

Ethyl Acetate Extracts Endophytic Fungi from the Medicinal Tree Fern *Cyathea Contaminans* (Hook) Copel with Antimicrobial Activity

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Abstract

Medicinal plants are a rich source of naturally occurring substances utilized in treating a wide range of diseases; as a result, they serve as the basis for discovering new drugs. Research has focused on the relationship between endophytic microorganisms and host plants, as they produce a variety of secondary metabolites with significant biological functions. This study aimed to extract the endophytic fungi from various plant parts, including the roots, barks, and leaves of the *Cyathea contaminans* (Hook) Copel, which was collected from Kerinci, Jambi, Indonesia. By employing direct planting and pouring techniques with fungal culture media, the fungi were separated from the roots, stems, hairs, and leaves of *C. contaminans* and grown on rice media. The secondary metabolite of the fungus was extracted using ethyl acetate. The extracts were then tested for antimicrobial activity using a disk diffusion assay against microbial pathogens *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC25922, and Methicillin-Resistant *Staphylococcus aureus* (MRSA). This research yielded 19 endophyte fungi. In the antibacterial activity screening results, four fungal strains (CBK3, CDK1, CDK4, and CAK2) were active against *S. aureus*, MRSA, and *E. coli*, with an inhibition zone in the range of 15.08 ± 0.854 to 23.52 ± 0.87 mm. All active fungal isolates were identified molecularly. CBK3 and CDK1 were *Paecilomyces subglobosus* PP510226 and *Penicillium citrinum* PP510227, respectively, while CDK4 and CAK2 were comparable to *Aspergillus terreus* PP510229 and *Aspergillus terreus* PP510228. The four endophytic fungi of *Cyathea contaminans* may be a new source of antibacterial compounds.

Keywords: Antimicrobial activity, Endophytic fungi, Internal Transcribed Spacer (ITS), *Cyathea contaminans*, *Paecilomyces subglobosus*, *Penicillium citrinum*, *Aspergillus terreus*

Introduction

Tree ferns are vascular plants that do not produce seeds and reproduce through spores. There are various species of tree fern or pole fern, including the plants *Cibotium barometz* and *Cyathea contaminans*. These plants have been traded internationally, so they are regulated by CITES regulations [1]. At 1st glance, these two plants are the same but differ in the hairs, scales, and spores. Many *Cyathea* species are grown and used for medicinal purposes. Several countries, such as India, Mexico, New Zealand, Venezuela, India, China, and Japan, use it as a medicinal plant [2-4]. Cyatheaceae (tree ferns) emerged from the time of the

dinosaurs, and several species still exist. This plant species is thought to survive because of the phytotoxic compounds it contains [5].

C. contaminans have been used in food, traditional medicine, plant ornamentation, crafting supplies, ornaments, and other benefits [2,3]. This fern's leaves, stems, hairs, and roots are utilized as medicinal ingredients. Several studies have reported that this fern has antimicrobial, anti-inflammatory, antioxidant, anti-seizure, flu medication, and hypocholesterolemia activities [8-10]. Cytotoxic and anticancer compounds are also found in *Cyathea* species [11]. Secondary metabolites contained in *C. contaminans* are saponins, alkaloids, flavonoids, tannins, and anthraquinones [4]. The new pteroflavonol compound is an acylated flavonol glycoside and is characterized chemically as a kaempferol-7-(6"-suxinal)-glucoside compound. This compound is reported to be distributed in 57 species of 11 genera in 3 families of plant ferns (Pterophyta) [13].

All plant species have endophytic fungi inside their tissues, which have been proven to create significant physiologically active compounds [5]. Endophytes are present in plant stems, roots, tubers, leaves, seeds, fruit, stem roots, and root [6]. The discovery of bioactive compounds from fungi associated with ferns (*C. contaminans*) is still limited, so ongoing research needs to be carried out.

Materials and methods

Identification of sample material

C. contaminans was collected in Sungai Bungkal, Jambi, Indonesia (Geographic coordinates: 2°3'32.1624"S and 101°21'56.2428"E) (**Figure 1**). The ANDA Herbarium identified the plant at the Department of Biology, Faculty of Mathematics and Natural Science, Andalas University in Padang, Indonesia. The letter number for plant authentication was 463/K-ID/ANDA/VII/2023, and the voucher number was 00051803 to 00051805.



