

Mycotoxins Extracted from Exophytic and Endophytic Fungi Isolated from *Catharanthus roseus* Plant and Their Toxicity Effect

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Abstract

Secondary metabolites such as mycotoxins can be produced by fungi on agricultural commodities in the field. These fungi can colonize medicinal plants, such as *Catharanthus roseus*. On the PDA medium, 18 isolates of exophytic and endophytic fungi isolated from *C. roseus*, including *Aspergillus*, *Penicillium*, and *Talaromyces* species, were tested for mycotoxin production. Thin layer chromatography indicated the formation of six mycotoxins from six isolates, including aflatoxin G₁, aflatoxin B₁, sterigmatocystin, citrinin, aflatoxin B₂, and penicillic acid. To measure the impact of *C. roseus* leaf extract on the generation of fungal mycotoxin, toxic isolates were cultivated on a PDA medium supplemented with the leaf extract. It was discovered that enriched PDA media could enhance the formation of certain mycotoxins. Toxicity tests were performed on brine shrimp larvae *Artemia salina* for fungal mycotoxin extraction and *C. roseus* leaf extraction. Selected fungal extracts at 10 µg/ml were also harmful to brine shrimp larvae, while leaf extracts at 6.25-200 µg/ml were very toxic.

Keywords: Mycotoxins, *Catharanthus roseus*, Exophytic fungi, Endophytic fungi, TLC

INTRODUCTION

Fungal populations live on the surfaces of plants (exophytic) and within their tissues (endophytic). Long-term evolution has resulted in sophisticated interactions between various species, resulting in a symbiotic connection rather than them continuing as independent entities [1]. Interactions between these microbes and plants are thought to benefit plant survival, biodiversity, and ecosystem function. Most plant species have varied microbial endo- and exophyte populations, as well as non-pathogenic fungi that colonize the inside or surface of plants. Fungi that are exophytic or epiphytic can be found on the surface of living, healthy plant components. The name epiphytes is derived from two Greek words: 'epi,' which means upon, and 'phyton,' which means plant. They vary in size, density, and kind based on biotic and abiotic elements that influence their growth and vitality. Endophytic fungi are organisms that can live in all healthy plant tissues without interfering with their physiological functions. These microorganisms can regulate phytopathogens and herbivorous insects and create growth-promoting chemicals [2].

Secondary metabolites produced by fungi include mycotoxins, antibiotics, and pigments. Mycotoxins are naturally formed on agricultural commodities by filamentous fungi in the field and during storage under a variety of environmental circumstances [3]. Different mycotoxigenic species create a wide range of mycotoxins. Food and feed safety is largely concerned with the harmful mycotoxins

generated by certain filamentous fungus fungal genera, such as *Aspergillus*, *Fusarium*, and *Penicillium*, which comprise several species that produce the most mycotoxins globally. These comprise ochratoxins, fumonisins, zearalenones, aflatoxins, and trichothecenes. The most significant mycotoxin that *Aspergillus* species produce is aflatoxin. It is significant to remember that aflatoxins come in four main forms: B₁, B₂, G₁, and G₂. The luminous blue and green colors on thin-layer chromatography (TLC) plates under UV light are the source of the designation "B" and "G." Thus far, hundreds of distinct mycotoxins have been identified, displaying a wide range of structural variations that lead to distinct chemical and physical characteristics [4]. Mycotoxicosis is the name given to the toxic reaction that

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How to cite this article: Alsubaie Sh, Bokhari F, Hassoubah Sh, Najjar A. Mycotoxins Extracted from Exophytic and Endophytic Fungi Isolated from *Catharanthus roseus* Plant and Their Toxicity Effect. Arch Pharm Pract. 2023;14(4):160-8. <https://doi.org/10.51847/EVSwmc9NCd>

occurs when mycotoxins are consumed. Mycotoxins have varied degrees of deleterious impact on animal health depending on the type, dose, and duration of ingestion. These compounds can have a wide range of harmful effects on both animals and humans, including cytotoxicity, nephrotoxicity, neurotoxicity, carcinogenesis, mutagenesis, immunosuppression, and estrogenic effects. Climate change, which has a major effect on fungal development, dispersion, and mycotoxin production, may enhance the hazards mycotoxigenic fungi pose to food and feed safety [5]. Fungi seek an environment in which most nutrients may be supplied in considerable quantities to proliferate and create secondary metabolites. Carbohydrates, proteins, and vital fatty acids are examples of these nutrients. While botanicals have a long history and are widely used as foods and traditional remedies, few investigations on molds and mycotoxins contamination have been undertaken, compared to numerous papers on the contamination of grains and oilseeds. Botanicals have been found to have high quantities of *Aspergillus*, *Penicillium*, and *Fusarium* species [6].

