Phytochemical Profiling and HPTLC Fingerprinting of Cassia fistula Leaf Extracts in Relation to In Vitro Antifungal Activity

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Abstract

Cassia fistula L. is a medicinally important species rich in secondary metabolites, yet systematic characterization of its solvent-dependent phytochemical composition and chromatographic fingerprints remains limited. This study aimed to compare the phytochemical profiles of aqueous, methanolic and acetone leaf extracts of *C. fistula* using standard qualitative assays and High-Performance Thin Layer Chromatography (HPTLC). Shade-dried, defatted leaf powder was subjected to Soxhlet extraction with water, methanol and acetone. Qualitative phytochemical screening was carried out for alkaloids, flavonoids, tannins, carbohydrates, reducing sugars and proteins, and HPTLC fingerprints were developed for each extract at 254 nm and 366 nm before and after derivatization, with Rf values and band characteristics recorded. Phytochemical tests revealed the presence of tannins, flavonoids and alkaloids in all three extracts, whereas carbohydrates, reducing sugars and proteins were predominantly detected in the aqueous extract, reflecting the high polarity of water. Methanolic and acetone extracts showed more intense reactions for phenolic and flavonoid constituents, consistent with their ability to solubilize moderately polar secondary metabolites. HPTLC profiles of the methanolic extract exhibited the greatest number and diversity of well-resolved bands over a wide Rf range, followed by the aqueous extract, while the acetone extract displayed comparatively fewer and less intense bands. Common bands at selected Rf positions indicated core constituents shared among solvents, whereas additional solvent-specific bands reflected differential extraction of distinct metabolite subsets. The close agreement between qualitative screening and chromatographic fingerprints confirms that *C. fistula* leaves possess a chemically diverse secondary metabolite repertoire and demonstrates that solvent polarity strongly shapes extract composition. These HPTLC fingerprints provide practical reference profiles for authentication, quality control and future marker-based standardization of *C. fistula* leaf extracts in herbal and phytopharmaceutical applications.

Keywords

Cassia fistula; phytochemical profiling; HPTLC fingerprinting; solvent extraction; phenolic compounds; herbal standardization

Introduction

Medicinal plants are recognized as rich reservoirs of structurally diverse secondary metabolites with important pharmacological and technological applications (Afu et al., 2020; Abid et al., 2016). Phenolics, flavonoids, tannins and related compounds contribute to antioxidant, anti-inflammatory and

antimicrobial properties, and also support intrinsic plant defence against biotic stresses. Systematic phytochemical profiling of such plants is essential to understand their chemical diversity, to support quality control of botanical preparations and to provide baseline data for future bioactivity-oriented studies (Selvaraj et al., 2019; Menon et al., 2019).

Cassia fistula L. (Fabaceae), commonly known as the golden shower tree, is a widely used medicinal species reported to exhibit hypolipidemic, antioxidant and other bioactivities attributed to its rich content of secondary metabolites (Abid et al., 2016; Afu et al., 2020). Different plant parts, including leaves, bark, pods and flowers, are traditionally used and have been shown to contain diverse groups of phytochemicals, indicating that extraction conditions and solvent systems can markedly influence the spectrum of compounds obtained (Abid et al., 2016; Alabi et al., 2022). Despite this importance, there is limited comparative information on how different solvents shape the phytochemical profiles and chromatographic fingerprints of C. fistula leaf extracts.

High-Performance Thin Layer Chromatography (HPTLC) has emerged as a powerful technique for generating reproducible chemical fingerprints of complex plant extracts, enabling simultaneous qualitative and semi-quantitative assessment of multiple constituents (Selvaraj et al., 2019; Thongkhao et al., 2020). HPTLC is widely applied for assessing phytochemical diversity, authenticating plant materials, detecting adulteration and standardizing herbal products by visualizing characteristic banding patterns of phenolics, flavonoids, alkaloids, terpenoids and related metabolites (Palve et al., 2015; Alexandar & Joy, 2022). Solvent polarity strongly influences the range and abundance of extracted compounds, with methanol often yielding richer HPTLC profiles than water or less polar solvents for many medicinal plants (Abaza et al., 2011; Garg & Dwivedi, 2021).

