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Exploring natural antimicrobial properties: cell-free culture filtrates of *Aspergillus niger* and *Rhizomucor pusillus*

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Abstract

Fungi are rich sources of medicinal bioactive metabolites. The fungi used in this study were identified as *Rhizomucor pusillus* and *Aspergillus niger* through multilocus gene sequencing. HPLC was used to analyze the cell-free culture filtrate (CFCF) of the fungi. The most prevalent organic acid in *A. niger* was tartaric acid, whereas the most predominant acid in *R. pusillus* was succinic acid. Furthermore, chlorogenic acid was the most abundant phenolic compound in both CFCFs. The impacts of the CFCFs as antibacterial agents against Gram (+) bacteria *Staphylococcus aureus*, *Enterococcus faecalis,* methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative *Staphylococcus sp.* (MRCoNS) and Gram (-) bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, a yeast *Candida parapsilosis* and a filamentous fungus *A. niger* were also investigated. CFCF of *A. niger* was found to exhibit significant antimicrobial activity by acting on a wider range of microorganisms than CFCF of *R. pusillus*. Especially, CFCFs of *A. niger* and *R. pusillus* demonstrated the highest levels of activity at a concentration of 0.125 mL/mL against MRSA and *C. parapsilosis*, respectively. This study offers the first comparison of organic acids and phenolics in R. pusillus and A. niger CFCFs, linking their distinct antimicrobial profiles to potential use in fungal-based antimicrobial formulations.

Introduction

Fungi, which constitute a major group of eukaryotic organisms, are ubiquitous in nature, exhibiting heterogeneity and biomass that is analogous to that of bacteria (Subhash et al., 2022). A robust metabolic relationship has been observed to exist between fungi and the substrate on which they reside. Fungi possess vast biosynthetic capabilities, resulting in the creation of molecules with intricate chemical structures that demonstrate significant biological activity (Sułkowska-Ziaja et al., 2023). Hence, fungi are considered a potential source for discovering and developing unique biologically active metabolites(Hashem et al., 2023). Their biosynthesis processes are distinguished by their ease and economy, demonstrating robust growth, extensive surface area coverage, and favourable chemical composition. Consequently, these procedures enable the development of reliable syntheses (Halkai, 2017). While the focus of fungal metabolites has been predominantly on their antibacterial properties, fungi have a notable capacity to form toxic secondary metabolites in response to predators, UV radiation, and competition from other bacteria (Baz et al., 2024).

As reported by the World Data Centre for Microorganisms (WDCM), approximately half of the identified *Aspergillus* species are of significant

pharmacological and commercial importance. (El-hawary et al., 2020; Gill et al., 2023). Given its remarkable diversity, *Aspergillus* continues to be one of the most prominent producers of secondary metabolites with potential therapeutic applications, including antibacterial, anti-inflammatory, anticancer, and antioxidant properties. (Mokhtar et al., 2022). *Rhizomucor pusillus*, a naturally occurring isolate and belonging to the order Mucorales, is a filamentous wild-type fungus. The genus *Rhizomucor* is restricted to thermophilic species with dark-colored and predominantly spherical sporangia (Scaife et al., 2023). The studies on the subject of the effect of *Rhizomucor pusillus* on antimicrobial activity are limited. The present study will provide significant information to the literature on this subject (Kenany, M. A., Youssef, M. M., & Gebreil, 2024; Sabado et al., 2018)

Fungi are known to produce a diverse array of compounds that demonstrate antibacterial properties. These compounds exhibit two distinct characteristics. Firstly, they display selective activity against specific bacterial strains. Secondly, they demonstrate a broad spectrum of activity (Agrawal & Verma, 2021; Sułkowska-Ziaja et al., 2023). In recent years, there has been an increased focus on the utilisation of natural products from new sources, with a particular emphasis on biological organisms that remain underexplored (Santra et al., 2024). As biological agents, either direct whole cell suspensions or their secondary metabolites can be used (Aydi Ben Abdallah et al., 2023; Ferreira & Musumeci, 2021).

