E-ISSN 2250-0944
ISSN 2250-1150
doi: 10.31838/ijprt/16.02.582

Research Article

Bioprospecting *Calotropis procera*-Associated Endophytic Fungi for Antifungal Bioactive Compounds

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Received: 18.07.2026, Revised: 21.08.2026, Accepted: 23.09.2026

ABSTRACT
Endophytic fungi associated with Medicinal Plants represent a promising source of biologically active Secondary Metabolites with potential applications in antimicrobial drug discovery. The present study aimed to isolate and evaluate Endophytic fungi associated with the roots of *Calotropis procera* for their phytochemical constituents and antifungal potential. Root tissues of C. procera were processed for the isolation of culturable Endophytic Fungi, resulting in the recovery of Six isolates designated EFM01, EFM02, EFM03, EFM04, EFM05, and EFM06. The isolates were cultured under in vitro conditions, and crude secondary metabolites were obtained by Methanolic extraction. Qualitative phytochemical screening revealed the presence of alkaloids, phenols, tannins, and steroids in all six fungal extracts. Anthraquinones were detected in EFM02, EFM04, and EFM06, whereas terpenoids were detected in EFM01, EFM04, and EFM05. Flavonoids and saponins were not detected in any of the tested extracts. The antifungal activity of the crude extracts was evaluated by the Agar well diffusion method against three fungal pathogens, namely *Candida albicans*, *Fusarium oxysporum*, and *Alternaria sp.*, at different concentrations. At 100 µg, all six extracts exhibited measurable antifungal activity, although the magnitude of inhibition varied among isolates and test fungi. Against C. albicans, EFM06 produced the highest inhibition zone (22 mm), followed by EFM04 (21 mm), while EFM06 showed 16.8 mm inhibition against *F. oxysporum*. Against *Alternaria* sp., EFM06 and EFM04 produced inhibition zones of 19.1 and 18.9 mm, respectively. EFM06 exhibited comparatively strong and broad-spectrum antifungal activity, while EFM04 also showed considerable activity against the tested fungi. These findings demonstrate that root-associated endophytic fungi of C. procera are a potential source of chemically diverse secondary metabolites with antifungal properties.

KEYWORDS: Calotropis Procera, Endophytic Fungi, Secondary Metabolites, Phytochemical Screening, Antifungal Activity, Candida Albicans, Fusarium Oxysporum, Alternaria Sp.

INTRODUCTION

Endophytic fungi are microorganisms that colonize the internal tissues of living plants without producing visible disease symptoms at the time of isolation. They represent an important component of plant-associated microbial communities and may establish complex interactions with their host plants, ranging from mutualistic and beneficial associations to latent pathogenicity [1,2]. Fungal Endophytes are widely distributed among terrestrial plants and have attracted considerable scientific interest because of their ecological functions and their ability to produce structurally diverse Secondary Metabolites [3–6]. Endophytic fungi are increasingly recognized as promising sources of Bioactive Natural Products with potential applications in Medicine, Agriculture, food Biotechnology and Pharmaceutical Research. Their secondary metabolites include alkaloids, terpenoids, phenolics, steroids, polyketides and other chemically diverse compounds exhibiting antimicrobial, antifungal, antioxidant, anticancer and other biological activities [7–12]. The discovery of taxol production by the endophytic fungus Taxomyces associated with Taxus brevifolia provided an important example of the biosynthetic potential of Endophytic Microorganisms and stimulated extensive research into Endophyte-derived natural products [9]. Subsequent investigations have demonstrated that Endophytic fungi can produce a wide variety of biologically active metabolites, making them valuable targets for natural-product discovery and bioprospecting [7,10–15]. Medicinal plants are particularly attractive hosts for the exploration of bioactive endophytes because they have evolved extensive chemical defence systems and may harbour microorganisms capable of producing chemically and biologically interesting metabolites [11,12]. Calotropis procera (Aiton) is a medicinally important plant belonging to the

