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BIOPHYSICAL CHARACTERIZATION OF EXUDATES OF ENDOPHYTIC FUNGI ISOLATED FROM *CENTELLA ASIATICA* AS A SOURCE OF BIOACTIVE COMPOUNDS

Vidya Holeyannavar and Vootla Shyam Kumar *

Department of Microbiology and Biotechnology, Karnatak University, Pavatenagar, Dharwad - 580003, Karnataka, India.

Keywords:

Fungal exudates, Secondary metabolites, Antimicrobials, GCMS, FTIR, Talaromyces verruculosus

Correspondence to Author:
Dr. Vootla Shyam Kumar

Professor,
Department of Microbiology and Biotechnology, Karnatak University, Pavatenagar, Dharwad - 580003, Karnataka, India.

E-mail: shyamkumarvootla@gmail.com

ABSTRACT: Fungal exudate is the phenomenon that involves the exudation of water and metabolites produced by fungal cells, Fungal exudates are rich sources of bioactive secondary metabolites with potential applications. Exudates are a prevalent phenomenon in the fungal domain, influenced by various factors, such as the composition of the growth medium and incubation temperature. Filamentous fungi, renowned for their extensive metabolic capabilities, release exudates that act as reservoirs for a wide range of bioactive compounds. These exudates, often underexplored, contain diverse secondary metabolites and proteins with significant biological activities. From *Centella asiatica*, 30 fungal isolates were obtained, of which 12 were analyzed based on morphological and cultural characteristics. Among these, *Talaromyces verruculosus* and *Penicillium chrysogenum* exhibited exudate production. SEM analysis highlighted the structural morphology of the fungi, while ATR- FTIR revealed the presence of diverse functional groups, indicative of a complex chemical composition, Exudates from *T. verruculosus* exhibited hydroxyl (-OH) stretch, isothiocyanate (-NCS), conjugated alkene, allenes, amines, and aliphatic iodo compounds. In contrast, *P. chrysogenum* exudates contained secondary amines, carbodiimides, and alkenes. GC-MS analysis further identified potential bioactive compounds. These findings underscore the importance of exudate-producing fungi as a reservoir of structurally diverse bioactive compounds with promising pharmaceutical applications.

INTRODUCTION: Exudates are a well-documented phenomenon in which water and dissolved chemicals actively exude from organisms without causing tissue damage [1, 2], while guttation droplets are most commonly associated with plants, they are also widely observed in fungi [1, 3]. For a long time, the ecological significance of these exudates remained largely overlooked and underappreciated. However, early investigations by McPhee and Colotelo in 1977 shed light on their potential roles. They proposed that guttation allows fungi to accumulate metabolite reserves while simult-aneously eliminating toxic metabolic by products. Additionally, it was suggested that guttation is linked to mycelium maturation and that the exudate may serve as a water reservoir, facilitating the sustained growth of aerial hyphae away from their substrate [5].

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Filamentous fungi are known to produce diverse bioactive metabolites, many of which have significant therapeutic relevance for humans. Exudates have been found to naturally contain both harmful substances, such as mycotoxins, cytotoxic agents, and carcinogens, as well as beneficial

compounds like antibiotics, fungicides, insecticides, and antineoplastic or antiviral agents [6, 7]. Detailed analyses of exudates have revealed their complex composition and biological roles. Studies by [3, 8] demonstrated the presence of a wide range of bioactive compounds within these secretions. These findings underscore the importance of exudates not only in fungal ecology but also in biotechnological and pharmaceutical applications.

Droplet exudation occurs during defined external conditions; it is different for every fungus and appears only during a certain period of culture duration. Although guttation is observed in nature, laboratory culture is most convenient for the elucidation of all promoting factors and parameters [8, 9]. Among the studies focused on exudate investigations, the potato dextrose agar, and malt extract agar media were [10, 11]. Potato sucrose agar [8, 12] indicated how big impact on a fungal guttation has the composition of sugars in growth medium. The study showed that a combination of more than one carbon sources, well-metabolized, and non-preferred sugars, could promote exudation [8]; thus, nutritional condition establishments were important in the experimental design. Culture temperature was of similar importance to culture media composition. The temperature range in which guttation occurred, most often, was from 20°C [12, 13].

