The Biological Activity of Peniciketal A produced by terrestrial *Aspergillus sydowii* 195

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Abstract

Food and feed spoilage caused by food-borne fungi and their toxic metabolites can be controlled using synthetic antioxidants to inhibit the production of reactive oxygen species (ROS) that cause rancidity and oxidative damage. However, the use of synthetic antioxidants raises health concerns, leading researchers to seek natural alternatives. This study focused on a terrestrial fungal strain, *Aspergillus sydowii* 195, isolated, purified, and identified morphologically and genetically. The active constituents and fractions produced by *A. sydowii* 195 were studied, and the most active fraction underwent chemical structure identification. The suggested chemical structure was a spiroketal compound named Peniciketal A. This research investigated the biological activity of Peniciketal A, including its antioxidant, antifungal, killing kinetics, and minimum inhibitory concentration. The results demonstrate the potent antifungal activity of Peniciketal A against food-borne fungi, and this could make it a useful alternative to synthetic antioxidants. This is the first study to show the production of Peniciketal A from terrestrial *A. sydowii* 195.

Keywords: *Aspergillus sydowii*,Food-borne fungi,Peniciketal A, Terrestrial fungi.

Introduction

In the healthcare system, food-borne diseases are a continuous problem that causes multiple economic and social burdens[1,2]. Initially, parasites, worms, and protozoa were considered the main cause of food-borne diseases [3]. However, recent studies have shown fungi can cause food-borne diseases [4]. Even though filamentous fungi cause costly and hazardous spoilage for foods and food products, their impact is often underestimated [5].

Previous studies have reported the ability of many fungal species, especially Penicillium and Aspergillus, which are two common storage fungal groups, to cause lethal mycosis by producing aflatoxin, one of the most potent mycotoxins [6]. Other toxinogenic fungal groups, such as Alternaria and Fusarium, can also grow on various human and animal foods, and domestic and commercial refrigeration methods cannot prevent their growth. Furthermore, some nontoxigenic fungal genera, such as Rhizopus, can cause food spoilage [7].

Various sources, including sunlight, ultraviolet light, metabolic processes, and chemical reactions, generate reactive oxygen species (ROS). ROS causes numerous pathological effects, including carcinogenesis, DNA damage, and cellular degeneration associated with aging [8].Antioxidants are used to protect foods and food products from oxidative damage and rancidity. Antioxidants inhibit the generation of ROS and/or scavenge the generated free radicals by preventing the propagation or initiation steps of oxidizing chain reactions [9]. The most widely used synthetic antioxidants are butylated hydroxyl toluene and butylated hydroxyl anisole, but recent studies have raised concerns about their potential toxic effects [10].Consequently, natural sources such as spices, herbs, plants, and microorganisms that

produce antioxidants as secondary metabolites have received much attention from scientists. Microorganisms are generally considered a cheap source of natural antioxidants [11].
In recent years, secondary metabolites produced by filamentous fungi have attracted significant attention due to their interesting biological properties and unique structures [12]. Recent studies have reported that multiple secondary metabolites possessing potent bioactivity and uncommon structures have been isolated from fungi and bacteria in various environments, including soil, sediments, and water [13]. One of these secondary metabolites is spiroketals, which originate from many living organisms, such as microbes, marine organisms, plants, and insects. Spiroketal moieties bind with other building blocks, producing different biologically active and structurally intriguing natural compounds. Liu et al. [14]demonstrated that *Penicillium raistrickii* isolated from saline soil can produce Peniciketal A containing a benzo-fused 2,8-dioxabicyclo [3.3.1] nonane moiety. Furthermore, many researchers have reported the production of spiroketals from various sources with different biological activities, such as promoting cancer cell death [15,16].
However, there have been very few studies and reports on the effects, mechanisms, and molecular mechanisms mediating the effects of Peniciketal A produced by fungi. Gao et al. [17,18]reported the ability of Peniciketal A to reduce cell reproduction in three different cell lines of leukemia, with the advantage of high selectivity for cancer cells and lower toxicity for normal cells (L02, MRC5, and MEFs). Due to its antitumor properties, more mechanistic studies were conducted, such as the global proteomic profile of Peniciketal A, which suggests that it may possess additional promising bioactivities [19].
Gillard and Brimble [20] studied the structure of Peniciketal A, which contains one phenyl ring fused to a [5,6]- or [6,6]-spiroketal, as well as to a 2,8-dioxabicyclo-[3.3.1] nonane moiety. They also reported the rare presence of benzannulated spiroketals, particularly those containing a [6,6]-spiro-ring.This study aims to identify the structure of an active ingredient isolated from local *Aspergillus sydowii* 195 and evaluate its antimicrobial activity against food-borne fungi and its antioxidant activity.

Materials and methods

Collection of samples

Clay soil areas without roots were selected as sources for sampling. The dilution plate technique was employed for the clay soil samples to isolate fungi. The isolated fungi were then cultured on slants containing Czapek's agar and stored in a refrigerator at 4°C for further experimentation.

Morphological identification of selected isolates

The isolated fungi were examined for morphological features. The morphological features include pigmentation, the color of conidia, the diameter of the colony, the reverse color of mycelium, and the extracellular exudates. Additionally, microscopic features such as the heads of conidia, the sporulation degree, the conidiogenous cells' homogeneity, and the fruiting bodies were observed using an Olympus CH40 optical light microscope (10×90) [21,22]. Once identified, the purified isolates were cultured using malt extract-agar (MEA) broth for 7–10 days at 28°C, after which they were stored in a refrigerator (4°C) for further experimentation.

