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Research Article

Evaluation of the Antifungal Effects of Indole Carboxylic Acid-Pyridine Derivatives on Clinical Isolates of *Aspergillus*

Mohammad karimi[1], Mojtaba Taghizadeh Armaki[2], Jalal Jafarzade[2], Asieh Khalilpour[3]

1. Student Research Committee, Babol University of Medical Sciences, Babol, I.R.Iran
2. Infectious Diseases and Tropical Medicine Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R.Iran
3. Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R.Iran

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*** Corresponding Author:**

Asieh Khalilpour
E-mail:
asieh.khalilpour@gmail.com
Tel: 011-32190101

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Abstract

***Background*:** Due to the increasing resistance of Aspergillus species to azole drugs, the development of new antifungal agents with fewer side effects and potent activity is critical. This study evaluates the antifungal activity of indole carboxylic acid–pyridine derivatives against clinical isolates of Aspergillus.

***Methods*:** This cross-sectional laboratory study examined the antifungal sensitivity of 100 clinical isolates of Aspergillus species using the broth microdilution method (CLSI M38-A2 protocol). The minimum inhibitory concentrations (MICs) of five derivatives (K1–K5) and itraconazole were determined visually after 48 hours of incubation at 35°C. Statistical analyses were performed using SPSS version 22, and significance was set at $p < 0.05$.

***Results*:** There was no statistically significant correlation between the MIC values of the tested derivatives and itraconazole (Wilcoxon signed-rank test, $p < 0.001$). None of the derivatives exhibited superior antifungal effects. Itraconazole consistently demonstrated lower MICs across all Aspergillus species compared to the tested derivatives.

***Conclusion*:** The indole carboxylic acid–pyridine derivatives exhibited significantly weaker antifungal activity compared to itraconazole. Further structural optimization is needed to improve their efficacy.

Keywords: Indole derivatives, pyridine scaffold, itraconazole, *Aspergillus*, antifungal resistance.

Introduction

Heterocyclic compounds are among the most significant structural classes in medicinal chemistry. The indole nucleus, with the chemical formula C_8H_7N (1), is a heterocyclic scaffold fundamental to many biologically active molecules (2). To date, over ten thousand indole derivatives with biological activity have been identified, with more than 200 currently in clinical use or trials (3). They encompass various therapeutic categories, including antihistamines (4), antifungals (5), antimicrobials (6), antioxidants (7), plant growth regulators (8), anti-HIV agents (9), anticonvulsants (10), anti-inflammatory (11), and analgesics (12).

Pyridine, with the formula C_5H_5N (13), is another important heterocycle found in numerous natural products, vitamins, alkaloids, and coenzymes, as well as in many drugs and pesticides (14, 15). Recent studies suggest compounds containing the pyridine nucleus exhibit notable antibacterial, antifungal, and antiviral properties, especially when combined with other heterocycles.

Increased incidence and mortality from fungal infections, particularly among immunocompromised patients (e.g., with cancer, tuberculosis, or HIV/AIDS), underscore the need for effective antifungal agents (16-19). *Aspergillus* infections, especially invasive aspergillosis, can have mortality rates as high as 80% in some populations. Resistance to azole drugs complicates treatment, reducing efficacy and increasing failure rates. Therefore, continuous research into heterocyclic derivatives, including indole and pyridine compounds, is essential to develop new therapies and combat drug resistance (20-23).

Despite limited local studies, the potential antifungal activity of these compounds warrants investigation. This study evaluates the antifungal effects of indole carboxylic acid-pyridine derivatives on clinical Aspergillus isolates collected from Babol, with the goal of informing future therapeutic strategies.

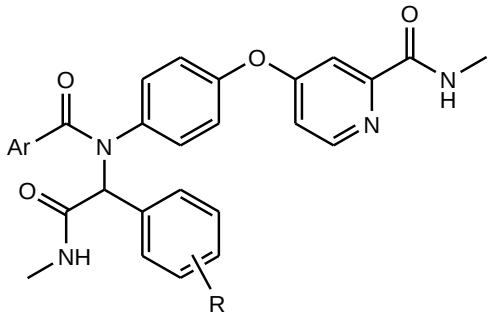
Methods

Isolates

In this experimental and laboratory study, 50 Aspergillus isolet: Aspergillus fumigatus (33), A. flavus (34), A. Niger (18), A. terreus (12), A. nidulans (3) were available in the reservebank of the Department of Parasitology and Mycology, Babol University of Medical Sciences. Fungal isolates were confirmed using the PCR–restriction fragment length polymorphism molecular technique (24). To perform the antifungal susceptibility test, fungal isolates were cultured on Sabouraud dextrose agar culture medium containing chloramphenicol and incubated for 2 to 5 days at 30 °C.

Synthesis of Indole Carboxylic Acid-Pyridine Derivatives

The synthesis involved multiple steps starting with the conversion of 2-picolinic acid to an amide intermediate via reaction with thionyl chloride and methylamine. This intermediate was then condensed with 4-amino-N-methylbenzamide at high temperature to yield a key amine compound. Subsequent alkylation with benzyl chloride derivatives in THF and potassium carbonate produced a set of intermediates, which were finally reacted with 2-chloroacetamide derivatives to yield the target indole carboxylic acid–pyridine compounds. Reaction progress was monitored using thin-layer chromatography (Fig1).