The general biological roles of guttation in fungi remain unknown. Exudate droplets have been shown to form in laboratories but not in the field, implying that they represent "the image" of secretory activity in these circumstances [14, 15]. While there are only speculations on some of the broad purpose of guttation (such as engagement in growth). However, these general goals may be as significant; for example, in early regions of aerial hyphae, dangers of desiccation are visible, thus keeping appropriate moisture via exudates should help maintain a steady development rate, even with adverse water potential [16].

MATERIALS AND METHODS:

Isolation of Endophytic Fungi: Healthy plants of *Centella asiatica* with a length of approximately 10 to 20 cm long shoots along with rhizomes were used for the isolation of endophytic fungi. Surface sterilization method for the isolation of endophytic fungi was carried out as described by [16, 17] with minor modifications. The plant samples were washed with running tap water, followed by a rinse with 250 ml of sterile distilled water with the addition of 2 to 4 drops of tween 80, and the explants were placed in 75% ethanol for 1 min. Later, the *Centella asiatica* sample was soaked in the 4% sodium hypochlorite for 5 minutes, following a repeated wash with distilled water, and followed by washing with 75% ethanol and sterile distilled water. The effectiveness of surface sterilization was tested by the method of [18]. Surface-sterilized explants of a medicinal plant were inoculated on potato dextrose agar (PDA) to isolate endophytic fungi, **Fig. 1.**

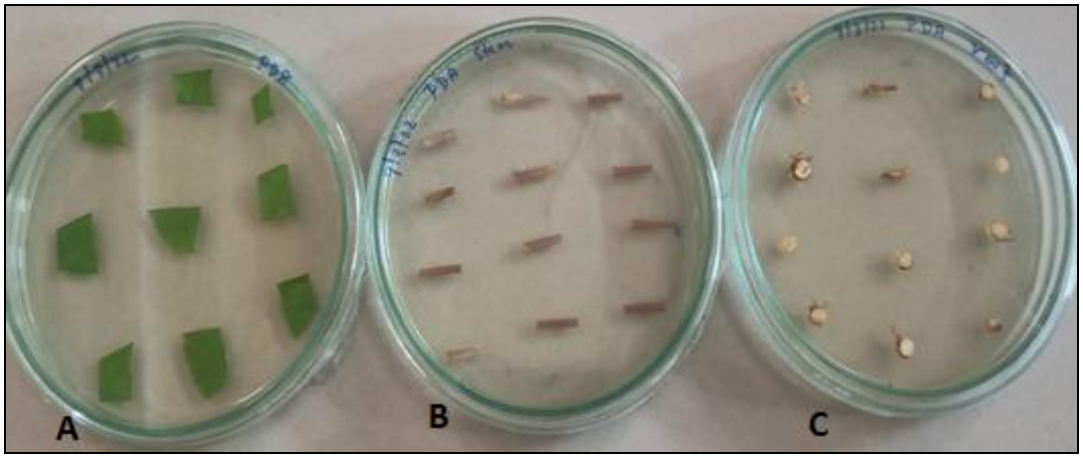

FIG. 1: SURFACE STERILIZED EXPLANTS ON POTATO DEXTROSE AGAR TO ISOLATE ENDOPHYTIC FUNGI, A-LEAF, B-STEAM, C-ROOT

Morphological and Molecular Identification: The endophytic fungi isolated from *Centella asiatica* were initially identified by their colony morphology characters. Microscopic analysis of structures like spores and hyphae further supported species-level identification, forming the basis for

molecular characterization and further functional studies. Further, 18S rRNA sequencing was done at Unigenome (Ahmedabad, India). The selected fungal DNA was isolated and quality was evaluated on 1.8% agarose gel, further, isolated DNA was amplified with 18s rRNA specific primer (18S_18A and 18S 1200R) using Veriti 96 well Thermal Cycler. Later, the PCR amplicon was purified and subjected to Sanger sequencing. Finally, the nucleotide sequence of the isolates was checked by BLAST analysis using the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was constructed by the neighbor-joining method using MEGA X software [20] Shown in **Fig. 2** and **Fig. 3.**

Collection of Fungal Exudates: Exudates were collected from fungal cultures grown on PDA by aseptically pipetting liquid droplets secreted onto the medium surface. The cultures were visually examined for their ability to form exudate droplets. The droplets were collected after 3 weeks of cultivation with a pipette and transferred into Eppendorf tubes. The volume was measured using a Hamilton syringe. Before subsequent analysis, the exudate was filtered using a 0.45-µmMillipore Polyvinylidene Difluoride (PVDF) hydrophilic membrane to remove spores.