For C. fistula leaves, a detailed comparison of aqueous, methanolic and acetone extracts using both qualitative phytochemical assays and HPTLC has been comprehensively documented. Such an approach can clarify the contribution of solvent choice to the recovery of major secondary metabolite classes and provide chromatographic reference profiles useful for authentication and standardization. The present study therefore aimed to: (i) compare the phytochemical profiles of aqueous, methanolic and acetone leaf extracts of C. fistula using standard qualitative tests; and (ii) develop and interpret HPTLC fingerprints for each extract under multiple detection conditions. The resulting solvent-specific chemical fingerprints are intended to serve as baseline tools for quality control and future marker-guided work on C. fistula-derived herbal products.

Materials and methods

Plant material and preparation of extracts

Fresh, healthy leaves of Cassia fistula were collected from the vicinity of Satna, Madhya Pradesh, India, taxonomically identified, and authenticated against herbarium reference material before use. Leaves were washed to remove surface contaminants, shade-dried at room temperature to constant weight, defatted as per the thesis protocol, ground to fine powder and stored in airtight containers until extraction.

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Fig 3.1 Collected leaf of *Cassia fistula*

Fig 3.2 Reference from Herbarium

Fig 3.3 Drying of *Cassia* leaves

Index in Cosmos

Dec 2023 Volume 13 ISSUE 4

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Fig 3.4 Drying of *Cassia* leaves after defattening

Fig 3.5 Grinding, Weighing and Storage of leaf powder

Soxhlet extraction was carried out using three solvents of differing polarity: distilled water (aqueous), methanol and acetone. A known amount of leaf powder was packed into a thimble and extracted with each solvent in a Soxhlet apparatus until the siphon solvent became colourless, indicating exhaustive extraction; extracts were then filtered, concentrated under reduced pressure and dried to constant mass. Extract yields for each solvent were calculated and the dried extracts were stored in sealed vials at low temperature until phytochemical and chromatographic analyses.

Table 1: Shows the solvent and its properties

Solvent	Polarity	Procedure	Target Compounds
Water (aqueous)	Highly polar	Percolation	polysaccharides, proteins, glycosides, and some phenolics
Methanol	Polar	Soxhlet Extraction	phenolics, flavonoids, saponins, and some alkaloids
Acetone	Low to medium polarity	Soxhlet Extraction	flavonoids, phenolics, and some alkaloids

Qualitative phytochemical screening

Qualitative phytochemical screening of aqueous, methanolic and acetone extracts was performed using standard colour reactions for major classes of secondary metabolites. Tests included Dragendorff's and

Mayer's reagents for alkaloids, Shinoda and alkaline reagent tests for flavonoids, ferric chloride reaction for tannins, Molisch's and Benedict's tests for carbohydrates and reducing sugars, and Biuret and xanthoproteic reactions for proteins and amino acids, following established pharmacognostic procedures. Observations were recorded as positive or negative based on colour development or precipitate formation for each extract–test combination.

HPTLC fingerprinting

HPTLC was employed to generate chromatographic fingerprints of the three C. fistula leaf extracts. Appropriate volumes of each extract were applied as bands on silica gel HPTLC plates using a suitable applicator, maintaining identical band width, spacing and application volume for all tracks (aqueous, methanolic and acetone). HPTLC of the test solutions of all samples were carried out on Silica Gel 60 F254 precoated plates (0.2 mm thickness; from Merck India Limited Mumbai). A TLC applicator from Camag Linomat-5 (Camag Switzerland 140443) was used for band application and photo documentation unit (Camag Reprostar-3: 140604) was used for documentation of chromatographic fingerprints.

Procedure: Applied 10 µl each of the test solutions as 10 mm bands and develop the plate in a solvent system toluene: ethyl acetate (7: 3) to a distance of 9 cm. Dry the developed plate in air and examined under ultraviolet light, at 254 nm and at 366 nm before derivatization. Derivatized the plate using 5% Methanolic-sulphuric acid reagent and heating at 105 0c till the bands are clearly visible and examined the plate under 366nm and under ultraviolet light. The Rf values and colours of the bands obtained were recorded.

After development, plates were air-dried and visualized under UV light at 254 nm and 366 nm to detect native fluorescent or quenching zones, followed by derivatization with a suitable reagent to enhance visualization of specific phytochemical groups, and re-visualization at the same wavelengths and under visible light. Rf values, band colours and intensities were recorded for each track under all detection conditions, and representative images were used to compare solvent-dependent profiles.