Catharanthus roseus is a tropical plant endemic to Madagascar and southern Asia that is classified as a member of the Apocynaceae family. *Catharanthus roseus* is an important medicinal plant that plays an important part in worldwide health. The medicinal plant *C. roseus*, according to researchers, has a group of alkaloids that are highly poisonous but have therapeutic potential. Plants were able to perform several biological activities and protect against predators such as insects, fungi, and herbivorous mammals due to their ability to synthesize a diverse range of chemical compounds. Various extracts and isolated compounds of *C. roseus* have been reported to have anticancer and cytotoxic, antibacterial, antidiabetic, antioxidant, anthelmintic, and larvicidal effects. Carbohydrates, flavonoids, saponins, and alkaloids are found in *Catharanthus roseus* [7]. The goal of this study was to look at the potential of fungal isolates to produce mycotoxin and their toxicity.

MATERIALS AND METHODS

Media Preparation

Two media were employed for fungal mycotoxin generation screening: PDA with 200 dextrose 20, agar15, and 1L distilled water. The second was a PDA medium supplemented with leaf extract containing 250 mL of PDA medium and 400 mg/L of *C. roseus* leaf extract.

Screening of Mycotoxin Extracted from Fungal Isolates Growing on PDA Medium

Exophytic and endophytic fungi, including *Aspergillus*, *Penicillium*, and *Talaromyces* species, were used to isolate 18 isolates. These selected isolates are capable of producing various mycotoxins. Fungal isolates were cultured in PDA medium for 10 days at 28°C [8]. Agar plugs were removed with a flame-sterilized stainless-steel cork borer (4 mm) at the colony's core. In a screw-capped bijou bottle, the plugs were

immersed in 2 ml of chloroform: methanol combination (2:1, v/v). For each extract, 20 ul of the mixture was spotted onto the application line TLC plates with a micropipette and dried with a cool domestic hairdryer. In the solvent system, individual plates containing extract spots were created. The organic layer was collected in amber vials and stored at -20 °C for later investigation.

Effect of Supplemented PDA Medium with Leaf Extract on Toxicogenic Isolates

Absolute ethanol was used to make the *C. roseus* leaf extract. Twenty g of the leaf samples were homogenized with 200 cc of the solvent. The crude preparation was placed in the shaker overnight at room temperature before being centrifuged at 4000 rpm for 20 minutes. After that, the leaf extract-containing supernatant was moved to a beaker that had been previously weighed. The solvent was then evaporated at 60°C to concentrate the extract. The crude extract was weighed and diluted in a predetermined volume of dimethyl sulfoxide (DMSO) and then refrigerated in a sterile bottle until utilized [9]. To assess the capacity of the fungal isolates to create mycotoxins, a PDA medium supplemented with *C. roseus* leaf extract was utilized as a supplementary nutritional source. Toxicogenic fungal isolates were plated on PDA supplemented with (400 mg/L) *C. roseus* leaf extract and cultured for 10 days at 28°C. The same approach was then used to record fungal mycotoxins. They were compared to isolates that had been plated on a PDA medium as a control.

Fungal Mycotoxin Extraction

Based on mycotoxin production, six fungal isolates were chosen from exophytic and endophytic fungi, including *Aspergillus*, *Penicillium*, and *Talaromyces* species. Fungal isolates were plated on PDA medium and PDA medium enriched with leaf extract and cultured at 28°C for 10 days [10]. Agar plugs were extracted from each medium plate using a flame-sterilized scalpel and a flame-sterilized stainless-steel cork borer (4 mm) that was used to cut out the plugs close to the colony's center. The plugs were placed in a screw-capped bijou bottle with 2 ccs of chloroform: methanol combination (2:1, v/v). For each extract, 20 l of the mixture was placed onto the application line TLC plates with a micropipette and dried with a domestic hairdryer (cold). In the solvent technique, individual plates dotted with extracts were created. The organic layer was collected in amber bottles and kept at -20 °C for subsequent study.

