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In Vitro Antifungal Activity of Cassia fistula Leaf Extracts Against Major Phytopathogenic Fungi and Their Phytochemical Characterization

Santosh Kumar[1], Dr. Neeraj Verma[2]

1. Research Scholar, Department of Biotechnology, AKS University, Satna, MP, India
2. Professor, Department of Agriculture Science, AKS University, Satna, MP, India

Corresponding Author: santosh_e1452@aksuniversity.com

Abstract

Fungal phytopathogens such as *Alternaria*, *Cercospora*, *Colletotrichum* and *Fusarium* cause substantial yield and quality losses, while increasing resistance and environmental concerns limit the long-term use of synthetic fungicides. This study evaluated the in vitro antifungal activity of aqueous, methanolic and acetone leaf extracts of *Cassia fistula* L. against these four fungi and related the activity to their phytochemical profiles. Shade-dried, defatted leaf powder was extracted by Soxhlet using water, methanol and acetone, followed by qualitative phytochemical screening and HPTLC fingerprinting. Antifungal activity was assessed by the poisoned food technique on potato dextrose agar, recording colony diameter at 3 and 5 days after inoculation and calculating percentage inhibition over control; data were analysed by ANOVA and Tukey's post hoc test. Tannins, flavonoids and alkaloids were detected in all extracts, whereas carbohydrates and proteins were predominantly present in the aqueous extract, and methanolic and acetone extracts showed stronger reactions for phenolic and flavonoid constituents. HPTLC profiles of methanolic and acetone extracts exhibited more numerous and intense bands than the aqueous extract, indicating a richer diversity of moderately polar compounds. All extracts significantly inhibited mycelial growth of the test fungi, but methanolic extract consistently produced the highest inhibition, followed by acetone, while the aqueous extract showed comparatively lower activity; inhibition increased with extract concentration and was generally higher at 5 than at 3 days after inoculation. *Alternaria* and *Cercospora* were relatively more sensitive, whereas *Fusarium* displayed lower susceptibility at equivalent treatment levels. The results suggest that *C. fistula* leaves, particularly methanolic and acetone extracts enriched in phenolic and flavonoid constituents, represent promising sources of botanical antifungal agents for integration into eco-friendly management of fungal plant diseases.

Keywords: Cassia fistula; antifungal activity; phytopathogenic fungi; poisoned food technique; phytochemicals; HPTLC fingerprinting

Introduction

Crop production worldwide is severely constrained by fungal phytopathogens that infect leaves, stems, fruits, and seeds, leading to substantial yield and quality losses (Achilonu et al., 2023; Adhikari et al., 2021; Al-Askar et al., 2022). Genera such as Alternaria, Cercospora, Colletotrichum, and Fusarium are among the most destructive, causing leaf spots, blights, anthracnose, and wilt diseases in a wide range of economically important crops (Achilonu et al., 2023; Adhikari et al., 2021). Management of these diseases has traditionally relied on synthetic fungicides, which, although effective, are increasingly associated with problems such as development of fungicide resistance, environmental persistence,

residues in food commodities, and adverse impacts on non-target organisms including humans (Adigun et al., 2020; Akarsh et al., 2016).

The search for safer and sustainable alternatives has therefore turned attention towards plant-derived antifungal agents, which are often biodegradable and may contain complex mixtures of bioactive compounds with multiple modes of action (Abdu et al., 2021; Akarsh et al., 2016). Medicinal plants and their extracts have been reported to possess significant antimicrobial activity, and many species used in traditional medicine are now being systematically investigated for their potential in plant disease management (Abdu et al., 2021; Afu et al., 2020). Among these, Cassia species are known to contain a rich array of secondary metabolites such as phenolics, flavonoids, tannins, and anthraquinones, many of which have documented antimicrobial properties (Abid et al., 2016; Afu et al., 2020).

Cassia fistula L., commonly known as the golden shower tree, is widely distributed in tropical regions and has been used in traditional systems of medicine for its laxative, anti-inflammatory, antioxidant, and antimicrobial properties (Abid et al., 2016; Alabi et al., 2022). Different parts of the plant, including leaves, bark, pods, and flowers, have been shown to contain diverse phytochemicals that may contribute to biological activity (Abid et al., 2016; Afu et al., 2020). However, systematic evaluation of the antifungal potential of Cassia fistula leaf extracts against major phytopathogenic fungi such as Alternaria, Cercospora, Colletotrichum, and Fusarium, along with detailed phytochemical and chromatographic profiling, remains limited.

Solvent extraction plays a critical role in isolating bioactive constituents from plant materials, and the polarity of the solvent influences both the yield and the spectrum of extracted compounds (Abaza et al., 2011; Alexandar & Joy, 2022). Studies with other plant species have demonstrated that methanol, acetone, and water differ markedly in their ability to extract phenolic and other bioactive constituents, resulting in corresponding differences in antioxidant and antimicrobial activities (Abaza et al., 2011; Afu et al., 2020). Therefore, a comparative evaluation of aqueous, methanolic, and acetone extracts of Cassia fistula leaves can provide insights into the relationship between solvent-specific phytochemical profiles and antifungal efficacy.

The present study was designed to evaluate the in vitro antifungal activity of aqueous, methanolic, and acetone extracts of Cassia fistula leaves against four important phytopathogenic fungi, namely Alternaria, Cercospora, Colletotrichum, and Fusarium, using the poisoned food technique (Akarsh et al., 2016). In addition, qualitative phytochemical screening and HPTLC fingerprinting were performed to characterize the major groups of secondary metabolites present in the extracts and to explore possible associations between chemical profiles and antifungal activity (Abaza et al., 2011; Alexandar & Joy, 2022). The overarching aim was to assess the potential of Cassia fistula leaf extracts as botanical fungicidal agents suitable for integration into eco-friendly plant disease management strategies (Abdu et al., 2021; Akarsh et al., 2016).

Materials and Methods

Plant material and authentication

Fresh, healthy leaves of Cassia fistula were collected from the vicinity of Satna, Madhya Pradesh, India, during the appropriate growing season. The plant material was taxonomically identified and

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authenticated by comparing with herbarium specimen from Botanical Survey of India, barcoded CAL0000191011. Collected leaves were washed with tap water to remove dust and debris, and then air-dried under shade at room temperature until constant weight was obtained. Dried leaves were then defatted as per the protocol established in the thesis, ground into fine powder using a mechanical grinder and stored in airtight containers until extraction.



Fig 3.1 Collected leaf of *Cassia fistula*



Fig 3.2 Reference from Herbarium

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Fig 3.3 Drying of *Cassia* leaves



Fig 3.4 Drying of *Cassia* leaves after defattening



Fig 3.5 Grinding, Weighing and Storage of leaf powder

Preparation of solvent extracts

Soxhlet extraction was employed to obtain aqueous, methanolic and acetone extracts from the Cassia fistula leaf powder. A known quantity of dried leaf powder was packed into a thimble and extracted successively or separately with each solvent in a Soxhlet apparatus, maintaining appropriate solvent volumes and extraction durations as standardized in the thesis. The extraction was continued until the solvent in the siphon tube of the apparatus became colourless, indicating exhaustive extraction. The resulting extracts were filtered and concentrated under reduced pressure using a rotary evaporator, and the residues were dried to yield crude extracts of each solvent type. Dried extracts were weighed to calculate percentage yield and stored at low temperature in airtight vials until use in antifungal assays and chemical analysis.

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Fig3.6. **Soxhlet Assembly for extraction of compounds**



Fig 3.7 Aqueous, Methanolic and Acetone extract

Qualitative phytochemical screening

Preliminary qualitative phytochemical screening of the aqueous, methanolic and acetone extracts was carried out using standard protocols to detect major classes of secondary metabolites. Tests were

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performed for alkaloids (Dragendorff's and Mayer's tests), flavonoids (Shinoda and alkaline reagent tests), tannins (ferric chloride test and others), saponins, terpenoids and other groups as described in the methods section of the thesis. The presence or absence of each phytochemical class in the different solvent extracts was recorded in a tabular format.

Table 3.1: 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

HPTLC fingerprinting

HPTLC fingerprinting was performed to obtain chromatographic profiles of Cassia fistula leaf extracts. Appropriate volumes of each extract were applied as bands on HPTLC plates coated with silica gel as stationary phase. Plates were developed in a suitable mobile phase system optimized in the thesis, and the developed plates were visualized under UV light at 254 nm and 366 nm, before and after derivatization with an appropriate reagent. Rf values and band intensities were recorded, and representative chromatograms and images were used to compare the chemical profiles among aqueous, methanolic and acetone extracts.

Fungal pathogens and culture maintenance

Four phytopathogenic fungi, namely Alternaria, Cercospora, Colletotrichum and Fusarium, were selected as test organisms based on their importance in causing foliar and other diseases in crop plants. Pure cultures of these fungi were obtained and maintained on potato dextrose agar (PDA) medium. Morphological characteristics of the colonies and spores were observed and documented, including colony texture, colour and microscopic features such as conidial shape and septation.