The objective of this research was to obtain cell-free culture filtrates (CFCFs) from *Rhizomucor pusillus* and *Aspergillus niger* to perform high-performance liquid chromatography (HPLC) analysis to identify organic and phenolic compounds present in CFCFs. Moreover, this study will assess the antimicrobial activity of these CFCFs on Gram-positive and Gram-negative bacteria and fungi.

Materials and Methods

Morphological and multilocus gene identification

The previously isolated thermophilic and thermotolerant fungi from hot spring waters Naşa thermal springs, the Simav district of Kütahya in Turkey (Coordinates: 39.13098, 28.95576) were initially identified at the genus level according to their microscopic and colonial characteristics (Samson et al., 2010; Samson et al., 2004; Samson et al., 2014). For molecular identification, the isolates were cultivated on Malt Extract Agar (MEA) for seven days at 25 °C before DNA extraction. The DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research), following the manufacturer's instructions. The molecular characterization of the isolates was performed using sequencing of the standard gene regions, which are internal transcribed spacer (ITS) regions, D1/D2 region of the rDNA genes, and calmodulin (CaM). The ITS regions were amplified using the described methods (Visagie et al., 2014). For CaM, the CL1 and CL2 primer sets were used (Serra et al., 2006). For the D1/D2 region, the LROR and LR3 primer sets were used (Sharma et al., 2008). Polymerase chain reaction (PCR) was conducted using a T100 Thermal Cycler (Bio-Rad®) as previously described (Visagie et al., 2014). The PCR products were separated by agarose gel electrophoresis (1% w/v in 1xTAE) and visualised by GelRed staining. Sequencing analyses were performed by a company through a service procurement. The sequences obtained in this study were compared with those deposited in the National Center for Biotechnology Information (NCBI) Database using BLAST for identification.

Cell-free culture filtrate production

Cell-free culture filtrates of *R. pusillus* and *A. niger* were produced by the submerged fermentation method for use in analytical and antimicrobial analysis. CFCF production was carried out in 250 mL Erlenmeyer flasks containing 100 mL of Sabouraud 2% Dextrose Broth medium for submerged fermentation. Each flask was inoculated using a 10 mm diameter agar disk cut from an actively growing fungal culture at 28ºC (for thermotolerant fungi) or 45ºC (for thermophilic fungi) for 4 days of incubation. The flasks were incubated at 28ºC or 45ºC for 7 days at 150 rpm. The obtained fungal biomass was centrifuged at 13,000 rpm (10,304 × g) for 15 min to separate the supernatant. The obtained supernatant was filtered through Whatman No. 1 filter paper and used in HPLC and antimicrobial analyses.

Analytical methods

The organic acids present in the cell-free culture filtrates of *R. pusillus* and *A. niger* were determined by utilizing high-performance liquid chromatography (Agilent 1260 Infinity Series). The HPLC system was primarily equipped with a HI-Plex H, 300 x 7.7 mm: PL1170-6830 column, and a diode-array detector (DAD). The analytical column was operated at 50 °C with 0.02 N H_2SO_4 in HPLC-grade water as the mobile phase. The analysis was conducted using isocratic elution with a mobile phase flow rate of 0.6 mL/min.

The phenolic compounds of the CFCFs of *R. pusillus* and *A. niger* were also determined by HPLC (Agilent 1260 Infinity Series). The HPLC system was primarily equipped with an Acegenerix 5c18 (4.6 x 250 mm) 5µm column and a DAD detector. The analytical column was operated at 30 °C with acetonitrile and 0.1% phosphoric acid as the mobile phase. Analysis was conducted using isocratic elution with a flow rate of 0.8 mL/min.

Quantification of metabolites was performed using internal standards appropriate for each compound class to ensure accuracy and reproducibility of peak identification and integration. All analyses were performed in triplicate, and the results were expressed as mean ± standard deviation; statistical significance

was evaluated to ensure the reliability and reproducibility of the findings.