family Apocynaceae and has traditionally been used for several therapeutic purposes. Different parts of the plant contain biologically active constituents, and its associated microorganisms have also attracted attention as potential sources of antimicrobial and other pharmacologically relevant metabolites [16–18].Previous investigations have demonstrated the occurrence of diverse endophytic fungi associated with C. procera. Nascimento et al. reported considerable diversity of endophytic fungi associated with C. procera and demonstrated their antimicrobial and pharmacological potential [16]. Mohamed et al. subsequently isolated endophytic fungi from C. procera and investigated their antimicrobial potential, further supporting the importance of this medicinal plant as a source of bioactive fungal endophytes [17]. More recently, Abo El-Souad et al. investigated endophytic fungi from C. procera for bioactive compounds and reported antimicrobial and anticancer activities together with chemical characterization of fungal metabolites [18]. The ability of endophytic fungi to synthesize secondary metabolites is influenced by fungal genotype, host plant, tissue type, environmental conditions and culture conditions [5,7,10]. Consequently, screening multiple isolates obtained from a single medicinal plant can reveal substantial differences in metabolite profiles and biological activities. Qualitative phytochemical screening provides an initial indication of the major classes of secondary metabolites present in fungal extracts, while antimicrobial assays provide a preliminary assessment of their biological potential [12–15]. Antimicrobial resistance and the continuing emergence of pathogenic microorganisms have increased the demand for new antimicrobial molecules. Endophytic fungi are considered promising candidates in this context because their secondary metabolites may possess antimicrobial activities against clinically and agriculturally important microorganisms [13,15,22]. Agar diffusion assays remain useful preliminary screening methods for comparing the antimicrobial activity of crude extracts, although inhibition-zone measurements should be interpreted as screening data rather than as direct measures of potency or minimum inhibitory concentration [19,20].

MATERIALS AND METHODS

Collection and Processing of Plant Material

Healthy and apparently disease-free roots of Calotropis procera were selected for the isolation of endophytic fungi. Plant material was collected carefully and transferred to the laboratory under sterile conditions. The samples were processed as soon as possible after collection to minimize contamination and changes in the endogenous microbial population.

Surface Sterilization and isolation of Endophytic Fungi

The Root samples were thoroughly washed under running tap water to remove adhering soil particles and other surface contaminants. The roots were subsequently cut into small segments and subjected to surface sterilization. The sterilization procedure was based on established methods for recovering endophytic fungi while minimizing the presence of epiphytic microorganisms [1,18].The root segments were immersed in 70% ethanol for 1 min, followed by treatment with 5% sodium hypochlorite for 15 min. The segments were subsequently rinsed several times with sterile distilled water to remove residual sterilizing agents. The sterilized tissues were dried aseptically on sterile filter paper and cut into approximately 5 × 5 mm pieces. The sterilized tissue segments were placed on potato dextrose agar (PDA) supplemented with an antibacterial agent to suppress bacterial growth. The plates were incubated under suitable fungal growth conditions and monitored regularly for the emergence of fungal hyphae from the plant tissue. Fungal growth emerging from the internal tissue was considered presumptive evidence of endophytic colonization. Surface sterilization procedures are particularly important in endophyte studies because inadequate sterilization may result in recovery of epiphytic rather than endophytic microorganisms [1].

Purification and Preservation of Fungal isolates

Individual fungal colonies emerging from the plant tissues were transferred aseptically to fresh PDA plates to obtain pure cultures. Repeated subculturing was performed whenever necessary until morphologically uniform colonies were obtained. Pure isolates were maintained on PDA slants/plates under appropriate storage conditions for subsequent experiments. The isolates were designated EFM01, EFM02, EFM03, EFM04, EFM05 and EFM06.

Morphological Characterization

The fungal isolates were initially characterized using macroscopic and microscopic characteristics. Colony morphology, colour, texture, growth pattern and pigmentation were recorded during culture. Microscopic examination was performed using lactophenol cotton blue staining to observe fungal hyphae and reproductive structures. Morphological characterization was used as a preliminary basis for comparison among isolates. Molecular identification should be performed in future work for definitive taxonomic assignment.

Selection of promising Endophytic Fungal isolates

All recovered isolates were initially screened for growth characteristics and secondary-metabolite-producing potential. Isolates exhibiting comparatively good growth and promising biological activity were considered candidates for subsequent metabolite extraction and antimicrobial screening. Based on the overall screening observations, EFM04 and EFM06 were identified as particularly promising isolates.

Production of fungal Secondary Metabolites

For secondary-metabolite production, selected fungal isolates were inoculated into potato dextrose broth (PDB). The cultures were prepared in 250-mL Erlenmeyer flasks containing 100 mL of culture medium. The inoculated flasks were incubated for approximately 15 days at 28 °C under shaking conditions at approximately 100 rpm. Endophytic fungi can alter their secondary-metabolite profiles depending on nutrient availability, incubation conditions, culture age and other environmental parameters [7,10,12]. Therefore, standardized culture conditions were maintained for comparative screening of the isolates.