Molecular identification of the isolate

1. Preparation of the fungal culture

For collecting the fungal spores of the fungal isolates, a sterile saline solution (5 ml) was added to each slant of 4-day-old cultures. The suspension was then inoculated into a flask containing 100 ml of CzapekDox's broth and incubated at 28°C for 4 days. Finally, the cultures were filtered after incubation, and the mats were gathered and washed with sterile distilled water.

2. DNA extraction

The method for extracting genomic DNA from the fungal isolates followed the protocol described by Ajay et al. [23].

3. PCR amplification

The primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ were used for the PCR reaction. The reaction mixture (30 µl) contained 20 ng of genomic DNA as the template and EF-Taq polymerase. The reaction conditions were carried out according to the protocol of Khoja et al. [24].

4. Fermentation and working up

Single colonies of *Aspergillus sydowii* 195 were inoculated onto slants containing MEA and incubated at 30°C for 7 days. After the incubation period, the slants were used to inoculate flasks containing 100 ml of GYMP medium (pH 6.0), and the flasks were incubated using a rotary shaker incubator at 28°C for 5 days. Following harvest, the fungal biomass (together with the medium) was homogenized at 16,000 rpm in a blender and filtered via a filter press. Ethyl acetate was used to extract the filtrate, and the resulting aqueous ethyl acetate was concentrated in-vacuo, following the protocol described by Serizawa et al. [25]. The ethyl acetate extracts of mycelium and supernatant showed high similarity on thin layer chromatography (TLC), so they were combined and then concentrated in-vacuo to obtain a brown oily crude extract. Biological screening was performed on the organic extracts obtained.

5. Isolation of the active constituents and spectral measurements

The column chromatography on silica gel G254 eluted by a CH_2Cl_2-MeOH (99.5:0.5, v/v) was used to isolate the active components as described by El-Sayed et al. (2015).The active fraction was loaded on top of a Sephadex LH-20 column, and CH_2Cl_2-MeOH (60:40, v/v) was used as the mobile phase for further purification. The obtained fractions were examined for their antioxidant activities. For elucidation of the chemical structures of the promising active compounds, GC-mass, infrared (IR), and nuclear magnetic resonance (NMR) spectroscopy techniques were employed following the protocol of El-Sayed et al. [26].

6. Biological activity

6.1. Antioxidant activity

The free radical scavenging activity of the isolated active secondary metabolite was evaluated by measuring the decolorization of a chloroform solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, which appears violet in color. The scavenging activity was measured using spectrophotometry at 517 nm, following the protocol described by Brand-Williams et al. [27]. The experiment was performed in triplicate, and the average result was used for calculations. The scavenging activity was calculated using the following equation:

$$\text{Scavenging ability (\%)} = \frac{(\text{A517 of control} - \text{A517 of sample})}{\text{A517 of control}}$$

6.2. Antifungal activity

The antifungal activity of Peniciketal A against four strains of food-borne fungi (*Aspergillus niger*, *Aspergillus flavus*, *Penicillium* sp., and *Rhizopus* sp.) was evaluated using the drop diffusion technique described by Hili et al. [28]. The food-borne fungi were inoculated on Petri dishes filled with potato-dextrose agar and given time to harden. Then, 20 µL of Peniciketal A was separately dropped in the center of each dish and incubated for 48 h at 30 °C. The diameter of the inhibitory zone was measured in millimeters following incubation. Three replicates of Petri dishes were used for each essential oil.

6.3. Minimum inhibitory concentration assay

A stock solution of Peniciketal A was prepared by diluting 0.3 g of Peniciketal A in a 20 ml dimethyl sulfoxide (DMSO) solution. The stock solution was added to the culture broth [Malt-yeast-glucose-peptone (MYGP)] to achieve final 100, 200, 300, 400, and 500 µg/ml concentrations. To 2.0 ml of MYGP broth, the required amount of the tested compound in DMSO and 200 µl of fungal isolates in MYGP broth were added. After 40 hours of incubation at 30 °C, the absorbance at 700 nm was measured for the samples and the control. The minimum inhibitory concentration (MIC) was defined as the concentration at which the measured absorbance was decreased by greater than 90%.

6.4. Killing potential assay

The method described by Diniz et al. [29]was slightly modified to study the killing potential of Peniciketal A. A 1.2 ml aliquot of each fungal stock grown at 30°C for 48 h in MYGP broth was added to 0.8 ml of Peniciketal A (40 mg/ml), resulting in a final concentration of 16 mg/ml (named the system). After the addition, 1 ml of each system was collected at 12 h intervals for three days and added to the same volume of sterile saline solution (0.85% NaCl) to count the viable cells. By comparing the fungal population at the start of the experiment (time = 0 min) and each 12 h interval after the addition of the active compound, the killing potential curve for each fungal strain with the active compound was obtained.

Results

A total of 200 isolates of filamentous fungi, comprising 22 genera and 83 species, as well as two species varieties, were screened for their antioxidant activity using the DPPH radical scavenging technique. The results of this screening had previously been published by Hamed et al. [30]. Based on these results, the research team selected isolate no. 195 as a promising candidate.