Scanning Electron Microscopy (SEM): SEM imaging was performed to study the structural morphology of fungal hyphae and their exudate-producing regions [21].

FTIR Analysis: Fourier-transform infrared spectroscopy (FTIR) was conducted to identify functional groups in the fungal exudates. Characteristic peaks were analyzed to infer the chemical functionalities present. The scanning range for FTIR was 400–4000 cm, [22].

GC-MS Analysis: Gas chromatography-mass spectrometry (GC-MS) analysis was performed to identify volatile and semi-volatile compounds in the fungal exudates. Identified compounds were compared against the NIST23 database for confirmation.

RESULTS AND DISCUSSION:

Fungal Isolation and Identification: A total of 30 isolates were obtained, of which 12 selected were further studied based on morphological and cultural traits, among 12 isolates, the fungi *Talaromyces verruculosus* and *penicillium chrysogenum* were identified as significant exudate producers. The identification of *Penicillium chrysogenum* had been previously reported in my early study [23].

While the molecular identification of *Talaromyces verruculosus* was conducted in this study, with sequences showing 99% similarity to reference strains. DNA was isolated and quality was evaluated on 1.8% agarose gel, Isolated DNA was amplified with ITS Specific Primer (ITS1Fand ITS4R) using Veriti® 96 well Thermal Cycler. A single discrete PCR amplicon band of ~650bp was observed **Fig. 2.**

The PCR amplicon was bead purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was performed with ITS1F and ITS4R primers using BDT v3.1 Cycle sequencing kit on ABI 3500Dx Genetic Analyzer.

BLAST analysis used the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the neighbor-joining method constructed a phylogenetic tree using MEGA X software **Fig. 3.**