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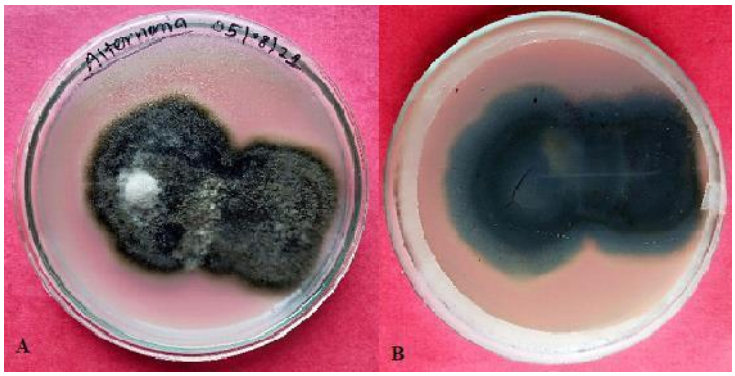


Fig 3.8 Pure culture *Alternaria* (A.) Front side (B.) Back side

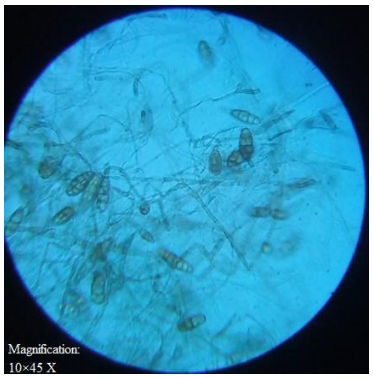


Fig 3.9 Spores of *Alternaria*



Fig 3.10 Pure culture *Cercospora* (A.) Front side (B.) Back side

Fig 3.11 Spores of *Cercospora*

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Fig 3.12 Pure culture *Colletotrichum* (A.) Front side (B.) Back side

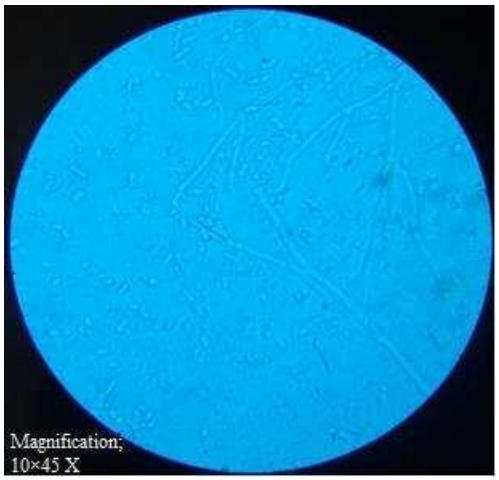


Fig 3.13 Spores of *Colletotrichum*

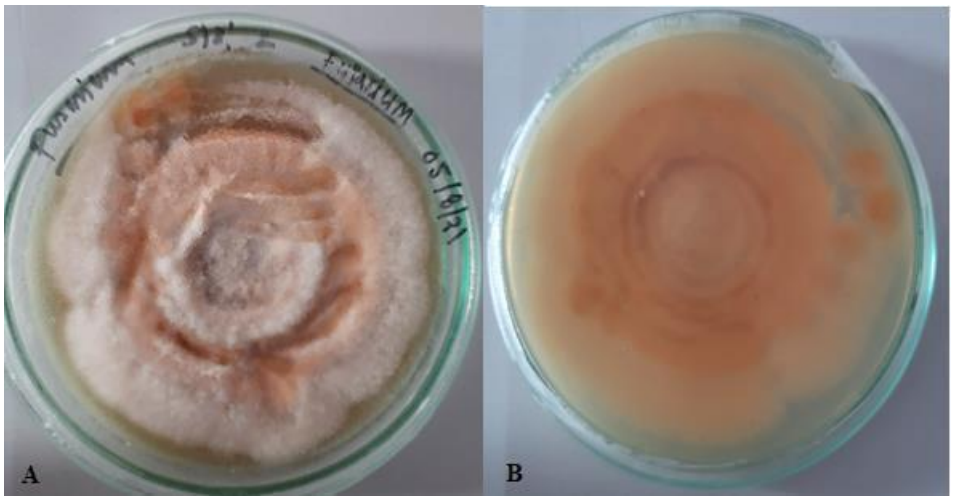


Fig 3.14 Pure culture *Fusarium* (A.) Front side (B.) Back side

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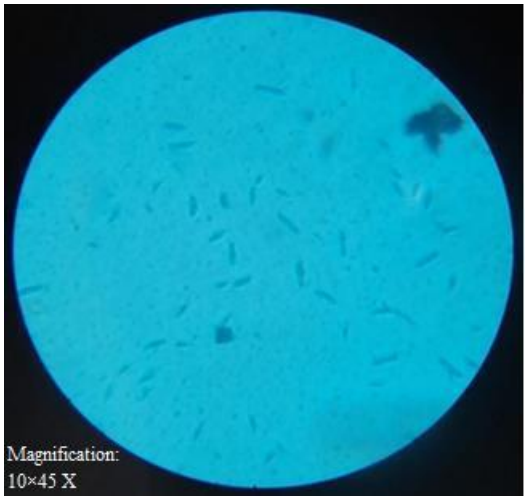


Fig 3.15 Spores of *Fusarium*

PDA medium was prepared as per standard formulation described in the thesis, and autoclaved prior to use.

Table 3.2: Composition of potato dextrose agar medium for 1 L

Component	Quantity
Potatoes (peeled and sliced)	200.0 gm
Dextrose powder	20.0 gm
Agar-agar powder	20.0 gm
Sterilized distilled water (to make up)	1000 ml

In vitro antifungal assay (poisoned food technique)

The antifungal activity of Cassia fistula leaf extracts was evaluated using the poisoned food technique. Required quantities of each extract were dissolved in a small volume of suitable solvent and incorporated into molten PDA medium at predetermined concentrations, followed by thorough mixing and pouring into sterile Petri plates. Control plates received PDA medium without extracts but with equivalent solvent, if any.

After solidification of the medium, mycelial discs of uniform diameter were cut from actively growing margins of 7-day-old fungal cultures and placed at the centre of each plate. Plates were incubated at appropriate temperature for fungal growth, and radial mycelial growth (colony diameter) was measured in two perpendicular directions at 3 and 5 days after inoculation (DAI). Percentage inhibition of mycelial growth over control was calculated using standard formula based on colony diameter. The

experiments were conducted in a randomized block design with multiple replications for each treatment, and data were tabulated accordingly.

Statistical analysis

Data on colony diameter and percentage inhibition were subjected to analysis of variance (ANOVA) to test the significance of differences among treatments, including effects of solvent type, extract concentration and their interaction for each fungus. Where significant F-values were obtained, Tukey's post hoc test was applied to compare treatment means. Statistical analyses were carried out as described in the thesis using appropriate software, and results were interpreted at a standard significance level (e.g., $p < 0.05$).

Results

Phytochemical screening of Cassia fistula leaf extracts

Qualitative phytochemical analysis indicated that Cassia fistula leaf extracts contain several classes of secondary metabolites, with some variation depending on the extraction solvent. Tannins, flavonoids and alkaloids were detected in all three extracts, while certain groups such as saponins or terpenoids showed solvent-dependent presence or intensity. Methanolic and acetone extracts generally exhibited a richer or more intense reaction for phenolic and flavonoid constituents than the aqueous extract, suggesting higher solubility of these compounds in organic solvents.

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 fingerprinting

HPTLC analysis produced distinct chromatographic fingerprints for aqueous, methanolic and acetone extracts of Cassia fistula leaves. At 254 nm and 366 nm, both before and after derivatization, multiple

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bands with different Rf values and intensities were observed, indicating the presence of several phytoconstituents. Methanolic and acetone extracts showed a greater number of prominent bands compared to the aqueous extract, which is consistent with their broader phytochemical profile. Some bands were common across all extracts, while others were solvent-specific, reflecting differential extraction of compounds.

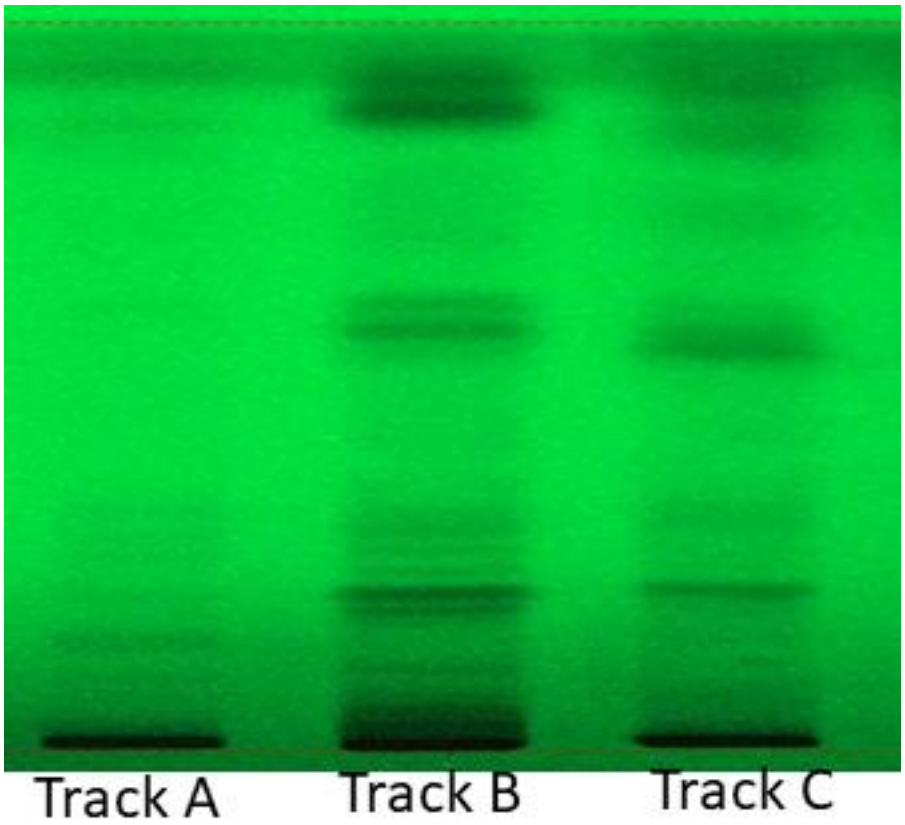


Fig 4.1: HPTLC fingerprints profile of test solutions at 254 nm before derivatization