Cultural and morphological characteristics

The colonies of strain No. 195 on Czapek's agar exhibited healthy growth, with a close, velvety texture due to crowded conidiophores and blue-green conidial heads arising from the substrate. Exudates were abundant, and the reverse showed shades of red. Conidial heads ranged from nearly globose to radiate and were 100–150 µm in diameter. The colorless, smooth, thick-walled conidiophores measured up to 500 µm in length by 5 to 8 µm in diameter. Vesicles were nearly globose and up to 20 µ in diameter, with sterigmata in two series. The conidia were globose to sub-globose. These characteristics indicate that strain No. 195 is *Aspergillus sydowii* 195, as described by Klich and Pitt [22] (Figure 1a & b).*Aspergillus sydowii* is a mesophilic soil saprobe known to contaminate food and occasionally act as an opportunistic pathogen in humans [29-32]. Geiser et al. [33] studied the differences between terrestrial and marine isolates of *Aspergillus*

sydowii, finding that terrestrial isolates were not pathogenic. They also reported that marine *A. sydowii* was pathogenic to sea fan corals.

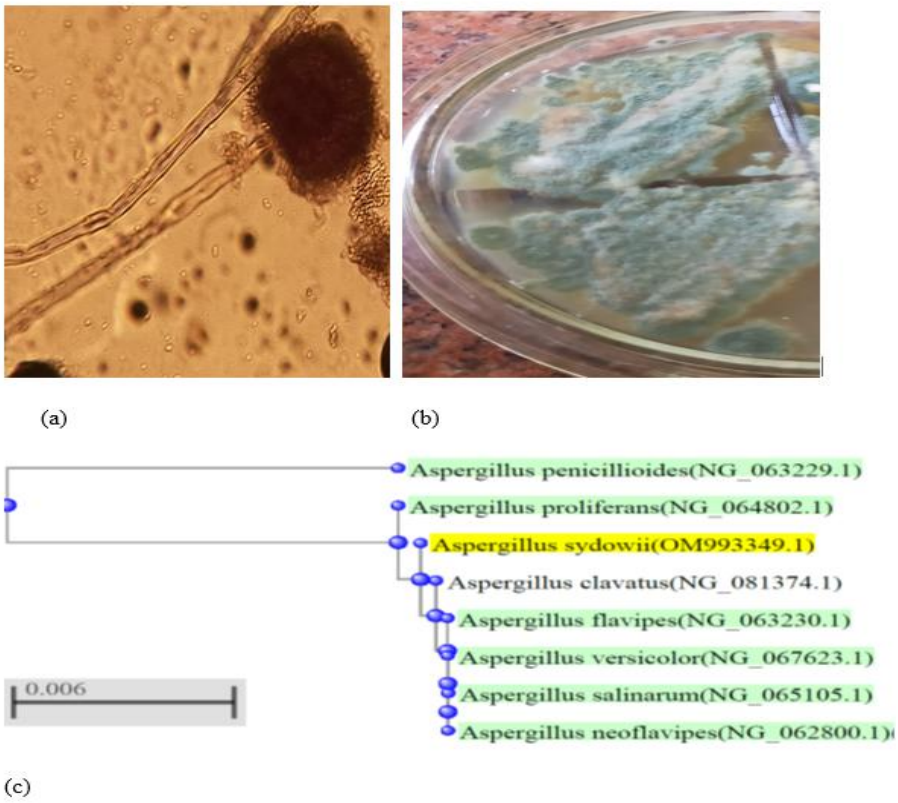

Figure 1. Microscopic features (a) and macroscopic features (b) of the colony on Czapek's agar media, and neighbor-joining phylogenetic tree (c) of *Aspergillus sydowii*.

Sequencing of the 18S rRNA gene of Aspergillus sydowii 195

The 18S rRNA gene sequence of *Aspergillus sydowii* 195 was determined, and a phylogenetic tree was constructed. Genomic DNA from *Aspergillus sydowii* 195 was used to enzymatically amplify the 18S rRNA gene, based on biochemical and staining properties, using universal fungal primers and Taq DNA polymerase. A phylogenetic analysis of *Aspergillus sydowii* (Figure 1c) and comparing closely related strains from databases revealed a clear identity to *Aspergillus sydowii*. The *Aspergillus sydowii* rRNA sequence has been deposited in the Gene Bank with the accession number OM993349 (http://www.ncbi.nlm.nih.gov/nuccore/OM993349).

Extraction and isolation of Peniciketal A

*Aspergillus sydowii*195 was cultivated in 100 Erlenmeyer flasks for 7 days at 30°C. Afterwards, the medium and fungal biomass were blended at 16,000 rpm and extracted using ethyl acetate. The organic phase was evaporated to yield a 5.0 g oily brownish extract. A silica gel column (CH_2Cl_2-MeOH; 99.5:0.5, v/v) was used to fractionate the extract, resulting in four main single fractions: F1, F2, F3, and F4. The single fractions were further fractionated using a Sephadex LH-20 column (CH_2Cl_2-MeOH; 60:40, v/v). The antioxidant activity of these four fractions was tested (Figure 2), and F2 exhibited the best scavenging ability.F2 was then subjected to

preparative TLC sub-fractionation using n-hexane-ethyl acetate (9:1 v/v), resulting in four pure sub-fractions (A, B, C, and D). An antioxidant activity test was conducted to screen the sub-fractions (Figure 3).

