Journal of Advances in Microbiology Research

E-ISSN: 2709-944X
P-ISSN: 2709-9431
JRM 2026; 7(1): 15-22

www.microbiojournal.com
Received: 08-11-2025
Accepted: 10-12-2025

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Isolation of *Paradendryphiella arenaria* from Pomegranate Fruit Rot (PMR) and its management with *Allium sativum* (garlic) and *Zingiber officinale* (ginger) extracts

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DOI: https://www.doi.org/10.22271/micro.2026.v7.i1a.295

Abstract
Pomegranate (*Punica granatum* L.) is a commercially and nutritionally significant fruit crop, but its cultivation is increasingly threatened by fruit rot disease, leading to substantial reductions in yield, fruit quality, and market value. This study is focussed on identification of the fungal pathogens associated with PMR disease and to evaluate the antifungal efficacy of aqueous extracts of *Allium sativum* (garlic) and *Zingiber officinale* (ginger) as potential biological control agents. The fungal isolate from fruit rot was isolated, identified and characterized as *Paradendryphiella arenaria* based on morphological features and molecular identification using internal transcribed spacer (ITS) regions of rDNA. The plant extracts were assessed for their antifungal activity using standard in vitro assays. The findings revealed key pathogenic fungi involved in rot disease and demonstrated significant antifungal effects of both garlic and ginger extracts. These results suggest that garlic and ginger extracts could serve as effective, eco-friendly alternatives for managing PFR disease contributing to the development of sustainable disease control strategies.

Keywords: Pomegranate (*Punica granatum* L.), fruit rot disease, *Paradendryphiella arenaria*, antifungal activity, *Allium sativum*, *Zingiber officinale*, plant extracts, biological control, sustainable disease management

Introduction
Punica granatum L (Pomegranate) is a valuable fruit crop that is commercially cultivated across various regions of India. In India, Karnataka is recognized as one of the major pomegranate-producing states, with leading cultivation occurring in the districts of Vijayapura, Bagalkot, Belagavi, Chitradurga, and Tumakuru [1]. The pomegranate tree is increasingly utilized in landscape greening owing to its high ornamental value and adaptability to various climatic conditions. In addition to its aesthetic appeal, the pomegranate fruit is of considerable nutritional and economic importance in the agricultural and food sectors, as it is rich in carbohydrates, vitamins, folic acid, potassium, flavonoids, iron, and other essential minerals. It is commonly consumed both as fresh fruit and in the form of juice [2, 3]. In addition, pomegranate fruit contains relatively high levels of pharmacologically and medicinally bioactive compounds, which contribute to its diverse health-promoting properties, including hypolipidemic, antioxidant, antiviral, anticancer, antibacterial, and vascular protective effects [4]. Consequently, the popularity of pomegranate fruit among consumers has been increasing, leading to a rising market demand in recent years. However, pomegranate trees and their fruit remain vulnerable to various bacterial and fungal diseases throughout the growth and development stages. Rot disease significantly weakens tree vigour, thereby diminishing the ornamental and greening value of pomegranate trees. Moreover, it can cause substantial reductions in both fruit yield and quality in commercial pomegranate cultivations. Overall, the ecological and economic benefits of pomegranate trees are severely limited by the impact of rot disease.
Several studies have identified different pathogens associated with rot diseases in pomegranate across the country. Reported causal agents include, *Botrytis cinerea* [5], *Botryosphaeria dothidea* [6], *Pilidiella granati* [7], *Neofusicoccum parvum*, and *Alternaria alternata* [8]. In this study, *Paradendryphiella arenaria* was identified as the pathogen associated with rot diseases in pomegranate fields across Srinivasapura, Kolar district.

Rot disease in pomegranate is becoming an increasingly serious threat to pomegranate production worldwide. Therefore, it is particularly urgent to isolate and identify the pathogens responsible, with a strong emphasis on developing effective disease prevention and control strategies. A precise approach for fungal molecular identification involves sequencing the nuclear ribosomal internal transcribed spacer (ITS) region [9]. By examining the sequence data from the ITS region, fungi can be rapidly and accurately identified and classified. Besides molecular techniques, morphological traits observed microscopically, helps delineate species boundaries. These features serve not only as important tools for species identification but also provide insights into the fungus's biological examination [10]. Traditional management of these diseases primarily involves the use of chemical fungicides. However, the extensive use of synthetic fungicides has raised concerns regarding environmental pollution, development of resistant fungal strains, and potential health risks to consumers. Consequently, there is a growing interest in exploring alternative, eco-friendly approaches for disease management. Plant-based antifungal agents, such as *Allium sativum* (garlic) and *Zingiber officinale* (ginger) extracts, have shown promising results in controlling various plant pathogens. Garlic contains bioactive compounds like allicin and ajoene, which exhibit strong antifungal activity against a range of fungi, including *Candida* and *Aspergillus* species. Similarly, ginger extract has inhibitory effects against fungi such as *Fusarium* spp., with its efficacy attributed to compounds like α-zingiberene and citral [11]. This study aims to isolate and identify the fungal pathogens associated with PFR disease in pomegranate and to evaluate the antifungal efficacy of aqueous garlic and ginger extracts as potential biological control agents. The findings could contribute to the development of sustainable disease management strategies for pomegranate cultivation.