Antimicrobial activity

The determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the cell-free culture filtrates (CFCFs) of *R. pusillus* and *A. niger* was conducted against Gram-positive and Gram-negative bacteria. The following Gram-positive bacterial strains were utilised in this study: *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591) and methicillin-resistant coagulase-negative *Staphylococcus sp.* (MRCoNS) (obtained from Kütahya Health Sciences University). Moreover, the following Gram-negative bacteria were utilised: *Pseudomonas aeruginosa* (ATCC 277853), *Escherichia coli* (ATCC 25922), yeast *Candida parapsilosis* (ATCC 22019) and filamentous fungus *Aspergillus niger* (obtained from Kütahya Health Sciences University) which have pathogenic effects in humans. Chloramphenicol (10 mg/mL) and Bacitracin (10 mg/mL) for bacteria and Ketoconazole (25 mg/mL) for yeast were used as control drugs. Experimental setups containing only medium and selected microorganisms were also used to test microbial growth as positive controls. The microdilution method was performed according to the Clinical and Laboratory Standards Institute (CLSI Guidelines, 2017, 2023). The stock solutions of CFCFs of *R. pusillus* and *A. niger* were prepared at a concentration of 2.5 mL/mL. The stock solutions of CFCFs were diluted to concentrations of 0.6, 0.5, 0.25, and 0.125 mL/mL. The dilutions of CFCFs of *R. pusillus* and *A. niger* were prepared in Muller Hilton broth (MHB) and Sabouraud Dextrose Broth (SDB). One hundred microliters of the various dilutions of CFCFs of *R. pusillus and A. niger* were transferred to the wells. A volume of 100 µL of the overnight culture, adjusted to a density of 0.5 McFarland units, was added to each well. The plates inoculated with bacteria were incubated at 37°C for a period of 24 hours. The plates inoculated with yeast were incubated at 28°C for 72 hours. Following the incubation period, the lowest concentration that demonstrated no growth was identified as the MIC value. The plates were inoculated with Mueller-Hinton Agar (MHA) for bacteria and Sabouraud Dextrose Agar (SDA) for yeast from all concentrations without growth, commencing with the lowest concentration at which no bacterial growth was observed. The plates were inspected to determine whether growth had occurred after incubation, and the lowest concentration that did not result in growth was noted as the minimum fungicidal concentration (MFC) or minimum bactericidal concentration (MBC). The experiments were conducted in triplicate, with growth being monitored and controlled using tetrazolium chloride (TCC).

Results and Discussion

Identification of fungi

With the morphological and molecular taxonomy study conducted within the scope of the study, it was determined that the thermophilic fungus was *R. pusillus* and the thermotolerant species was *A. niger* (Figure 1). The use of natural products and components derived from them has been used extensively in improving human health, with a particular focus on the management of cancer and microbial infections. A significant proportion of these substances are derived from bacterial and fungal sources. Fungi are primarily responsible for the production of bioactive substances (Baz et al., 2024).

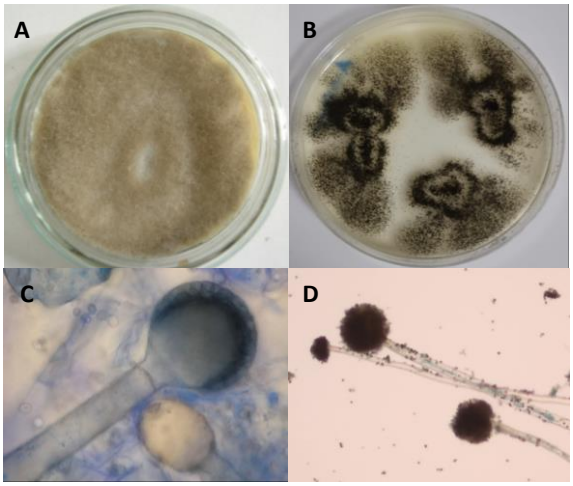

Figure 1. *R. pusillus* view on MEA media **A)** and microscopic view, light microscope 100x **C)**; *A. niger* view on MEA media, **B)** and Microscopic view, light microscope 10x **D)**.

