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RESEARCH ARTICLE

Formulation development, optimization and evaluation of *Aloe vera* gel for the management of post-harvest disease of mango anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

Theradimani M[1*], Senthil K[2], Rajinimala N[1], Sheela J[1], Juliet Hepziba S[1] & Ayyandurai M[3]

[1]Department of Plant Pathology, V.O. Chidambaranar Agricultural College and Research Institute, Tamil Nadu Agricultural University, Killikulam 628 252, Tamil Nadu, India

[2]Department of Soil Sciences and Agricultural Chemistry, Anbil Dharmalingam Agricultural College and Research Institute, Tamil Nadu Agricultural University, Trichy 620 009, Tamil Nadu, India

[3]Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India

*Correspondence email - mtheradi@gmail.com

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Abstract

The Mango (*Mangifera indica* L.), one of the most economically important fruit crops, is produced predominantly in India, the world's leading mango producer. India grapples with anthracnose, a major farm and post-harvest disease that significantly limits mango production, particularly in high-humidity regions of India. The diseased mangoes collected from Kumbakarai, Periyakulam and nearby Madurai in Tamil Nadu, India, were used to identify a virulent strain of *C. gloeosporioides* through molecular analysis. Sequencing of the 560 bp PCR product yielded the GenBank Accession Number OI468719. Phylogenetic analysis of the sequence showed 99 % identity with *C. gloeosporioides* sequences in the database. *A. vera* dry gels were prepared and diluted to concentrations of 10 %, 20 %, 30 %, 40 % and 50 % using potato dextrose agar medium. *In vitro* efficacy of different concentrations of *A. vera* gel against *C. gloeosporioides* was tested. Among the 10 %, 20 %, 30 %, 40 % and 50 % concentrations, significant inhibition was recorded at 10 %. Several antimicrobial compounds were identified from *A. vera* gel, including 1-dodecanol, 1-tetradecene, 1-hexadecanol, heneicosane, dibutyl phthalate and 1-heptacosanol, by GC-MS technique. An emulsifiable concentrate of *A. vera* gel was subsequently formulated and evaluated for its efficacy against the pathogen, offering a potential alternative approach for postharvest disease management.

Keywords: *Aloe vera* gel; *Colletotrichum gloeosporioides*; emulsifiable concentrate; GC-MS; mango

Introduction

Mango is one of the five most economically important fruit crops worldwide, with production occurring in most countries in the tropics and subtropics (1,2). Anthracnose caused by *Colletotrichum* spp. is the most important disease of mango in almost all production areas, because it attacks leaves, twigs, flowering panicles and fruits (3-6). Yields are drastically reduced when the inflorescence is attacked. Disease occurs as quiescent infections on immature fruit, but the damage is more economically significant in postharvest fruits (7-9). Anthracnose pathogen initially resides in host tissues as an endophyte, lying dormant (10). However, when the moist and rainy season arrives, it becomes active and leads to severe infections. The pathogen creates lesions on leaves, fruits and panicles. As it penetrates deeper into the host tissue, the lesions darken and form a distinctive concentric ring pattern. The blossom and peduncle blight phase are particularly destructive, impacting fruit set and ultimately reducing the overall yield.

The incidence of mango anthracnose can reach almost 100 % in fruit produced in areas with high humidity during the flowering period, particularly in poorly managed orchards (11, 12). The mango anthracnose pathogen is highly dangerous, despite the availability of various management strategies. *A. vera* (*A. vera.* (L.) Burm.f.) gel-based emulsifiable concentrations have proven to be the most effective for managing this disease. *A. vera* gel can effectively combat the postharvest fruit pathogens such as *Penicillium digitatum*, *Penicillium expansum*, *Botrytis cinerea* and *Alternaria alternata* (13).