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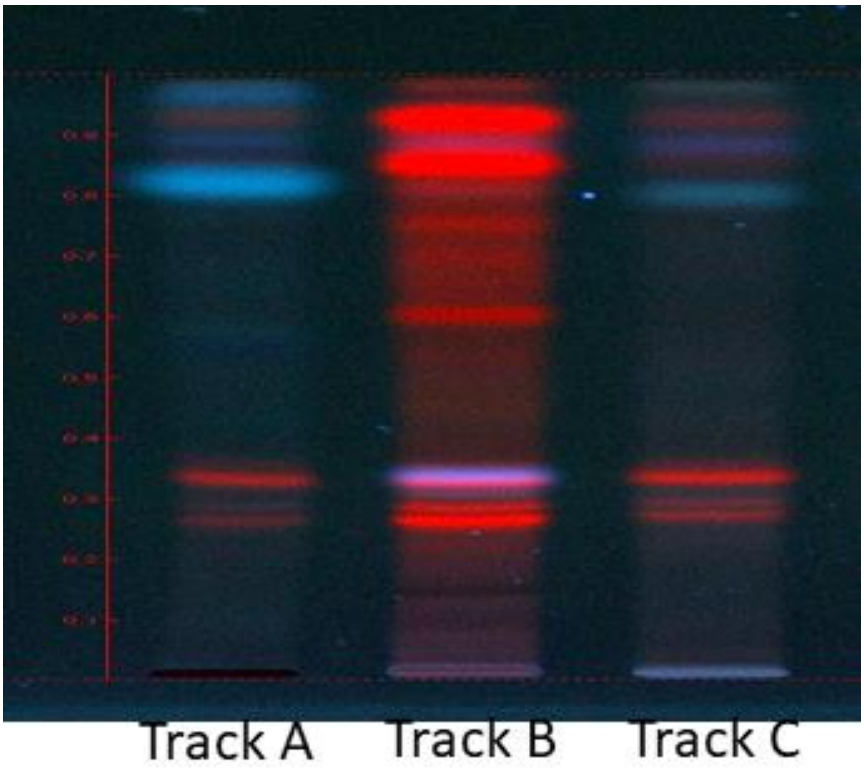


Fig 4.2: HPTLC fingerprints profile of test solutions at 366 nm before derivatization

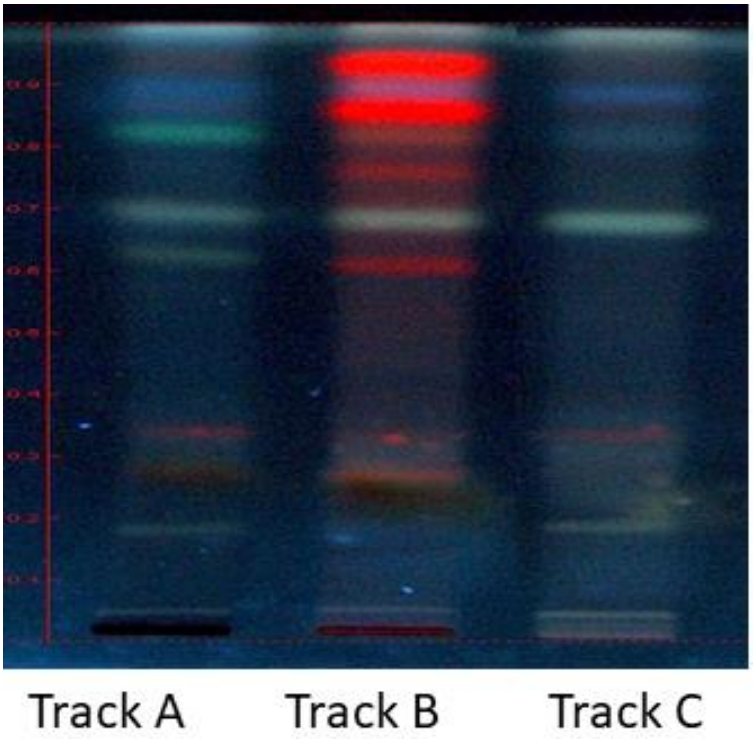


Fig 4.3: HPTLC fingerprints profile of test solutions at 366 nm after derivatization

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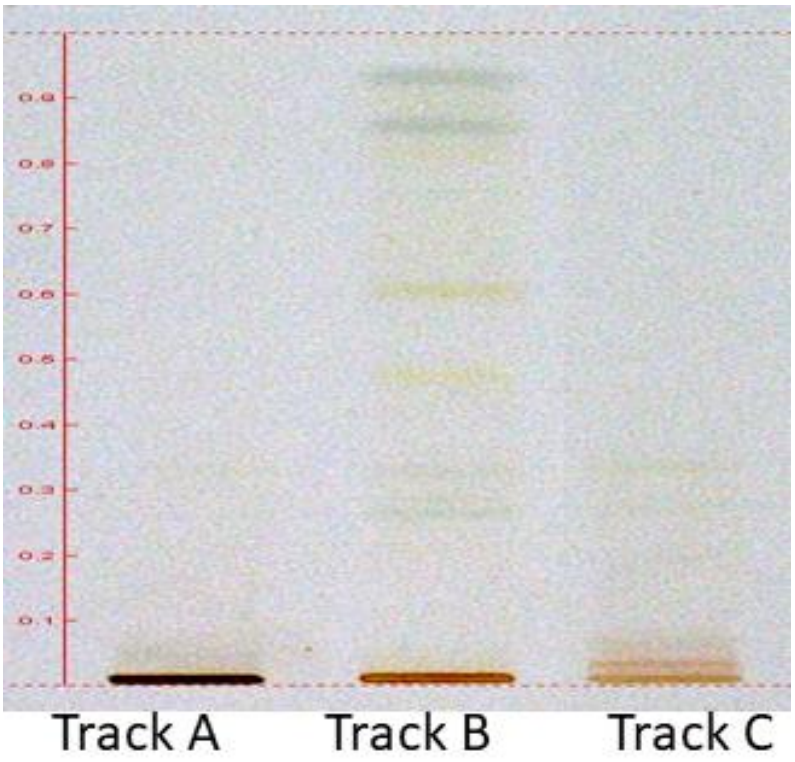


Fig 4.4: HPTLC fingerprints profile of test solutions at UV light before derivatization

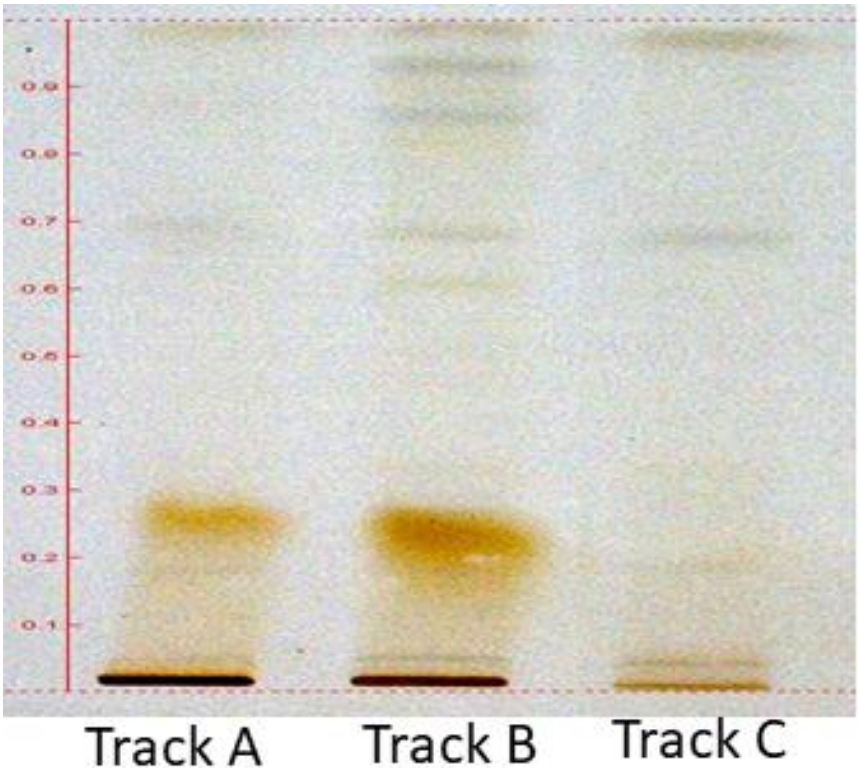


Table 4.2: Rf values of HPTLC fingerprints profile of test solutions at 254 nm before derivatization

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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	-

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	-

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

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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	-

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	-

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	-	-	-

Antifungal activity against Alternaria

Cassia fistula leaf extracts significantly inhibited the mycelial growth of Alternaria compared to control, with differences among solvent types and concentrations. At both 3 and 5 DAI, methanolic and acetone extracts produced greater reductions in colony diameter than the aqueous extract, and inhibition generally increased with extract concentration. Percentage inhibition values were higher at 5 DAI than at 3 DAI, indicating sustained or enhanced activity over time.

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ANOVA revealed significant effects of extract type and concentration on mean colony diameter, and Tukey's test grouped methanolic and acetone treatments at higher concentrations into statistically superior categories relative to aqueous extract and control.

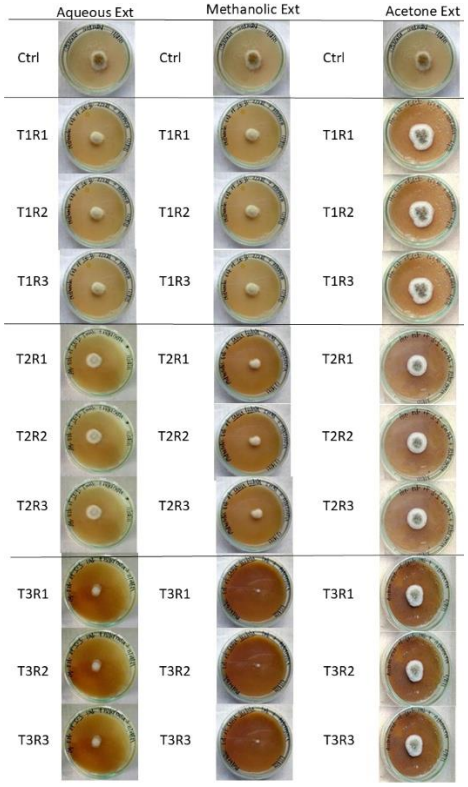


Fig 4.6. *Alternaria* treatment at 3 DAI against different extract.

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Fig 4.6. *Alternaria* treatment at 5 DAI against different extract.