Qualitative phytochemical screening

The crude fungal extracts were subjected to qualitative phytochemical screening to detect major classes of secondary metabolites. Tests were performed for alkaloids, phenolic compounds, tannins, steroids, anthraquinones, terpenoids, flavonoids and saponins using standard qualitative phytochemical procedures. The appearance of characteristic colour reactions, precipitates or other positive reactions was recorded as evidence for the corresponding metabolite class. Qualitative phytochemical screening is useful as an initial chemical characterization step, although chromatographic and spectrometric analyses are required for definitive identification of individual compounds [12,14].

Test Microorganisms

The antifungal activity of the crude fungal extracts was evaluated against *Candida albicans*, *Fusarium oxysporum and Alternaria* sp. These organisms were selected to represent yeast and filamentous fungal targets relevant to medical and agricultural microbiology.

Antifungal activity assay

The antifungal activity of the crude extracts was evaluated by an agar diffusion assay. Appropriate agar medium was inoculated with the respective test organism, and wells were prepared aseptically in the agar surface. Defined quantities of fungal crude extracts were introduced into the wells. The extracts were evaluated at a concentration of 100 µg per test. Appropriate positive controls were included according to the experimental design. Following incubation under suitable conditions for the respective test organisms, the diameter of the growth-inhibition zones surrounding each well was measured in milli metres. Diffusion-based antimicrobial assays are useful for preliminary comparison of antimicrobial activity; however, the resulting inhibition zones are influenced by factors such as diffusion characteristics, extract concentration, agar composition, inoculum density and incubation conditions [19,20]. Therefore, the results obtained in the present study were interpreted primarily as comparative screening data.

Data recording and interpretation

All observations were recorded systematically, and inhibition-zone diameters were expressed in millimetres. Comparative interpretation was performed among EFM01–EFM06 for each test organism. Isolates showing larger inhibition zones against one or more target fungi were considered comparatively more promising for further investigation. For future confirmation of antifungal potency, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), chromatographic profiling and compound-level structural characterization are recommended.

RESULTS AND DISCUSSION

Isolation and Recovery of Endophytic Fungi

Six morphologically distinct endophytic fungal isolates were recovered from the surface-sterilized root tissues of Calotropis procera and designated EFM01, EFM02, EFM03, EFM04, EFM05 and EFM06. The recovery of multiple fungal morphotypes from the root tissue is consistent with previous reports demonstrating that medicinal plants can harbour diverse endophytic fungal communities [11,12,16]. The successful recovery of fungal isolates following

surface sterilization indicates that the isolated fungi were associated with the internal plant tissues rather than being readily recoverable surface contaminants. Nevertheless, surface sterilization does not absolutely establish an endophytic lifestyle, and molecular confirmation together with rigorous sterilization controls is recommended for definitive characterization [1,2].The occurrence of several fungal isolates within the same host plant may reflect differences in fungal colonization ability, tissue specificity, host genotype and environmental factors. Endophytic fungal communities are known to vary according to plant species, tissue, developmental stage, geographical location and environmental conditions [2,5,16].

Figure. 3.1 Screening of Endophytes isolates

Morphological Characterization and Preliminary Selection

The recovered isolates displayed differences in colony morphology, pigmentation, texture and growth characteristics, demonstrating phenotypic diversity among the fungal cultures. Microscopic examination using lactophenol cotton blue provided additional morphological characteristics for preliminary differentiation. Among the six isolates, EFM04 and EFM06 demonstrated comparatively promising characteristics during preliminary screening and were therefore considered priority isolates for further secondary-metabolite investigation. Such isolate-to-isolate variation is expected because endophytic fungi possess distinct biosynthetic capabilities and may produce different metabolite profiles even when recovered from the same host plant [7,10,12].Morphological characteristics alone, however, are insufficient for reliable species-level identification. Therefore, molecular identification using ITS rDNA sequencing and phylogenetic analysis should be performed before assigning definitive taxonomic names to EFM01–EFM06.