A previous study examined the efficacy of *A. vera* gel, both alone and in combination with garlic oil (GO), against anthracnose in banana fruit and reported that significant antifungal activity was demonstrated by AV (*A. vera*) + GO 0.1 % treatment, inhibiting mycelial growth as well as spore germination by 87.7 % and 91.2 % respectively (14). Also, this combination *in vivo* had substantially reduced the anthracnose

incidence (92.5 %) and severity (81.0 %) in banana fruit while preserving key quality attributes and enhancing antioxidant properties (14). *A. vera* contains various bioactive compounds including polysaccharides, glycoproteins, anthraquinones (aloins), vitamins (A, C, E, B12), enzymes (amylase, lipase), minerals (calcium, magnesium, zinc) and amino acids. These components possess the antimicrobial activity against the pathogens (15). This investigation delves into the formulation of an emulsifiable concentrate derived from *A. vera* gel, assessing its efficacy against *C. gloeosporioides* under controlled *in vitro* conditions as well as during the storage of mango fruits.

Materials and Methods

Isolation and purification of the pathogen

Diseased mango fruits were collected from Kumbakarai (10° 10' 28.80" N, 77° 31' 29.99" E), Periyakulam (10° 07' 12.00" N, 77° 32' 60.00" E) and nearby Madurai tracts (9° 55' 2.46" N, 78° 07' 10.63" E) of Tamil Nadu, India. The most common cultivated varieties are Kalapadi, Neelam, PKM-1, PKM-2 and Banganapalli. Pathogen isolates were isolated using the tissue isolation technique described below. The diseased fruits were first washed with running water. Inside the laminar airflow chamber a small amount of disease along with the healthy leaf tissues (0.5 to 1 mm diameter) were cut off, surface sterilized with 0.1 % mercuric chloride ($Hgcl_2$) for 30 sec, rinsed thrice in sterile water and blot dried. Then, the sterilized leaf tissues were transferred to a sterile Petri dish containing Potato dextrose agar (PDA) medium and incubated at room temperature (27 ± 2 °C) for 6 to 7 days. The mycelial growth of the pathogen was observed periodically. After seven days of incubation, fungal hyphae grown from the infected tissues were subcultured aseptically by the single hyphal tip method to maintain pure culture (16).

Pathogenicity test

Pathogenicity tests were carried out on detached mango fruits at the first colour break stage of ripening. Mango fruits were thoroughly washed under running water, surface disinfested in 1 % sodium hypochlorite solution (NaClO) for 2 min followed by immersion in 70 % ethanol for 2 min, rinsed two times in sterile distilled water and dried in a laminar flow hood. The fruits were inoculated using the colonized agar plug method because some isolates had no satisfactory sporulation on the culture medium. Fruits were wounded in two allocated areas with a sterile toothpick (3 mm in depth). A mycelial plug (5 mm in diameter) removed from the margin of a 6-day-old PDA culture was placed onto the fruit surface on each wound. A noncolonized agar plug was placed on the wounds of 10 fruits used as the control. The fruits were incubated at 25 °C in the dark on plastic trays lined with two layers of paper towel moistened with sterile distilled water and enclosed in a plastic bag. Occurrence of symptoms was observed (2).

Molecular characterization of *C. gloeosporioides*

Genomic DNA extraction

A 9 mm disc of fifteen days old C. *gloeosporioides* culture was inoculated into the 250 mL of PDA broth and incubated at 25 ± 20 °C. After fifteen days of incubation, the harvested mycelial mat was ground with CTAB (Cetyltrimethylammonium bromide) buffer using a pestle and mortar. Finely grounded mixture was transferred into 2 mL centrifuge tubes. It was vortexed and incubated in a water bath at 60 °C for 30 min. After incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10000 rpm for 15 min. The supernatant was collected, added with 300 µL of ice-cold isopropanol and stored overnight at 40 °C. After incubation, it was centrifuged at 10000 rpm for 15 min. The supernatant was removed and the pellet was added with 300 µL of ice-cold ethanol. It was centrifuged at 10000 rpm for 5 min and the supernatant was removed. The pellet was air-dried for 30 min, 40 µL of nucleus-free water was then added and stored at 40 °C (17).