Table 4.13 Mean Colony Diameter and Percentage Inhibition (*Alternaria*)

Solvent	Treatment	Mean Diameter (mm), 3 DAI	Mean Diameter (mm), 5 DAI	Percentage Inhibition 3 DAI (%)	Percentage Inhibition 5 DAI (%)
Aqueous	T1	11.00	25.88	30.47	13.75
Aqueous	T2	9.73	22.60	38.95	24.67
Aqueous	T3	8.58	21.20	46.35	29.33
Methanolic	T1	9.82	25.88	38.64	13.75
Methanolic	T2	6.78	22.60	57.61	24.67
Methanolic	T3	1.00	21.20	93.75	29.33
Acetone	T1	12.13	25.97	24.22	13.44
Acetone	T2	10.77	18.50	32.71	38.33
Acetone	T3	9.58	16.40	40.10	45.33

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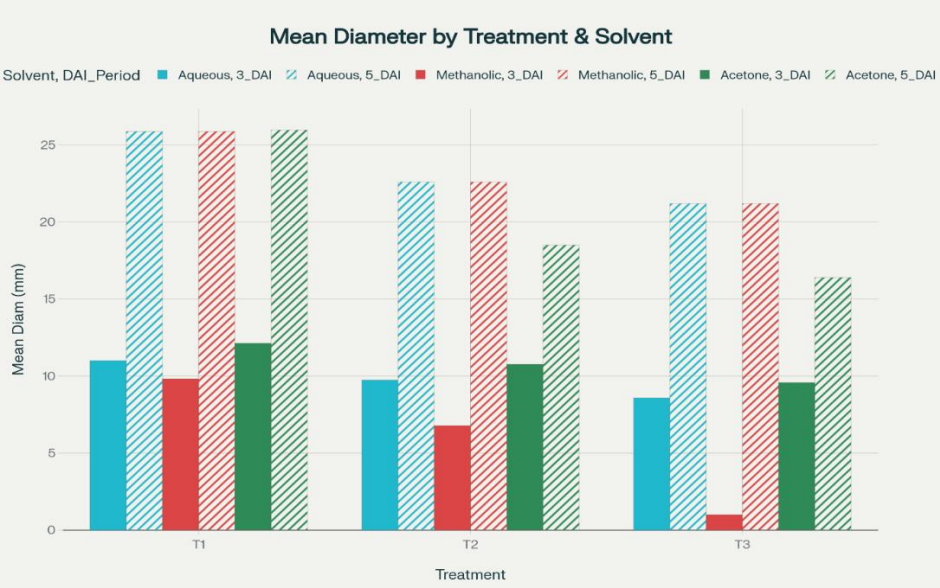


Fig 4.8 Mean Diameter Comparison Across Treatments and Days

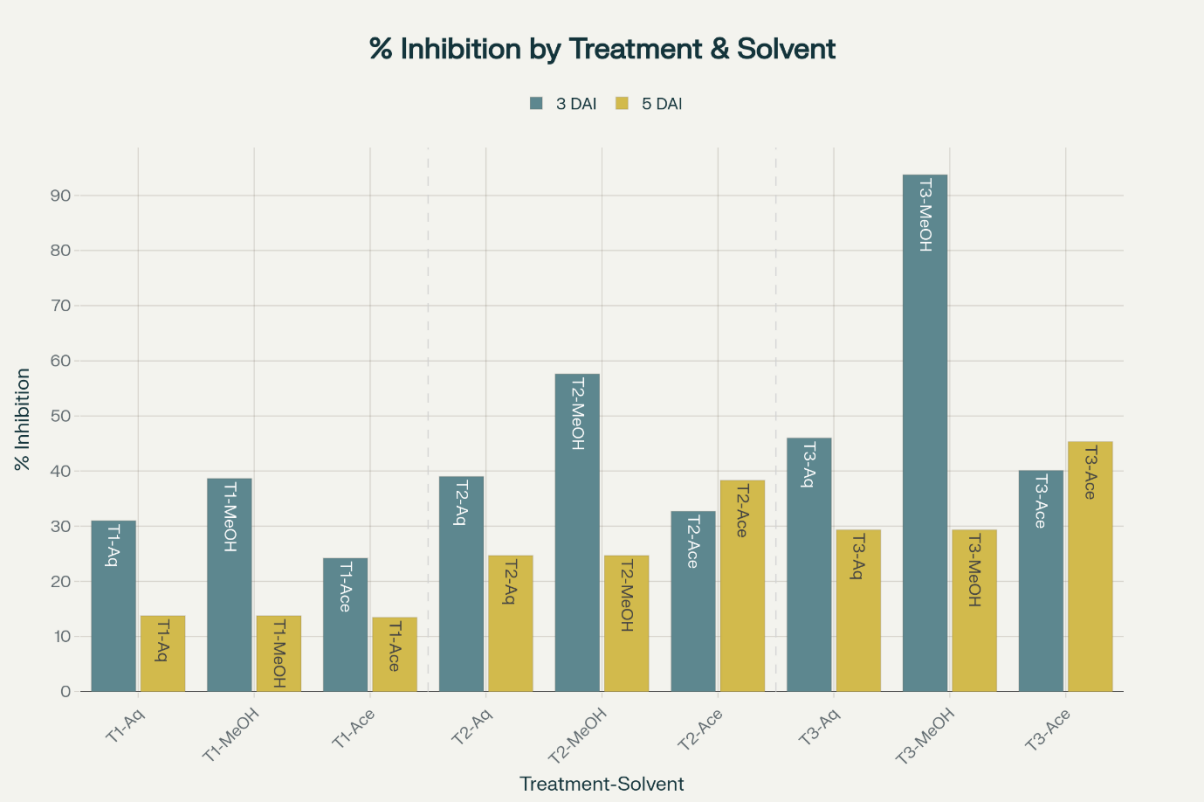


Fig 4.9 Percentage Inhibition Comparison Across Treatments and Days

Table 4.14 ANOVA Table –Mean Colony Diameter for *Alternaria*

Solvent	Day	Source	df	F-statistic	p-value

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Aqueous	3 DAI	Between Groups	2	197.96	<0.001
Aqueous	5 DAI	Between Groups	2	194.14	<0.001
Methanolic	3 DAI	Between Groups	2	19698.82	<0.001
Methanolic	5 DAI	Between Groups	2	194.14	<0.001
Acetone	3 DAI	Between Groups	2	197.96	<0.001
Acetone	5 DAI	Between Groups	2	1739.03	<0.001

Here,

- df = degrees of freedom (between groups: 2; within groups: n (total) - 3)
- F-statistic and p-value as reported for each test
- All p-values are highly significant (<0.001)

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-1.3056	0.0013	-1.8793	-0.7318	True
T1	T3	-2.5181	0.0013	-3.0918	-1.9443	True
T2	T3	-1.2125	0.0013	-1.7862	-0.6387	True

Tukey's Post hoc Aq 3DAI Alternaria

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.0333	0.0013	-3.0932	-2.9735	True
T1	T3	-8.8333	0.0013	-8.8932	-8.7735	True
T2	T3	-5.8000	0.0013	-5.86	-5.7402	True

Tukey's Post hoc Meth 3DAI Alternaria

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-1.3562	0.0013	-1.5142	-1.1981	True
T1	T3	-2.5625	0.0013	-2.7206	-2.4044	True
T2	T3	-1.2062	0.0013	-1.3642	-1.0481	True

Tukey's Post hoc Acetone 3DAI Alternaria

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.1889	0.0013	-3.6492	-2.7286	True
T1	T3	-4.6556	0.0013	-5.1159	-4.1953	True
T2	T3	-1.4667	0.0013	-1.9269	-1.0064	True

Tukey's Post hoc Aq 5DAI Alternaria

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.1861	0.0013	-3.4711	-2.9011	True
T1	T3	-5.1861	0.0013	-5.4711	-4.9011	True
T2	T3	-2.0000	0.0013	-2.2850	-1.7150	True

Tukey's Post hoc Meth 5DAI Alternaria

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-7.4667	0.0013	-7.6663	-7.2671	True
T1	T3	-9.5667	0.0013	-9.7663	-9.3671	True
T2	T3	-2.1000	0.0013	-2.2996	-1.9004	True

Tukey's Post hoc Acetone 5DAI Alternaria

Fig 4.16 Tukey's Post hoc analysis (*Alternaria)*

Antifungal activity against Cercospora

All three Cassia fistula leaf extracts exhibited inhibitory effects on Cercospora, though the magnitude varied with solvent and concentration. Methanolic extract consistently produced the highest inhibition, followed by acetone extract, while aqueous extract showed comparatively lower but still significant activity relative to control. As seen with Alternaria, inhibition against Cercospora was more pronounced at 5 DAI, reflecting progressive suppression of mycelial growth.

Statistical analysis confirmed significant differences among treatments, with methanolic extract at higher concentrations forming a distinct group with the lowest mean colony diameters in Tukey's post hoc comparison.