Analytical methods

The field of aromatic metabolism in fungal species has been the subject of extensive research over several decades (Lubbers et al., 2019; Mäkelä et al., 2015; Tel-Çayan et al., 2023). The HPLC analytical results in this study demonstrated the presence of a range of organic acids and phenolic compounds in the CFCF of *A. niger*. These included shikimic acid, tartaric acid, succinic acid, lactic acid, acetic acid, fumaric acid, and propionic organic acids. Additionally, phenolic compounds of chlorogenic acid, caffeic acid, 4-hydroxybenzoic acid, p-coumaric acid, and trans-ferulic acid were identified. The HPLC results showed the presence of a range of organic acids and phenolic compounds in the CFCF of *R. pusillus*. These included quinic acid, citric acid, tartaric acid, malic acid, succinic acid, lactic acid, fumaric acid, and propionic organic acids. Additionally, phenolic compounds of chlorogenic acid and p-coumaric acid were identified. The HPLC results indicated that tartaric acid (1921.29 ± 0.62 ng/µL) was the most abundant organic acid in *A. niger*, whereas succinic acid (3495.89 ± 0.11 ng/µL) was the most abundant organic acid in *R. pusillus*. Furthermore, among the identified phenolic compounds, chlorogenic acid was found to be the most abundant, with concentrations of 18.34 ± 0.23 ng/µL

Table 1. Organic and phenolic compounds of CFCFs of *R. pusillus* and *A. niger*

A. niger		*R. pusillus*	
Organic Acids	**Amount (ng/µL)**	**Organic Acids**	**Amount (ng/µL)**
Shikimic acid	9.35 ± 0.06	Shikimic acid	34.36 ± 0.05
Tartaric acid	1921.29 ± 0.62	Quinic acid	1160.78 ± 0.14
Succinic acid	1005.52 ± 0.37	Citric acid	1043.04 ± 0.05
Lactic acid	409.55 ± 0.05	Tartaric acid	562.68 ± 0.31
Acetic acid	654.29 ± 0.07	Malic acid	370.11 ± 0.04
Fumaric acid	39.07 ± 0.43	Succinic acid	3495.89 ± 0.11
Propionic acid	85.76 ± 0.20	Lactic acid	1883.38 ± 0.12
		Fumaric acid	22.85 ± 0.13
		Propionic acid	196.89 ± 0.12
Phenols	**Amount (ng/µL)**	**Phenols**	**Amount (ng/µL)**
Chlorogenic acid	18.34 ± 0.23	Chlorogenic acid	37.11 ± 0.17
Caffeic acid	0.06 ± 0.00	p-Coumaric acid	0.83± 0.02
4-Hydroxy benzoic acid	0.48 ± 0.01		
p-Coumaric acid	2.87 ± 0.04		
trans-Ferulic acid	6.74 ± 0.07		

and 37.11 ± 0.17 ng/µL in both *A. niger* and *R. pusillus*, respectively (Table 1.).

Aromatic compounds are used in many industries, including food, cosmetics, and pharmaceuticals. These compounds are predominantly produced from non-renewable petroleum resources, which are gradually diminishing in supply. Consequently, alternative sources, such as plant biomass, are being investigated as potential substitutes. An example is the release of feruloyl esterases from the fungus *Aspergillus niger* and hydroxycinnamic acid ferulic acid from sugar beet pulp (Benoit et al., 2006; Lesage-Meessen et al., 1999). A study was conducted in which the mycochemical screening of *A. niger* and *R. pusillus* ethanolic extracts and fungal residue was undertaken. This demonstrated the presence of flavonoids and terpenoids. Additionally, ethanolic extracts of *A. niger* and *R. pusillus* were shown to contain tannins and alkaloid bioactive components, respectively (Sabado et al., 2018).