Agarose gel electrophoresis

A total of 500 mg of agarose powder was taken and weighed. The powder was mixed with 50 mL of 1X TAE (Tris-acetate-EDTA) buffer in a conical flask and melted in a microwave oven. Rubber wrap was used to cast the gel on trays. Afterwards, agarose mixed with 3 µL of 10 mg/mL ethidium bromide was melted and poured after cooling. To make wells, the comb was placed on the gel casting tray. The melted gel was poured into the gel casting tray and solidified for 30 to 45 min. After solidification, the comb was gently removed from the gel and with the casting tray, the gel was placed in an electrophoresis tank with 1X TAE buffer. The 6X loading dye was mixed with the DNA sample before being loaded into the wells with a micropipette and electrical leads attached to the tank. The gel was run at a constant voltage of 5.8 V/cm for 30 min. The gel was examined under UV light and the image was captured using the Gel Doc.

ITS (Internal Transcribed Spacer) sequencing of *C. gloeosporioides*

The PCR reaction was performed in a 15 µL volume of PCR mixture that contained 7 µL of master mix, 4 µL of double distilled water, 1 µL of forward primer (ITS1), 1 µL of reverse primer (ITS4) and 2 µL of template DNA. The intermediate 5.8S ribosomal gene, as well as the ITS region was amplified using the ITS1/ITS4 primers by following PCR protocol consisting of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 72 °C for 2 min. A final extension is performed at 72 ° C for 7 min. The process concludes with an infinite hold at 40 °C. PCR products were tested using agarose gel electrophoresis at 1 %. The PCR products are sequenced at Eurofins Genomics India Pvt. Ltd. in Bangalore. The primers used for ITS region amplification were ITS1 - 5' CCG AGG GAACC GCGG 3' ITS4 - 5' CC CCGC A GA A GC 3'. The full length of the ITS sequence obtained was BLAST searched at the NCBI database for similarity and phylogenetic tree was constructed.

Evaluation of *A. vera* gel extract against *C. gloeosporioides* under *in vitro*

Preparation of *A. vera* gel extracts and fungal inoculation

A. vera gel was extracted and dried. 10 g, 20 g, 30 g, 40 g and 50 g of *A. vera* gel powders were mixed with 90 mL, 80 mL, 70 mL, 60 mL and 50 mL of sterilized PDA medium to get 10 %, 20 %, 30 %, 40 % and 50 % concentration respectively. A 20 mL mixture was poured into the sterile Petri plate and allowed to solidify. A 9 mm mycelial disc of *C. gloeosporioides* was cut from a 10-day-old culture using a sterile cork borer and placed at the center of the Petri plate containing the melted medium. Three

replications were maintained for each treatment. PDA medium without plant extract served as control. The plates were incubated at 28 ± 2 ˚C for seven days. The diameter of the mycelial growth of the pathogen was recorded and the percentage of inhibition was calculated seven Days After Inoculation (DAI).

Identification of antimicrobial compounds from *A. vera* gel

Collection and preparation of extract

25g of the powdered sample was filled in the thimble and extracted using the solvent ethyl acetate in Soxhlet extractor for 48 hr. The extracts were concentrated under reduced pressure and preserved at 5 ˚C in an airtight bottle for further analysis.

GC-MS (Gas Chromatography-Mass Spectroscopy) analysis

The GC-MS analysis was carried out on a Shimadzu Gas chromatography (QP 2020 with Rxi 5 MS Column) equipped with a mass detector Turbo mass gold containing an Elite- 1 (100 % dimethyl polysiloxane), 30 m × 0.25 mm ID employing the following conditions: carrier gas, helium (1 mL/min). The oven temperature was programmed from 110 ˚C (2 min) ending with a 9 min isothermal at 280 ˚C; injector temperature 250 ˚C. Total GC running time was 45 min. A 1 mL aliquot sample was injected into the chromatograph. The major constituents were identified with the aid of a computer-driven algorithm and then by comparing the mass spectrum of the analysis to that of a National Institute of Standards and Technology (NIST) library (Version 2.0, 2005). The software used for GC-MS was Turbo mass -5.1 (18). This work was done at the Centre of Innovation for Excellence, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai.

Phylogenetic analysis

The evolutionary history was deduced using the Neighbour-Joining method (19). A bootstrap consensus tree based on 1000 replicates was used to depict the evolutionary relationships among the analyzed taxa. Branches that were not supported in at least 50 % of the bootstrap replicates were collapsed. The tree also displays the percentage of bootstrap replicates supporting the clustering of the taxa, indicated beneath each branch. Evolutionary distances were calculated with the Maximum Composite Likelihood method and are expressed as the number of base substitutions per site. The analysis included nine nucleotide sequences, covering 1st, 2nd, 3rd and non-coding codon positions. Ambiguous positions were excluded from each sequence pair using the pairwise deletion option. The final dataset comprised 555 positions. These evolutionary analyses were performed using MEGA11 software (20).

