±0.15	22±0.12	10±0.12	20±0.12	21±0.23
<i>Bacillus subtilis</i>	23±0.15	17±0.12	22±0.15	14±0.15	19±0.22	22±0.14
<i>P.aeruginosa</i>	22±0.12	19±0.10	20±0.12	19±0.12	19±0.13	23±0.11
Fungi						
<i>Aspergillus niger</i>	21±0.15	19±0.12	19±0.12	17±0.14	12±0.12	20±0.12
<i>Candida albicans</i>	20±0.15	19±0.15	20±0.15	18±0.3	12±0.10	18±0.22

DISCUSSION

The chemical analysis of bark of *Cassia fistula* L. showed the presence of various phytoconstituents, reported the presence of anthraquinones in *C. fistula* leaf [19]. The presence of flavanoids, saponins and alkaloids in the leaf extract of *C. tora* [20]. Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure². Practitioners make use of water primarily as a solvent. The results of the present study revealed that methanol extract was much better and powerful mainly due to the better solubility of the active compounds in organic solvents [2]. The present study also supplement the folkloric usage of the studied

plant which possesses several known and unknown bioactive compounds with bioactivity. In recent times in addition to morphological characters in plant taxonomy, anatomical, cytological, biochemical and molecular markers are also being used to classify the plants.

HPTLC finger printing profile is useful as phytochemical marker and also a good estimation of genetic variability in plant population. HPTLC is a valuable tool for reliable identification. It provides chromatographic finger prints that can be visualized and stored as electronic images which can be used several times without any errors and change [4, 21]. Further research is necessary to determine the identity of the compounds from this plant and also determine their full spectrum of efficacy. However, the present study form a primary platform for further phytochemical and pharmacological studies.

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