Detection of Mycotoxin by TLC Protocol

(aluminum-backed, DC-Alufolien-Kieselgel silica gel matrix) was cut to 10 × 10 cm and spotted 1.5 cm from the bottom with 10 l aliquots of extracts or standards for mycotoxin extracts analysis. The plates were developed at room temperature in the solvent solution (toluene, ethyl acetate, and 90% formic acid, T: E: F, 5:4:1 v/v) until the solvent front reached a line indicated 1cm from the plate's top. The plates were taken from the solvent and air-dried in a fume cabinet without any extra chemical reagents after

development. The plates are analyzed in a Chromato-Vue cabinet (Model UVP Upland, CA, USA) using UV light at short (254 nm) and long (366 nm) wavelengths, as reported by Samson *et al.* (2004) [11]. Colored chemicals were studied under visible light, while fluorescent substances were examined under UV light. Sarker *et al.* (2018) [12] previously detailed the derivation of the retention factor (Rf) value for mycotoxin toxicity.

Toxicity Test

In this part, six fungal mycotoxin extractions and six *C. roseus* leaf extractions were performed to assess their toxicity on the brine shrimp *A. salina*.

Brine Shrimp Larvae Bioassay

Five grams of brine shrimp *A. salina* eggs were hatched in a 300-milliliter Erlenmeyer flask with 100 milliliters of natural seawater for toxicity testing. The eggs were then incubated at 28°C for 24 to 30 hours under gentle aeration on a rotary shaker that ran at 140 revolutions per minute. Following their hatch, the larvae were gathered and kept at room temperature in fresh saltwater. The population density was adjusted so that each petri dish had roughly 20 larvae in 1 ml of solution [13]. For six fungal mycotoxin extractions, concentrations of 2, 6, and 10 µg/ml were utilized. For leaf extract, concentrations of 0.7, 1.5, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/ml were used. Additional petri plates were filled with dimethyl sulfoxide (DMSO) and incubated at 28°C as controls. The average number of larval deaths in each concentration was calculated after 6 hours. Harwig and Scott [14] assessed the toxicity of each extract. the following: 0-9% death rate for non-toxic (NT), 10-49% mortality rate for slightly toxic (ST), 50-89% mortality rate for toxic (T), and 90-100% mortality rate for extremely toxic (VT). Furthermore, Fauziah *et al.* (2022) [15] calculated the lethal concentration (LC₅₀) that killed fifty percent of the larvae. Agha *et al.* (2022) [16] used the following equation to compute the percentages of deaths:

$$\text{Mortality\%} = \frac{\text{no. of dead larvae}}{\text{no. of larvae introduced}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Screening of Mycotoxins Extracted from Fungal Isolates Growing on PDA Medium

Exophytic isolates *A. caespitosus*, *A. flavus*, *A. Janus*, *A. nidulans*, *A. niger 1*, *A. niger 2*, *Aspergillus* sp.1, *Aspergillus* sp.2, *Aspergillus* sp.3, *P. brevicompactum*, *P. commune*, and *T. purpurogenus*, and endophytic isolates *A. caespitosus*, *A. elegans*, *Aspergillus* sp. 4, *A. versicolor*, *P. chrysogenum* and *Penicillium* sp. 1 were grown on PDA medium and tested for mycotoxin productions as shown in **Table 1**. Four of the twelve exophytic fungal isolates tested positive for mycotoxin production, accounting for 33% of the total. These isolates were *A. caespitosus*, *A. flavus*, *A. nidulans*, and *P. commune*. TLC plates exposed to UV light revealed that only three *Aspergillus* species and one *Penicillium* species