Statistical analysis

Data were analyzed using ANOVA (One way) at significant levels (P = 0.05) and averages were compared using Duncan's Multiple Range Test (DMRT) (21). The statistical programs utilized for ANOVA were SPSS and AGRES (Statistical Package of TNAU). The *in vitro* and *in vivo* studies employed Completely Randomized Design (CRD) and Randomized Block Design (RBD) respectively.

Results

Isolation and purification of pathogens

The isolated pathogen was purified and morphologically confirmed based on elongated conidia with oil globule.

Molecular characterization of *C. gloeosporioides*

In addition to morphological confirmation, the pathogen was confirmed at molecular level also. Using the CTAB method, the genomic DNA was isolated. Subsequent amplification of the ITS region using ITS1 and ITS4 primers yielded a specific amplicon with a size of 560 base pairs (Fig. 1).

DNA sequence analysis of ITS region and Phylogenetic analysis

The output data showed matching sequences of *C. gloeosporioides* already available in the database. Hence, the virulent isolate of *C. gloeosporioides* used in this study was confirmed as *C. gloeosporioides* and its accession number is Ol468719**.** The phylogenetic tree displays bootstrap support and posterior probability values above the branches. The combined datasets revealed seven moderately well-supported clades corresponding to previously described *C. gloeosporioides*. The study isolate *C. gloeosporioides* THERADI MDU1 was clustered within the clade corresponding to *C. gloeosporioides* with a moderate bootstrap value of 63 % *C. truncatum* CcI1 and 47 % *C. gloeosporioides* 391 (Fig. 2).

Evaluation of *A. vera* gel extract against *C. gloeosporioides* under *in vitro*

From the results of the evaluation of *A. vera* extract at 10 %, 20 %, 30 %, 40 % and 50 % concentrations, it has been found out that there is significant inhibition at 10 % (Table 1). At 8 DAI, *A. vera* gel concentrations ranging from 10 % to 50 % effectively reduced mycelial growth of the pathogen compared to the control, with significant reductions particularly noted at higher concentrations (40 % and 50 %), each achieving a growth measurement of 2.11 cm.

Identification of antimicrobial compounds through GC-MS

The extracts of *A. vera* were analysed and the chemical constituent of *A. vera* with different retention period were identified. The results showed that various compounds with potential antimicrobial activities were derived from the GC-MS analysis of *A. vera* gel contains the following compounds *viz*. 1-tetradecene, 1- hexadecanol, heneicosane, dibutyl phthalate and 1-1 heptacosanol (Table 2, Fig. 3).

Discussion

In this study, we conducted a series of investigations to elucidate the antifungal properties of *A. vera* against the mango anthracnose pathogen, *C. gloeosporioides* and to characterize the pathogen using molecular techniques. Initially, the pathogen was isolated from infected mango fruits and purified using the single hyphal tip method to ensure a pure culture for subsequent analyses.

Our results on the molecular characterization of *C. gloeosporioides* show a successful extraction of genomic DNA using the CTAB method, with the integrity of the DNA confirmed by the presence of a single, clear band on an agarose gel. Visualizing a single band of intact genomic DNA on the agarose gel indicates the purity and integrity of the extracted DNA, ensuring the reliability of subsequent molecular analyses. This finding is crucial as it ensures the reliability of further