Materials and Methods

Sample Collection

Pomegranate fruits exhibiting rot symptoms were collected from local orchards in Srinivasapura Taluk, Kolar District, Karnataka, India. Fresh and healthy pomegranate fruits were also collected from a well-managed orchard in the same area for use in pathogenicity testing. Mature, healthy bulbs of garlic (*Allium sativum* L.) and fresh ginger (*Zingiber officinale* R.) were purchased from Krishnarajendra Market, Bengaluru, Karnataka, India, for preparation of plant extracts used in the study.

Isolation of Fungal pathogens

The epidermal tissues were excised from the margin of the rotted areas of infected pomegranate fruits [12]. The tissue samples were surface-sterilized with 0,5% Sodium hypo-chloride solution for a few seconds, then rinsed with sterile distilled water to remove residual sodium hypo chloride solution. The sterilized tissues were cut into small pieces (approximately 5 -10 mm in diameter) and aseptically transferred onto various solid agar media [13]. The inoculated plates were incubated at 28 °C for 7 days to allow for optimal fungal growth. from the plates showing maximum mycelial growth, actively growing hyphal tips were carefully collected from the edge of the colonies and transferred to fresh solid agar plates for purification. This sub-culturing process was repeated thrice to ensure the isolation of pure fungal cultures. Finally, single colonies representing each distinct fungal isolate were maintained for further morphological and molecular identification.

Evaluation of Fungal Growth on Different Culture Media

The isolated fungus *Paradendryphiella arenaria* was cultured on different mycological solid agar media to determine the medium supporting optimum mycelial growth. Four types of media were tested: Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Czapek-Dox Agar (CZA), and Richard's Synthetic Agar (RSA). The PDA medium was prepared using a commercial dehydrated formulation (HiMedia Laboratories, India) following the manufacturer's instructions. The remaining media were prepared according to standard formulations: SDA (peptone 10 g L^{-1}, dextrose 40 g L^{-1}, agar 15 g L^{-1}, pH 5.6 ± 0.2), CZA (sucrose 30 g L^{-1}, $NaNO_3$ 2 g L^{-1}, K_2HPO_4 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g L^{-1}, agar 15 g L^{-1}, pH 7.2), and RSA (sucrose 50 g L^{-1}, KNO_3 10 g L^{-1}, KH_2PO_4 5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2.5 g L^{-1}, $FeCl_3$ trace, agar 15 g L^{-1}, pH 6.0). All media were sterilized by autoclaving at 121 °C for 15 min and poured aseptically (~25 mL per 90 mm Petri plate). A 5 mm diameter mycelial disc was cut from the actively growing margin of a 7-day-old culture of *P. arnariea* and placed in the middle of each plate under aseptic conditions. Each medium was tested with five replicate plates, and the experiment was repeated three times independently to ensure reproducibility. The plates were incubated in the dark at 28 ± 1 °C for 7 days. Colony diameter was measured every 24 h using the cross method, and the mean radial growth rate (mm day^{-1}) was calculated by averaging two perpendicular measurements [14-18]. Uninoculated plates served as controls to ensure sterility of media.

Identification of the pathogen by Koch's postulates

Spores or mycelial fragments were obtained from the third subculture of purified fungal isolates and used to prepare spore suspensions or mycelial inoculum. The spore concentration was standardized to approximately 1×10^6 spore mL^{-1} with sterile double-distilled water. healthy pomegranate fruits were surface-disinfected with 0.5% sodium hypo chloride solution, followed by thorough rinsing with sterile distilled water [19]. The disinfected fruits were air-dried using sterile blotting paper, and uniform wounds were made on the surface with a sterile surgical blade. Each wound site was inoculated with 100 µL of the prepared spore suspension or a small mycelial plug, while control fruits received sterile water. The inoculated fruits were incubated in a dark chamber at 28 ± 2 °C under controlled conditions to facilitate infection. Lesion progression was monitored daily, and symptom expression was recorded 7-10 days after inoculation. When typical rot symptoms resembling those from naturally infected fruits appeared, tissue sections from the interface of diseased and healthy regions were excised and re-cultured on potato dextrose agar (PDA). The morphological characteristics of the recovered colonies were compared with the original isolates to validate pathogenicity according to Koch's postulates.