Figure 1 Tree fern *Cyathea contaminans*.

Isolation of endophytic fungi from branch stem, root, scale, and leaves of *C. contaminans*

Endophytic fungi are isolated in 2 ways: By pouring and direct planting. The pouring method is done by cleaning each sample with distilled water, sterilizing the surface of the plant part with 70 % alcohol by dipping for 1 min, rinsing again with distilled water, then cutting the sample into small pieces or grinding it as much as 10 g and placing it in an Erlenmeyer bottle and adding 100 milliliters of purified water. Subsequently, dilutions were carried out up to a concentration of 10^{-6} , incubated at 25 - 30 °C for 5 - 7 days after being inoculated on SDA medium. Direct planting is done by scraping or splitting plant parts and sterilizing the surface. Spattered and sterilized plant parts are placed on Sabouraud dextrose agar (SDA) media. Samples were cultured for 5 - 7 days at 25 - 30 °C. Isolates are colonies distinct from other colonies in terms of color and form. The isolate was transferred to a fresh medium and purified until a pure isolate was obtained [7,8].

Cultivation and extraction of the secondary metabolites

Pure fungus isolates are cultured in rice media and incubated for 4 - 6 weeks. Endophytic fungal isolates that had grown optimally had their secondary metabolites extracted with ethyl acetate by maceration for 24 h, repeating the maceration until the extract solution was clear. A rotary evaporator was used to evaporate the resulting extract solution. The antibacterial activity of the extracted material was examined [7,9,10].

Assay of antibacterial activity

Staphylococcus aureus ATCC29213, *Escherichia coli* ATCC25922, and clinical strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA) are pathogenic bacteria used in screening the antibacterial activity of ethyl acetate extract of endophytic fungi. The ethyl acetate extract was made at a concentration of 5 % w/v in dimethylsulfoxide (DMSO). Each of 10 µL was dropped onto a 6 mm sterile disk (Advantec®) and placed on Nutrient Agar media (Merck®) containing 0.5 McFarland bacterial suspension. A sterile disk containing 30 µg/mL chloramphenicol (Oxoid®) was used as a positive control. The test media was incubated at 37 °C for 24 h at 37 °C. The diameter of the inhibition zone was measured using a caliper, and the antibacterial activity was displayed based on data on the diameter of the inhibition zone (mm) [11].

Phytochemical assays

Four ethyl acetate extracts from endophytic fungi with the inhibition zone with the highest antibacterial activity were carried out by phytochemical examination to determine the content of secondary metabolites, namely alkaloids, flavonoids, phenolics, terpenoid, and steroid extracts, following the procedures carried out by previous research [12,13].

Detection of alkaloids

1 mL extracts are dissolved in dilute hydrochloric acid and filtered. The filtrate was added to 4 drops of Mayer reagent. A yellow precipitate formation indicates the presence of alkaloids.

Detection of Flavonoids

1 mL extract was diluted with 5 mL of hydrochloric alcohol in the test tube. Two to three magnesium chips are added. The addition of three drops of isoamyl alcohol intensifies a pink-to-orange color, indicating the presence of flavonoids.

Detection of phenolic

1 mL extract was dissolved in 0.5 mL diluted hydrochloric acid (10 %) and then filtered. Three to four drops of ferric chloride acid solution were added to the extract. A blue color indicates the presence of phenolic.

Detection of steroid and terpenoid

1 mL extract was dissolved with chloroform solvent and filtered. The filtrate was added with 1 to 2 drops of acetic anhydride (99 %) and concentrated sulfuric acid. The formation of blue to violet color of the filtrate indicates the presence of steroid, while the reddish-brown colour of the filtrate will show positive terpenoid.

Fungi identification

Macroscopic and microscopic identification

Macroscopic observation of endophytic fungi is used to identify them based on their morphology, namely the color and shape of the colony. Lactophenol solutions were used to observe endophytic fungus under a microscope. The experiment was seen under a microscope after spreading lactophenol on a single fungal loop on a glass surface and covering it [14,15].