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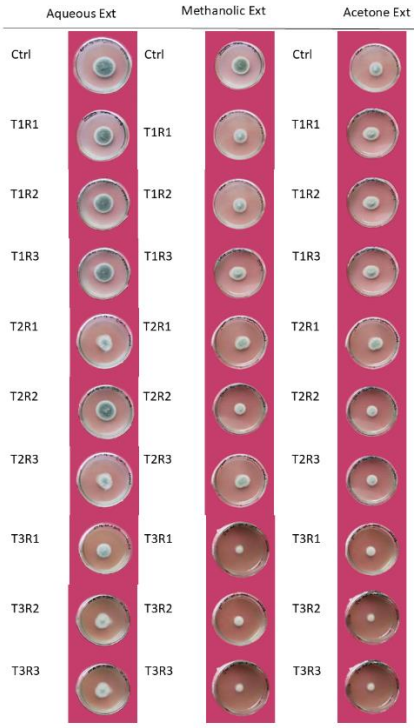


Fig 4.17 *Cercospora* treatment at 3 DAI against different extract

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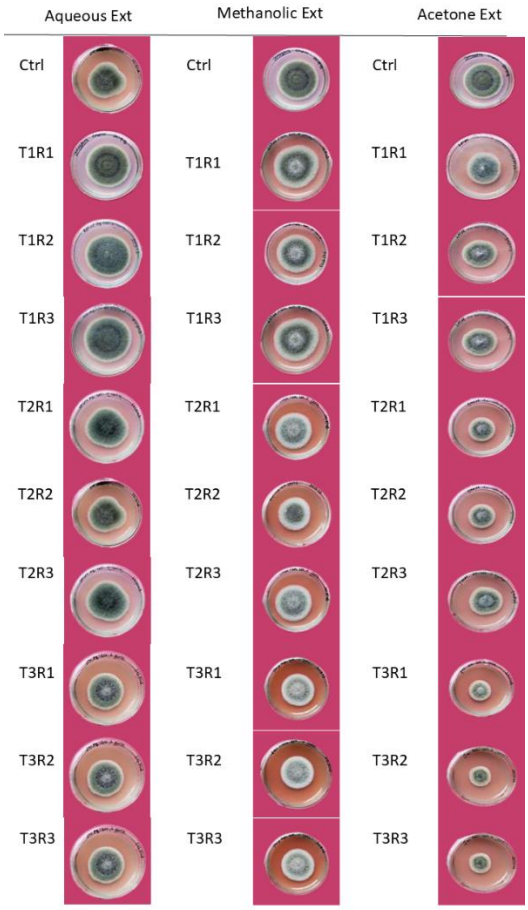


Fig 4.18 *Cercospora* treatment at 5 DAI against different extract

Table 4.21. Mean Colony Diameter and Percentage Inhibition (*Cercospora)*

Solvent	Treatment	Mean Diameter (mm), 3 DAI	Mean Diameter (mm), 5 DAI	Percentage Inhibition 3 DAI (%)	Percentage Inhibition 5 DAI (%)
Aqueous	T1	23.25	43.08	3.13	4.26
Aqueous	T2	20.15	41.4	16.04	8
Aqueous	T3	16.45	34.95	31.79	22.33
Methanolic	T1	21.18	43.1	11.74	4.22
Methanolic	T2	18.5	35.75	22.92	20.56
Methanolic	T3	12.5	32.5	47.92	27.78

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Acetone	T1	16.6	31.68	30.83	29.59
Acetone	T2	13.9	28.9	42.08	35.78
Acetone	T3	7.36	22.28	69.31	50.48

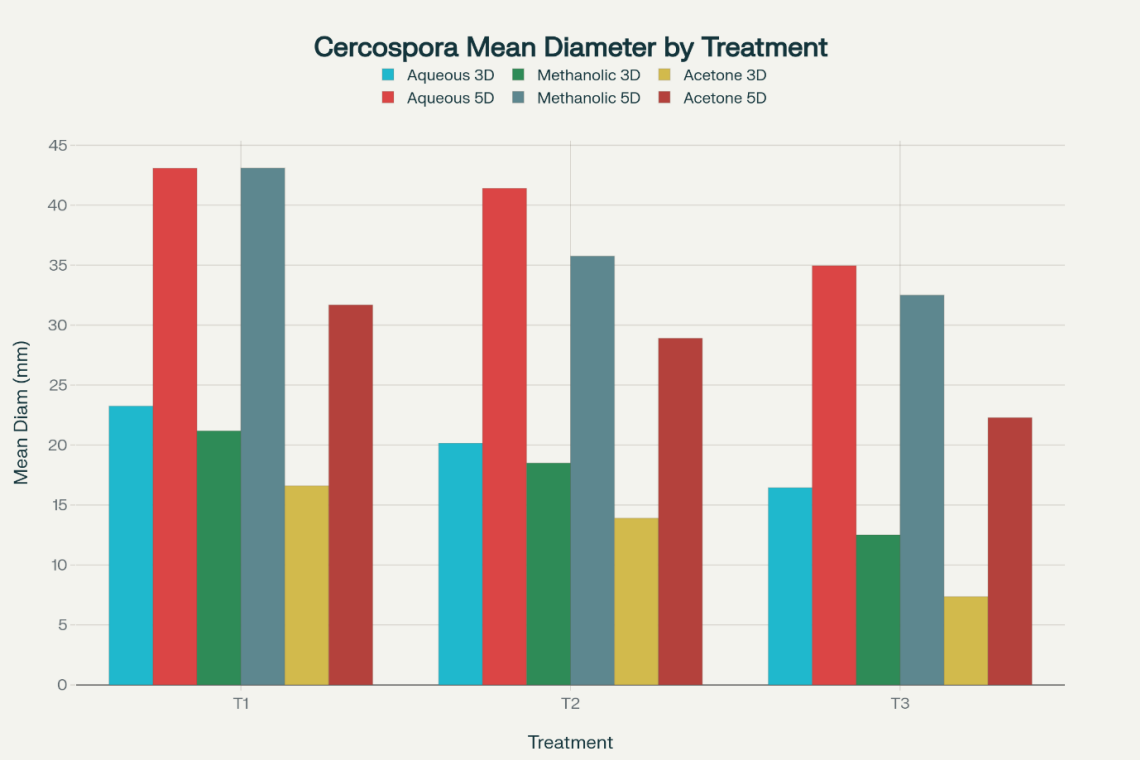


Figure 4.19 Mean Diameter Comparison Across Treatments and Days (*Cercospora)*

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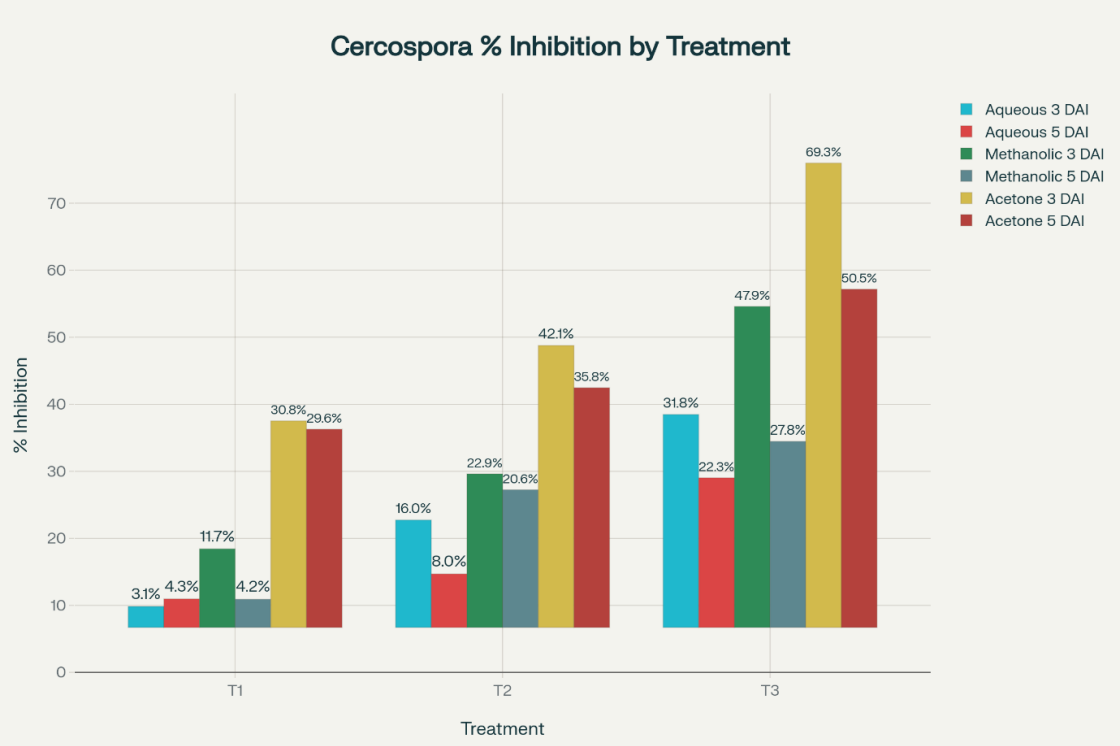


Figure 4.20 Percentage Inhibition Comparison Across Treatments and Days (*Cercospora)*

Table 4.22 ANOVA Table –Mean Colony Diameter for *Alternaria*

Extract	Day	F-statistic	p-value
Aqueous	3 DAI	1303.88	<0.001
Aqueous	5 DAI	240.99	<0.001
Methanolic	3 DAI	864.3	<0.001
Methanolic	5 DAI	2166.67	<0.001
Acetone	3 DAI	918.54	<0.001
Acetone	5 DAI	1027.74	<0.001

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Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.1333	0.001	-3.2061	-3.0606	True
T1	T3	-6.7000	0.001	-6.7728	-6.6272	True
T2	T3	-3.5667	0.001	-3.6394	-3.4939	True

Tukey Aqueous 3DAI

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-2.6667	0.001	-2.7741	-2.5593	True
T1	T3	-8.5556	0.001	-8.6630	-8.4481	True
T2	T3	-5.8889	0.001	-5.9963	-5.7815	True

Tukey Methanolic 3DAI

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-2.2000	0.001	-2.3074	-2.0926	True
T1	T3	-9.2467	0.001	-9.3541	-9.1393	True
T2	T3	-7.0467	0.001	-7.1541	-6.9393	True

Tukey Acetone 3DAI

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-1.4700	0.001	-1.5533	-1.3867	True
T1	T3	-8.4817	0.001	-8.5650	-8.3983	True
T2	T3	-7.0117	0.001	-7.0950	-6.9283	True

Tukey Aqueous 5DAI

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-7.4556	0.001	-7.5630	-7.3481	True
T1	T3	-10.6667	0.001	-10.7741	-10.5593	True
T2	T3	-3.2111	0.001	-3.3185	-3.1037	True

Tukey Methanolic 5DAI

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-2.7833	0	-3.4369	-2.1298	TRUE
T1	T3	-9.4	0	-10.0536	-8.7464	TRUE
T2	T3	-6.6167	0	-7.2702	-5.9631	TRUE

Tukey Acetone 5DAI

Fig 4.27 Tukey's Post hoc analysis (*Cercospora)*

Antifungal activity against Colletotrichum

Cassia fistula extracts also reduced mycelial growth of Colletotrichum compared to untreated control. Methanolic extract again showed superior antifungal activity, followed by acetone extract, whereas the aqueous extract exhibited moderate inhibition. Percentage inhibition values for Colletotrichum were generally comparable to or slightly lower than those observed for Alternaria and Cercospora, indicating species-specific variation in sensitivity.

