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Qualitative and Quantitative estimation of Phytoconstituents and Antimycobacterial property of ethanolic extract of *Cassia fistula* leaf against H37RV test organism

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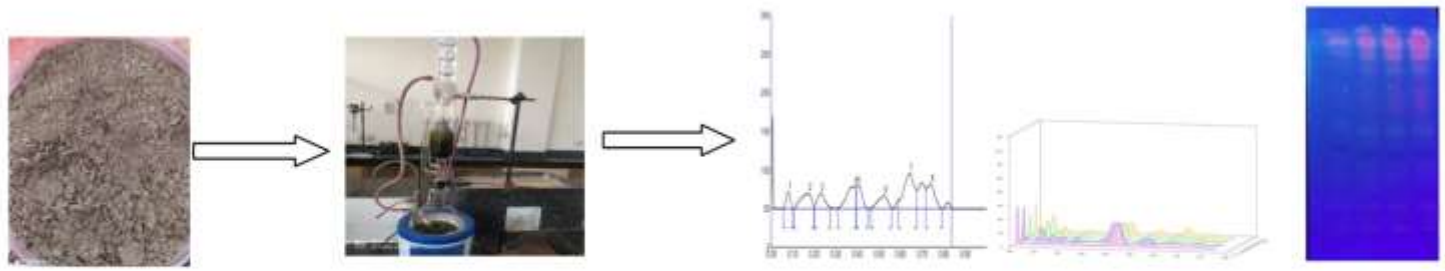
Abstract



Powder of *Cassia fistula* Leaves

Extraction by Hot Percolation Method

a) Chromatogram of ethanol extract of *Cassia fistula* leaf at 366 nm
b) Three dimensional representation of HPTLC chromatogram of ethanol extract of *Cassia fistula* leaf measured at 366 nm
c) HPTLC Plate of ethanol extract of *Cassia fistula* leaf shown at 366 nm

Cassia fistula belongs to the family Caesalpinaceae commonly known as "Golden shower tree" has been used in different traditional system of medicines for various ailments since ancient times. *Cassia fistula* grows throughout in Bangladesh and in many other Asian countries such as India, China, Hong Kong, Philippines, Malaysia, Indonesia, and Thailand. Sequential extraction of leaves of *Cassia fistula* was carried out by using different solvents like petroleum ether, chloroform, ethanol, methanol and water in Soxhlet apparatus. Ethanolic extract was investigated for qualitative and quantitative phytochemical studies. Ethanolic extract was also analysed for its antimycobacterial property. Results of the study showed highest yield of the leaf extract in ethanol. Phytochemical studies revealed the presence of secondary metabolites like carbohydrate, proteins, amino acids, steroids, saponins, alkaloids, tannins, phenols and flavanoids. Ethanolic leaf extract showed good inhibitory activity against H_{37}RV test organism. The minimum inhibitory concentration was found to be 2.5µg/ml. Furthermore, the ethanol extract was subjected to LCMS, TLC and HPTLC fingerprinting. Results of LCMS, TLC and HPTLC showed the presence of alkaloids, flavonoids and phenols.

Keywords: *Cassia fistula*, Phytochemical Screening, LCMS, TLC, HPTLC, Antimycobacterial property

Introduction

Cassia species are the well-known medicinal plants have different medicinal properties. The genus Cassia comprises of 600 species of herbs, shrubs and trees (Danish *et al*., 2011). Traditionally, *Cassia fistula* is one of the most commonly used plants in Ayurveda, Siddha, Unani and Homoeopathy (Kumar et al., 2017). *Cassia fistula* is

distributed and used as a traditional herbal medicine in India, China, Hong Kong, Philippines, Malaysia, Indonesia, and Thailand (Sandai, 2019; Rahmani, 2015). *Cassia fistula* is the national tree of Thailand and its flower is the national flower of Thailand (Danish *et al*., 2011; Kumar et al., 2017; Sandai, 2019).