developed mycotoxins. Aflatoxin G₁ (green fluorescence) was created by *A. caespitosus*; aflatoxin B₁ (blue fluorescence) was produced by *A. flavus*; sterigmatocystin (light green fluorescence) was produced by *A. nidulans*; and citrinin (yellow-green fluorescence) was produced by *P. commune*. Additionally, two of the six endophytic fungal isolates, *A. elegans*, and *P. chrysogenum*, showed evidence of producing mycotoxin. TLC plates under UV light revealed that only one species of *Aspergillus* and *Penicillium* produces mycotoxins. Aflatoxin B₂ (blue fluorescence) was created by *A. elegans*, while penicillic acid (green fluorescence) was produced by *P. chrysogenum*. Three-quarters of the endophytic isolates (two out of six) were toxic. Thin-layer chromatography is an analytical method of separation used for qualitative analysis that makes it possible to identify unknown chemicals and see how complicated combinations of analytes react [17]. It is the most popular and successful analytical technique; it is inexpensive but time-consuming. Aflatoxin was discovered in 50% of the samples and was the most common mycotoxin produced by toxigenic isolates from exophytic and endophytic isolates on the PDA medium, totaling six mycotoxin productions. The findings demonstrated that *A. caespitosus* developed aflatoxin G₁ on a PDA medium for exophytic isolates. Contrary to our findings, Rabie *et al.* (1977) [18] observed that *A. caespitosus* generated fumitremorgin B on the PDA medium. On PDA medium, *A. flavus* developed aflatoxin B₁, which is consistent with findings reported by and Al-warshan *et al.* (2023) [19].

Furthermore, in the PDA medium, *A. nidulans* produced sterigmatocystin, which is consistent with the findings reported by Keller *et al.* (2000) [20]. *Penicillium commune* produced citrinin on the PDA medium in our investigation. Lešić *et al.* (2021) [21], who discovered that *P. commune* produced cyclopiazonic acid in a PDA medium, disagree with this result. Our research on endophytic isolates revealed that *A. elegans* generated aflatoxin B₂ on the PDA medium. In contrast to our findings, Palumbo *et al.* (2007) [22] discovered that *A. elegans* generated ochratoxin A on PDA media. Additionally, *P. chrysogenum* generated penicillic acid in PDA media; however, citrinin was reported to be synthesized by Devi *et al.* [23] in PDA medium. Nonetheless, exophytic fungi from various medicinal plants were found to harbor distinct mycotoxins, including aflatoxin, ochratoxin A, and citrinin, as reported by Ahmad *et al.* (2014) [24]. Prior studies have demonstrated that a wide variety of toxic exophytic organisms frequently infect plants [25]. Herbal medicine was found to have mildew infestations, which led to widespread fungal growth and mycotoxin accumulation, hence compromising the product's quality and effectiveness. When many mycotoxins are combined, their combined negative impacts on agricultural produce and animal health outweigh their individual effects. For instance, ochratoxin A does not have a significant death rate when used alone, but when combined with aflatoxins, it increases their damaging effects. Numerous studies have shown that mycotoxins produced by fungus endophytes deter insects and other grazing animals. By influencing plant communities and

having a major impact on the structure of such communities, these fungi can have a dramatic impact on the ecology and evolution of plants [26]. Other research revealed that mycotoxins could be produced by endophytic fungus, as described by Saini *et al.* (2021) [27]. Rodriguez and colleagues (2009) [28] categorized endophytes into four classes according to their ecological interactions and mode of transmission. Endophytic fungi may produce mycotoxins due to transmission processes. The endophytic isolate of the same species did not discover mycotoxin production in the current investigation, whereas the exophytic isolate *A. caespitosus* generated aflatoxin G₁. These findings corroborate those of Zainudin and Perumal (2015) [29], who discovered that although fungi are members of the same species, their distinct strains differ physiologically from one another. Thus, various fungal strains within the same species may be unable to create

mycotoxins. According to Dong *et al.* (2023) [30], strain-specific variations in the corresponding toxins' production are noticeable, potentially because of their disparate genetic backgrounds and other regulatory factors. According to Zhang *et al.* (2020) [31], fungal mycotoxin production may be enhanced by mutant genes. Fungal host-selective toxins (HSTs) are poisonous only to the host plants of the fungus that produces the toxin, according to research by Tsuge *et al.* (2013) [32]. By training a toxin-sensitive plant to be a good host, the producer can enter and colonize host cells thanks to the potential of this process. HST-producing isolates can only directly enter epidermal cells in plants that are susceptible to toxins. Both the germination of conidia and the hyphae's invasion of the host produce fungal HSTs. Nonspecific toxins, however, can harm a wide variety of plants, whether they are hosts of the pathogen that is creating them.