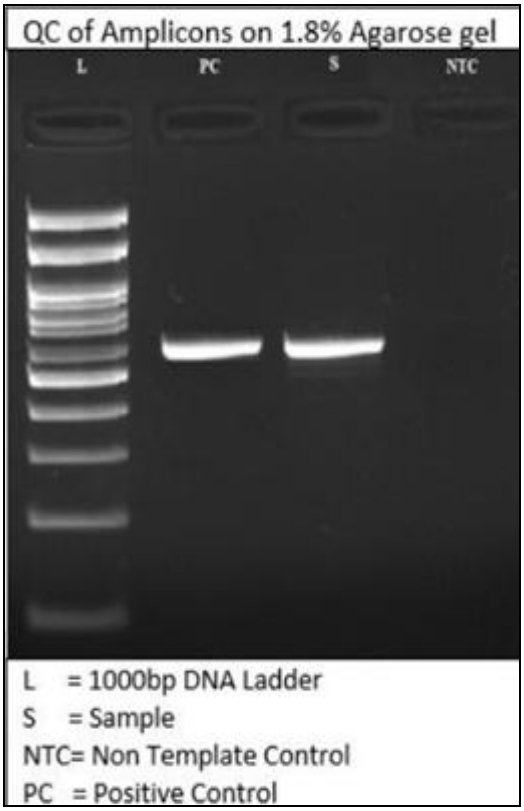

FIG. 2: 1.8% AGAROSE GEL SHOWING SINGLE ~650BP OF ITS AMPLICON

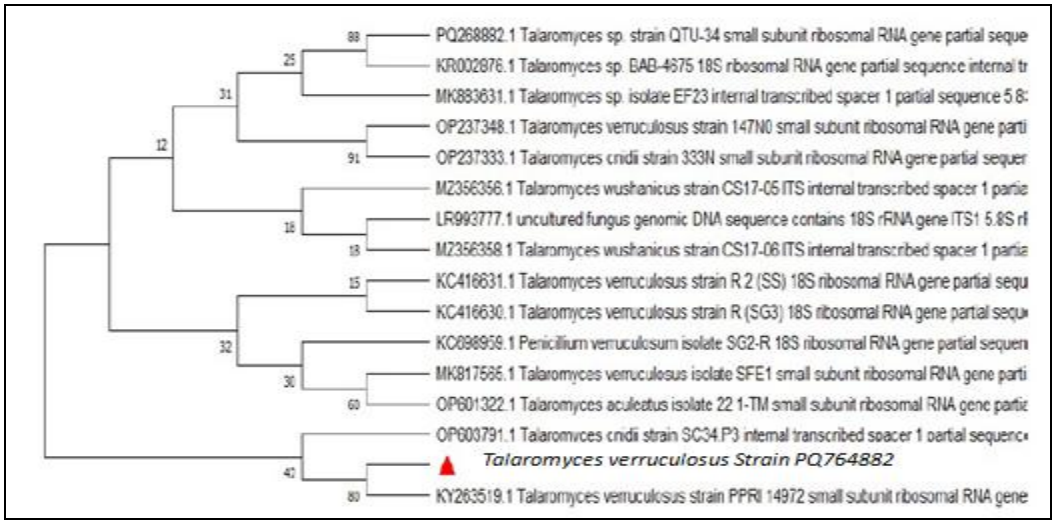

FIG. 3: PHYLOGENY CONNECTION OF TALAROMYCES VERRUCULOSUS BASED ON THE 18S RRNA SEQUENCE AMONGST SIMILAR SEQUENCES RETRIEVED FROM THE NCBI SERVE

Morphological and Structural Analysis (SEM): SEM imaging of *T. verruculosus* revealed dense hyphal networks with distinct surface projections, suggesting active exudate secretion. These structural adaptations are indicative of the fungal ability to produce and release secondary metabolites **Fig. 4.**

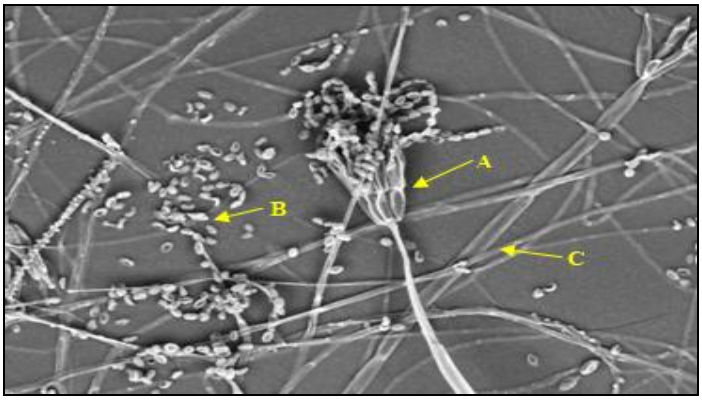

FIG. 4: SCANNING ELECTRON MICROSCOPY IMAGES OF ENDOPHYTIC FUNGI *T. VERRUCULOSUS* ISOLATE. A-CONIDIOPHORES, B- CONIDIA, C-MYCELIUM

Collection and Formation of Exudates: Formation of exudates on fungal culture is observed as in **Fig. 5A** and **5B**. The exudates droplets were collected after 3 weeks of cultivation with a pipette and transferred into Eppendorf tubes. The volume was measured using a Hamilton syringe.

FIG. 5: A - EXUDATES OF *T. VERRUCULOSUS AND B- PENICILLIUM CHRYSOGENUM*

FTIR Analysis: FTIR analysis of *T. verruculosus* exudates revealed: Hydroxyl (-OH) stretch indicating the presence of phenolic or alcohol compounds. Isothiocyanate (-NCS) groups associated with antimicrobial activity. Conjugated alkene and amine groups, indicating the presence of unsaturated and nitrogen-containing compounds. These functional groups highlight the diverse chemical nature of the exudates, reflecting their bioactive potential showed in **Fig. 6A** and **6B.**