The plant is widely used in traditional Indian medicinal system reported to possess hepatoprotective, anti-inflammatory, antitussive, antifungal, antibacterial, antimicrobial and also used to improve wounds healing (Hanif *et al*., 2007; Kabila *et al*., 2017). Traditionally, it has been also used in the treatment of diabetes, hematemesis, leucoderma, pruritis, intestinal disorder, antipyretics, antioxidant, antimutagenic, antitumor, analgesic and laxative (Kiritikar *et al*., 2006). *Cassia fistula* contain various phytoconstituents viz. tannins, flavonoids, glycosides, carbohydrates, linoleic acid, oleic, stearic acid, oxalic acid, tannins, oxyanthraquinones, anthraquinones derivatives. *Cassia fistula* also contain rhein glycosides, fistulic acids, sennosides A and B, anthraquinones, flavanoid-3-ol derivatives, ceryl alcohol, kaempferol, bianthraquinone glycosides, fistulin, essential oils, volatile components (Kumar *et al*., 2017; Sharma *et al*., 2021). *Cassia fistula* is also used as an excipient by imparting properties like binding agent, coating agent, film forming, thickening agent, gelling agent, film former and suspending agent. Various novel formulations have been prepared by using *Cassia fistula* like monometallic nanoparticles, bimetallic nanoparticles, microspheres, beads, nanoemulsions, topical gels, etc. The present research paper highlights qualitative and quantitative determination of Phytoconstituents, characterization and antimycobacterial property of ethanolic extract of *Cassia fistula* leaf.

MATERIAL AND METHODS

Collection and authentication of plant

Cassia fistula complete plant parts were collected within the kangra District (H.P.). The Herbarium of plant was subjected to authentication from National Herbarium of Cultivated Plants, New Delhi. The plant was identified by Dr. Anjula Pandey Principal Scientist at National Herbarium of cultivated Plants, New Delhi.

Preparation of Plant Material

Leaves were washed with Distilled water to remove dirt and then shade dried. After drying leaves were crushed into coarse powder in a mechanical grinder and stored into a well closed container for further studies (Mandoli *et al*., 2018).

Extraction of Plant Material

About 60 g of dry leaf powder of *Cassia fistula* were sequentially extracted using petroleum ether, chloroform, ethanol, methanol, and aqueous solution in Soxhlet apparatus. Place the thimble inside the extractor and pour the solvent sequentially in Round bottom flask. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation on water bath. The yield of each extract was calculated and stored in self-sealing bag for further use (Panda *et al*., 2011).

Table1. Procedure for phytochemical screening of *Cassia fistula* leaf extract.

S. NO.	Phytoconstituents	Test Procedure
	Carbohydrates	***Molisch's test:*** To 2-3 ml of extract solution, few drops of alpha-naphthol solution in alcohol was added and shaken well. Concentrated sulphuric acid was added from sides of the test tube and formation of violet ring was observed at the junction of two liquids. ***Barfoed's test:*** Equal volume of Barfoed's reagent and test dispersion were mixed and heated for 1-2 min in boiling water bath. Formation of red color precipitate was observed.
	Proteins	***Biuret test:*** To 3 ml test Solution, 4% sodium hydroxide and few drops of 1% copper sulphate solution were added, and reaction mixture was observed for violet or pink color. ***Million's test:*** To 3 ml test Solution, 5 ml Million's reagent was added and observed for appearance of white precipitate. On warming precipitate should turns brick red or the precipitate dissolves giving

	red colored solution.
Starch	***Iodine test:*** To 3 ml of test Solution, few drops of dilute iodine solution was added and observed for the appearan- ce of blue color. Blue color disappeared on boiling and reappeared on cooling.
Alkaloids	Extract solution was evaporated and residue was collected. To the residue dilute hydrochloric acid was added and filtered. Filtrate was collected and following tests were performed: ***Murexide test for purine alkaloids:*** To 3-4 ml test dispersion, 3-4 drops of concentrated sulphuric acid was added; and evaporated to dryness. Residue was cooled, two drops of ammonium hydroxide was added and observed for the appearance of purple color. ***Wagner's test:*** To 2-3 ml filtrate, few drops of Wagner's reagent was added and observed for the appearance of reddish brown color precipitate. ***Hager's test:*** To 2-3 ml filtrate, few drops of Hager's reagent was added and observed for the appearance of yellow color precipitate. ***Mayer's test:*** To 2-3 ml filtrate, few drops of Mayer's reagent was added and observed for the appearance of precipitate. ***Dragendroff's test:*** To 2-3 ml of filtrate, few drops of Dragendroff's reagents was added and observed for the appearance of orange- brown precipitate
Glycosides	***Cardiac glycoside*** *Baljet's test:* A dispersion of mucilage was observed for appearance of yellow to orange color with sodium picrate. ***Anthraquinone glycosides*** ***Borntrager's test:*** To 3 ml dispersion of mucilage, equal volume of dilute hydrochloric acid was added, boiled and filtered. To cold filtrate, equal volume of chloroform was added and shaken well. Then organic layer was separated and ammonia was added to it. Appearance of pink or red color in ammonia layer confirms the presence of glyco- sides.
Flavanoids	To each extract add NaOH and observed for yellow coloration.
Saponin	***Foam test:*** Each extract was shaken vigorously with distilled water in a test tube and observed for the appea-rance of foam.
Steroids	***Salkowski reaction*****:** To 2 ml of extract dispersion, chloroform (2 ml) and concentrated sulphuric acid (2 ml) were added and shaken well. Reaction mixture was observed for the separation of chloroform layer and greenish yellow fluorescence in acid layer
Tannins and Phenols	***$FeCl_3$ (5%) solution*****:** To 2-3 ml of alcoholic dispersion of mucilage, few drops 5% ferric chloride solution was added, and reaction mixture was observed for the appearance of deep blue-black color.