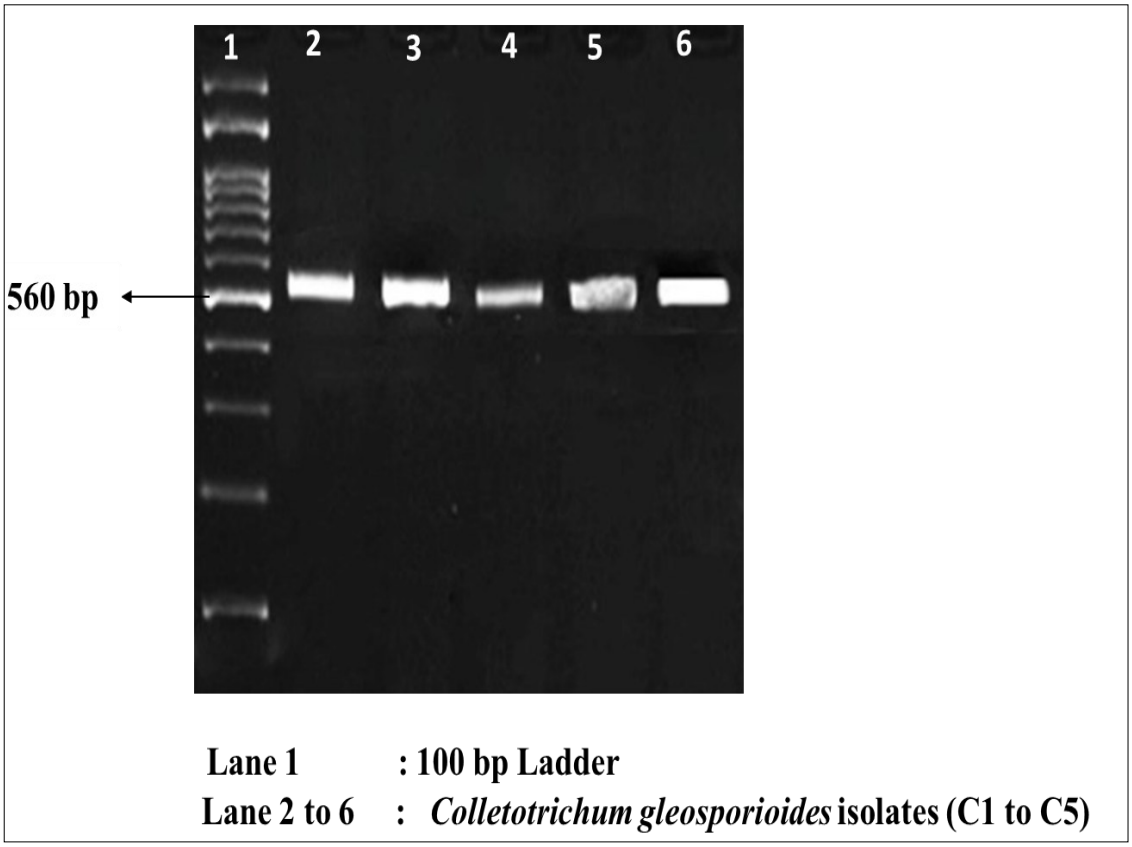


Fig. 1. PCR amplification of ITS region of *C. gloeosporioides*.