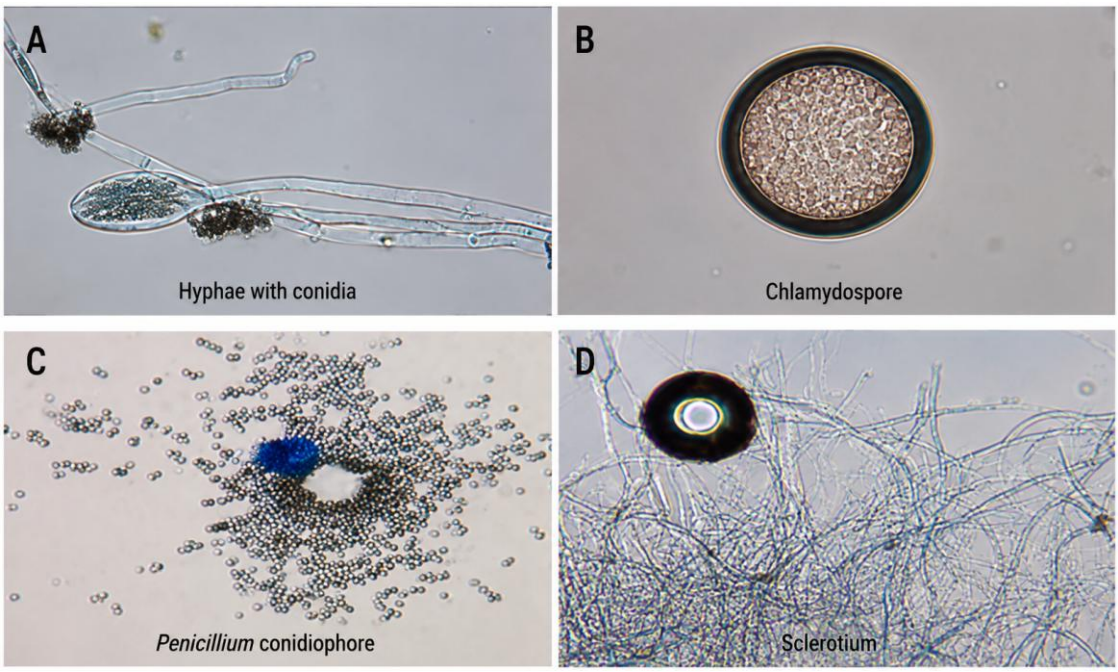

Figure. 3.2 Diagram showing endophytic microorganisms residing within the internal tissues of the root of *Calotropis procera.* (fungal staining).

Production of crude Secondary Metabolites

The Six isolates were capable of producing crude extracellular/intracellular metabolite extracts under the tested culture conditions. The ability of endophytic fungi to synthesize secondary metabolites in artificial culture is one of the major reasons for their importance in natural-product research [3,4,6,7].Endophytic fungi are recognized as prolific producers of chemically diverse secondary metabolites, including compounds with antimicrobial and antifungal properties [10–15]. The biological activity observed in the present study therefore supports the use of C. procera-associated endophytic fungi as a source of candidate bioactive metabolites.However, the crude extracts obtained in the present investigation represent complex mixtures.

Qualitative Phytochemical Screening

Qualitative phytochemical analysis demonstrated considerable variation in the secondary-metabolite profiles of the six fungal isolates. Alkaloids, phenolic compounds, tannins and steroids were detected in all six isolates (EFM01–EFM06). Anthraquinones were detected in EFM02, EFM04 and EFM06, whereas terpenoids were detected in EFM01, EFM04 and EFM05. Flavonoids and saponins were not detected in any of the six fungal extracts under the conditions employed. The detection of multiple classes of secondary metabolites is consistent with the recognized metabolic diversity of endophytic fungi [7,10–15]. Endophytic fungi can produce structurally diverse metabolites, and their metabolite profiles may vary considerably among isolates because of differences in biosynthetic pathways and ecological interactions with the host plant [2,5,10]. The presence of phenolic compounds, alkaloids, terpenoids, steroids and anthraquinones is particularly noteworthy because several members of these chemical classes are associated with antimicrobial and other biological activities. However, a positive qualitative phytochemical reaction only indicates the possible presence of a chemical class and does not establish the identity, concentration or biological contribution of an individual compound [12,14].

Table: 3.1 Qualitative Phytochemical screening

Phytochemical Screening of isolated fungal extracts.									
S.No	**Endophytes**	***Alk***	***Ant***	***Flav***	***Phe***	***Sap***	***Ste***	***Tan***	***Ter***
1.	EFM-01	+	-	-	+	-	+	+	+
2.	EFM-02	+	+	-	+	-	+	+	-
3.	EFM-03	+	-	-	+	-	+	+	-
4.	EFM-04	+	+	-	+	-	+	+	+

5.	EFM-05	+	-	-	+	-	+	+	+
6.	EFM-06	+	+	-	+	-	+	+	-

Qualitative Phytochemical screening: (+): Presence, (-): Absence, Alk.: Alkaloïds, Ant.: Anthraquinone, Phe.: Phenols, Tan.: Tannins, Flav.: Flavonoïds, Sap.: Saponins, Ter.: Terpenoïds.