Qualitative Phytochemical Studies

The extracts were investigated for the presence of carbohydrates, proteins, amino acids, steroids, glycosides, saponins, alkaloids, glycosides, tannins and flavonoids (Table 1) (Kokate *et al*., 2009; Kokate *et al*., 2019).

Quantification of Phytochemicals

Ethanolic leaf extract of *Casssia fistula* was analysed for total phenolic content, total tannin Content, total Alkaloid content and total flavonoid content (Selvakumar *et al*., 2019; Kumar *et al*., 2017)

Total content of Alkaloids

Plant extract (1 mg) was dissolved in dimethylsulphoxide and 1ml of 2N HCl added and filtered. This solution was transferred to a separating funnel; add 5ml of bromocresol green solution and 5ml of phosphate buffer. The mixture was shaken with 1, 2, 3 and 4 ml of chloroform by vigorous shaking and collected in a 10 ml volumetric flask and diluted to the volume with the chloroform.

A set of reference standard solutions of Atropine (10, 20, 30, 40 and 50 µg/ ml) were prepared in the same manner as described above. The absorbance for standard solutions and test solutions were determined on the reagent blank at 470 nm with an UV/Visible spectrophotometer. The content of alkaloids was expressed as mg of AE/g of plant extract.

Total Flavonoids content

Colorimetric assay was used to determine the total content of flavonoids using aluminium chloride. In 10 ml flask 1ml of Plant extract and 4 ml of distilled water was taken. Add 0.30 ml of 5% sodium nitrite and after 5minutes, 0.3 ml of 10 % Aluminium chloride was mixed in the flask. 5 minutes later, 2 ml of 1M NaOH was treated and diluted using 10 ml distilled water. A set of standard solutions of Quercitin (20, 40, 60, 80 and 100 µg/ml) were prepared as mentioned above. The absorbance was measured for test and standard solutions using reagent blank at 510 nm wavelength by UV-Visible spectrophotometer. The total content of flavonoid was denoted as mg of QE/g of extract.

Total Tannin content

Folin-Ciocalteu method was used for the quantification the tannin total content. About 0.1ml of plant extract was added in 10 ml of volumetric flask containing the distilled water of 7.5ml and Folin-Ciocalteu phenol reagent of 0.5ml, 35% Na_2CO_3 solution of 1 ml and diluted to 10ml using distilled water. The reagent mixture was well shaken and kept at 30°C temperature for 30 min. A set of gallic acid solutions (20, 40, 60, 80 and 100 µg/ml) were prepared as mentioned earlier. Absorbance of standard and test solutions was analyzed with blank at 725 nm wavelength using UV-Visible spectrophotometer. The tannin total content of tannin was expressed as mg of GAE/g of extract.

Total Phenolic Content

The phenolic compounds concentration in extract was quantified by Spectrophotometry method. Folin-Ciocalteu method was employed for the quantification of total phenolic content. The reaction mixture contains 1 ml of plant extract and 9 ml of distilled water. 1 ml of Folin-Ciocalteu phenol reagent was treated with the mixture and well shaken. After 5minutes, 10 ml of 7 % $Na_2 CO_3$ solution was treated with the mixture. The volume was 25 ml. A set of gallic acid standard solutions (20, 40, 40, 60, 80 and 100 µg/ml) were prepared as earlier. Incubated for 90 min at 30°C and absorbance was analyzed for test and standard solutions with reagent blank at 550 nm with using UV Visible spectrophotometer. The content of total phenolic compound was denoted as mg of GAE/g of extract.

Characterization of leaf extract of *Cassia fistula*

Ultraviolet visible spectroscopy analysis

For UV-vis spectrophotometer (LabIndia 3000^+) analysis, the ethanolic extract of *Cassia fistula* leaf filtered through Whatmann No.1 filter paper by using a high-pressure vacuum pump. The sample was diluted to 1:10 with the same solvent. The extracts were scanned in the wavelength ranging from 200-800 nm using Spectrophotometer and the characteristic peaks were detected (Mishra *et al*., 2018; Joshi *et al*., 2014).