	K1	K2	K3	K4	K5
R:	H	F	Br	OCH3	CH3

Fig1: Indole Carboxylic Acid-Pyridine Derivatives

Antifungal susceptibility testing

In vitro, antifungal susceptibility testing of *Aspergillus* isolates was done using the broth microdilution method as recommended by the protocol CLSI M38-A3 (25). Itraconazole (Sigma-Aldrich USA) was used as a control drug to compare the antifungal activity of new derivatives. First, 2.3 mg of pure powder of drug and derivatives were dissolved in 1 mL of DMSO, and serial dilutions were prepared for final concentrations ranging from 16 to 1024 µg/mL for Indole Carboxylic Acid-Pyridine derivatives, and 0.032 to 16 for Itraconazole. In the next step, 200 µL of derivatives and itraconazole were seeded into the first column of a flat-bottomed 96-well plate, and then 100 µL of RPMI medium (Sigma-Aldrich, USA) was added to the remaining wells (except the first column) and serial dilution was done. Columns 11 and 12 were considered the negative control (drug only, no organism) and the positive control (organism only, no drug). The suspension was adjusted spectrophotometrically to ODs between 80% to 83% transmission at a 530 nm wavelength.

Lastly, 100 µL of the fungal suspensions prepared were added to all columns except the negative control column, and the plates were incubated at 35 °C for 48 hours. After incubation, the minimum inhibitory concentration (MIC) was visually determined as the lowest drug concentration that inhibited fungal growth by 100% or more. The reference strains of *Candida parapsilosis* (ATCC 22019) and *Candida Kruse* (ATCC 6258) were used as quality control for each new set of isolates. All

antifungal susceptibility tests were replicated to ensure reproducibility.

Statistical analysis

The data were analyzed using SPSS software, version 27 (IBM) software. The independent t-test was used to analyze quantitative results, and the chi-squared test was used to analyze qualitative variables, with P≤0.05 considered significant. Also, using Excel version 2020, MIC50, MIC90, and GM (geometric mean) were calculated for all isolates.

Results

Based on descriptive statistics of the frequency and percentage of the examined species among 100 *Aspergillus* isolates, the following were identified: *Aspergillus fumigatus* (33 isolates), *Aspergillus flavus* (34 isolates), *Aspergillus Niger* (18 isolates), *Aspergillus terreus* (12 isolates), and *Aspergillus nidulans* (3 isolates). In a separate analysis of each derivative compared to the drug itraconazole, the Wilcoxon test yielded a P-value of <0.001, indicating no significant relationship between them. This suggests that the derivatives exhibit lower antifungal activity against itraconazole. Moreover, no statistically significant differences were observed in the average MIC values of the derivatives when compared to one another (see Tables 1 and 2).

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Table 1: Results of the Wilcoxon Signed-Rank Test

Sample	MIC (µg/ml)	MIC (µg/ml)	Control	P-value
K1	356.90 ± 476.80	0.179042 ± 0.22235		<0.001
K2	361.06 ± 480.00	0.179042 ± 0.22235		<0.001
K3	286.85 ± 417.92	0.179042 ± 0.22235		<0.001
K4	308.44 ± 423.68	0.179042 ± 0.22235		<0.001
K5	311.53 ± 440.96	0.179042 ± 0.22235		<0.001

Table 2: Descriptive statistics of drug sensitivity results

Species	K1	K2	K3	K4	K5	Itraconazole
Aspergillus fumigatus						
Minimum	64	128	64	128	128	0.031
Maximum	1024	1024	1024	1024	1024	0.5

MIC (µg/ml)50	256	512	512	256	512	0.125
MIC (µg/ml) 90	1024	1024	1024	1024	1024	0.5
GM	309.27	441.99	381.55	381.25	381.55	0.132
Aspergillus flavus						
Minimum	128	64	64	64	64	0.031
Maximum	1024	1024	1024	1024	1024	0.5
MIC 50	512	256	256	512	256	0.25
MIC (µg/ml)50	1024	1024	1024	1024	1024	0.5
MIC (µg/ml) 90	400.88	326/.95	307.307	326.95	298/309	0.155
Aspergillus niger						
Minimum	128	128	256	64	64	0.031
Maximum	1024	1024	1024	1024	1024	0.5
MIC (µg/ml)50	512	256	256	512	256	0.25
MIC (µg/ml) 90	1024	1024	1024	1024	1024	0.5
GM	335/20	348/36	376/25	310/35	322/53	0.16
Aspergillus terreus						
Minimum	128	64	64	128	256	0.031
Maximum	1024	1024	512	1024	1024	0.5
MIC (µg/ml)50	256	256	256	256	512	0.125
MIC (µg/ml) 90	512	512	512	1024	512	0.25
GM	304.43	241.63	203.18	287/35	406/37	0.11
Aspergillus nidulans1	1024	256	512	1024	512	0.25
Aspergillus nidulans2	256	512	128	256	128	0.125
Aspergillus nidulans3	1024	256	1024	512	512	0.25