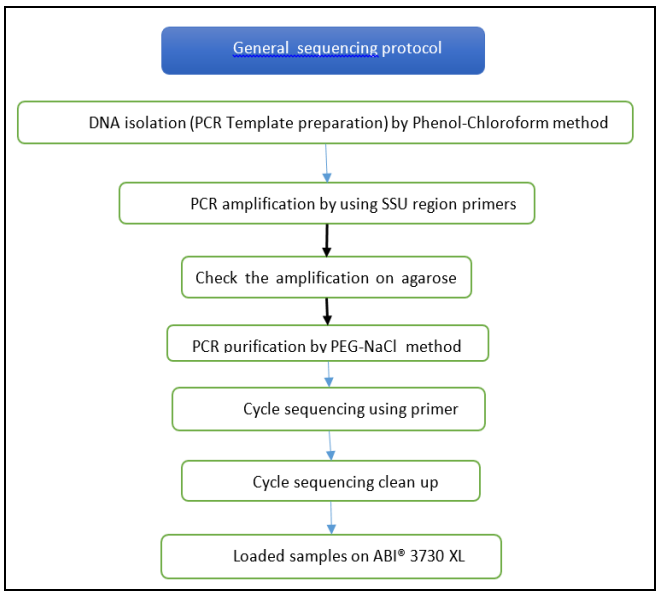
Morphological identification of pathogen

The colonies obtained from the third successive generation

of purified isolates were subcultured on fresh potato dextrose agar (PDA) plates and incubated at 28 °C for 12 days. Colony morphology, including colour, texture, and growth pattern, was recorded daily. The micro morphological features such as spore shape, size, and hyphal structure were examined under a compound light microscope using lacto-phenol cotton blue staining. The observed morphological characteristics were then compared with published descriptions from previous studies to facilitate taxonomic identification of the pathogens [20-21].

DNA sequencing

The identification of isolates was carried out at the sequencing facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method, followed by PCR amplification of the SSU regions using universal primers NS1 [5'- GTAGTCATATGCTTGTCTC -3'] and NS8 [5'-TCCGCAGGTTCACCTACGGA-3']. The amplified SSU PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Bio systems, Inc., Foster City, CA) as per manufacturer's instructions [22-24]. Essentially, sequencing was carried out from both ends so that each position was read at least twice. Assembly was carried out using Laser gene package



Pathogenicity test

To confirm the pathogenic potential of the isolated fungal pathogen, a pathogenicity test was conducted on fresh, healthy pomegranate fruits. The surface of each fruit was sterilized using 70% ethanol, applied with sterile cotton, to eliminate surface contaminants. After air drying under aseptic conditions, wounds measuring approximately 4 mm in length, breadth, and depth were made on the fruit surface using a sterile surgical blade. A 4 mm diameter mycelial plug taken from a freshly prepared pure culture of the test fungus was placed onto wound site to serve as the inoculated treatment. For the control treatment, sterile cotton plug without fungal mycelium were placed on the wounds following the same procedure. Both inoculated and control fruits were then incubated at 28 °C under aseptic conditions to promote disease development. The appearance of symptoms and lesion development were recorded on the 3rd, 7th, and 10th days post-inoculation. The lesion diameter was measured using the cross-measurement method, where the average of two perpendicular diameters of each lesion was calculated to determine the mean lesion size [25].

$$\text{Lesion diameter} = \frac{\text{(Lesion diameter A+Diameter B perpendicular to diameter)}}{2}$$