Fourier Transform Infrared spectroscopy

Dried powder of the plant extracts of *Cassia fistula* was used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample discs. The powdered sample of each extracts was loaded in FTIR spectrophotometer (Simadzu IR Affinity), with a Scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}(Pavia *et al*., 2014).

Liquid Chromatography Mass Spectroscopy (LCMS)

Qualitative identification of phytochemicals was carried out using a waters, Synapt XSHDMS (2.1mm x 100mm, 1.7µm; C18 waters, Acquity BEH) LC column operated at 120°C and the chromatographic separation was performed using a mobile phase of solvent A: 0.1% formic acid in water solvent A /0.1% formic acid in ACN solvent B with a constant flow rate of 0.15mL/ min. Full screen mass scpectra detection was carried out in electrospray positive ES+ in a mass range m/z of 50-1300 and using a capillary voltage of 3.22 keV and collision energy +4.0 eV. The source parameters were identical for all of the analytes (Mishra *et al*., 2018).

Thin Layer Chromatography (TLC)

TLC studies were carried out to select the solvent system capable of showing better resolution and different solvent systems of different polarities were prepared. Analytical TLC of the ethanolic extract was carried out using a silica gel glass plate (5×10 cm, 0.25 mm thick). Plant sample (10 µl) was loaded on TLC plate as line spots. Capillary tubes were used while applying plant extracts on pre-coated TLC plates using Toluene: Ethyl acetate: Formic acid (5:4:1) as mobile phase. Compounds under the influence of the mobile phase travel over the stationary phase. Thus, separation of compound occurs. Plates were sprayed with Anisaldehyde reagent. After color development, the plates were air dried and also observed using UV light (Wagner *et al*., 1966). Values were determined using retention factor and calculated.

$$Rf = \frac{\text{Distance travelled by the Solute Front}}{\text{Distance Travelled by the Solvent Front}}$$

Where; Rf- Retention factor

High Pressure Thin Layer Chromatography (HPTLC) Profile

Chromatographic fingerprint profile of ethanolic extract of *Cassia fistula* was studied by HPTLC (Wagner *et al*., 1966).

Sample Preparation and Application

Sample of *Cassia fistula* leaf extract was dissolved in HPTLC grade methanol. Prepared sample of *cassia fistula* leaf extracts was applied on TLC aluminium sheets silica gel 60 F 254 (Merck) 0.2 µl each with band length of 6.0 mm using Linomat 5 sample applicator set at a speed of 150 nl/sec.

Developing Solvent System

A number of solvent systems were used, for 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.

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.

Scanning and Detection of spots

The air dried plates were viewed in ultraviolet radiation to mid-day light 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 120°C for 5 minutes) and then visualized in visible light range 400-600 nm. Scanning was performed by CAMAG TLC Scanner 3 (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 5.0 X 0.45 macro. The Rf values and colour of the resolved bands were noted.

Antimycobacterial property

Take 1 mg sample, 1 ml solvent and sterile D/W so that its final concentration is 1000 microgram/ml then dilute it by serial dilution method. DMSO used as 2% diluted as per CLSI Guidelines and so its activity against bacteria was nil. L.J Medium was used for study. In screening 100, 50, 12.5, 6.25. 3.125, 10, 5, 2.5, 1.25, 8, 4, 2, 1, 0.5, 0.25 µg/ml concentrations of the ethanolic extract of *Cassia fistula* leaf were taken. It was added on L. J medium as per ml concentration. The ethanolic extract of *Cassia fistula* leaf found in this primary screening were further tested in a second set of dilution against Strains. The highest dilution showing at least 99 % inhibition was taken as MIC. The result of this was much affected by the size of the inoculum. The test mixture should contain 10^8 organism/ml. It was compare with MacFarnald standard. Results were read by observing visual growth on L.J media. The Standard strain *M.tuberculosis*, H_{37} RV was tested with each new batch of medium. The recommended

drug concentrations are 4 mg/l for streptomycin, 0.2 mg/l for isoniazide, 40 mg/l for Rifampicin and 2 mg/ l for ethambutol (Andrews, 2001).

RESULTS AND DISCUSSION

Percent yield of *Cassia fistula* extract

The percent yield of *Cassia fistula* leaf with different Solvents were 15, 8, 13, 18, 21.6 and 42.6%, with chloroform, petroleum ether, methanol, distilled water, and ethanol solvents, respectively. The percentage yield of the extract was found to be more in ethanol as shown in Table 2.