ANOVA results indicated significant treatment effects, and post hoc analysis distinguished methanolic extract at higher concentrations as the most effective treatment.

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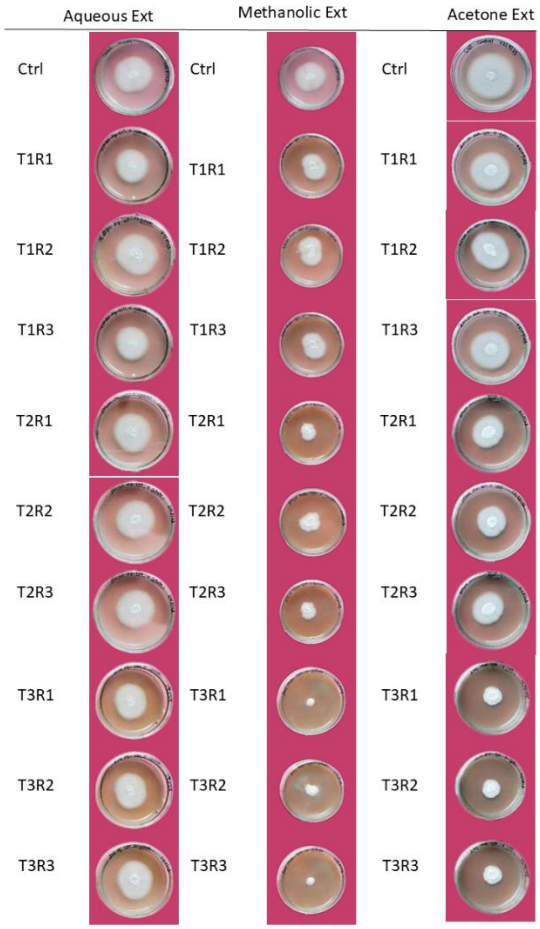


Fig 4.28 *Colletotrichum* treatment at 3 DAI against different extract

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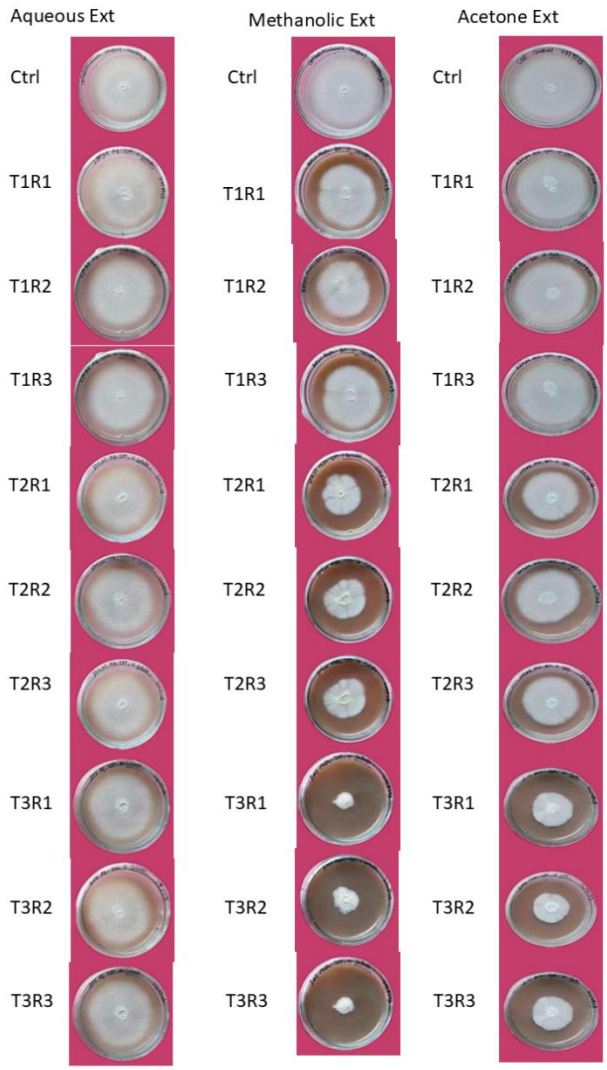


Fig 4.29 *Colletotrichum* treatment at 5 DAI against different extract

Table 4.29 Mean Colony Diameter and Percentage Inhibition

Solvent	Treatment	Mean Diameter (mm), 3 DAI	Mean Diameter (mm), 5 DAI	Percentage Inhibition 3 DAI (%)	Percentage Inhibition 5 DAI (%)
Aqueous	T1	31	53.1	7.7	2.4
Aqueous	T2	27	46.7	18.5	13
Aqueous	T3	24.5	41.8	27	23.4
Methanolic	T1	28.4	48	15.7	8.7

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Methanolic	T2	18.5	31.7	44.9	41.8
Methanolic	T3	7.9	17	75.6	67.4
Acetone	T1	26	44.3	21.8	19.3
Acetone	T2	26.4	40.5	21.3	25.6
Acetone	T3	15.2	27.2	54.3	49.9

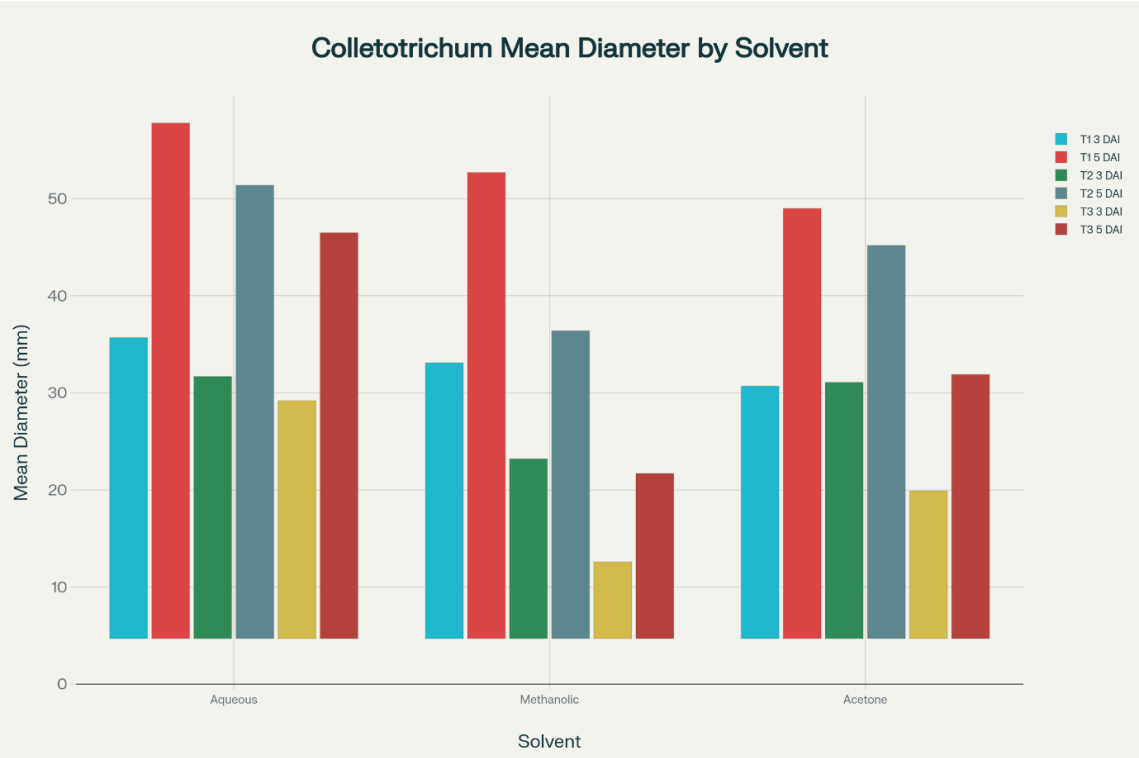


Fig 4.30 Mean Diameter Comparison Across Treatments and Days (*Colletotrichum)*

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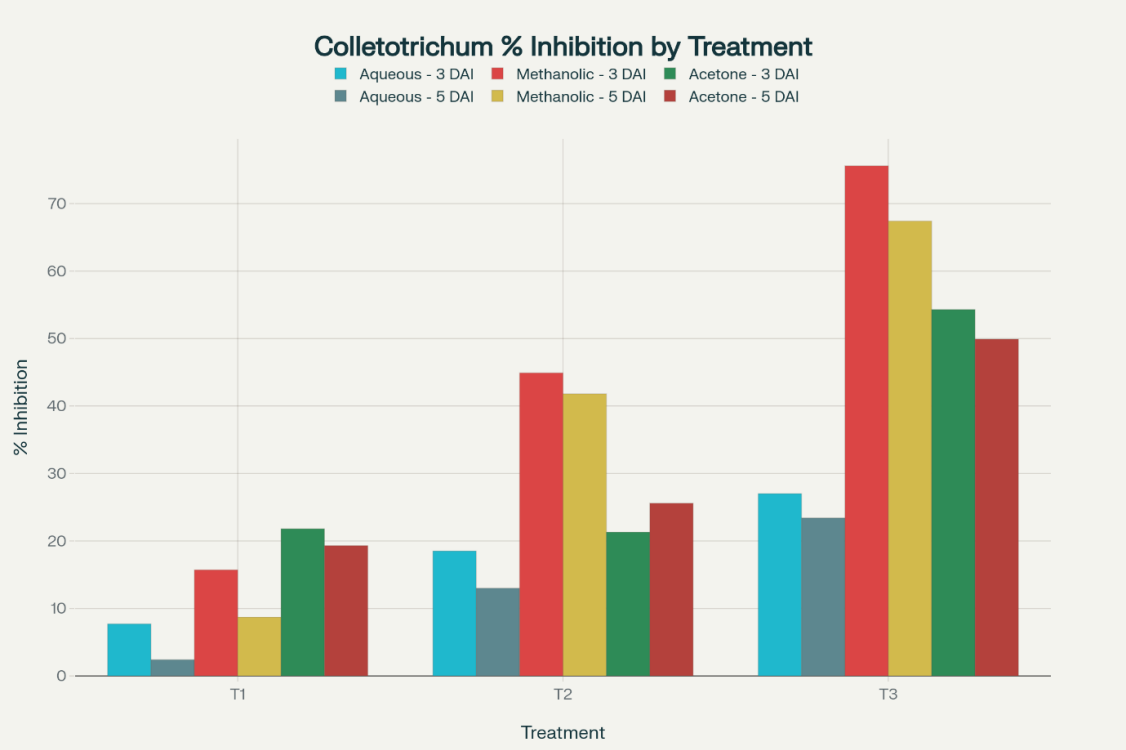