Preparation of Aqueous Garlic and Ginger Extracts and Antifungal Assay

Fresh garlic and ginger roots were peeled, sliced into pieces of approximately 2 mm thickness, and dried using a freeze dryer (LABCONCO) for 72 hours [26]. The dried materials were finely ground into powder by using sterile pastel and mortar. Precisely 100 g of each powder (garlic and ginger) was transferred into separate Erlenmeyer flasks containing 1000 mL of sterile distilled water. The mixtures were incubated at 28 °C with shaking at 200 rpm for 3 hours. After incubation, the extracts were filtered through four layers of sterilized gauze to obtain the crude aqueous extracts, which were used immediately. The crude extracts were distributed into 50 mL centrifuge tubes and centrifuged at 2000 rpm for 10 minutes at 25 °C. The resulting supernatants were collected and mixed with sterilized potato dextrose agar (PDA) medium before pouring into Petri dishes, to obtain final concentrations equivalent to 1, 5, 10, 25, 50, and 100 mg of ginger powder per mL of medium and 1,5,10,15 and 20 % of garlic powder per ml of medium [27]. PDA without extracts served as the control for both garlic and ginger treatments. Each Petri dish contained approximately 15 mL of medium. 0.4 mm mycelial plug, cut from the edge of a 5-day-old actively growing fungal colony, was placed at the centre of each plate [28]. The dishes were sealed with Para film and incubated at 28 °C. For each fungal pathogen, six Petri dishes for ginger and 5 petri dishes for garlic were prepared per concentration, and each treatment was replicated three times. When the fungal mycelium in the control plate had completely covered the culture dish, the colony diameter of each treated plate was measured using the cross-measurement method. Subsequently, the minimum inhibitory concentration (MIC) was determined, and the inhibition rate was calculated using the following formula.

$$\text{Inhibition rate} = \frac{\text{The growth of control} - \text{the growth of tratment group}}{\text{The growth of control group}} \text{ x } 100\%$$

Results

Selection of the rot symptoms

The infected pomegranate fruits exhibited characteristic PFR, beginning as small, dark brown necrotic spots on young fruits and progressively expanding with fruit development. The lesions became sunken, dry, and dark brown to black, eventually covering a substantial portion of the fruit surface. Symptom progression was uniform across all developmental stages, indicating a continuous infection pathway initiated at the calyx region (Fig.1). Advanced stages showed extensive tissue discoloration and shrivelling, consistent with fungal-induced fruit rot. These morphological features collectively confirm severe pathogenic colonization throughout fruit maturation. After purification, the pathogen isolated from the rotten pomegranate fruits was identified as *Paradendryphiella*

arenaria, the causal agent of dry rot.

Fig 1: Symptoms of rot at various developmental stages of pomegranate fruits observed under natural field conditions.

Morphological identification of the pathogen

The pathogen exhibited vigorous mycelial growth on PDA at 28 °C. Colonies initially appeared white at the actively growing margins, while the central region gradually developed brown-grey to dark olivaceous pigmentation. After 7 days of incubation, the colony surface became distinctly darker, often showing a sooty to dark brown appearance, with the reverse side turning dark olive-brown to blackish (Fig.2).

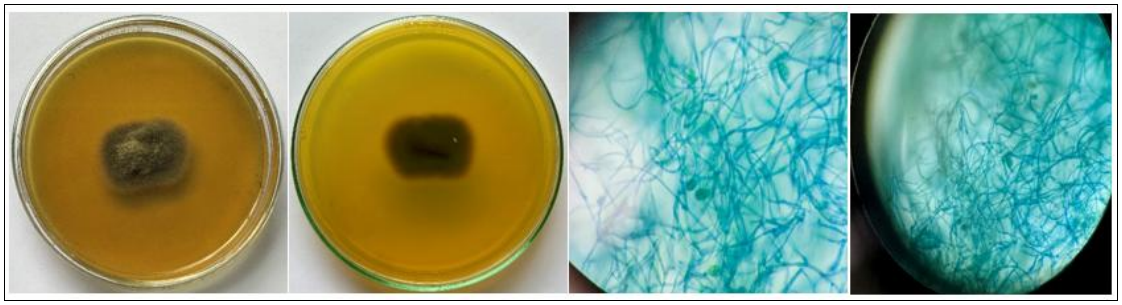


Fig 2: Morphology of pathogen (7 days) on PDA plates and under the microscope. Front, back (a, b) and (c, d) vesicles, Hyphae of pathogen (5 days)

The microscopic examination revealed hyphae are septate, branched**,** and pale to dark brown. multicellular conidial heads borne in short chains on geniculate conidiophores. The conidiophores were dark and became distinctly geniculate following spore formation, showing characteristic scars at the points of conidial detachment. The conidia were free-forming, brownish to pale brown in colour, smooth-walled, and multicellular. These morphological traits collectively support the identification of the pathogen.

Effect of different culture media on mycelial growth of *Paradendryphiella arenaria*

Colonies grown on PDA and RSA were dense, cottony, and showed vigorous radial expansion with uniform margins, while those on SDA and CZA were comparatively thinner and exhibited reduced aerial mycelium (Fig.3). Statistical analysis (one-way ANOVA, $p<0.05$) confirmed significant differences among the tested media, with PDA supporting the maximum mean colony diameter, followed by RSA, SDA, and CZA, respectively. These results suggest that PDA medium, due to its rich carbohydrate composition, is most suitable for *P. arenaria* culture and further experimental studies.