Results

Qualitative phytochemical screening

Qualitative phytochemical screening revealed that Cassia fistula leaf extracts contained multiple classes of secondary metabolites, with clear solvent-dependent differences in detection patterns. Tannins, flavonoids and alkaloids were detected in all three extracts, indicating that these groups are widely distributed in the leaf matrix and can be recovered by both aqueous and organic solvents (Abid et al., 2016; Afu et al., 2020). In contrast, carbohydrates, reducing sugars and proteins showed strong positive reactions only in the aqueous extract, while methanolic and acetone extracts were negative for these tests, reflecting the higher affinity of water for highly polar primary metabolites. Methanolic and acetone extracts exhibited more intense colour reactions for phenolics and flavonoids, consistent with their capacity to solubilize moderately polar secondary metabolites (Abaza et al., 2011; Garg & Dwivedi, 2021).

Table 4.1 Phytochemical screening of solvent extracts of aqueous extract of *Cassia fistula*

Name of Test	Target Compound	Aqueous Extract	Methanolic Extract	Acetone Extract

Dragendorff's Test	Alkaloids	Negative	Positive	Positive
Shimoda Test	Flavonoids	Negative	Positive	Positive
Ferric Chloride Test	Tannins	Positive	Positive	Positive
Molisch's Test	Carbohydrates	Positive	Negative	Negative
Benedict's Test	Reducing Sugars	Positive	Negative	Negative
Biuret Test	Protein	Positive	Negative	Negative
Xanthoproteic Test	Protein/Amino acids	Positive	Negative	Negative
Sudan III Test	Lipids	Negative	Negative	Positive

Here, Positive = Present - : Negative = Not Present,

HPTLC fingerprints at 254 nm

HPTLC profiles at 254 nm before derivatization showed distinct banding patterns for aqueous, methanolic and acetone extracts of C. fistula leaves. The methanolic extract (Track B) displayed the highest number of well-resolved bands across low, mid and high Rf regions (approximately 0.08–0.92), indicating a broad spectrum of UV-absorbing compounds extracted by this solvent (Selvaraj et al., 2019; Menon et al., 2019). The aqueous extract (Track A) exhibited a moderate number of bands, mainly in the mid to high Rf range, while the acetone extract (Track C) presented fewer and generally less intense bands, suggesting lower abundance or diversity of strongly UV-absorbing constituents in this fraction.
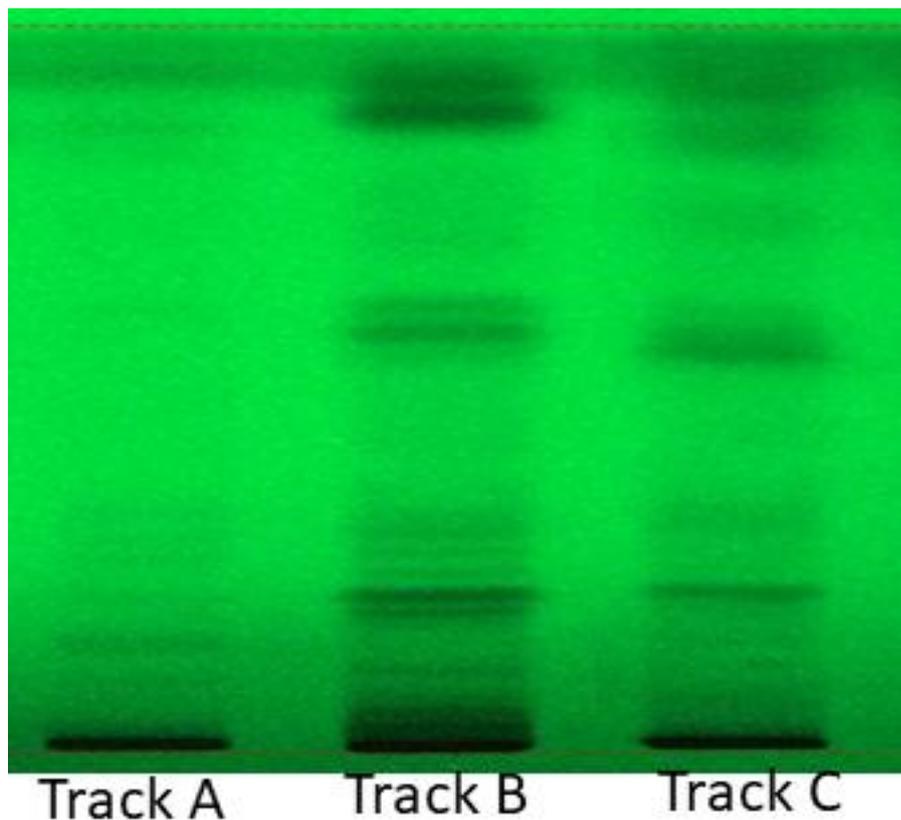
Fig 4.1: HPTLC fingerprints profile of test solutions at 254 nm before derivatization