Figure 4.31 Percentage Inhibition Comparison Across Treatments and Days *Colletotrichum)*

Table 4.30 ANOVA Table –Mean Colony Diameter for *Alternaria*

Extract	Day	F-statistic	p-value
Aqueous	3 DAI	674.28	<0.001
Aqueous	5 DAI	861.52	<0.001
Methanolic	3 DAI	2192.43	<0.001
Methanolic	5 DAI	4299.84	<0.001
Acetone	3 DAI	466.2	<0.001
Acetone	5 DAI	5452.7	<0.001

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Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandi	p-adj	lower	upper	reject
T1	T2	-3.6	0	-4.143	-3.057	TRUE
T1	T3	-6.483	0	-7.026	-5.941	TRUE
T2	T3	-2.883	0	-3.426	-2.341	TRUE

Tukey's Post hoc Aque 3DAI Colletotrichum

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandi	p-adj	lower	upper	reject
T1	T2	-6.433	0	-7.274	-5.593	TRUE
T1	T3	-11.33	0	-12.17	-10.49	TRUE
T2	T3	-4.9	0	-5.74	-4.06	TRUE

Tukey's Post hoc Meth 3DAI Colletotrichum

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandi	p-adj	lower	upper	reject
T1	T2	0.333	0.716	-0.943	1.61	FALSE
T1	T3	-10.83	0	-12.11	-9.557	TRUE
T2	T3	-11.17	0	-12.44	-9.89	TRUE

Tukey's Post hoc Ace 3DAI Colletotrichum

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandi	p-adj	lower	upper	reject
T1	T2	-6.433	0	-7.274	-5.593	TRUE
T1	T3	-11.33	0	-12.17	-10.49	TRUE
T2	T3	-4.9	0	-5.74	-4.06	TRUE

Tukey's Post hoc Aque 3DAI Colletotrichum

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandi	p-adj	lower	upper	reject
T1	T2	-17.04	0	-18.09	-15.99	TRUE
T1	T3	-31.72	0	-32.77	-30.67	TRUE
T2	T3	-14.67	0	-15.72	-13.62	TRUE

Tukey's Post hoc Meth 3DAI Colletotrichum

Multiple Comparison of Means - Tukey HSD, FWER=0.05

Group1	Group2	Mean D	p-adj	Lower	Upper	Reject
T1	T2	-3.75	0	-4.277	-3.224	TRUE
T1	T3	-17.05	0	-17.58	-16.52	TRUE
T2	T3	-13.3	0	-13.83	-12.77	TRUE

Tukey's Post hoc Ace 5DAI Fusarium

Fig 4.38 Tukey's Post hoc analysis (*Colletotrichum)*

Antifungal activity against Fusarium

Fusarium growth was significantly inhibited by Cassia fistula leaf extracts, though this pathogen tended to be relatively less sensitive than some of the other fungi tested. Methanolic extract provided the highest inhibition of Fusarium mycelial growth, followed by acetone extract, while the aqueous extract showed the lowest level of activity among the three solvents. As with other pathogens, inhibition increased with extract concentration and was greater at 5 DAI compared to 3 DAI.

ANOVA and Tukey's tests demonstrated that methanolic extract at higher concentrations formed a significantly superior group in terms of reducing mean colony diameter, whereas aqueous extract at lower concentrations did not differ markedly from control in some cases.

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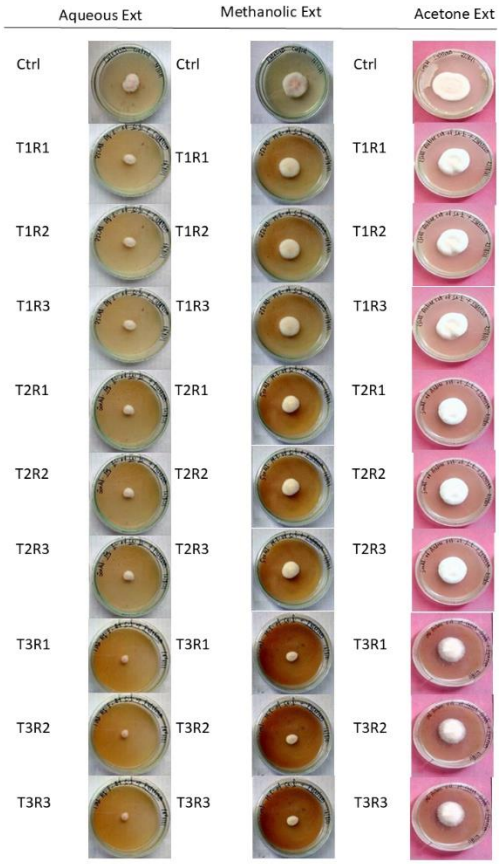


Fig 4.39 *Fusarium* treatment at 3 DAI against different extract

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Fig 4.40 *Fusarium* treatment at 5 DAI against different extract

Table 4.37 Mean Colony Diameter and Percentage Inhibition

Solvent	Treatment	Mean Diameter (mm), 3 DAI	Mean Diameter (mm), 5 DAI	Percentage Inhibition 3 DAI (%)	Percentage Inhibition 5 DAI (%)
Aqueous	T1	16.27	35.35	32.22	11.63
Aqueous	T2	13.15	25.32	45.21	36.71
Aqueous	T3	9.48	21.23	60.49	46.92
Methanolic	T1	16.28	35.18	32.15	12.04
Methanolic	T2	11.05	26.13	53.96	34.67
Methanolic	T3	6.57	16	72.64	60

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Acetone	T1	17.27	24.87	28.06	37.83
Acetone	T2	14.15	20.92	41.04	47.71
Acetone	T3	10.48	17.12	56.32	57.21

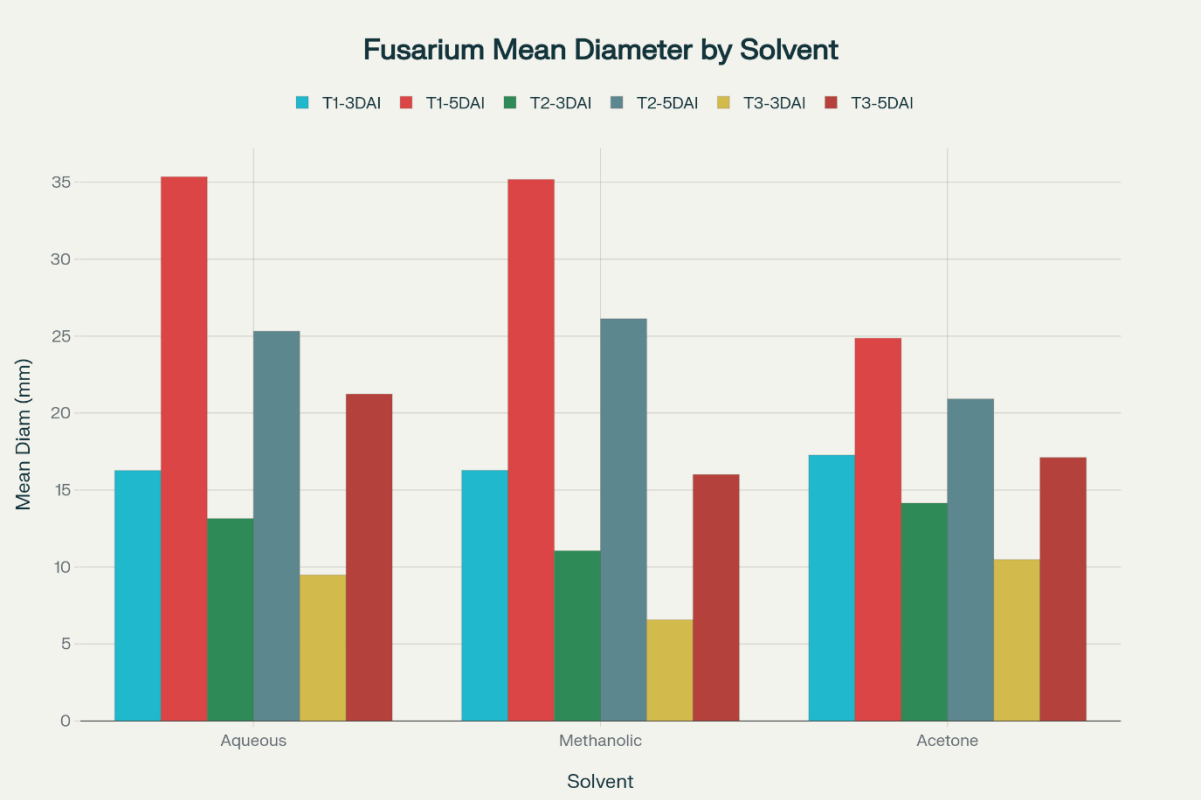


Fig 4.41 Mean Diameter Comparison Across Treatments and Days (*Fusarium*)