Table 2. Percent yield of Cassia fistula leaf extract in different Solvents.

S. No.	Solvent	Wt. of dried powder (g)	Wt. of dried Extract (g)	% Yield
1.	Chloroform	60	5	8
2.	Petroleum ether	60	8	13
3.	Methanol	60	11	18
4.	Distilled water	60	13	21.6
5.	Ethanol	60	28	42.6

Phytochemical screening of *Cassia fistula* leaf extract

Phytochemical screening of *Cassia fistula* leaf extract with different solvents showed the presence of various secondary metabolites as shown in Table 3. The ethanolic extract showed the presence of protein, amino acids, steroids, saponins, alkaloids, tannins, phenols, and flavonoids. Methanolic extract showed the presence of protein, amino acids, saponins, alkaloids, tannins & phenols, and flavonoids. Petroleum ether extract showed the presence of Saponins only. Aqueous extract showed the presence of Carbohydrates, proteins, amino Acids, saponins, alkaloids, tannins, phenols, and flavonoids. Chloroform extract showed the presence of tannins, phenols and flavonoids.

Table 3. Preliminary phytochemical screening *Cassia fistula* leaf extract in different solvents.

Test	Results				
	Ethanol	**Methanol**	**Petroleum ether**	**Distilled Water**	**Chloroform**
Carbohydrates	-	-	-	+	-
Protein	+	+	-	+	-
Amino Acids	+	+	-	+	-
Glycosides	-	-	-	-	-
Steroids	+	-	-	-	-
Saponins	+	+	+	+	-
Alkaloids	+	+	-	+	-
Tannins & Phenols	+	+	-	+	+
Flavanoids	+	+	-	+	+

Quantification of Phytochemicals

The results of quantitative estimation of total phenolic content, total tannin Content, total Alkaloid content and total Flavonoids content (as shown in Table 4) along with the standard curves plotted (by using the standard equation of the curve: $y= m x + c$, R^2 value) have been depicted in Figure 1.

Table 4. Quantification of phytoconstituents present in the ethanolic leaf extract of *Cassia fistula.*

Plant Part	Total Alkaloidal Content (mg of AE/g)	Total Flavonoids Content (mg of QE/g)	Total Tannins Content (mg of GA/g)	Total Phenols Content (mg of GA/g)
Leaf	0.431	0.342	0.609	0.00682

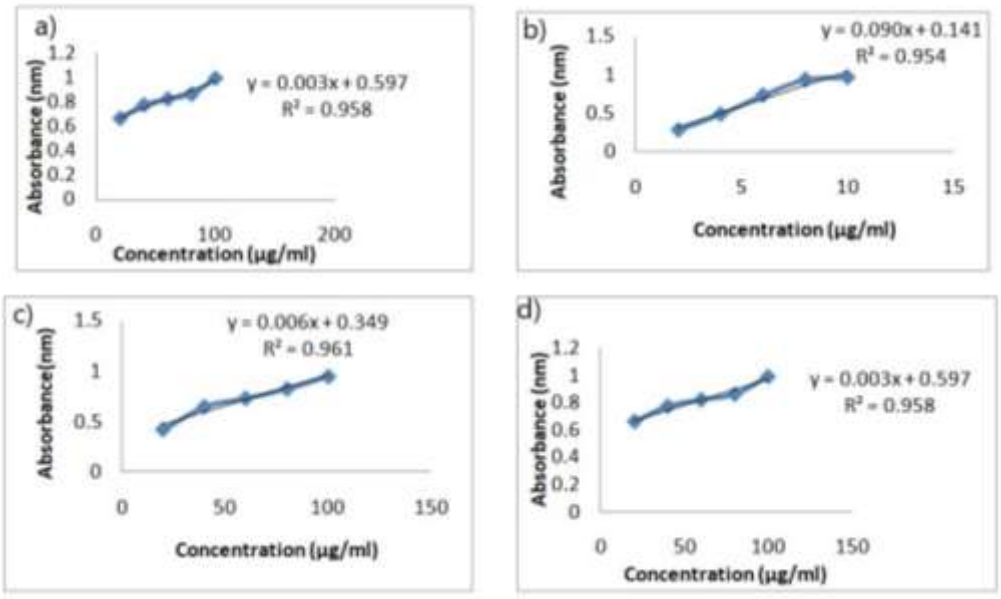


Figure 1. Calibration curve for a) Alkaloids b) Phenols c) Flavonoids d) Tannins.