Antifungal activity against *Candida albicans*

All six crude fungal extracts demonstrated inhibitory activity against *Candida albicans* at the tested concentration of 100 µg. The inhibition zones were 18.0 mm for EFM01, 17.4 mm for EFM02, 19.2 mm for EFM03, 21.0 mm for EFM04, 19.0 mm for EFM05 and 22.0 mm for EFM06.Among the isolates, EFM06 exhibited the greatest inhibition zone against C. albicans (22.0 mm), followed by EFM04 (21.0 mm). EFM02 showed the lowest inhibition zone (17.4 mm). The positive-control values were higher than those observed for the crude extracts, indicating that the fungal extracts exhibited promising but comparatively lower activity than the reference antimicrobial agent. The ability of endophytic fungal extracts to inhibit pathogenic fungi is consistent with previous studies reporting antimicrobial metabolites from endophytic fungi [13,15,17,18]. The comparatively high activity of EFM04 and EFM06 may be associated with differences in the quantity or composition of their secondary metabolites.

Table 3.2 Zone of inhibition against fungus *Candida albicans* in potato dextrose agar after 3days

Fungal Isolates	**Zone of Inhibition against *Candida albicans***					
	Standard	100	75	50	25	Blank
EFM01	29	18	15	13.3	11.2	0
EFM02	30	17.4	13	12	10	0
EFM03	30	19.2	16.8	14	13.1	0
EFM04	28	21	19.8	17.2	15.3	0
EFM05	29	19	17.1	15.2	14	0
EFM06	27	22	22	20	18.5	0

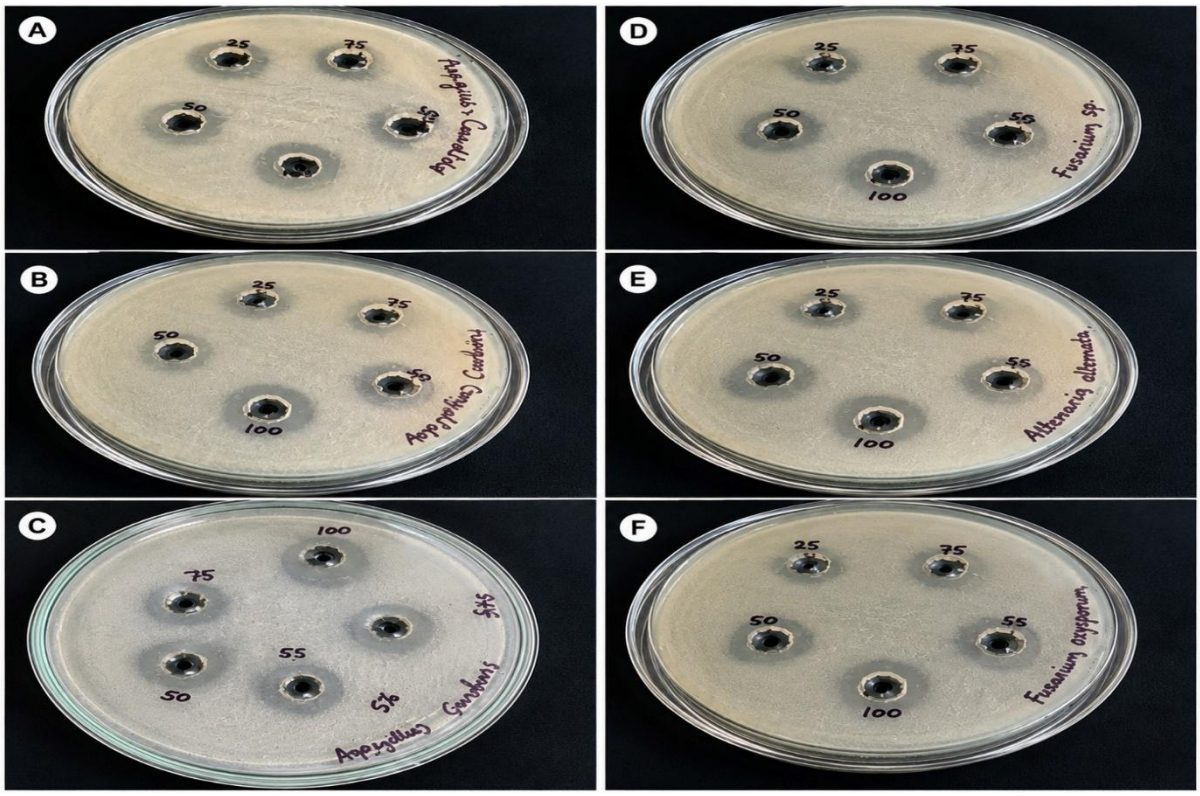

Figure 3.3. Antifungal activity of the extracts (A, B, and C) was evaluated against *Candida* spp. using the agar well diffusion method on Potato Dextrose Agar (PDA), and the inhibition zones were recorded after 3 days of incubation.