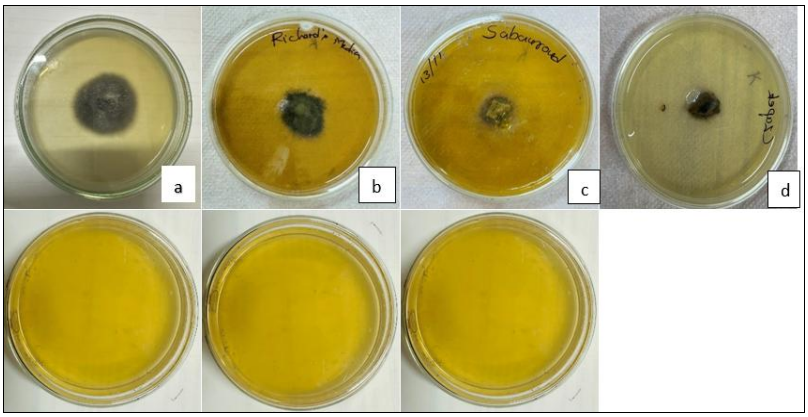


Figure 3. Morphology and optimum growth of pathogen on PDA after 5 days. The optimum growth observed (a) Richardsens agar media (c)Czapak agar media(d) Sabarroad agar media.

Identification of pathogenicity of the pathogen

The purified fungal isolates were re-inoculated into healthy

pomegranate fruits to verify pathogenicity. After inoculation, the fruits developed internal symptoms identical to those observed in naturally infected samples (Fig. 4). As the infection progressed, lesions formed at the inoculation sites, and the extent of rot gradually increased. In fruits affected by dry rot, the internal tissues exhibited pale yellow to off-white discoloration of the seeds (Fig. 4d). The same pathogens were successfully re-isolated from the symptomatic tissues, confirming that *Paradendryphiella arenaria* isolate were the causal agents of pomegranate rot.

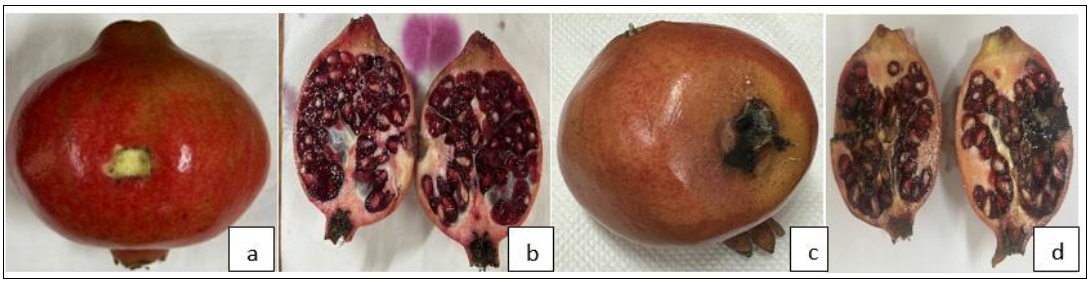


Fig 4: Pathogenicity status of Control pomegranate (a & b), infected by pathogen (c & d) after 7 days in incubation chamber.

Molecular Identification of Fungal isolate from PFR

The fungal rDNA ITS region of the pathogen was cloned and sequenced at the National Centre for Microbial Resource (NCMR), National Centre for Cell Science (NCCS), Pune. The amplified ITS fragment measured approximately 1200 bp, and NCBI-BLAST analysis revealed a maximum sequence similarity of 99.25% with *Paradendryphiella arnariae* strain CBS 1881.58, confirming the identity of the isolate.