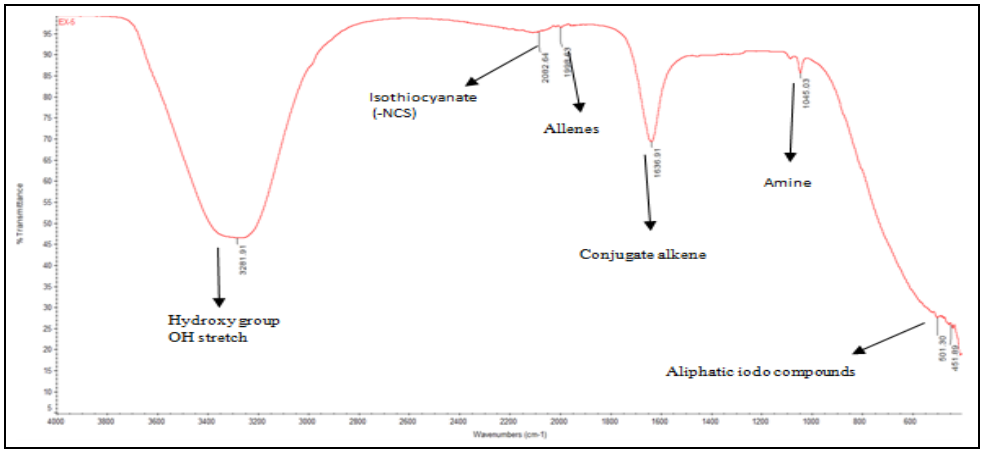

FIG. 6A: ATR-FTIR ANALYSIS OF FUNGI EXUDATES OF *TALAROMYCES VERRUCULOSUS* STRAIN EF-L1 PQ764882

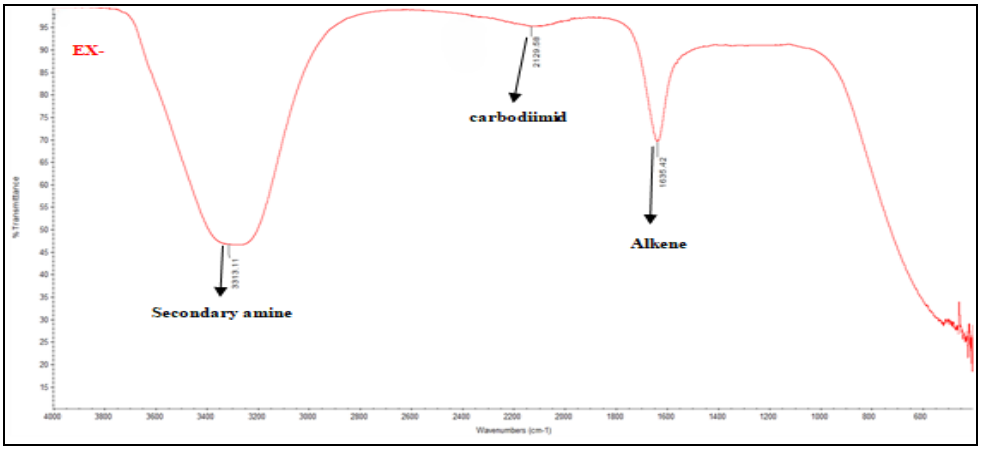

FIG. 6B: ATR-FTIR ANALYSIS OF FUNGI EXUDATES OF *PENICILLIUM CHRYSOGENUM* STRAIN EF- S1 (PQ555271)

GC-MS Analysis: GC-MS analysis identified key bioactive compounds in the exudates of *T. verruculosus*, including Cyclopropene, 3-Butyn-1-ol, and 3-Butynoic acid. These metabolites underscore the pharmaceutical relevance of fungal exudates and their potential for novel drug discovery, **Fig. 7A, 7B** and **Table 1, Table 2**, and **Table 3** relieve the present compounds.