Antifungal activity against *Fusarium oxysporum*

All six extracts also inhibited *Fusarium oxysporum*. The inhibition zones were 15.0 mm for EFM01, 16.0 mm for EFM02, 15.0 mm for EFM03, 17.0 mm for EFM04, 15.0 mm for EFM05 and 16.8 mm for EFM06.EFM04 demonstrated the highest inhibition zone against *F. oxysporum* (17.0 mm), closely followed by EFM06 (16.8 mm). The comparatively similar activities of EFM02 and EFM06, despite differences in their phytochemical profiles, indicate that qualitative phytochemical screening alone cannot adequately explain antifungal potency.The inhibition of *F. oxysporum* is of particular interest because this species is associated with important plant diseases. Endophytic fungi have previously been investigated as potential sources of metabolites with activity against plant-pathogenic fungi, supporting their possible application in biological control and agricultural biotechnology [5,13,15].

Table 3.3 Zone of inhibition produced by the fungal extract against *Fusarium oxysporum* on Potato Dextrose Agar (PDA) after 3 days of incubation.

Endophytes isolates	**Zone of Inhibition against *Fusarium oxysporum***					
	Std	100	75	50	25	Blank
EFM01	24	15	13	10	8.5	0
EFM02	22	16	14	12	11	0
EFM03	23	15	14	13.5	11.1	0
EFM04	22	17	15	13	12	0
EFM05	22	15	13	11.8	11	0
EFM06	23	16.8	16	14	12	0

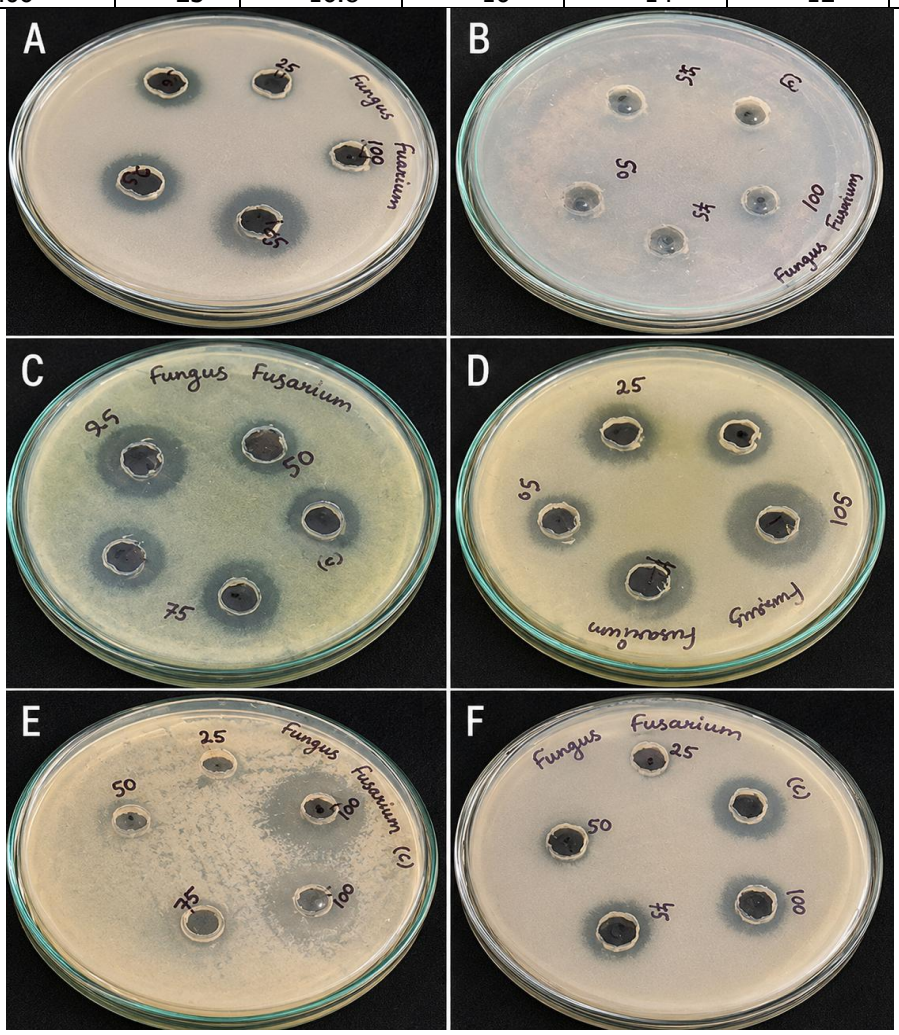

Figure 3.4. Antifungal activity (A, B, C, D, E and F) of Endophytic isolates using well diffusion method against fungus *Fusarium*.