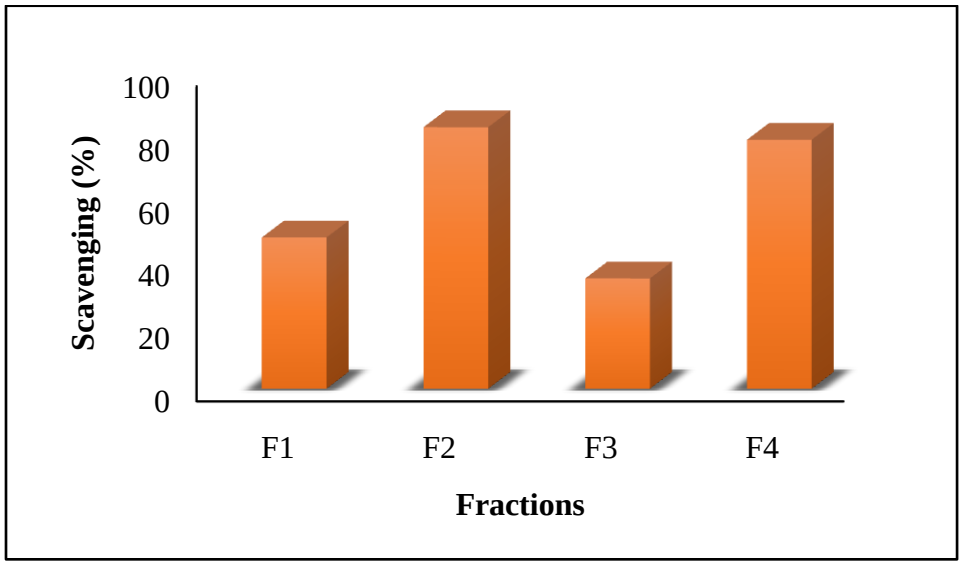

Figure 2. The scavenging ability of different fraction extracts of *A. sydowii* 195.

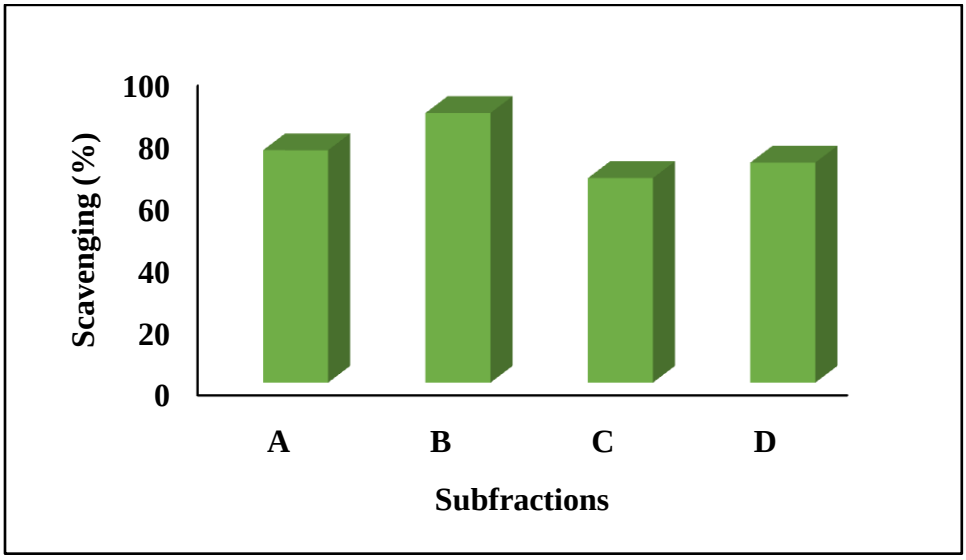

Figure 3. The scavenging ability of different purified sub-fractions from fraction 2 of *A. sydowii* 195.

Elucidation of sub-fraction B structure

The active sub-fraction B was isolated from the ethyl acetate extract of *Aspergillus sydowii*195 and subjected to structure elucidation. Sub-fraction B is light yellowish, water-insoluble, and soluble in most organic solvents, including ethyl acetate, ethanol, chloroform, and hexane. It appears as a single spot on TLC Merck Kieselgel 60 F254 (Rf:0.25) using ethyl acetate: n-hexane (1:9) solvent system under long wavelength UV light (Figure 4). The molecular weight of sub-fraction B was determined to be 539.2279 by EI-MS. Its molecular formula was

$C_{30}H_{36}O_9$, [M+] 279.2, with quadrilateral carbons, seven methylenes (one oxygenated), four methines (three O2-bearing), and two secondary methyl groups (Figure 5). The UV spectra (Ethanol) at λ_{max}= 206 nm are shown in Figure 5.
The IR spectrum showed a carbon band (C=) at 1600, 1628 cm^{-1}, a strong band of C-O at 1123 and 1274 cm^{-1}, a band of C-H at 2958, 2926, and 2858 cm^{-1}, and a strong O-H band at 3425 (Figure 6). The 1HNMR and 13CNMR spectra were obtained in a $CDCl_3$ solution at 500 MHz, suggesting the following structures: two phenyl rings (one hexasubstituted, and a second pentasubstituted), seven methylenes (one oxygenated), four methines (three O_2-bearing), quaternary carbons (two doubly oxygenated), four methines (three O_2-bearing), two aromatics, and two secondary methyl groups with four additional rings. Based on the UV, IR, 13CNMR, 1HNMR, and EI-MS results, sub-fraction B could be Peniciketal A as represented in Figure 7.