Characterization of leaf extract of *Cassia fistula*

Ultraviolet-visible spectroscopy analysis

UV-VIS spectrum of this plant extract has absorption peaks at 412.00, 323.00, 291.0, 272.0 214.0 nm with the absorption of 2.204, 2.262, 2.405, 2.405 and 2.356, respectively as shown in (Table 5 & Figure 2). These absorption bands were characteristic of Alkaloids, flavonoids and tannins (Pavia et al., 2014).

Table 5. UV- Spectroscopy analysis of ethanolic extract of *Cassia fistula*

S. No.	Wavelength (nm)	Absorption Value	Result	Reference Values
1	412.00	2.204	Alkaloids (Catechin)	350-450
2	323.00	2.262	Flavonoids (Quercitin, Kaemferol)	300-350
3	291	2.405	Alkaloids (Isoquinilones)	291
4	272.0	2.356	Tannins (Gallic Acid)	204-284

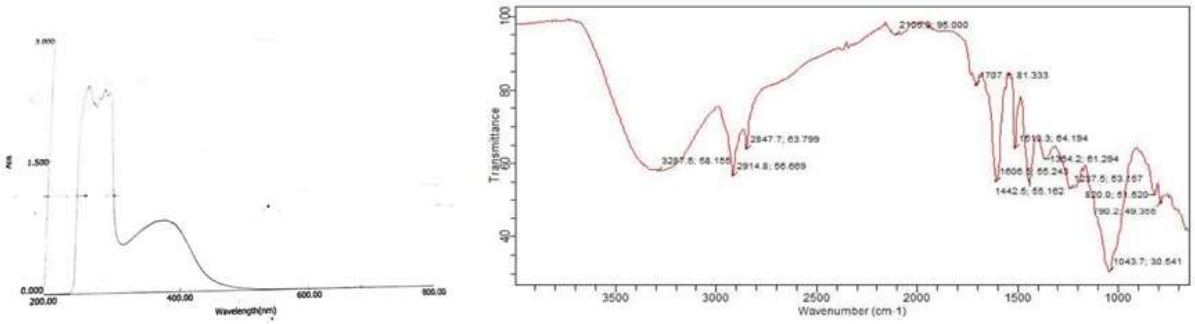


Figure 2. UV and FTIR spectrum of ethanolic leaf extract of *Cassia fistula.*

Fourier Transform Infrared spectroscopy

The FTIR analysis (Table 6 & Figure 2) revealed the presence of a carboxylic group, Phenols, aldehydes, alkenes, allenes, alkanes, esters, Amines, Aromatics and alcohol compounds. FTIR Spectroscopy analysis showed that Alkaloids, Flavonoids, Tannins and Saponins were present in the ethanolic extract of *Cassia fistula* (Pavia et al., 2014).

Table 6. FTIR- Spectroscopy analysis of ethanolic extract of *Cassia fistula.*

Frequency Range (cm^{-1})	**Observed Frequency (cm^{-1})**	**Functional Group**
3400-2400	2914.8	-OH (Carboxylic group)
3500-3100	3287.5	N-H Stretch (Phenols)
2900-2800	2847.7	C-H (Aldehydes)
2270-1940	2105.9	X=C=Y (Alkenes, Allenes)
1725-1705	1707.1	C=O (Carboxylic Acid)
1450-1375	1442.5	CH3 bend (Alkanes)
1640-1550	1606.5	N-H (Primary and Secondary Amine Bend)
1550-1350	1513.3	N=O (Nitro Group)
1375-1300	1364.2	S=O (Sulfones, Sulfoxides, Sulfonyl Chloride, Sulfate, Sulfonamides)
1300-1000	1237.5	C-O (Esters) C-N (Amines)
900-690	820	C-H (Aromatic)
900-690	790	C-H (Aromatics)
1400-1000	1043.7	C-O (Alcohols)

Liquid Chromatography Mass Spectroscopy (LCMS)

The LC-MS analysis of phytochemicals in the leaf extracts of *Cassia fistula* showed the presence of various bioactive components. The identification of the phytochemicals was confirmed based on the molecular mass and its fragmentation pattern. The results showed the presence of 17 bioactive compounds. The identified compounds include Six phenols, six alkaloids, and four flavonoids. The LC-MS chromatogram was shown in Figure 3. Chromatogram LC-MS analysis of the ethanolic extract of *Cassia fistula* showed the presence of certain different peaks and the components corresponding to the peaks were determined as shown in Table 7.