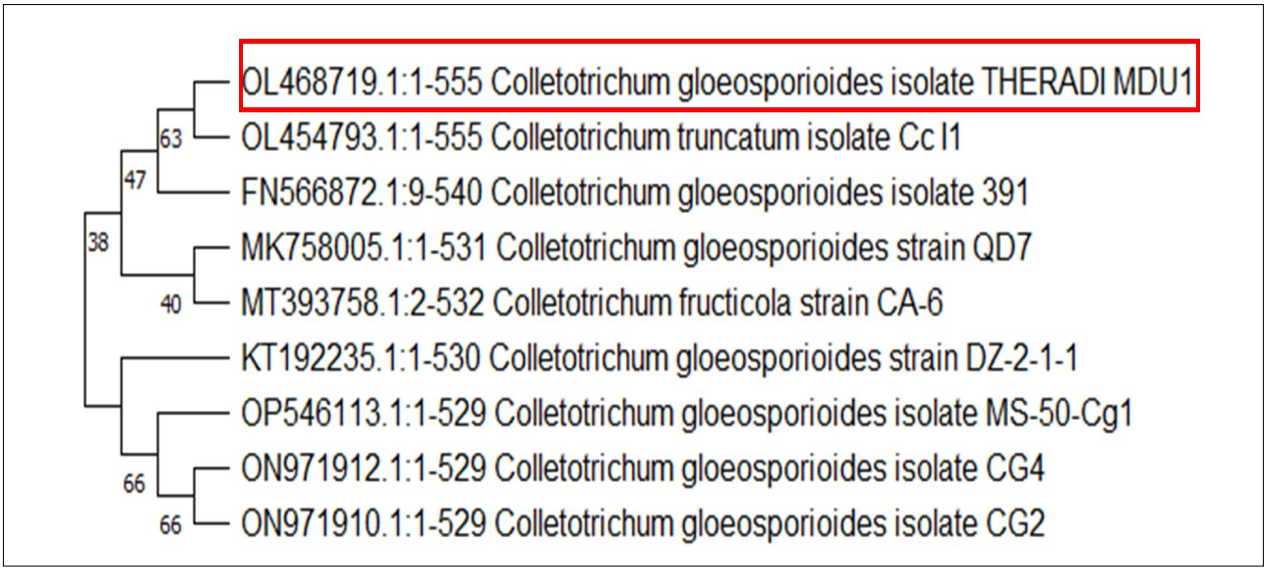


Fig. 2. Phylogenetic analysis of *C. gloeosporioides* THERADI MDU1.

The isolates of this study were indicated with a red mark.

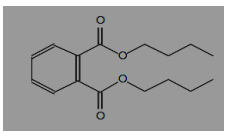
Table 1. Effect of *A. vera* gel on mycelial growth of *C. gloeosporioides*

Sl. No	*A. vera* gel concentration	Mycelial growth (cm) 8 DAI*	PROC
1.	10	8.56	4.88^{a} (12.72)
2.	20	8.62	4.22^{ab} (11.80)
3.	30	8.75	2.77^{bc} (9.47)
4.	40	8.81	2.11^{c} (8.19)
5.	50	8.81	2.11 (8.19)
6.	Control	9.00	-
	CD (*P*=0.05)	**3.202**	

* Values in the Parentheses are arc Sine transformed value, Mean of three replication

PROC- Per cent Reduction Over Control. CD: Critical Difference

Table 2. Phytocompounds identified from *A. vera* gel extract through GC-MS analysis

Sl. No.	Name of the phytocompound	Structure	Chemical formulae	MW g/mol	Specific role
1.	1- Tetradecene		$C_{14}H_{28}$	196.37	Antimicrobial activity
2.	1- Hexadecanol		$CH_3(CH_2)_{15}OH$	242.44	Antifungal activity
3.	Heneicosane		$CH_3(CH_2)_{19}CH_3$	296.57	Antimicrobial activity
4.	Dibutyl phthalate		C_6H_4-1,2-$[CO_2(CH_2)_3CH_3]_2$	278.34	Antimicrobial activity
5.	1-1 Heptacosanol		$C_{27}H_{56}O$	396.73	Antibacterial activity

MW: Molecular Weight.