Molecular identification

DNA extraction was done by modifying Fatimawali *et al.* [16]. The internally transcribed spacer (ITS1 and ITS2) DNA barcode segments were used to identify four endophytic fungal strains with the highest antibacterial activity. The Ferrer *et al.* [17] approach was used to carry out the amplification reaction. First, Base Malaysia received the PCR products for sequencing. The neighbor-joining approach was used to build the phylogenetic tree on the MEGA 7.0 program, and the bootstrap value for the *p*-distance model was adjusted to 1,000 replications [18]. The accession number was then obtained by depositing GenBank's nucleotide sequence data of possible endophyte fungal strains.

Results and discussion

Our current research focuses on endophytic fungi in the *C. contaminans* plant. The fern is native to Jambi, Indonesia. The study yielded 19 endophytic fungi isolated from various plant tissues. Each fungal strain was named CDK, CBK, CBIK, and CAK (C represents *C. contaminans*).

In the process of extracting secondary metabolites from endophytic fungi, ethyl acetate is used to maximize the extraction of secondary metabolites, which are generally semipolar, and minimize the extraction of other polar compounds, for example, carbohydrates, protein, minerals, and others from the rice medium [7]. The ethyl acetate extract of endophytic fungi was tested for antibacterial activity. Four of the 19 fungal isolates showed potential antibacterial activity against MRSA: CBK3, CDK1, CDK4, and CAK2. Their average inhibitory diameters were 23.52 ± 0.87 , 18.8 ± 0.131 , 17.39 ± 0.878 and 16.85 ± 0.199 mm, respectively. For *S. aureus*, the average inhibitory diameters were 21.36 ± 0.795 , 15.08 ± 0.854 , 19.52 ± 0.702 and 18.07 ± 0.56 mm. Against *E. coli*, CBK3, and CDK1 showed potential antibacterial activity with average inhibitory diameters of 19.755 ± 0.463 and 17.567 ± 0.743 mm, respectively.

The antibacterial activity of each fungal extract was tested at a 5 % concentration, as shown in **Tables 1 and 2**. The ethyl acetate extract from the endophytic fungus CBK3 demonstrates potent antibacterial activity against pathogenic and antibiotic-resistant bacteria. An inhibition zone diameter of 10 to 20 mm indicates firm inhibitory activity, while a diameter of ≥ 20 mm is excellent [19].

Table 1 Antibacterial activity of endophytic fungi from *C. contaminans* against pathogenic bacteria.

No	Fungus code	Inhibition zone (mm)		
		MRSA	<i>S. aureus</i>	<i>E. coli</i>
1	CBIK1	-	-	-
2	CBIK2	10.45	9.20	-
3	CBK1	10.66	9.35	-
4	CBK2	8.00	7.83	-
5	CBK3	24.51	20.48	19.48
6	CDK1	18.76	16.06	17.22
7	CDK2	8.00	6.62	-
8	CAK1	13.60	10.80	-
9	CDK3	-	-	-
10	CDK4	16.47	19.12	-
11	CDK5	8.91	7.50	-
12	CDK6	-	-	-
13	CAK2	16.71	17.43	-
14	CAK3	9.91	7.90	-
15	CBK4	-	-	-
16	CBK5	9.05	9.09	7.80
17	CAK4	-	-	8.50
18	CBK6	11.97	9.23	8.31
19	CAK5	8.83	-	-

Note: - = no inhibition

Table 2 Antibacterial activity of selected fungus isolates.

No	Fungus code	Inhibition zone (mm) \pm Standard Deviation		
		MRSA	<i>S. aureus</i>	<i>E. coli</i>
1	CBIK2	9.67 \pm 0.68	n	n
2	CBK1	10.85 \pm 0.62	n	n
3	CBK3	23.52 \pm 0.87	21.36 \pm 0.80	19.76 \pm 0.46
4	CDK1	18.80 \pm 0.13	15.08 \pm 0.85	17.57 \pm 0.74
5	CAK1	14.54 \pm 0.86	10.70 \pm 0.17	n
6	CDK4	17.39 \pm 0.88	19.52 \pm 0.70	n
7	CAK2	16.85 \pm 0.20	18.07 \pm 0.57	n
8	CBK6	11.27 \pm 0.83	n	n