Table 7. LCMS analysis of ethanolic extract of *Cassia fistula* leaf

S. No.	Retention time	m/z ratio	Compound	Class	Molecular formula
1.	1.24	217.0681	1,4-Dihydroxyanthraquinone	Phenol	$C_{14}H_8O_4$
2.	1.445	266.136	1,4-bis(methylamine)anthraquinone	Phenol	$C_{16}H_{14}N_2O_2$
3.	10.75	123.646	Nicotinic Acid	Alkaloid	$C_6H_5NO_2$
4.	11.216	563.156	Kaemferol rhamnosyl xyloside	Flavanoid	$C_{26}H_{28}O_{14}$
5.	11.83	139.041	6-hydroxynicotinic acid	Alkaloid	$C_6H_5NO_3$
6.	12.679	287.055	Dihydro Kaemferol	Flavanoid	$C_{15}H_{12}O_6$
7.	14.074	395.206	Quinine hydrochloride hydrate	Alkaloid	$C_{20}H_{29}ClN_2O_4$
8.	14.815	379.117	Quinine dihydrochloride Dehydrate	Alkaloid	$C_{20}H_{25}ClN_2O_2$
9.	15.658	515.321	3,4,di-o-Caffeoylquinic acid	Phenol	$C_{25}H_{24}O_{12}$
10.	17.364	351.21	Hydraquinine,2'-cyano	Alkaloid	$C_{21}H_{25}N_3O_2$
11.	20.586	822.361	Catharine	Alkaloid	$C_{46}H_{54}N_4O_{10}$
12.	21.550	550.321	Syringetin3-acetylglucoside	Flavanoid	$C_{25}H_{26}O_{14}$
13.	22.60	301.108	Quercitin	Flavanoid	$C_{15}H_{10}O_7$
14.	24.428	277.216	1,8-Dichloroanthraquinone	Phenol	$C_{14}H_6Cl_2O_2$
15.	24.428	678.458	3,4,5-Tri-O-Caffeoylquinic acid	Phenol	$C_{34}H_{30}O_{15}$
16.	32.092	531.410	9-Naphthalen-2yl-10-phenyl-2-(3-phenylphenyl) anthracene	Phenol	$C_{42}H_{28}$
17.	32.998	965.800	Unknown	-	-

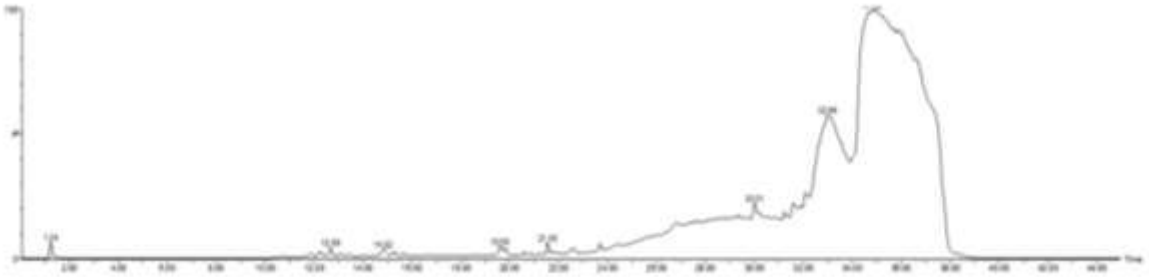


Figure 3. LCMS Chromatogram of ethanolic extract of *Cassia fistula* leaf.

Thin Layer Chromatography

The TLC results showed a total of 3 spots with Rf values of 0.93, 0.84, and 0.76. The TLC plate of ethanol extract showed yellow, blue, and green spots which indicated the presence of flavonoids as shown in Table 8 & Figure 4(Wagner *et al*., 1966).

Table 8. TLC of *Cassia fistula* leaf ethanolic extract.

S. No.	Rf Value	Reference Values	Compound
1	0.93	-	Flavonoids
2	0.84	0.87	Flavonoids (Kaemferol)
3	0.76	0.75	Flavonoids (Quercitin)

Figure 4. TLC Plate of *Cassia fistula* leaf ethanolic extract

High-Pressure Thin Layer Chromatography (HPTLC) Profile

The study revealed that the ethanolic leaf extract of *Cassia fistula* had the best results in Toluene: Ethyl Acetate: Formic acid::5:4:1 solvent system. After scanning and visualizing the plates in absorbance mode at 254 nm, 366 nm and visible light range (400-600 nm after spraying with anisaldehyde sulphuric acid reagent) best results were shown at 366 nm (Figure 5c). The results from the HPTLC finger print scanned at wavelength 366 nm for ethanolic extract of *Cassia fistula* leaf revealed the presence of 8 phytoconstituents (Table 9). The Rf values ranged from 0.10 to 0.79 in which the highest percent area of the phytoconstituents was found to be 26.37% and its corresponding Rf value was found to be 0.68. The bands revealed the presence of one greenish, one purple, three pink, one bluish and two yellowish bands showing the presence of tannins, flavonoids, phenols and alkaloids (Figure 5) after spraying with anisaldehyde sulphuric acid reagent.