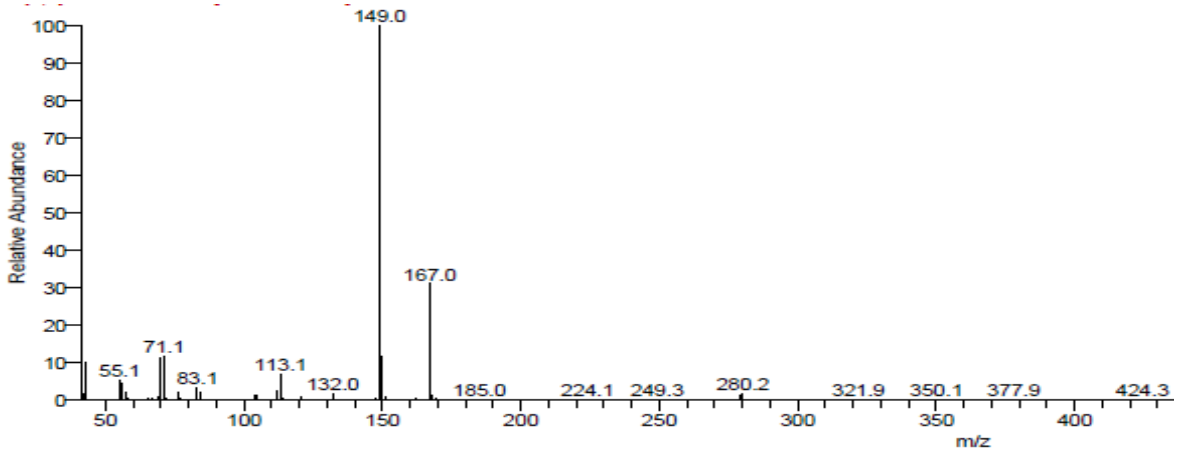

Figure 4. EI-MS of active sub-fraction B.

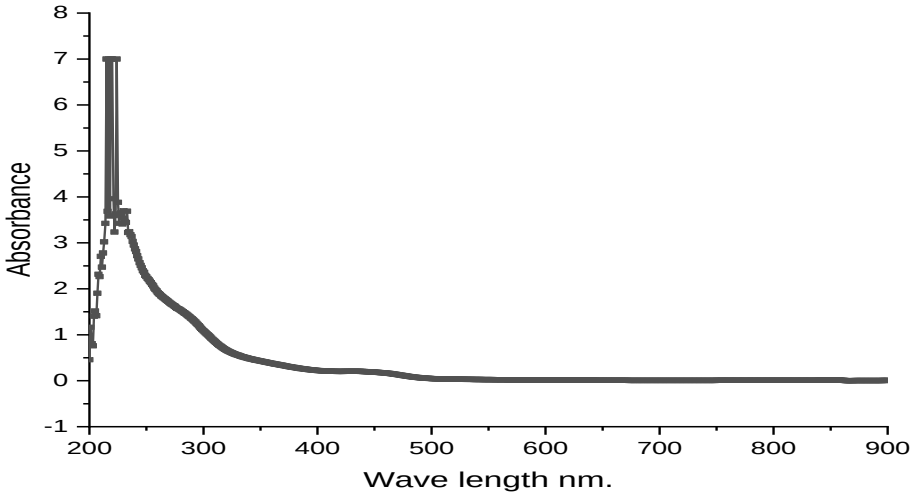

Figure 5. UV spectra of active sub-fraction B.