Table 1. Screening of mycotoxins extracted from fungal isolates under UV light growing on PDA medium at 28°C for 10 days.

SI no.	Fungal type	Fungal genus	Fungal species	Mycotoxin productions	Mycotoxin identified fluorescence under UV light	Other pigmentations	
1			<i>A. caespitosus</i>	+	Aflatoxin G ₁ Green	+	
2			<i>A. flavus</i>	+	Aflatoxin B ₁ Blue	+	
3			<i>A. janus</i>	-	-	-	
4			<i>A. nidulans</i>	+	Sterigmatocystin Light green	+	
5	Exo	<i>Aspergillus</i>	<i>A. niger 1</i>			+	
6			<i>A. niger 2</i>			+	
7			<i>Aspergillus</i> sp.1			-	
8			<i>Aspergillus</i> sp.2			-	
9			<i>Aspergillus</i> sp.3			+	
10			<i>Penicillium</i>	<i>P. brevicompactum</i>			-
11				<i>P. commune</i>	+	Citrinin Yellow-green	+
12			<i>Talaromyces</i>	<i>T. purpurogenus</i>	-	-	+
Total count			12	4	33%		
13	Endo	<i>Aspergillus</i>	<i>A. caespitosus</i>	-	-	+	
14			<i>A. elegans</i>	+	Aflatoxin B ₂ Blue	+	
15			<i>Aspergillus</i> sp.4			-	
16			<i>A. versicolor</i>	-	-	-	
17			<i>Penicillium</i>	<i>P. chrysogenum</i>	+	Penicillic acid Green	+
18				<i>Penicillium</i> sp.1	-	-	-
Total count			6	2	33%		

Effect of Supplemented PDA Medium with Leaf Extract on Toxicogenic Isolates

Aspergillus caespitosus, *A. flavus*, *A. nidulans*, *A. elegans*, *P. commune*, and *P. chrysogenum* were among the exophytic and endophytic toxic fungal isolates that were able to thrive on both control and supplemented PDA media containing *C. roseus* leaf extract. As seen in **Figure 1**, the effects of the supplemented PDA medium and the control PDA medium on the growth of various fungi, such as *A. flavus*, *P. commune*, and *P. chrysogenum*, are comparable. As seen in **Figure 2**,

the augmented PDA was able to promote the growth of various fungi, including *A. caespitosus*, *A. nidulans*, and *A. elegans*. Furthermore, **Figure 3** illustrates how the addition of leaf extract to the PDA medium enhanced the synthesis of mycotoxin in toxicogenic isolates when compared to control PDA media exposed to UV light. Aflatoxin G₁ (green fluorescence) and aflatoxin B₁ (blue fluorescence) were produced on the control PDA medium by exophytic isolates *A. caespitosus* and *A. flavus*, respectively. Verruculogen (greenish-blue fluorescence) and cyclopiazonic acid (red-