The antimicrobial properties of organic acids are based on their ability to cross the cell membrane due to their lipophilic nature. This enables them to alter proton and associated anion concentrations in the cytoplasm, which in turn affects purine bases and essential enzymes, reducing bacterial viability (Dibner & Buttin, 2002; Gómez-García et al., 2019; Warnecke & Gill, 2005).

Phenolics play a pivotal role in maintaining health due to their capacity to neutralize free radicals that can occur in the body and lead to a range of age-related diseases, as well as lung, autoimmune, and heart diseases (Crozier et al., 2009; Ren et al., 2017). Phenolic acids are chemically hydroxylated derivatives of benzoic and cinnamic acids. The most common hydroxycinnamic acid derivatives are p-coumaric, caffeic, chlorogenic, and ferulic acids (Kumar & Goel, 2019). Our study revealed that chlorogenic acid was the most prevalent phenolic compound in both CFCFs. It has been documented that this acid and its associated hydrolysates function as antioxidants. Although chlorogenic acid was the most abundant phenolic compound detected, its concentration in both CFCFs (18–37 ng/µL) falls well below the MIC range typically reported for pure chlorogenic acid (20–80 µg/mL), indicating that its antimicrobial effects in this context may depend on synergistic interactions with organic acids (Lou et al., 2011). In addition, it has been demonstrated to possess hepatoprotective properties, with the capacity to inhibit cancer and facilitate the management of chronic hepatitis B infection (Yang et al., 2022).

Antimicrobial activity

In this study, the broth microdilution method was employed to assess the antimicrobial susceptibility of CFCFs of *R. pusillus* and *A. niger* against a range of microorganisms, including *E. faecalis*, *S. aureus*, MRSA, MRCoNS, *E. coli*, *P. aeruginosa*, *A. niger*, and *C. parapsilosis*. The MBC/MFC values of CFCFs are given in Table 2. The MIC values of CFCF of *R. pusillus* were determined to be 0.125 mL/mL against *C. parapsilosis*, 0.25 mL/mL against *E. faecalis*, MRSA, and *E. coli*, and 0.6 mL/mL against *S. aureus*. The MBC/MFC values of CFCF of *R. pusillus* were determined to be 0.6 mL/mL against *E. faecalis*, *C. parapsilosis*, and>0.6 mL/mL against *S. aureus*, MRSA, and *E. coli*. Among the microorganisms tested against *R. pusillus* CFCF, MRCoNS, *P. aeruginosa*, and *A. niger* were found to be resistant. The tested doses of CFCF were found to be ineffective against these organisms. The resistance of MRCoNS and *P. aeruginosa* to both CFCFs may be attributed to intrinsic defense mechanisms, particularly the action of broad-specificity efflux pumps, which actively export antimicrobial compounds and contribute to multidrug resistance (Gaurav et al., 2023; Nikaido & Pagès, 2012).

The MIC values of CFCF of *A. niger* were determined as 0.125 mL/mL against MRSA, and *A. niger*, 0.25 mL/mL against *E. faecalis*, *S. aureus*, *E. coli*, and *C. parapsilosis*, and 0.6 mL/mL against MRCoNS, and *P. aeruginosa*. The MBC/MFC values of CFCF of *A. niger* were determined to be 0.25 mL/mL against *A. niger*, 0.25 mL/mL against *E. faecalis*, *S. aureus*, MRSA, *E. coli*,