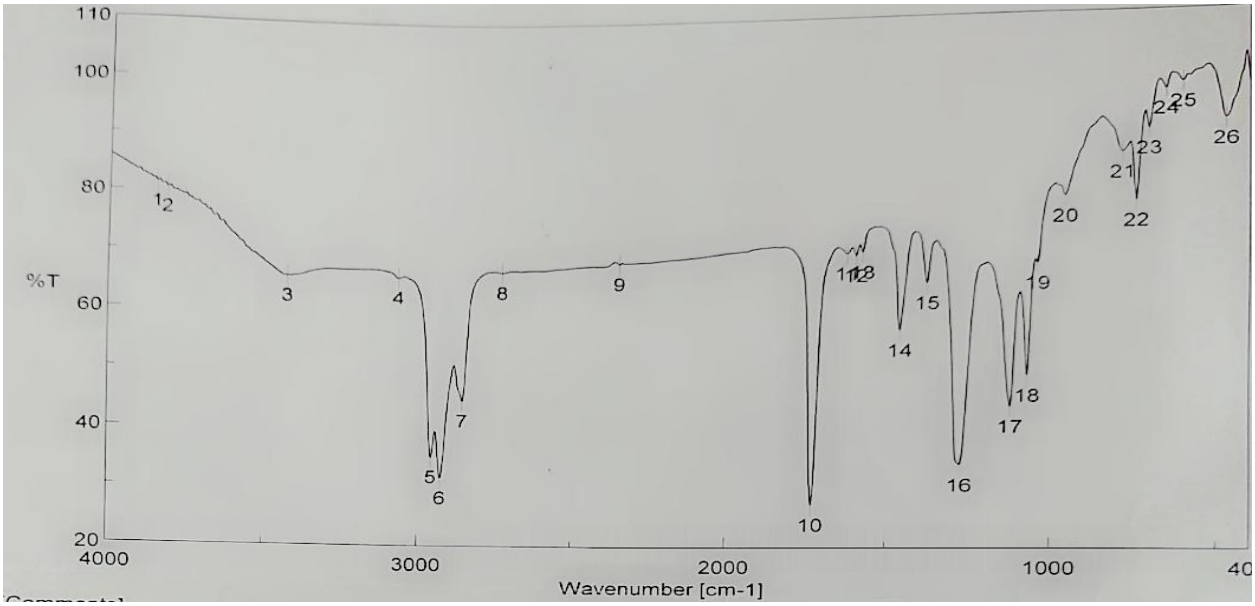

Figure 6. The IR spectrum of active sub-fraction B.

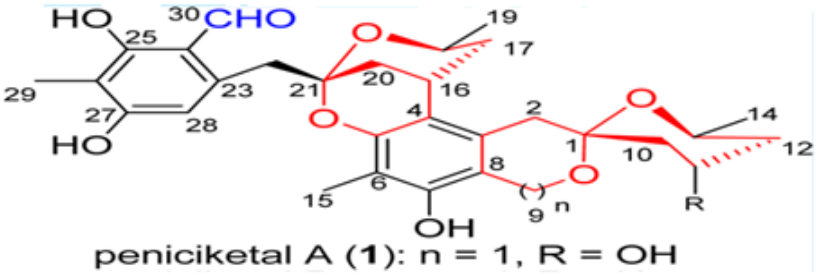

Figure 7. The sub-fraction B suggested chemical structure.

Biological evaluation of Peniciketal A

1. Antioxidant activity

The antioxidant activity (scavenging effect) of Peniciketal A was evaluated using DPPH at 517 nm absorbance, revealing a proportional relationship between the antioxidant activity and the concentration of Peniciketal A (Figure 8). The antioxidant activity increased as the concentration of Peniciketal A increased from 2.0 $mg.ml^{-1}$ to 12.0 $mg.ml^{-1}$. The maximum antioxidant activity of 89.98% was observed at 12.0 $mg.ml^{-1}$. The results showed that the IC_{50} of Peniciketal A was 2.0 $mg.ml^{-1}$ after 90 min.

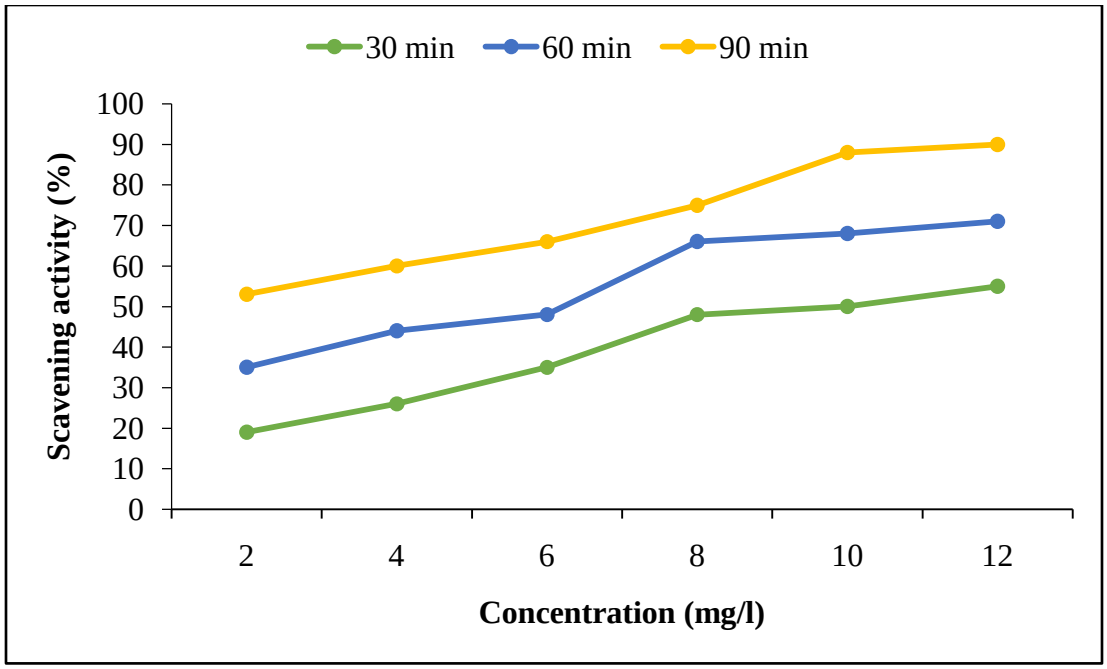

Figure 8. Antioxidant activity of peniciketal A.

2. Antifungal activity

The antifungal activity of Peniciketal A was assessed using the drop diffusion method against four isolated food-borne fungal species (*A. flavus*, *A. niger*, *Penicillium* sp., *Rhizopus* sp.). The results in Table 1 revealed significant antifungal activity of Peniciketal A against all the tested food-borne fungal species. The antifungal potency of Peniciketal A was strong against *Penicillium* sp., *Rhizopus* sp., *A. niger*, and *A. flavus*.The minimum inhibitory concentration (MIC) of Peniciketal A against food-borne fungi was also examined. The results in Table 2 demonstrated that Peniciketal A had variable MIC values ranging between 400 μgml^{-1} and 500 $\mu g.ml^{-1}$, indicating its antifungal activity. The results obtained from the drop diffusion method and MIC measurements indicated the antifungal properties of Peniciketal A against the tested food-borne fungal species.