Amplified ITS Fragment sequence

CTCTAATGACCGAGTTTGACGAACTTTCCGGCTTGA
GGTGGTCGTTGCCAACCTCCTCGAGCCAGTCCGAA
GGCCTCACTGAGCCATTCAATCGGTAGTAGCGACG
GGCGGTGTGTACAAAGGTCAGGGACGTAATCAACG
CATGCTGATGACACGCGCTTACTAGGCATTCCTCGT
TGAAAAGCAATAATTGCAATGCTTTATCCCCAGCA
CGACAGAGTTTAACAAGATTACCCAATTCTTTCGA
ACAAGGAAAAGAACTCGTTGGCTCTGTCAGTGTAG
CGCGCGTGCGGCCCAGAACATCTAAGGGCATCACA
GACCTGTTATTGCCTCAAACTTCCATCAACTTGAGT
TGATAGTCTCTCTAAGAAGCCGGCGACCAACCAAA
GTTAGCCTGGCTATTTAGCAGAGTAAGGTCTCGCT
CGTTATCGCAATTAAGCAGACAAGTCACCCCACGA
ACTAAGAACGGCCATGCACCACCACCTGAAAAATC
AAGAAAGAGCTCTCAATCTGTCAATCCTTATTTCAT
CTGGACCTGGTGAGTTTCCCCGTGTTGAGTCAAATT
AAGCCGCAGGCTCCACGCCTGGTGGTGACCTTCCG
TCAATTTCTTTAAGTTTCAGCCTTGCGACCATAATC
CCCCCAGAACCCAAAAACTTTGATTTCTCGTAAGG
TGCCGAGCGAGTCAGAAAAAGAACATCGCCCGATC
CCTAGTCGGCATAGTTTACGGTTAAGACTACGACG
GTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCA
CTGATTAATGAAAACATCCTTGGCAAATGCTTTCG
CAGTAGTTAGTCTTCAGTAAATCCAAGAATTTCAC
CTCTGACAACTGAATACTGATGCCCCCGACTGTTCC
TGTTAATCATTGCGGCGTCTCTAGAAACCAACAAA
ATAGAAACGCACGCCCTATTTTATTATTCCATGCTA
ACGTATTCGAGCAAAGGCCTGCTTTGAACACTCTA
ATTTTTTCAAAGTAAAAGTCCTGATTCCCCAGCACG
CCCAGTGAAGGGCATGAGGTTCTTCAGAAGGAAGG
CCCGGCCGGACGAGTGCACGCGGTGAGGCGGACC
CGCCAGCCAGGCCCAAGTTTCAACTACGAGCTTTT
TAACTGCAACAACTTTAATATACGCTATTGGAGCT
GGAATTACCGCGGCTGCTGGCACCAGACTTGCCCT
CCAATTGTTCCTCGTTAAGAGGTTTAAATTGTACTC
ATTCCAATTACAAGACCCAAAAGAGCCCTGTATCA
GTATTTATTGTCACTACCTCCCCGTGTCGGGATTGG
GTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGT
AGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCC
TAATTCCCCGTTACCCGTTGAAACCATGGTAAGCC
AATACCTTACCATCGAAAGTTGATAGGGCAGAAAT
TTGAATGAACCGTCGCCAGCGCAAGGCTATGCGAT
CCGTAAAGTTATCATGAATCACCAAAAAGCCCCGA
AGGGCATTGGTTTTTTATCTAATAAACACATCCCTT
CCGAAGTCGGGATTTTCAGCATGTATTAGCTCTAG
AATTACCACGGTTATCCAAGTAGTAAGGTATTATC
AAATAAACGATAACTGATTTAATGAG
CCATTCGCAGTTCACGGTATA

Pathogenicity test

During the first two days after inoculation, pomegranates infected with the isolated fungi showed no visible symptoms. However, by the ninth day, lesions caused by *Paradendryphiella arenaria* had expanded significantly, reaching a diameter of about 20 mm.

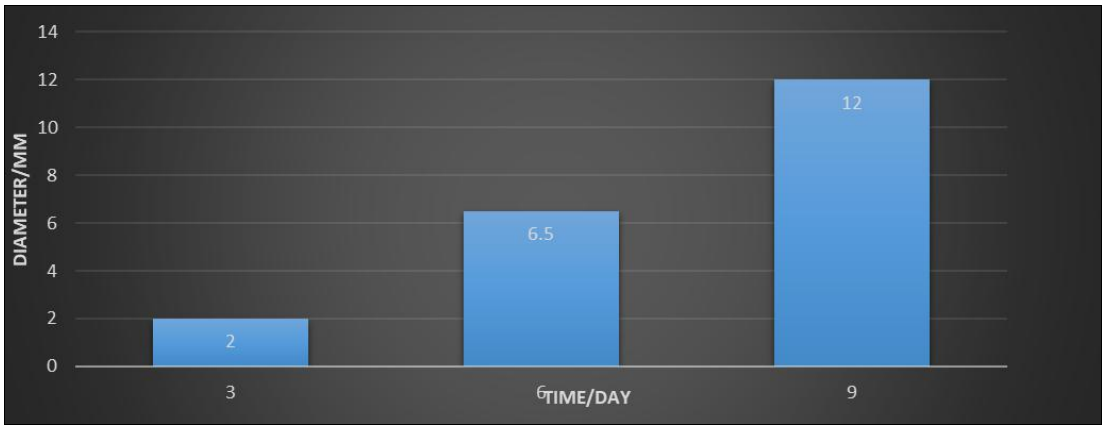


Fig 5: Variation in lesion diameter from pomegranate infected by *Paradendryphiella arenaria*

Antifungal assay

The aqueous extracts of garlic and ginger exhibited a pronounced inhibitory effect on the mycelial growth of *P. arenaria* under in vitro conditions (Fig. 6, 7). The antifungal activity increased progressively with increasing the extract concentrations. A significant reduction in mycelial growth was observed in treated samples compared to the control (Fig. 6a & 7a).