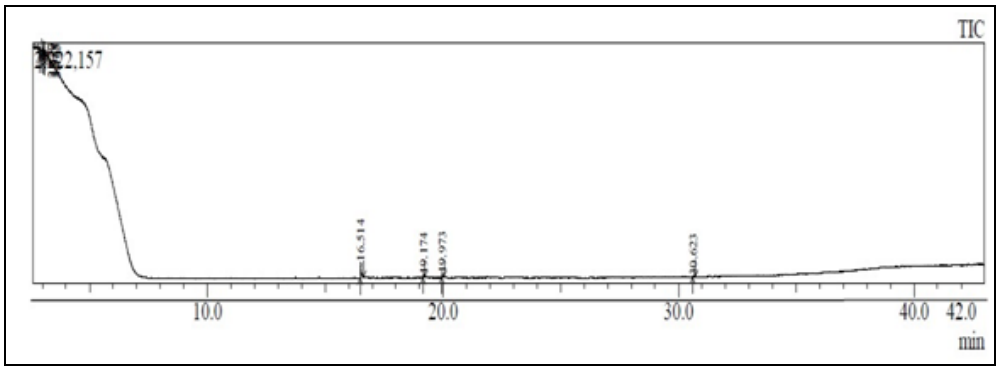

FIG. 7A: CHROMATOGRAM OF THE EXUDATES COLLECTED FROM *TALAROMYCES VERRUCULOSUS* STRAIN EF-L1 PQ764882

TABLE 1: COMPOUNDS DETECTED THROUGH GCMS ANALYSIS OF THE EXUDATE OF FUNGI *T. VERRUCULOSUS* STRAIN EF-L1 PQ764882

Peak #	R Time	Name of Compound	Molecular formula	Molecular weight	Area%	Activity
1.	2.870	Cyclopropene	C3H4	40	24.82	Antimicrobial agents [24]
2.	3.045	3-Butyn-1-ol	C_4H_6O	70	10.88	antioxidant and antimicrobial properties[13]
3.	3.105	3-Butynoic acid	$C_4H_4O_2$	84	20.06	antioxidant and antimicrobial properties[25]
4.	16.514	2(3H)-Benzothiazolone	C_7H_5NOS	151	21.48	antimicrobial and anti-inflammatory properties, [26]
5.	19.174	2-Butyl-1,2-benzisothiazolin-3-one	$C_{11}H_{13}NOS$	207	3.25	Biocidal agent in industrial and agricultural applications[27]
6.	19.973	Benzothiazole, 2-(2-hydroxyethylthio)-	$C_9H_9NOS_2$	211	4.88	No activity

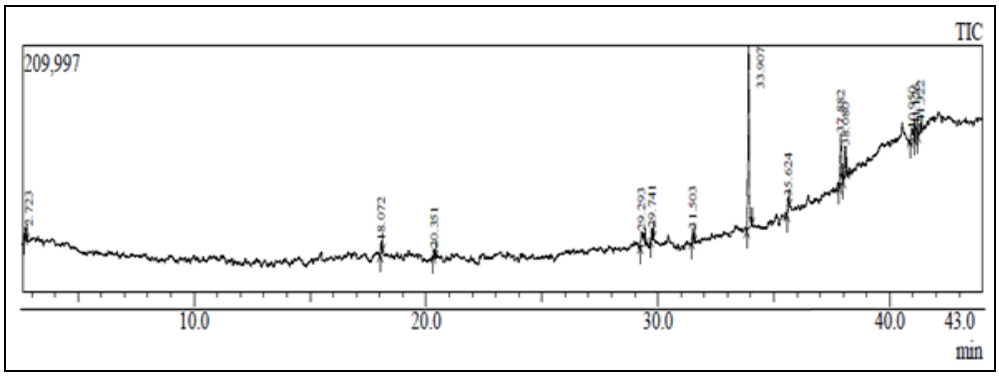

FIG. 7B: CHROMATOGRAM OF THE EXUDATES COLLECTED FROM *PENICILLIUM CHRYSOGENUMEF_S1 (PQ555271) STRAIN*

TABLE 2: COMPOUNDS DETECTED THROUGH GCMS ANALYSIS OF THE EXUDATE OF FUNGI *PENICILLIUM CHRYSOGENUM* STRAIN PQ555271

Peak #	R Time	Name of Compound	Molecular formula	Molecular weight	Area%
1	2.723	2,2-Dimethoxybutane	C6H14O2	118	2.10
2	18.072	2-Ethylhexyl salicylate	$C_{15}H_{22}O_3$	250	2.46
3	20.351	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.46
4	29.293	6-Methylheptyl palmitate	$C_{24}H_{48}O_2$	368	8.23
5	29.741	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	2.43
6	31.503	Octocrylene	$C_{24}H_{27}NO_2$	361	2.77
7	33.907	Squalene	$C_{30}H_{50}$	410	45.85
8	35.624	Glycerol tricaprylate	$C_{27}H_{50}O_6$	470	2.65
9	37.882	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	$C_{28}H_{45}ClO_2$	448	13.14
10	38.080	2-(Decanoyloxy)propane-1,3-diyl dioctanoate	$C_{29}H_{54}O_6$	498	7.59
11	40.950	9-Hexadecenoic acid, eicosyl ester, (Z)-	$C_{36}H_{70}O_2$	534	4.45
12	41.322	Docosanoic acid, docosyl ester	$C_{44}H_{88}O_2$	648	2.82