green fluorescence) were only presented and identified on supplemented PDA medium with *C. roseus* leaf extract. While the control PDA medium only detected aflatoxin B₂ by endophytic isolate *A. elegans*, the supplemented PDA medium containing *C. roseus* leaf extract was able to stimulate the production of aflatoxin B₂ with blue fluorescence, ochratoxin A with blue-green fluorescence, and sterigmatocystin with light green fluorescence. However, as **Figure 4** illustrates, the effects of the supplemented and control media are comparable in identifying the production of sterigmatocystin with light green fluorescence, citrinin yellow-green fluorescence, and penicillic acid with green fluorescence by the exophytic isolates *A. nidulans*, *P. commune*, and *P. chrysogenum*, respectively. In the current study, we found more compounds on TLC plates derived from fungal extracts that were grown on a PDA medium that was supplemented with *C. roseus* leaf extract. We proposed that plant chemicals induced fungal secondary metabolites to produce additional bands on TLC plates, which could be the consequence of pigments originating from plants. According to Frisvad (2012) [25], plant secondary metabolites stimulate the production of fungal secondary metabolites through epigenetic remodeling. Consequently, adding plant extracts to various media frequently increases the production of fungal secondary metabolites. As was covered in the preceding section, mycotoxin was formed at varying rates when fungal toxigenic isolates were cultivated on a PDA medium that was both controlled and supplemented with leaf extract. Toxigenic isolates on the leaf extract enriched PDA medium developed nine mycotoxins. On supplemented PDA media containing leaf extract, several mycotoxins were induced to be produced by certain toxigenic isolates, such as *A. caespitosus*, *A. flavus*, and *A. elegans*. Our findings for exophytic isolates demonstrated that *A. caespitosus* produced verruculogen on leaf extract-supplemented PDA medium but not on the control PDA medium. The findings of Schroeder *et al.* (1975) [33], who claimed that growing verruculogen on cracked field corn required specific nutrient needs to identify the verruculogen, were consistent with this conclusion. Furthermore, on a PDA medium enriched with leaf extract, *A. flavus* produced both cyclopiazonic acid and aflatoxin B₁, whereas only aflatoxin B₁ was formed on the control PDA medium. These findings corroborated those of Astoreca *et al.* (2014) [34] and Bíro *et al.* (2009) [35], who found that *A. flavus* produced more aflatoxin B₁ and cyclopiazonic acid when the Czapek yeast agar medium (CYA) and corn extract agar medium (CEM) had a high moisture content and were rich in carbohydrates and protein. Our results showed that *A. elegans* produced aflatoxin B₂, ochratoxin A, and sterigmatocystin on supplemented PDA medium with leaf extract, however only aflatoxin B₂ was formed on a control PDA medium for endophytic isolates. Furthermore, on yeast extract sucrose medium (YES), *A. elegans* produced ochratoxin A, according to reports by Batista *et al.* (2003) [36] and Akbar *et al.* (2016) [37]. There is no published material that we are aware of that discusses aflatoxin B₂ and sterigmatocystin generated by *A. elegans*. Furthermore, mycotoxin production was higher in PDA medium

supplemented with *C. roseus* leaf extract due to the availability of nutrients such as protein, fats, carbohydrates, and essential elements such as nitrogen, sulfur, phosphorus, potassium, and silicon for supporting fungal growth and secondary metabolite production [38]. Other toxigenic isolates, including *A. nidulans* (exophytic), *P. commune* (exophytic), and *P. chrysogenum* (endophytic), produced the same mycotoxins on both media in this experiment. *Aspergillus nidulans*, *P. commune*, and *P. chrysogenum* can all produce mycotoxins on diverse media, including YES, Malt extract agar (MEA), and Sabouraud dextrose agar (SDA) [39, 40]. Although *C. roseus* leaf extract is toxic, fungal isolates used in this study can consume toxic leaf extract and produce mycotoxins.