Table 1. Antimicrobial activity of peniciketal A against food-borne fungi using the drop diffusion method.

Fungal strains	Diameter of inhibition zone*
A.flavus	66
A. niger	69
Penicillium sp.	75
Rhizopus sp.	52

*Mean diameter of inhibition of growth in mm.

Table 2. Minimum inhibitory concentration (MIC) of peniciketal A against food-borne fungi.

Fungal strains	MIC for peniciketal A (µg/ml)
A.flavus	400
A. niger	500

Penicillium sp.	400
Rhizopus sp.	400

3. Killing potential assay

The colony-forming units (CFUs) of food-borne fungi, such as *Aspergillus flavus*, *A. niger*, *Rhizopus* sp., and *Penicillium* sp., were monitored at 12-hour intervals for 72 h following exposure to Peniciketal A (16 mg.ml^{-1}). A sterile saline solution, which did not affect fungal growth, was used as a control. The results indicated that Peniciketal A had notable killing power against the tested fungi (Figure 9).Furthermore, after 60 h of contact, Peniciketal A reduced the fungal counts by half.

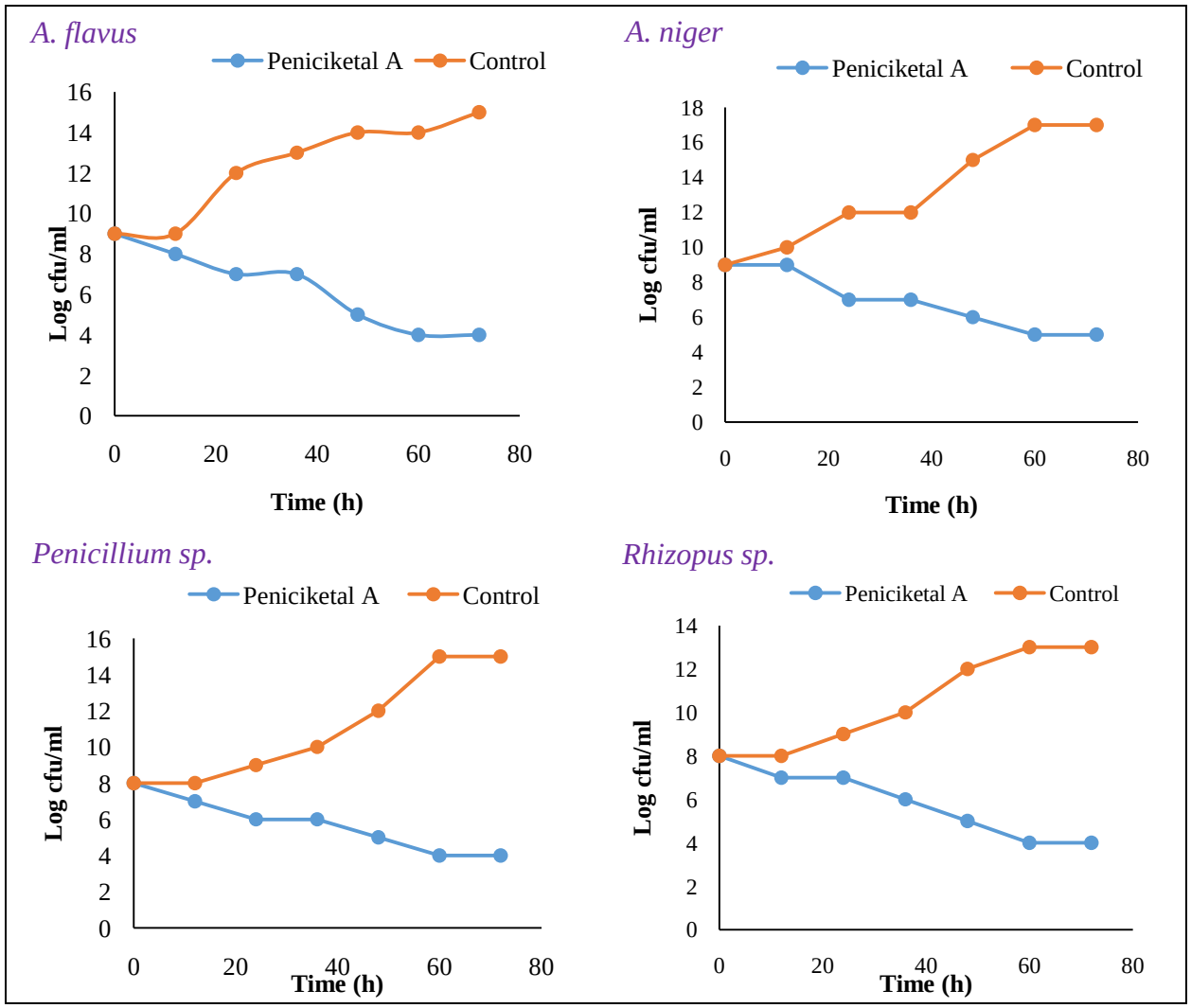

Figure 9. Killing kinetics of peniciketal A against food-born fungi.