Table 4.2: Rf values of HPTLC fingerprints profile of test solutions at 254 nm before derivatization

Rf values	Track A (Aqueous)	Track B (Methanolic)	Track C (Acetone)
Rf1	0.10 black	0.08 black	0.18 black
Rf2	-	0.18 black	0.26 black
Rf3	-	0.26 black	0.64 black
Rf4	-	0.64 black	0.66 black
Rf5	-	0.66 black	0.90 black
Rf6	-	0.90 black	0.92 black

Rf7	-	0.92 black	-

HPTLC fingerprints at 366 nm before and after derivatization

Under 366 nm UV light before derivatization, methanolic and acetone extracts showed several fluorescent or quenching bands, particularly in mid to high Rf regions, whereas the aqueous extract displayed fewer fluorescent bands of lower intensity. Bands with red or pink fluorescence were more prominent in the methanolic extract, consistent with the presence of flavonoids and other conjugated phenolics known to exhibit characteristic fluorescence at this wavelength (Palve et al., 2015; Thongkhao et al., 2020).

After derivatization, all extracts exhibited enhanced band visibility at 366 nm, but the methanolic extract again showed the richest pattern, with multiple red, blue and brown bands spanning a wide Rf range. In the aqueous extract, bands were fewer and largely concentrated in the mid-Rf zone, while the acetone extract displayed an intermediate pattern with limited but distinct bands at specific Rf positions. The appearance of red to pink bands is indicative of flavonoids, blue bands suggest phenolic acids and related compounds, and brown or yellow bands are associated with tannins, terpenoids and glycosides, respectively (Alexandar & Joy, 2022; Tessema et al., 2023).
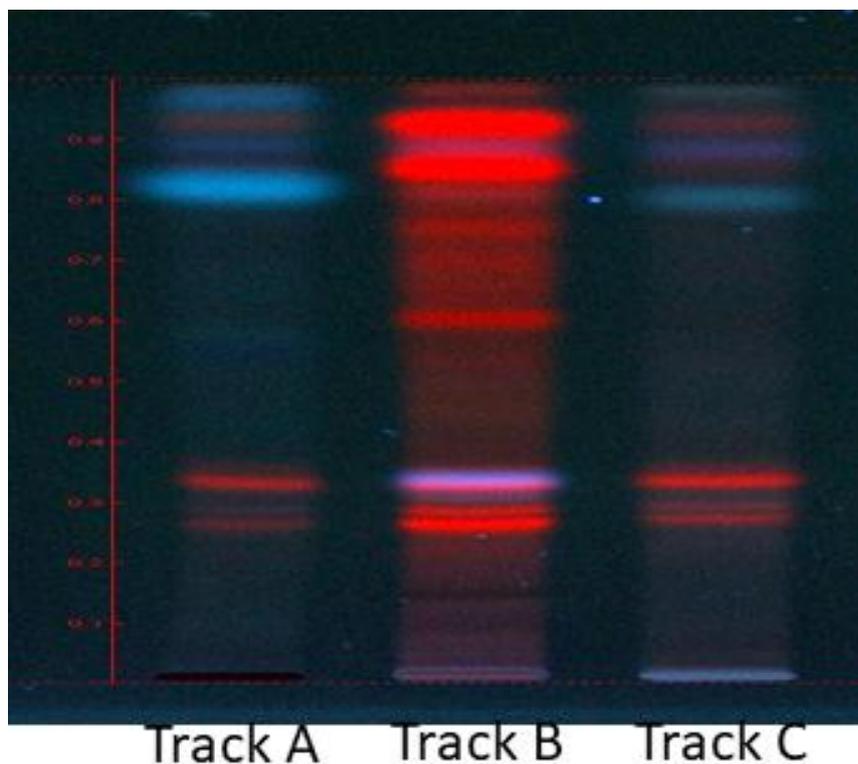