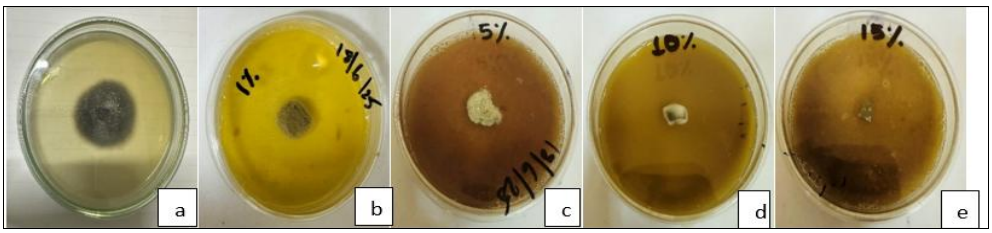


Fig 6: Growth of *P. arenaria* (5 days) on treatment with different concentrations of aqueous garlic extract. (a) control (b) 1mg/ml (c) 5mg/ml (d) 10mg/ml(e) 15 mg/ml

After five days of incubation, treatment with aqueous garlic extract resulted in colony diameters of 10 mm, 4 mm, and 1 mm at concentrations of 1%, 5%, and 10%, corresponding to inhibition rates of 50.5%, 80.2%, and 95.0%, respectively, compared with the control (20.22 mm). The garlic extract untreated fungus rapidly expanded and grown maximum in medium.

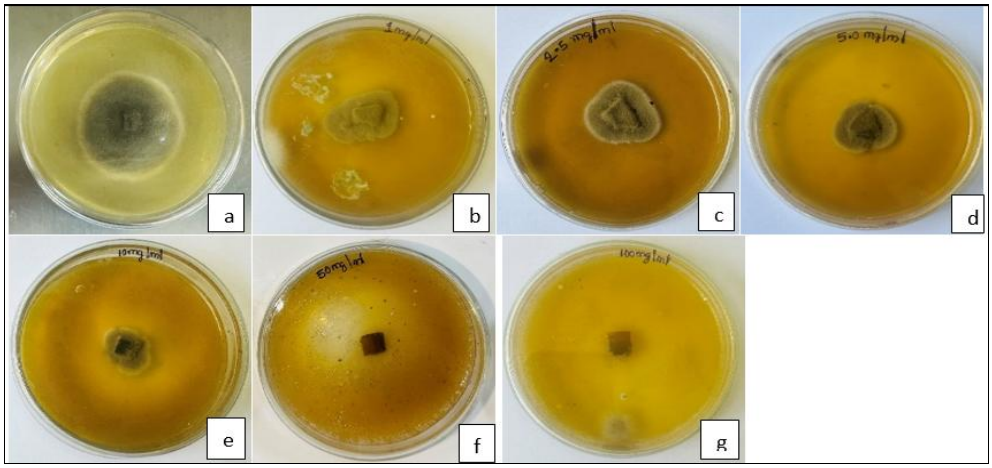


Fig 7: In vitro growth status of *P arenaria* (5 days) under different concentrations of aqueous ginger extract. (a) control; (b) 1 mg/mL; (c) 2.5 mg/mL; (d) 5 mg/mL; (e) 10 mg/mL; (f) 50 mg/mL; (g) 100 mg/ml.

Similarly, aqueous ginger extract significantly suppressed fungal growth, with colony diameters of 15 mm, 10 mm, 8 mm, 5 mm, and 2 mm recorded at 1 mg/mL, 2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 50 mg/mL. These values correspond to inhibition percentages of 40%, 60%, 68%, 80%, and 92%, respectively, compared with the control diameter of 25 mm. Statistical analysis indicated a clear concentration-dependent reduction in fungal growth for both extracts ($p<0.05$), confirming their significant antifungal efficacy against *P. arenaria*.