- **MIC 50**: *Minimum Inhibitory Concentration at which 50% of the organisms are inhibited*
- **MIC 90**: *Minimum Inhibitory Concentration at which 90% of the organisms are inhibited*
- **GM**: *Geometric Mean*

Discussion

Fungal pathogens are responsible for a wide spectrum of diseases, ranging from life-threatening invasive infections such as fungemia, meningitis, and pneumonia, to chronic conditions including allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis. A significant proportion of these infections arise in immunocompromised individuals, where the associated mortality rates are alarmingly high. Although early diagnosis and administration of antifungal therapy are critical to clinical outcomes, the current arsenal of antifungal agent's remains limited (26). Moreover, the rise of antifungal resistance, particularly among *Aspergillus* species, has created a pressing need for the development of new antifungal compounds with novel mechanisms of action (27).

Heterocyclic compounds, especially those containing indole and pyridine rings, have drawn significant attention in drug development due to their broad-spectrum biological activities. These scaffolds are commonly found in therapeutic agents with antifungal, antibacterial, antiviral, anti-inflammatory, and anticancer properties.

Previous studies have demonstrated the antifungal potential of indole-based compounds. For instance, Vaca et al. (2020) evaluated several indole alkaloid derivatives and reported that compounds bearing free amino groups showed potent antifungal activity against phytopathogenic fungi, such as *Moniliophthora Roreri* (28). Similarly, Shirinzadeh et al. (2018) synthesized indole derivatives substituted with triazole, thiadiazole, and carbothioamide moieties, and found that compound 3d (an indole-triazole) exhibited superior activity against *Candida krusei* compared to fluconazole, largely due to the presence of electron-withdrawing substituents like m-chlorophenyl (29).

Kokorekin and colleagues developed aryl thiocyanates derived from indole frameworks and demonstrated their strong antifungal activity against *Candida albicans*, *Candida krusei*, and *Aspergillus Niger* (MIC = 0.12 µg/mL). Their findings indicated that small changes in side chains, such as replacing a methyl nitrogen group with a phenyl ring, could drastically affect antifungal potency. These results collectively emphasize that subtle structural variations—particularly in the substitution pattern of the indole nucleus—play a critical role in determining antifungal efficacy (30).

In the current study, we synthesized a series of indole carboxylic acid–pyridine derivatives through a multi-step process. This involved the initial formation of 4-(4-aminophenylamino)-N-methylpicolinamide via the reaction of 2-picolinic acid with thionyl chloride and methylamine, followed by condensation with 4-amino-N-methylbenzamide. Subsequent alkylation using benzyl chloride derivatives and coupling with 2-chloroacetamide analogs yielded

the final target compounds. The structural integrity of intermediates was confirmed, and all reaction stages were monitored using thin-layer chromatography.

Despite successful synthesis, the antifungal evaluation revealed that the tested derivatives (K1–K5) exhibited significantly higher MIC values (≥256 µg/mL) than itraconazole (MIC_{50} = 0.125 µg/mL), indicating markedly lower antifungal potency. Statistical analysis confirmed this finding ($p < 0.001$). These results are in contrast to previous reports on indole-based compounds with improved efficacy. The observed discrepancy may be attributed to differences in substitution groups (e.g., absence of thiocyanate or halogenated moieties), the nature of the linker units, and the overall hydrophobicity or steric hindrance imposed by the side chains.

Furthermore, variations in the synthetic pathway, such as solvent-free heating conditions and non-functionalized terminal moieties, might have influenced the bioactivity of the final compounds. It is also possible that the electronic and spatial orientation of the carboxylic acid–pyridine fragment relative to the indole core did not favor optimal interaction with fungal cell targets.

Taken together, while the current derivatives were structurally inspired by known bioactive scaffolds, their limited antifungal activity suggests the need for further structure–activity relationship (SAR) optimization. Future studies should focus on introducing electron-withdrawing substituents (e.g., halogens, nitro groups), exploring triazole or thiocyanate hybrids, and modifying the central scaffold to enhance membrane permeability and binding affinity.

Conclusion

This cross-sectional study examined the prevalence of internet addiction among high school students in Babol, Iran, and revealed that 27.9% exhibited moderate levels of internet addiction. The results indicate significant associations between internet addiction and factors such as gender, educational field, and family structure, with female students and those in technical fields showing higher rates of moderate addiction. The study also underscores the importance of the home environment and parental employment status in influencing students' internet usage and potential addiction. Understanding these characteristics is crucial for developing targeted interventions aimed at reducing internet addiction among adolescents by addressing specific demographic influences.

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Availability of data and material: Not applicable.

Author's contribution

Study concept and design: RH, SJ, MEH, and HS. Analysis and interpretation of data: RH, MEH, SH. Critical revision of the manuscript RH, MEH, SSM. Data collection: MAL, Data collection: SSM, Writing an article: MEH. RH. All the authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Consent for publication: Not applicable.

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