Note: The value is expressed as the mean \pm standard deviation (SD); n = 3

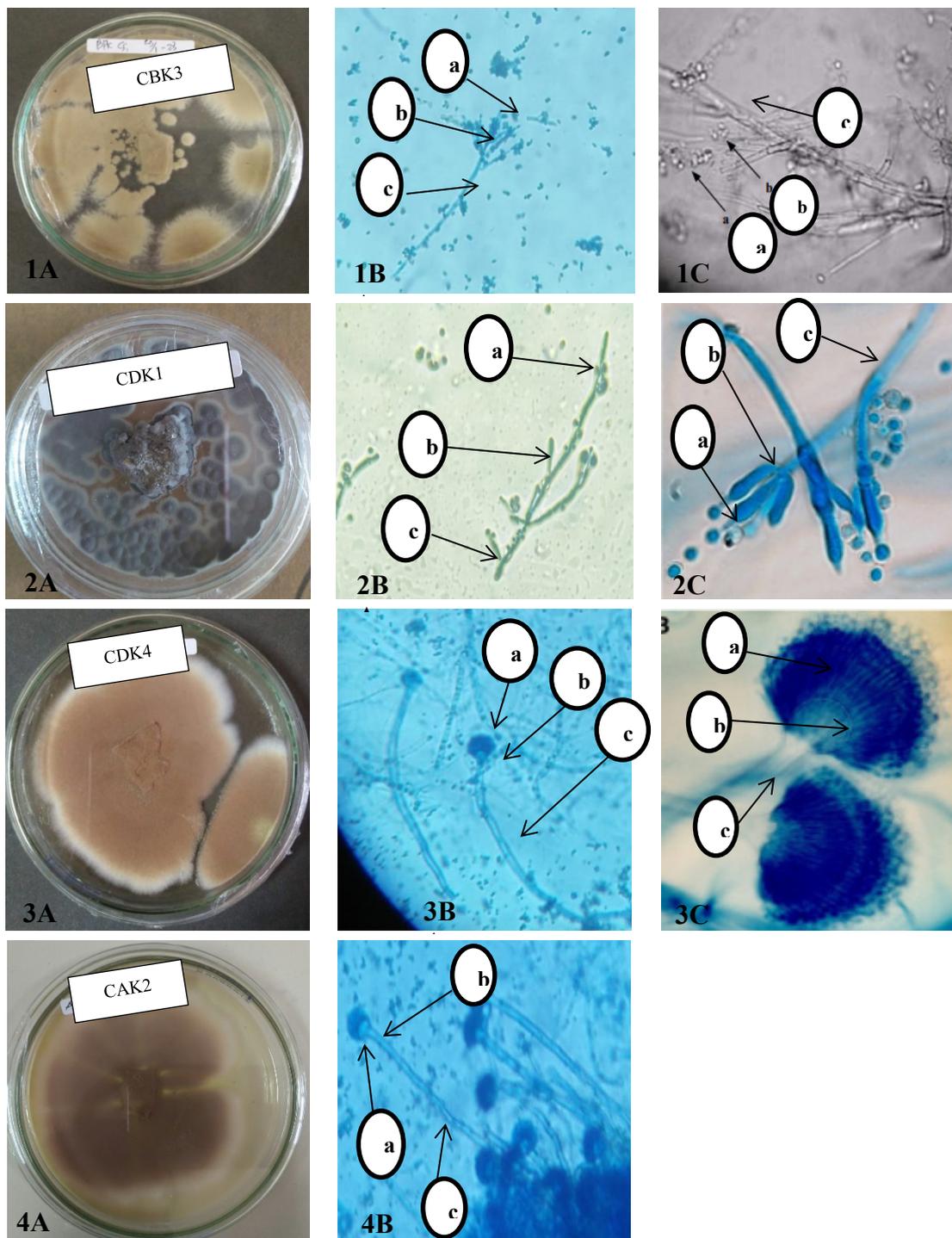
Table 3 Phytochemical assays result from selected extracts of endophytic fungi from *C. contaminans*.

No.	Fungus code	Phytochemical Assays			
		Alkaloid	Flavonoid	Phenolic	Steroid
1	CBK3	-	-	+	-
2	CDK1	+	+	-	-
3	CDK4	-	-	+	-
4	CAK2	-	-	+	-

Endophytic fungus extracts exhibit antibacterial activity that can potentially affect pathogenic bacteria through variations in inhibition zones among different fungus strains. Subsequent antibacterial activity testing was conducted on the extract of endophytic fungi with an inhibitory diameter equal to or greater than 10 mm (see **Table 2**).