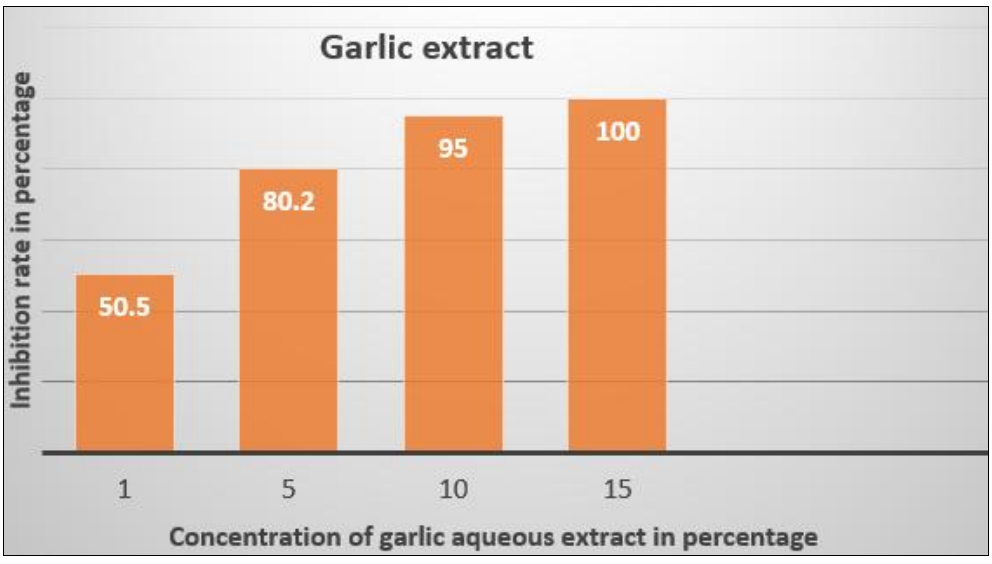


Fig 8: The inhibition rate of the Garlic extract on *P. arenaria*. (The values are means and error bars indicate the standard deviation)

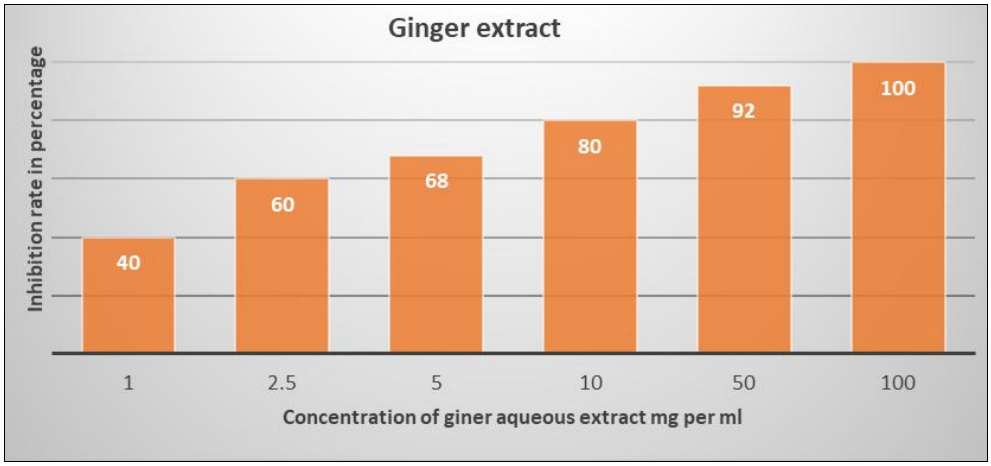


Fig 9: The inhibition rate of the ginger extract on *P. arenaria*. (The values are means and error bars indicate the standard deviation).

The findings indicate that the 10% garlic extract and the 50 mg/mL ginger extract produced the highest inhibitory effects on *P. arenaria*. Moreover, when comparing the two treatments, garlic extract exhibited a lower minimum inhibitory concentration (MIC) than ginger extract, demonstrating its stronger antifungal efficacy against the pathogen.

Conclusion

India, being one of the major global producers of pomegranate, faces significant yield and quality losses due to PFR disease. In this study, the causal pathogen was identified as *Paradendryphiella arenaria* through ITS sequencing, marking its association with pomegranate rot in the region. The strong inhibitory activity exhibited by aqueous and lyophilized extracts of garlic and ginger against the pathogen demonstrates their potential as promising eco-friendly alternatives to synthetic fungicides. These findings underscore the importance of further research to isolate the active antifungal constituents and to elucidate the mechanisms underlying their inhibitory effects. Such advancements may contribute to the development of sustainable and safer disease-management strategies for pomegranate cultivation.

Acknowledgement

Not available

Author's Contribution

Not available

Conflict of Interest

Not available

Financial Support

Not available

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How to Cite This Article
Krishnamurthi AV, Karigar CS. Isolation of *Paradendryphiella arenaria* from Pomegranate Fruit Rot (PMR) and its management with *Allium sativum* (garlic) and *Zingiber officinale* (ginger) extracts. Journal of Advances in Microbiology Research. 2026; 7(1): 15-22.