Antifungal activity against *Alternaria sp.*

The crude extracts also demonstrated inhibitory activity against *Alternaria sp*. The inhibition zones were 13.0 mm for EFM01, 14.8 mm for EFM02, 15.2 mm for EFM03, 18.9 mm for EFM04, 16.0 mm for EFM05 and 19.1 mm for EFM06.EFM06 showed the strongest activity against *Alternaria sp*. with an inhibition zone of 19.1 mm, while EFM04 showed the second-highest activity at 18.9 mm. EFM01 exhibited the lowest inhibition zone (13.0 mm). The relatively strong activity of EFM04 and EFM06 against both C. albicans and Alternaria sp. suggests that these isolates may possess metabolites with a comparatively broad antifungal spectrum. Similar variation in antimicrobial activity among endophytic fungal isolates has been reported in studies of medicinal plants and C. procera-associated endophytes [16–18].

Table 3.4. Zone of inhibition against fungus *Alternaria* in potato dextrose agar

Endophytes isolates	Zone of Inhibition against *Alternaria*					
	Std	100	75	50	25	Blank
EFM01	24	13	12.1	10	9	0
EFM02	24	14.8	13.2	12	10.1	0
EFM03	22	15.2	13.8	12.8	11	0
EFM04	23	18.9	17	15.8	13	0
EFM05	25	16	15.1	13.8	11.8	0
EFM06	25	19.1	17.8	15.9	12.2	0