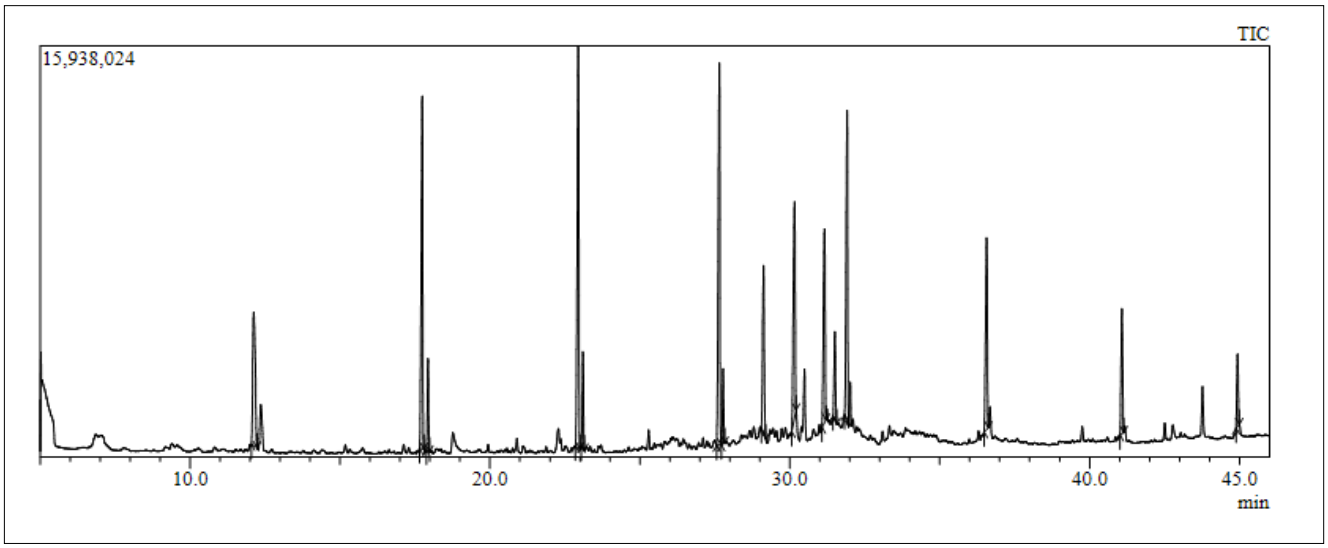


Fig. 3. GC-MS analysis of *A. vera* gel extract.

molecular analyses, such as the amplification of the ITS region. Amplification using ITS1 and ITS4 primers produced a specific 560 bp amplicon, closely aligning with the findings of an earlier study that reported a similar amplicon size of 550 bp in their identification of *Colletotrichum* species (22). This consistency in amplicon sizes across different studies adds significant credibility to the molecular identification methods used for *C. gloeosporioides* (23).

Additionally, the *in vitro* antifungal assays of *A. vera* extract revealed significant inhibitory activity against *C. gloeosporioides* at a concentration of 10 %. This observation is particularly noteworthy and is supported by literatures indicating the antimicrobial efficacy of *A. vera*. For instance, studies reported that *A. vera* leaf gel could inhibit the growth of gram-positive bacteria such as *Shigella flexneri* and *Streptococcus pyogenes* (24, 14). Similarly, other studies have shown that different fractions and extracts of *A. vera* possess inhibitory effects against various fungal pathogens, highlighting its broad-spectrum antimicrobial properties (25, 4). *A. vera* gel hinders the germination and mycelial growth with notable sensitivity observed in *P. digitatum* and *A. alternata*. Even at a low concentration of 1 µL/L, the gel inhibits colony growth by 67 - 69 % for *P. digitatum, A. alternata* and *B. cinerea* and by 19 % for *P. expansum* (13).

The GC-MS analysis of *A. vera* gel further provided insights into the specific compounds responsible for these antimicrobial effects. Compounds *viz*. 1- tetradecene, 1- hexadecanol, heneicosane, dibutyl phthalate, 1-1 heptacosanol were identified. Previous studies (26, 27) have suggested that specific plant compounds in *A. vera*, such as anthraquinones and dihydroxyanthraquinones have direct antimicrobial activities. These findings support the hypothesis that the antimicrobial properties of *A. vera* are likely due to these bioactive compounds, which contribute to the gel's efficacy in inhibiting fungal growth (28, 29).

Collectively, these results underscore the potential of *A. vera* as a natural antifungal agent against *C. gloeosporioides* and possibly other microbial pathogens. The consistency of molecular data across studies provides a robust framework for

the identification of this pathogen, while the antimicrobial assays highlight the practical applications of *A. vera* in plant disease management strategies (13, 10). Further studies are warranted to explore the full spectrum of bioactive compounds in *A. vera* and their specific mechanisms of action against various plant pathogens (29, 30).

Conclusion

In conclusion, this study confirmed the identity of a virulent isolate of *C. gloeosporioides* through molecular characterization. *A. vera* gel extract exhibited significant antifungal activity, particularly at a 10 % concentration, supported by identifying various potentially bioactive compounds. These findings suggest the potential of *A. vera* as a natural antifungal agent, warranting further exploration for practical applications in plant disease management. Additionally, the phylogenetic analysis contributes to our understanding of the evolutionary relationships among related taxa.

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Authors' contributions

TM contributed to the writing of the original draft and conceptualization of the research work. SK, RN, JHS, SJ and AM were involved in revising the draft, including the incorporation of tables and figures and proofreading. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

Grammarly AI tool was used to improve language and readability, with caution.

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