Discussion

Scientists have long focused on well-known bacterial, parasitic, and viral food-borne pathogens while overlooking fungal diseases. Food-borne infections pose a serious and ongoing problem for healthcare systems, leading to enormous economic and social burdens. As the primary decomposers, Fungi are widely distributed in different types of soils and play a major role in biogeochemical cycles. They also can produce many secondary metabolites with therapeutic properties, which are essential to the pharmaceutical industry [34].

In many countries, most fungal pathogens can cause severe problems for the healthcare system, resulting in severe damage to human health and, in many cases, death [3]. Recently, scientists and researchers have become increasingly interested in fungal secondary metabolites, which have distinct chemical structures and fascinating biological activities and characteristics.The first discovery of Peniciketal A (Pe-A), a new spiroketal compound, was reported by Liu et al. [14]. They extracted three new types of spiroketals, Peniciketals A, B, and C, from the saline soil fungus *Penicillium raistrickii* fermented in a seawater-based culture medium [18,34].

As part of our ongoing efforts to discover novel fungal secondary metabolites, we have extracted and identified a new spiroketal from *Aspergillus sydowii* 195. This spiroketal has a benzo-fused 2,8-dioxabicyclo [3.3.1] nonane moiety called Peniciketal A (Pe-A). The Peniciketal structure comprises a phenyl ring linked with either [6,6]- or [5,6]-spiroketal and a 2,8-dioxabicyclo-[3.3.1] nonane moiety.

Peniciketal A produced by *Penicillium raistrickii* exhibits cytotoxicity against HL-60 cells, with IC50 values of 3.2 μM [35].Peniciketal A has promising potential as an anticancer drug and exhibits many biological activities[15]. Extracted from *Penicillium raistrickii*, Peniciketal A has been found to have notable activity against human cancer cells [18]. However, the molecular mechanisms underlying these effects are still poorly understood.

Zhang et al. [36] proposed a possible biogenetic pathway for Peniciketal A, as shown in Figure 10. Biotransformation of malonyl CoA and acetyl malonyl produces the common precursor (1). The common precursor (1) is then methylated and cyclized to form intermediate compound (2), which then transforms through different reaction pathways into intermediate compound (3). Finally, intermediate compound (3) is converted into Peniciketal A through electrophilic aromatic substitution and ketal formation.

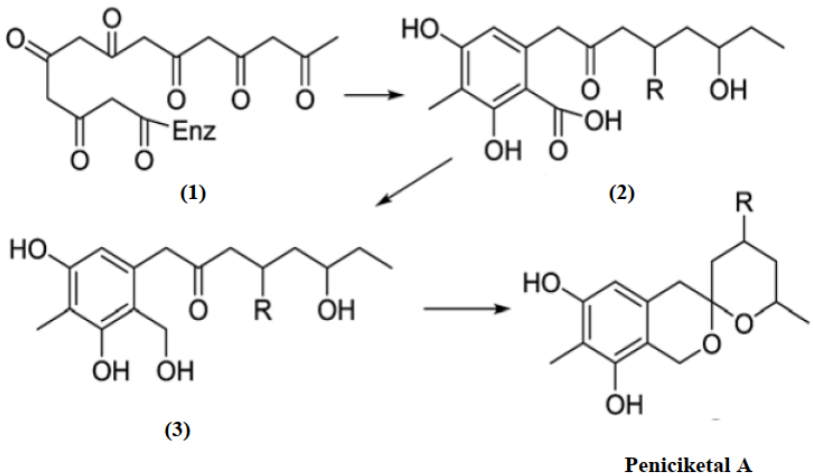

Figure 10. The possible biogenetic pathway for peniciketal A (modified from Zhang et al. [36]).

Pe-A has been found to exhibit cytotoxicity and some anticancer activity in cell lines. For instance, Pe-A reduces cell proliferation in leukemia and inhibits/proliferate human lung cancer [35]. Importantly, Pe-A showed considerably lower toxicity for normal cells, indicating high selectivity for cancer cells. Due to its high antitumor activity, studies such as the global proteomic profile for Pe-A have been carried out, suggesting that Pe-A is a natural secondary metabolite with additional bioactivities that make it a promising anticancer drug [35].
This study is the first to demonstrate that Pe-A possesses novel biological activities, including antioxidant and antibacterial activities against food-borne fungi. Some researchers have suggested that the variation in Pe-A's bioactivities may be attributed to an increase in the expression level of the Bax protein and a decrease in the level of the Bcl-2 protein. This, in turn, can increase the membrane permeability of mitochondria in the cell, inhibiting cell reproduction and proliferation [37-39].
Zhu et al. [9]discussed the contributory role of initially reduced ROS, supplemented by N-acetyl cysteine as an antioxidant. They reported that ROS with N-acetyl cysteine incombination with polyphenon enhanced the negative effect of Pe-A on cell viability. However, further studies and research are needed to fully understand the potential effects and applications of Peniciketal A and develop useful antifungal and anticancer drugs.

Conclusions

This study is the first to isolate and chemically identify Peniciketal A from *Aspergillus sydowii* 195 isolated from soil. The unique structure of Peniciketal A and its multiple bioactivities, including its antitumor activity, make it a promising candidate for cancer treatment. Furthermore, based on published data, the present study demonstrated both the antioxidant and antimicrobial activities of Peniciketal A against some food-borne fungi for the first time.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Funding statement

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