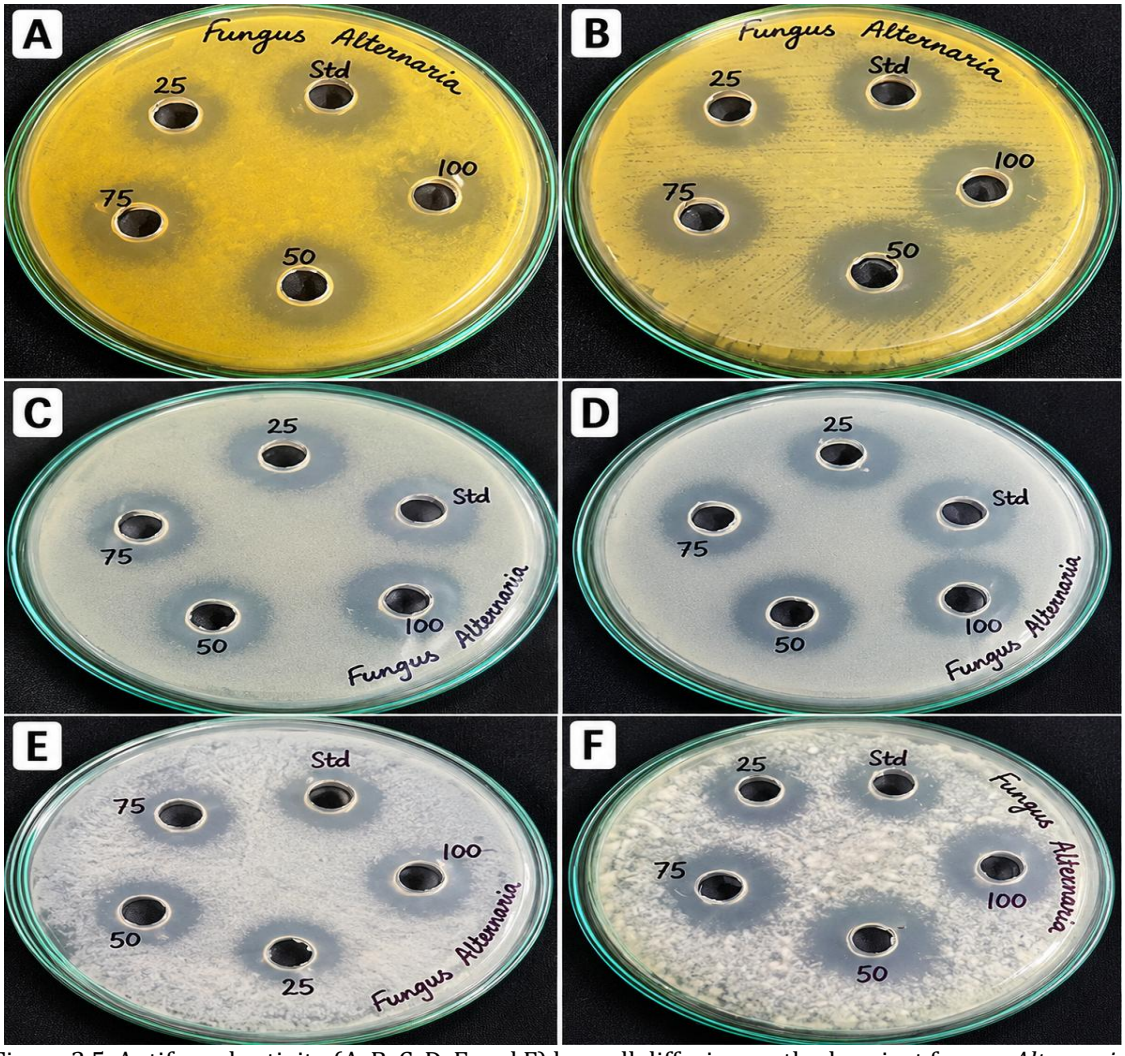

Figure 3.5. Antifungal activity (A, B, C, D, E and F) by well diffusion method against fungus *Alternaria*.

3.7 Comparative antifungal potential of EFM04 and EFM06

A comparison of the three test organisms demonstrated that EFM04 and EFM06 were the most promising isolates overall .EFM06

produced inhibition zones of 22.0 mm against *C. albicans,* 16.8 mm against *F. oxysporum* and 19.1 mm against *Alternaria sp*. EFM04 produced inhibition zones of 21.0, 17.0 and 18.9 mm against the same organisms, respectively.

Thus, EFM06 exhibited the highest activity against C*. albicans and Alternaria sp*., whereas EFM04 showed the highest activity against *F. oxysporum*. This isolate-specific variation suggests that the two fungi may possess different antimicrobial metabolite profiles.The observation is consistent with the concept that endophytic fungi represent chemically diverse microbial communities in which individual isolates can differ substantially in biosynthetic capacity [2,5,7]. Previous studies have likewise reported substantial variation in antimicrobial activity among fungal endophytes isolated from medicinal plants [12,13,16,17].

CONCLUSION

The present study demonstrated that the roots of *Calotropis procera* harbour culturable endophytic fungi with considerable potential for the production of bioactive secondary metabolites. Six endophytic fungal isolates, designated EFM01–EFM06, were successfully isolated and evaluated for their phytochemical characteristics and antifungal activity. Qualitative phytochemical screening revealed the presence of several classes of secondary metabolites, including alkaloids, phenols, tannins, steroids, anthraquinones, and terpenoids, with considerable variation among the isolates. All six fungal extracts exhibited inhibitory activity against the tested fungal pathogens, namely *Candida albicans*, *Fusarium oxysporum*, and *Alternaria* sp., although the magnitude of inhibition varied depending on the isolate, test organism, and extract concentration. Among the isolates, EFM04 and EFM06 demonstrated comparatively stronger and more consistent antifungal activity. EFM06 showed the highest inhibition against *C. albicans* (22.0 mm) and *Alternaria* sp. (19.1 mm) at 100 µg, whereas EFM04 showed the highest inhibition against *F. oxysporum* (17.0 mm) and also exhibited strong activity against *C. albicans* (21.0 mm) and *Alternaria* sp. (18.9 mm). The results indicate that *C. procera*-associated endophytic fungi, particularly EFM04 and EFM06, represent promising candidates for further investigation as sources of antifungal secondary metabolites. However, the present study provides preliminary evidence based on crude extracts and qualitative phytochemical screening. Further studies involving molecular identification of the promising isolates, bioassay-guided fractionation, MIC determination, HPLC/LC-MS analysis, FTIR and NMR characterization, and mechanistic investigations are required to identify the active compounds and establish their biological potential., the findings contribute to the growing evidence that medicinal plants and their associated endophytic fungi can serve as valuable resources for the discovery of novel bioactive metabolites

Acknowledgments

The Authors acknowledge ITM University, Gwalior, for providing instrumental facilities and technical assistance during the experimental studies.

Disclosure Statement

The Authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Funding & Ethical Approval

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Ethical approval was not required for this study.

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