Phytochemical tests were employed to determine the secondary metabolite compounds in the four antibacterial fungal isolates. The test results indicate that the extracts contain alkaloids, phenolics, and flavonoids, as shown in **Table 3**. The phytochemical analysis was used to screen the chemical components obtained before isolating the bioactive ingredient of the fungus. It should present the discoveries of the research.

Figure 2 displays macroscopic and microscopic observations of 4 bioactive fungi. The hyphae and characteristics of the colony surface, texture and color were noted during the macroscopic examination. The light microscope examined the spore or conidial characteristics and reproductive structure.



Note: a. Conidia, b. Fialid, c. Conidiophores

Figure 2 The macroscopic (A), microscopic (B), and microscopic fungi observations by other studies (C) [10,20,21] from 4 potential endophytic fungi, namely: 1. *Paecilomyces subglobosus*; 2. *Penicillium citrinum*; 3 and 4 *Aspergillus terreus* fungal strains are similar.

The fungus CBK3 isolate displayed macroscopic characteristics of a yellowish-brown color, a thin and smooth surface, and fading edges. The fungus exhibited conidia and elongated oval hyphae. The molecular analysis confirmed that the fungus matched the *Paecilomyces subglobosus* strain CBS 125145 (Figure 3). The fungus CDK1 strain displayed a uniform, slightly thick, bold grey color with flat borders on a large scale. CDK4 and CAK2 both exhibit a dark surface powder characteristic. The BLAST analysis indicates that the fungus is *Aspergillus terreus* strain YH Yeh I0108 (Figure 3).

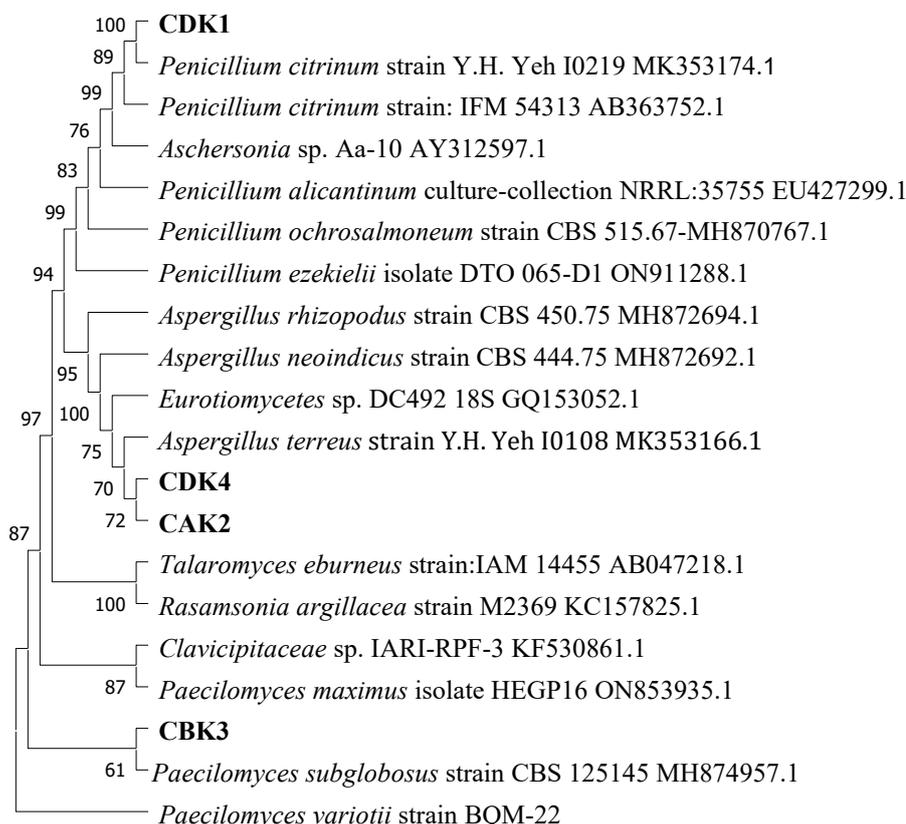


Figure 3 The phylogenetic tree inferred using the neighbor-joining method of ITS sequence of fungus derived from *C. contaminans* and its allied taxa.