Table 9. HPTLC fingerprint of ethanol extract of *Cassia fistula* leaf at 366 nm

Peak	Rf values	Area%	Assigned Compounds
1	0.10	5.15	Tannins
2	0.20	12.09	Alkaloids
3	0.27	8.59	Phenols
4	0.39	14.88	Alkaloids
5	0.45	9.27	Flavanoids
6	0.56	9.38	Phenols
7	0.68	26.37	Flavanoids
8	0.79	14.35	Flavanoids

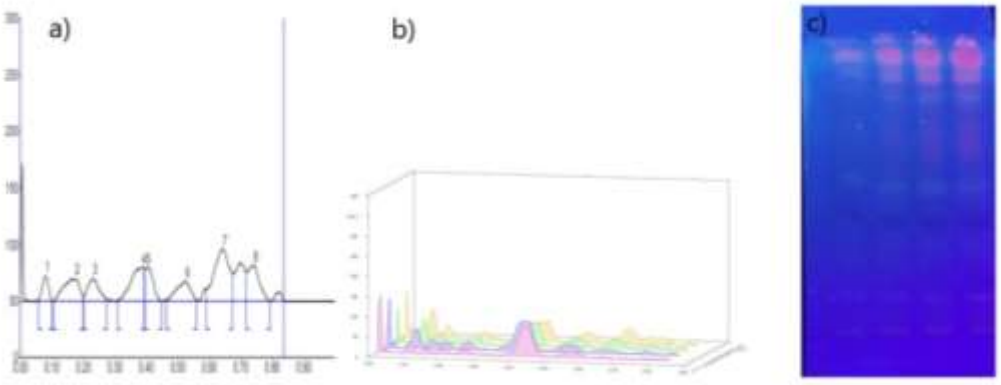


Figure 5. a) Chromatogram of ethanol extract of *Cassia fistula* leaf at 366 nm b) Three dimensional representation of HPTLC chromatogram of ethanol extract of *Cassia fistula* leaf measured at 366 nm. c) HPTLC Plate of ethanol extract of *Cassia fistula* leaf shown at 366 nm.

Antimycobacterial property

The Ethanolic leaf extract of *Cassia fistula* was screened for its *in-vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv by L.J. Slope Method. It was observed that the lowest dilution of extract had no growth on media with positive control and MIC of Ethanolic leaf extract of *Cassia fistula* was found to be 2.5µg/ml. MIC of Ethanolic leaf extract of *Cassia fistula was* compared with the first-line treatment drugs used in Tuberculosis which show remarkable results for the *Cassia fistula* extract (Table 10).

Table 10. Anti tuberculosis activity of ethanolic leaf extract of *Cassia fistula.*

Sample/Standard	**MIC µg/ml**
Sample (Ethanolic extract of *Cassia fistula* Leaf)	2.5
Standard Drugs	
Isoniazid	0.20
Rifampicin	40
Streptomycin	4
Ethambutol	2

Conclusion

The results of preliminary screening reveals that ethanolic extract of *Cassia fistula* leaf posses various Phytochemical constituents like protein, amino acids, steroids, saponins, alkaloids, tannins & phenols, flavanoids. UV spectroscopy and FTIR Spectroscopy analysis showed that alkaloids, flavonoids, tannins and saponins were present in the ethanolic extract of *Cassia fistula.* LCMS and HPTLC studies showed the presence of alkaloids, phenols and flavonoids. The ethanolic extract of *Cassia fistula* leaf showed the presence of many chemical constituents which are responsible for antimycobacterial activity.

List of Abbreviations

AE- Atropin
QE- Quercitin
GA- Gallic Acid
HCl- Hydrochloric acid

NaOH- Sodium Hydroxide
UV spectroscopy- Ultra Visible Spectroscopy
FTIR- Fourier Transform Infra Red Spectroscopy
TLC- Thin Layer Chromatography
HPTLC- High Pressure Thin Layer Chromatography
LCMS- Liquid Column Mass Spectroscopy
CSIL- Clinical and Laboratory Standards Institute
D/W- Distilled Water

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