Fig 4.2: HPTLC fingerprints profile of test solutions at 366 nm before derivatization

Table 4.3: Rf values of HPTLC fingerprints profile of test solutions at 366 nm before derivatization

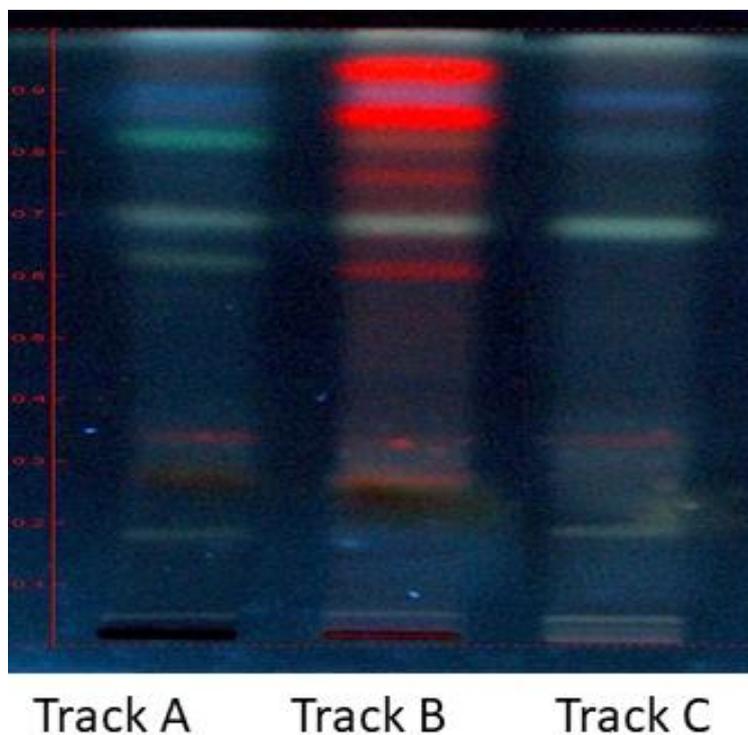| Rf values	Track A (Aqueous)	Track B (Methanolic)	Track C (Acetone)
Rf1	0.26 red	0.08 pink	0.26 red
Rf2	0.35 red	0.12 pink	0.35 pink
Rf3	0.80 sky blue	0.26 red	0.80 blue
Rf4	0.92 red	0.35 pink	0.84 pink
Rf5	0.94 red	0.60 red	0.90 pink
Rf6	-	0.70 red	0.92 blue
Rf7	-	0.84 red	-
Rf8	-	0.90 red	-
Rf9	-	0.92 red	-

Fig 4.3: HPTLC fingerprints profile of test solutions at 366 nm after derivatization

Table 4.4: Rf values of HPTLC fingerprints profile of test solutions at 366 nm after derivatization

Rf values	Track A (Aqueous)	Track B (Methanolic)	Track C (Acetone)
Rf1	0.18 brown	0.26 pink	0.08 brown
Rf2	0.36 pink	0.36 pink	0.20 brown
Rf3	0.64 brown	0.64 pink	0.36 pink
Rf4	0.70 brown	0.70 brown	0.70 brown
Rf5	0.84 brown	0.75 red	0.80 sky blue
Rf6	-	0.80 pink	0.90 sky blue
Rf7	-	0.86 red	0.92 brown
Rf8	-	0.92 red	-