TABLE 3: COMPOUNDS WITH POTENTIAL ACTIVITY FROM THE DETECTED COMPOUNDS THROUGH GCMS ANALYSIS OF THE EXUDATE OF FUNGI *PENICILLIUM CHRYSOGENUM* STRAIN PQ555271

Peak #	R Time	Name of Compounds	Molecular formula	Molecular weight	Area%	Activity
1.	20.35	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.46	antioxidant and antimicrobial[28].
2.	33.907	Squalene	C30H50	410	45.85	Anticancer, Antioxidant, drug carrier, detoxifier, skin hydrating, [29].

DISCUSSIONS: The present study demonstrates the metabolic potential of *Talaromyces verruculosus* and *Penicillium chrysogenum*, particularly their ability to secrete exudates rich in bioactive compounds. Using GC-MS analysis, compounds such as Cyclopropene, 3-Butyn-1-ol,

and 3-Butynoic acid were identified. Cyclopropene has been associated with applications in organic synthesis and the development of antimicrobial agents, as noted by [24]. Similarly, 3-Butyn-1-ol and 3-Butynoic acid are intermediates for synthesizing biologically active molecules with reported antioxidant and antimicrobial properties, as described by [13, 25].

Other identified compounds, including 2(3H)-Benzothiazolone, 2-Butyl-1,2-benzisothiazolin-3-one, and Benzothiazole, 2-(2-hydroxyethylthio)-, underscore the pharmaceutical potential of fungal exudates. 2(3H)-Benzothiazolone exhibits significant antimicrobial and anti-inflammatory properties, as demonstrated by [26]. Meanwhile, 2-Butyl-1,2-benzisothiazolin-3-one is known for its efficacy as a biocidal agent in industrial and agricultural applications, as highlighted [27]. Furthermore, benzothiazole derivatives are well-documented for their broad-spectrum antimicrobial activity and emerging anticancer potential, as discussed by Kumari and Verma (2020) and exudate metabolite of *Penicillium chrysogenum* exudate n-Hexadecanoic acid antioxidant and antimicrobial activities [28], Squalene has anticancer, antioxidant, drug carrier, detoxifier, skin hydrating, and emollient activities [29].

Structural insights from SEM revealed morphological adaptations associated with metabolite secretion. These findings resonate with the observations of O'Donnell and Sutton (2018), who reported similar structural changes in fungi during exudate production. Additionally, FTIR analysis detected functional groups such as hydroxyl (-OH), isothiocyanate (-NCS), conjugated alkenes, and secondary amines, which are often implicated in biological activities, according to [31].

These functional groups support the potential applications of fungal exudates in developing antimicrobial and antioxidant formulations. By integrating GC-MS, FTIR, and SEM analyses, this study provides a comprehensive understanding of the bioactive potential of *Talaromyces verruculosus* exudates. These findings pave the way for future research aimed at optimizing culture conditions to enhance metabolite production and exploring these compounds for novel therapeutic applications.

CONCLUSIONS: This study demonstrates the significant potential of *Talaromyces verruculosus* exudates as a source of bioactive compounds. GC-MS analysis identified several compounds, including Cyclopropene, 3-Butyn-1-ol, benzothiazole, Squalene and n-Hexadecanoic acid, which exhibit antimicrobial, antioxidant, and anti-inflammatory activities. FTIR analysis further confirmed the presence of functional groups associated with biological activity, while SEM revealed structural adaptations related to exudate secretion. These findings emphasize the therapeutic potential of fungal exudates for pharmaceutical and biotechnological applications. This research lays the groundwork for further studies to optimize exudate production and explore these metabolites for novel drug development.

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CONFLICT OFINTEREST: The authors hereby declare no conflict of interest.

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