Table 2. MIC and MBC/MFC values (mL/mL) of CFCFs of *R. pusillus* and *A. niger*

Microorganisms		Gram positive bacteria			Gram negative bacteria			Fungi	
		E. faecalis	***S. aureus***	**MRSA**	**MRCoNS**	***E. coli***	***P. aeruginosa***	***A. niger***	***C. parapsilosis***
MIC values (mL/mL)	CFCF- *A. niger*	0.25	0.25	0.125	0.60	0.25	0.60	0.125	0.25
	CFCF - *R. pusillus*	0.25	0.60	0.25	-	0.25	-	-	0.125
MBC/MFC values (mL/mL)	CFCF - *A. niger*	0.60	0.60	0.60	>0.60	0.60	>0.60	0.25	>0.60
	CFCF - *R. pusillus*	0.60	>0.60	> 0.6	-	>0.60	-	-	0.60
Chloramphenicol (10 mg/mL) and Bacitracin (10 mg/mL) / Ketoconazole (25 mg/mL)		-	-	-	-	-	-	-	-
(+)-Control		+	+	+	+	+	+	+	+

and greater than 0.6 mL/mL against MRCoNS, *P. aeruginosa*, and *C. parapsilosis* (>0.6 mL/mL). Considering the results obtained in HPLC analyses, it is thought that the CFCFs of *R. pusillus* and *A. niger* may have an effect on the bacterial membrane because it is associated with the formation of free radicals and oxidative stress.

Antioxidants ability to scavenge free radicals has been demonstrated to delay or prevent cellular damage (Tumilaar et al., 2024). A survey of the extant literature revealed that the *Aspergillus niger* strain exhibited the highest antioxidant activity, with a range of 50% to 80% (Yadav et al., 2014). A study conducted by Hameed et al., 2017 demonstrated that Mucor strains are a rich source of secondary metabolites and antioxidants that can be employed in the development of natural antioxidants and nutraceuticals. The capacity of mushroom extracts to scavenge free radicals is associated with their secondary metabolites, including tannins, terpenoids, and flavonoids. In a study conducted to screen the chemical components and biological activities, particularly antibacterial and antioxidant activities, of ethanolic extracts of *A. niger* and *R. pusillus* and fungal residue, *S. aureus*, and *E. coli* were employed as test organisms in an antibacterial assay. The findings of the study demonstrate that ethanolic extracts of *R. pusillus* and *A. niger* exhibit antibacterial properties against *E. coli* and *S. aureus*. Moreover, it was indicated that the ethanolic extracts of *A. niger* and *R. pusillus* demonstrated the highest capacity for free radical scavenging. It is thought that elucidation of these properties may facilitate the use of *A. niger* and *R. pusillus* in the synthesis of pharmaceuticals (Sabado et al., 2018).

Conclusion

This study analyzed the content and antimicrobial properties of CFCFs of *R. pusillus* and *A. niger*. HPLC analysis of their CFCFs revealed the presence of a wide array of organic acids and phenolic compounds, with tartaric acid and succinic acid being the most abundant in *A. niger* and *R. pusillus*, respectively. Chlorogenic acid emerged as the predominant phenolic compound in both species, highlighting their potential as natural sources of bioactive molecules. Both CFCFs showed strong antimicrobial activity against bacterial and fungal strains including *S. aureus*, MRSA, *E. coli*, and *C. parapsilosis*. The MIC and MBC/MFC results indicated that both fungi, particularly *A. niger*, exhibited broader-spectrum antimicrobial effects. These antimicrobial effects are likely associated with the ability of organic acids to disrupt microbial membrane integrity and the antioxidant potential of phenolic compounds. Moreover, the detection of secondary metabolites such as flavonoids, terpenoids, tannins, and alkaloids in ethanolic extracts supports the antioxidant activity observed, particularly in *A. niger*, which aligns with prior literature. The free radical scavenging capacity of these fungal extracts suggests their promising role in the development of natural antioxidants and antimicrobial agents.

Overall, the findings underscore the biotechnological potential of *R. pusillus* and *A. niger* as sustainable sources of antimicrobial and antioxidant compounds. Further investigations into optimization of the production, purification, structural elucidation, *in vitro* and *in vivo* efficacy of these CFCFs could contribute to the development of novel therapeutic agents derived from fungal sources.

Author Contributions

DB: Supervision, Conceptualization, Methodology, Visualization and Writing -original draft; and ŞG: Formal Analysis, Investigation, Writing -review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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