HPTLC fingerprints under UV/visible light after derivatization

Visualization of derivatized plates under UV/visible light further emphasized solvent-dependent differences in phytochemical diversity. The methanolic extract repeatedly showed a dense array of bands from low to high Rf values, indicating high extractive efficiency for a broad range of secondary metabolites, while the aqueous extract exhibited a simpler pattern with fewer prominent bands and the acetone extract displayed the lowest band density among the three. At specific Rf values (for example, 0.28 and 0.70), bands were common to all extracts, suggesting shared core constituents, whereas additional bands unique to methanolic or acetone tracks reflected solvent-specific components. The combined interpretation of band colour and Rf position supported the presence of flavonoids, phenolic acids, tannins, terpenoids and glycosides, in agreement with the qualitative screening results.
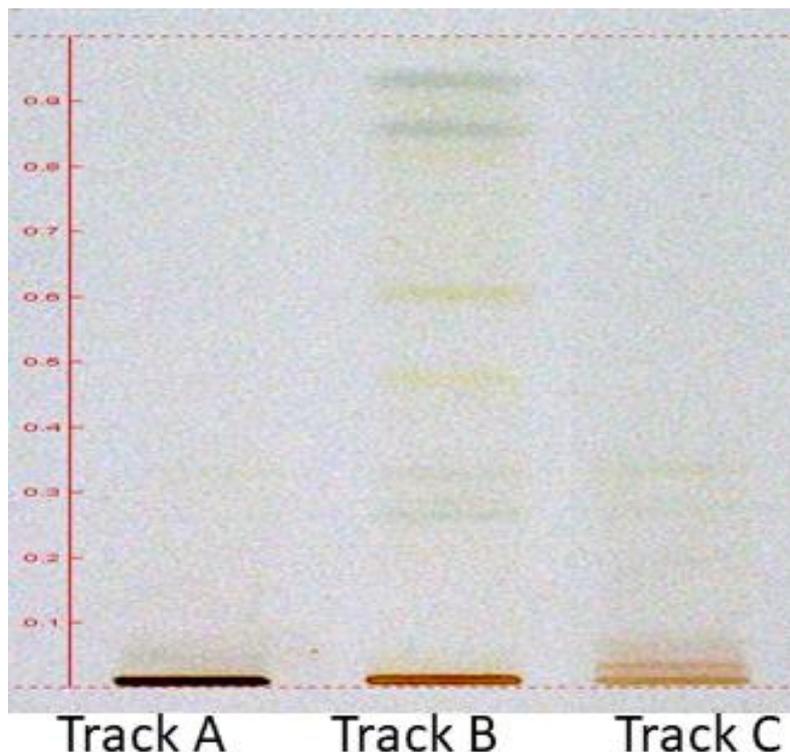
Fig 4.4: HPTLC fingerprints profile of test solutions at UV light before derivatization

Table 4.5: Rf values of HPTLC fingerprints profile of test solutions at UV light before derivatization

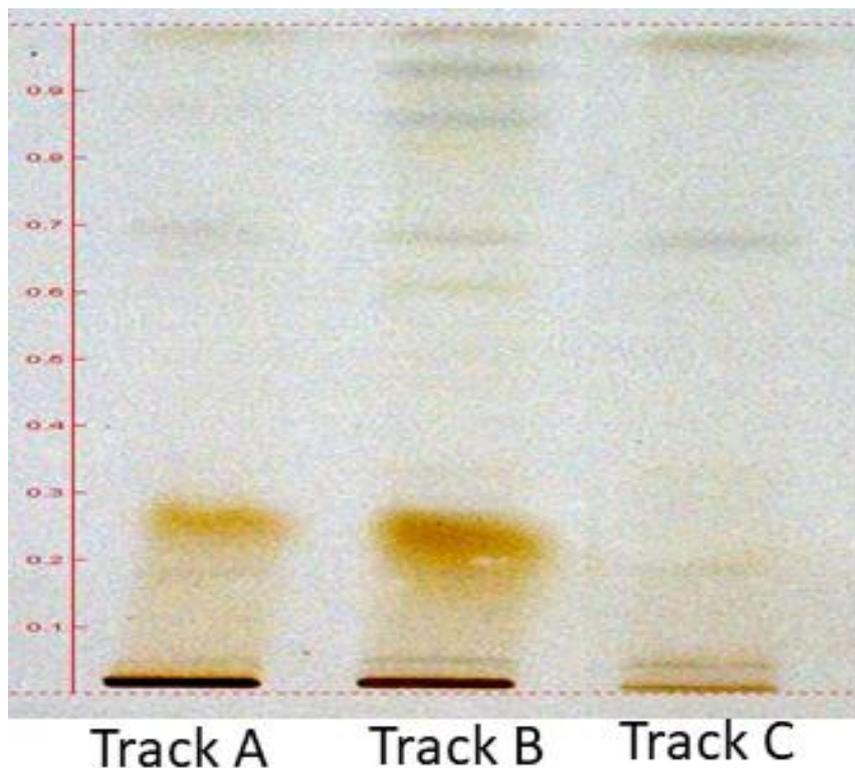| Rf values	Track A (Aqueous)	Track B (Methanolic)	Track C (Acetone)
Rf1	-	0.26 green	0.04 brown
Rf2	-	0.50 yellow	-
Rf3	-	0.60 yellow	-
Rf4	-	0.86 green	-
Rf5	-	0.92 green	-