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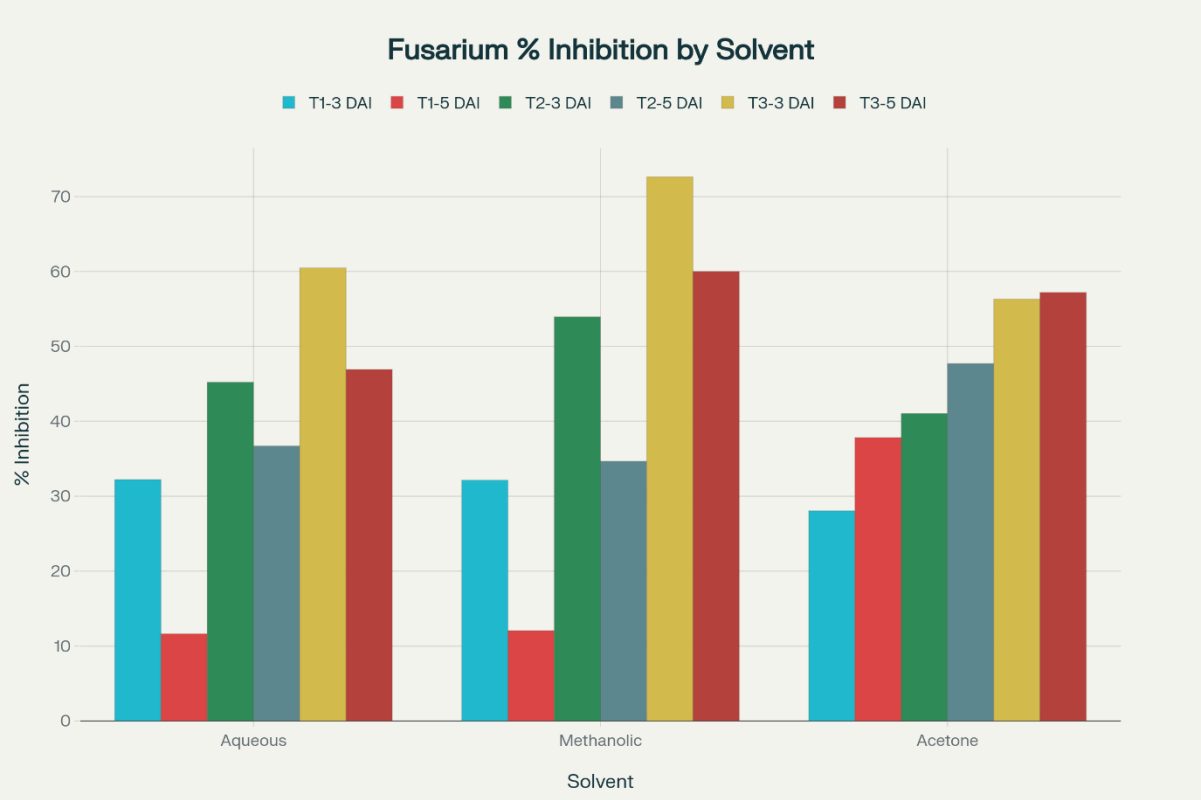


Fig 4.42 Percentage Inhibition Comparison Across Treatments and Days (*Fusarium*)

Table 4.38 ANOVA Table –Mean Colony Diameter for *Alternaria*

Extract	Day	F-statistic	p-value
Aqueous	3 DAI	1617	<0.001
Aqueous	5 DAI	3412.69	<0.001
Methanolic	3 DAI	1995.49	<0.001
Methanolic	5 DAI	6543.8	<0.001
Acetone	3 DAI	1617	<0.001
Acetone	5 DAI	1150.28	<0.001

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Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.1167	0.0013	-3.2935	-2.9398	True
T1	T3	-6.7833	0.0013	-6.9602	-6.6065	True
T2	T3	-3.6667	0.0013	-3.8435	-3.4898	True

Tukey's Post hoc Aq 3DAI Fusarium

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-5.2167	0.0013	-5.5296	-4.9037	True
T1	T3	-9.7167	0.0013	-10.0296	-9.4037	True
T2	T3	-4.5000	0.0013	-4.8129	-4.1870	True

Tukey's Post hoc Meth 3DAI Fusarium

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.1167	0.001	-3.3183	-2.9150	True
T1	T3	-6.7833	0.001	-6.9850	-6.5817	True
T2	T3	-3.6667	0.001	-3.8683	-3.4650	True

Tukey's Post hoc Ace 3DAI Fusarium

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-10.0333	0.0013	-10.1901	-9.8765	True
T1	T3	-14.1167	0.0013	-14.2735	-13.9598	True
T2	T3	-4.0833	0.0013	-4.2401	-3.9265	True

Tukey's Post hoc Aq 5DAI Fusarium

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-9.0833	0.0013	-9.4264	-8.7402	True
T1	T3	-19.1833	0.0013	-19.5264	-18.8402	True
T2	T3	-10.1000	0.0013	-10.4431	-9.7569	True

Tukey's Post hoc Meth 5DAI Fusarium

Multiple Comparison of Means - Tukey HSD, FWER=0.05

group1	group2	meandiff	p-adj	lower	upper	reject
T1	T2	-3.9667	0.001	-4.1771	-3.7562	True
T1	T3	-7.7333	0.001	-7.9438	-7.5229	True
T2	T3	-3.7667	0.001	-3.9771	-3.5562	True

Tukey's Post hoc Ace 5DAI Fusarium

Fig 4.49 Tukey's Post hoc analysis (*Fusarium*)

Comparative pattern across fungi and solvents

Overall, methanolic and acetone extracts of Cassia fistula leaves consistently exhibited stronger antifungal activity than the aqueous extract against all four test fungi. Among the pathogens, Alternaria and Cercospora tended to be more sensitive, while Fusarium appeared relatively more tolerant, as reflected by higher residual colony diameters and lower percentage inhibition at comparable concentrations. The trend of increased inhibition at 5 DAI suggests that the active components in the extracts maintained or enhanced their effectiveness over the incubation period.

Discussion

The present study demonstrates that Cassia fistula leaf extracts possess considerable in vitro antifungal activity against four major phytopathogenic fungi, with methanolic and acetone extracts being particularly effective. This aligns with reports that plant-derived preparations can suppress pathogenic fungi and offer alternatives to synthetic fungicides in disease management (Abdu et al., 2021; Akarsh et al., 2016). The observed inhibition of Alternaria, Cercospora, Colletotrichum and Fusarium supports the wider evidence that medicinal plants rich in secondary metabolites can be developed as botanical fungicidal agents for crop protection (Abdu et al., 2021; Afu et al., 2020).

The superior activity of methanolic and acetone extracts compared to the aqueous extract can be attributed to differences in solvent polarity and extractive capacity for key bioactive constituents. Methanol, and to some extent acetone, efficiently solubilize a broad spectrum of phenolic compounds and flavonoids, which are frequently implicated in antimicrobial activity (Abaza et al., 2011; Garg & Dwivedi, 2021). This is consistent with the qualitative phytochemical tests and HPTLC fingerprints, which showed richer and more diverse band patterns for methanolic and acetone extracts than for the aqueous extract, indicating higher abundance of moderately polar secondary metabolites (Selvaraj et al., 2019; Alexandar & Joy, 2022).

The antifungal effects observed against all four test fungi are likely mediated by multiple mechanisms attributable to plant secondary metabolites. Tannins and phenolic compounds are known to interact

with fungal cell walls and membranes, altering permeability and causing leakage of cellular constituents, while flavonoids can interfere with key enzymes and oxidative balance in fungal cells (Adigun et al., 2020; Afu et al., 2020). Alkaloids and related constituents may further disrupt metabolic pathways, and the combined action of these compounds in crude extracts can reduce the likelihood of rapid resistance development compared to single-site synthetic fungicides (Abdu et al., 2021; Akarsh et al., 2016).

Differences in sensitivity among the four fungi, with Alternaria and Cercospora generally showing higher inhibition than Fusarium, suggest species-specific variations in cell wall architecture, membrane composition and detoxification systems. Similar differential responses to plant-derived compounds have been documented in studies on Alternaria and other phytopathogens, where variation in pathogenicity and stress tolerance reflected underlying genetic and physiological diversity (Achilonu et al., 2023; Adhikari et al., 2021). These findings emphasize the importance of evaluating botanical extracts against multiple target pathogens to assess their broader applicability and limitations in plant disease management (Abdu et al., 2021).

The increase in percentage inhibition from 3 to 5 days after inoculation indicates that the active compounds in C. fistula extracts maintain their stability and bioactivity over the incubation period in vitro. Sustained or enhanced inhibition over time has also been reported for other plant-based antifungal preparations and suggests that longer exposure may be beneficial for maximizing efficacy (Abdu et al., 2021; Akarsh et al., 2016). However, the present work is limited to in vitro assays using crude extracts, and translation of these effects to in vivo plant–pathogen systems must be approached cautiously, as environmental factors and plant metabolism can influence performance under field conditions (Adigun et al., 2020).

Future research should focus on fractionating the most active extracts, isolating and characterizing individual antifungal constituents, and elucidating their specific mechanisms of action. Integration of chromatographic and spectrometric methods such as HPTLC, HPLC and LC–MS has been recommended for comprehensive phytochemical characterization and marker-based standardization of medicinal plants (Khushwaha, 2023; Menon et al., 2019). In addition, in vivo experiments on detached leaves or whole plants, followed by greenhouse and field trials, are required to evaluate efficacy, phytotoxicity, persistence and compatibility of C. fistula-based formulations under realistic agricultural conditions (Abdu et al., 2021; Akarsh et al., 2016).

Despite these limitations, the results clearly indicate that C. fistula leaf extracts, particularly those obtained with methanol and acetone, hold promise as sources of botanical fungicidal agents. Their development and integration into integrated disease management strategies could help reduce reliance on synthetic fungicides, mitigate negative environmental impacts and contribute to more sustainable crop protection systems (Akarsh et al., 2016; Adigun et al., 2020).

Conclusion

Cassia fistula leaf extracts exhibited significant in vitro antifungal activity against Alternaria, Cercospora, Colletotrichum and Fusarium, with methanolic and acetone extracts performing better than the aqueous extract across concentrations and incubation periods (Abdu et al., 2021; Akarsh et al., 2016). Phytochemical screening and HPTLC fingerprinting revealed a rich profile of secondary

metabolites—particularly phenolic and flavonoid constituents—in organic solvent extracts, providing a plausible chemical basis for the observed antifungal effects (Abaza et al., 2011; Alexandar & Joy, 2022). These findings support C. fistula as a promising source of plant-derived antifungal agents and justify further work on fractionation, compound identification and formulation development to enable its use in sustainable crop disease management and integrated plant health strategies (Abdu et al., 2021; Adigun et al., 2020).

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