The research is centered on plant endophytic fungi to uncover bioactive natural compounds. Experts found a symbiotic relationship of mutual benefit and equilibrium between endophytic fungi and the defense mechanisms of the host plant. Further investigation of this mutually beneficial relationship can yield bioactive compounds from endophytic fungi without disrupting the natural ecosystem, all while preserving plant biodiversity [22].

The CBK3 fungus isolate is a genetic match to *Paecilomyces subglobosus* strain CBS 125145. Information regarding the bioactive secondary metabolite compounds of this fungal species is limited. From the same genus, this fungus is known to have roles in biological control and applications in food, medicine, and environmental protection. The bioactive compounds produced by this fungus can be categorized as polyketides, sterols, quinones, pyrones, alkaloids, peptides, terpenoids, and fatty acids based on their chemical structure. Various biological actions have been proven, including nematicide, herbicide, anticancer, insecticide, antiplasmodial, antimalarial, and enzyme inhibitory properties [23].

Three dimeric xanthenes, Phomoxanthone A, Phomoxanthone B, and Dicerandrol B, were isolated

from the endophytic fungus *Paecilomyces* sp. EJC01.1 obtained from plants. Phomoxanthone A exhibits antimicrobial properties [24]. Two new compounds were obtained from the fungus *Paecilomyces* sp. KMU21009, originating from rhizosphere soil, is related to *Delphinium yunnanense*, which is a new hybrid sorbicillinoid class that has a rare sorbicillinoid urea unit and contains β -D-ribofuranose, named paeciureallin and a new monomeric sorbicillinoid named paecilkyetide. Both compounds have anti-inflammatory and cytotoxic activity *in vitro*. Paeciureallin showed moderate cytotoxicity against SW480 and A549 cell lines [25].

Indoleacetic acids and siderophores are metabolites produced by *Penicillium citrinum*, a phytopathogenic fungus found in plant-derived drugs [21]. The marine fungus *Penicillium citrinum* ZSS-9 synthesized penijanthe E, a new indole-diterpenoid compound and a previously identified analog, exhibiting antiviral properties against influenza [26]. Neotricitrinols A - C are novel compounds derived from a citrinin trimer found in *Penicillium citrinum* W23, which exhibit anti-osteoporosis properties. The source of these compounds is the deep sea [27]. The endophytic fungus *Penicillium citrinum* QJF-22 in mangroves yielded two benzopyran derivatives and a novel aliphatic chemical (S)-(3E,5Z,10E)-8-hydroxytridaka-3,5,10,12-tetraen-2-1. The samples were gathered on the island of Hainan [28].

Four compounds of a new chlorinated biphenyl, Aspergetherins A-D, were extracted from the *Aspergillus terreus* 164018 symbiotic fungus, which thrives on sea sponges. Evaluating the antibacterial potential of 4 new compounds against two generations of Methicillin-Resistant *Staphylococcus aureus* (MRSA) [29]. *Aspergillus terreus* GZU-31-1 was chemically manipulated to produce five new butanolide derivatives and four known diphenyl ether derivatives. This compound demonstrates anti-inflammatory properties [30].

Novel meroterpenoid terretonin O was isolated from Marine *Aspergillus terreus* LGO13 methanol extracts and thermophilic *Aspergillus terreus* TM8. Terretonins M and N, compounds extracted from the fungus LGO13, have demonstrated potential antimicrobial and cytotoxic properties [31]. *Aspergillus terreus* produced two new compounds: 2,2-dimethyl-3-hydroxychroman-6-aldehyde, a benzopyran derivative, and terreinlactone C, a spirocyclic lactone. Two compounds were evaluated for their cytotoxic effects on human cancer cells [32].

Conclusions

Four fungi with antibacterial properties have been isolated from different parts of the tree fern *C. contaminans*. The four fungi were identified as *Paecilomyces subglobosus* CBK3, *Penicillium citrinum* CDK1, and *Aspergillus terreus* CDK4/CAK2. Additional research on its bioactive compounds is necessary to better understand its potential as an effective antibacterial agent in the pharmaceutical industry.

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