Fig4.5: HPTLC fingerprints profile of test solutions at UV light after derivatization

Table 4.6: Rf values of HPTLC fingerprints profile of test solutions at UV light after derivatization

Rf values	Track A (Aqueous)	Track B (Methanolic)	Track C (Acetone)
Rf1	0.28 yellow	0.28 yellow	0.06 brownish blue
Rf2	0.70 black	0.70 black	0.70 black
Rf3	-	0.84 black	0.92 black
Rf4	-	0.92 black	-
Rf5	-	-	-

Discussion

The comparative phytochemical and HPTLC data generated in this study demonstrate that solvent polarity exerts a strong influence on the composition and apparent diversity of Cassia fistula leaf extracts. Methanol, as a polar protic solvent, yielded the richest set of bands across all HPTLC detection modes, indicating efficient extraction of both polar and moderately non-polar secondary metabolites such as phenolics and flavonoids, which are widely reported from Cassia species (Abaza et al., 2011; Garg & Dwivedi, 2021). Aqueous extraction, by contrast, favoured highly polar constituents including

carbohydrates, reducing sugars and proteins, resulting in a simpler chromatographic profile, while the acetone extract showed an intermediate pattern with fewer but distinct semi-polar constituents.

The strong concordance between qualitative phytochemical tests and HPTLC fingerprints reinforces the reliability of both approaches for characterizing C. fistula leaf chemistry. Detection of tannins, flavonoids and alkaloids in all extracts by colour reactions, together with red, blue, brown and yellow bands at characteristic Rf positions after derivatization, supports the presence of these classes as core components of the leaf metabolome (Selvaraj et al., 2019; Palve et al., 2015). The more complex and intense patterns observed for methanolic and, to a lesser extent, acetone extracts align with observations from other medicinal plants where methanol-derived HPTLC fingerprints correlate with higher levels of bioactive phenolics and flavonoids (Alexandar & Joy, 2022; Tessema et al., 2023).

From a phytochemical perspective, the presence of multiple solvent-common bands alongside solvent-specific bands suggests that C. fistula leaves contain both ubiquitous core constituents and additional metabolites that are selectively enriched depending on solvent polarity. Common bands at Rf values such as 0.28 and 0.70 likely represent shared backbone phenolic or glycosidic structures, whereas unique bands in methanolic or acetone tracks may correspond to more hydrophobic flavonoid derivatives or other semi-polar compounds preferentially extracted by these solvents. This pattern is consistent with previous reports highlighting methanol as a solvent capable of capturing a broad spectrum of secondary metabolites in herbal matrices (Abaza et al., 2011; Menon et al., 2019).

Beyond simple profiling, the HPTLC fingerprints established here have practical implications for quality control and standardization of C. fistula-based products. The solvent-specific fingerprints can serve as reference profiles for authentication of C. fistula leaf raw material and for monitoring batch-to-batch consistency in extract preparation, in line with earlier reports advocating HPTLC as a core method for herbal drug standardization and adulteration detection (Thongkhao et al., 2020; Alexandar & Joy, 2022). Selection of characteristic marker bands—particularly those consistently observed in methanolic extract—could support development of chromatographic specifications for regulatory or pharmacopoeial monographs.

The present work is limited by its reliance on qualitative assays and unassigned HPTLC bands, without isolation or structural elucidation of individual constituents. Further studies integrating HPTLC with HPLC or LC–MS, as recommended in recent analytical workflows, would enable more precise identification and quantification of key compounds in C. fistula leaves (Khushwaha, 2023). Bioassay-guided fractionation of the chemically richer methanolic and acetone extracts in future work could then be used to link specific chromatographic zones to defined biological activities, supporting mechanism-oriented studies and development of standardized formulations.

Overall, the findings clearly show that C. fistula leaves possess a diverse phytochemical repertoire, with methanolic extract providing the most complex and information-rich HPTLC fingerprint. Solvent-dependent differences captured by these fingerprints offer a robust basis for authentication, comparative evaluation of extraction protocols and future marker-based standardization of C. fistula-derived herbal products.

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