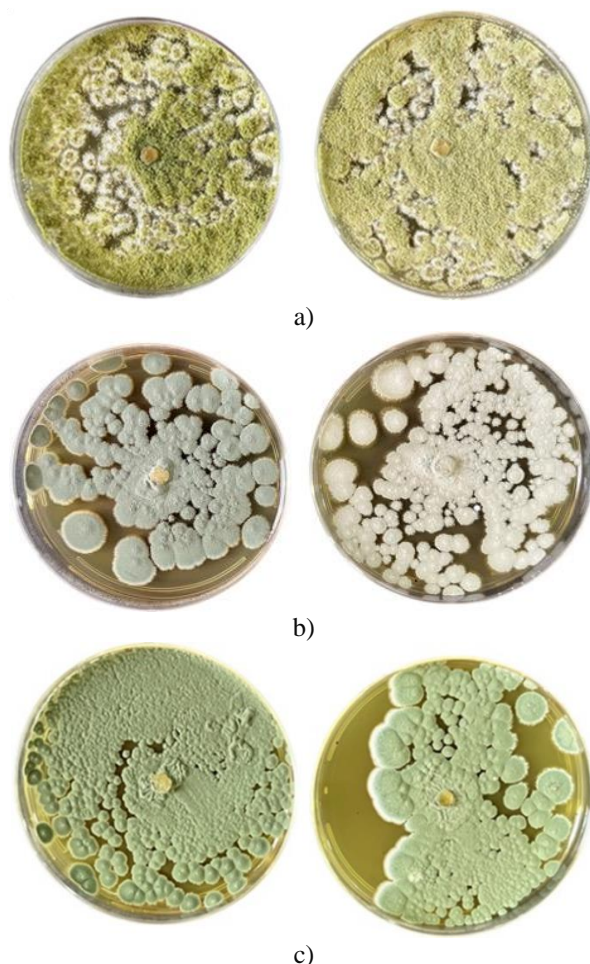


Figure 1. The control PDA medium (left) and supplemented PDA medium (right) have similar growth for (a) *A. flavus*, (b) *P. commune*, and (c) *P. chrysogenum* at 28°C for 10 days of incubation.

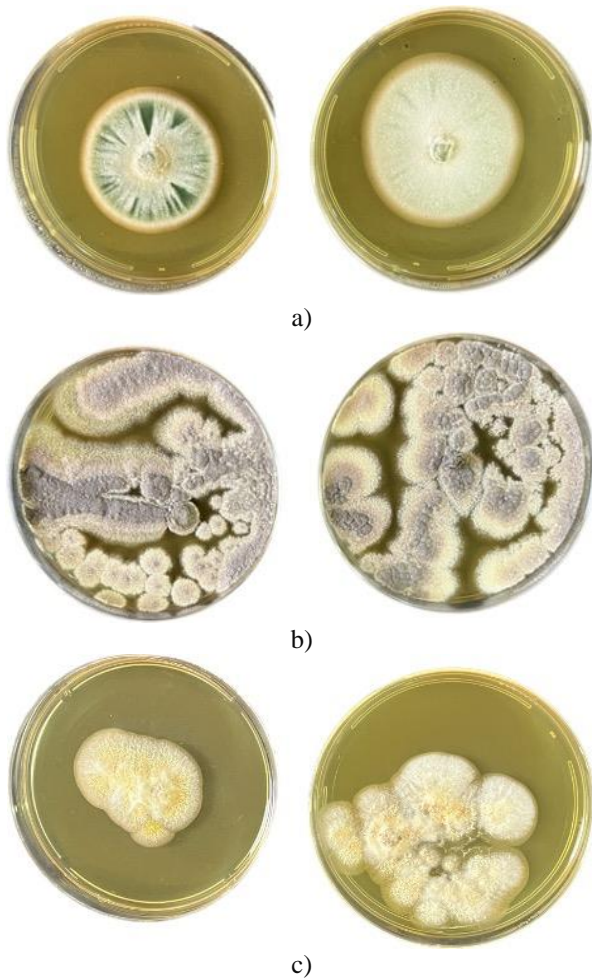


Figure 2. The growth of (a) *A. caespitosus* (b) *A. nidulans* and (c) *A. elegans* were greater on the supplemented PDA medium (right) compared to the control PDA medium (left) at 28°C for 10 days of incubation.

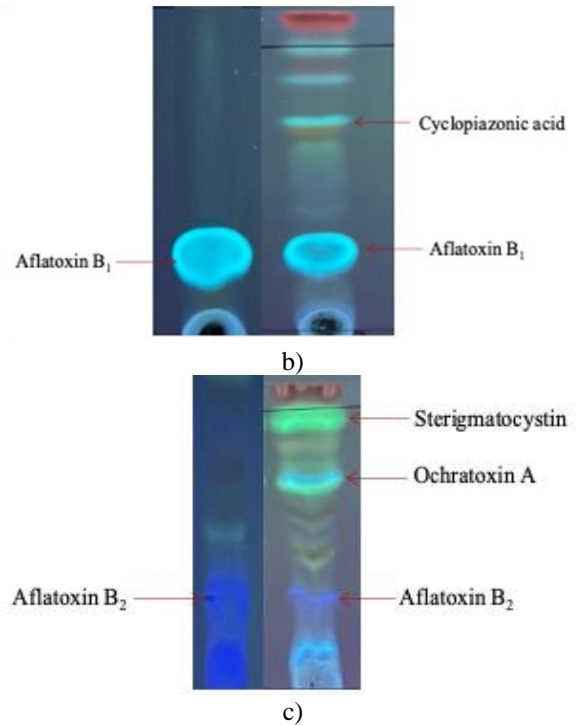
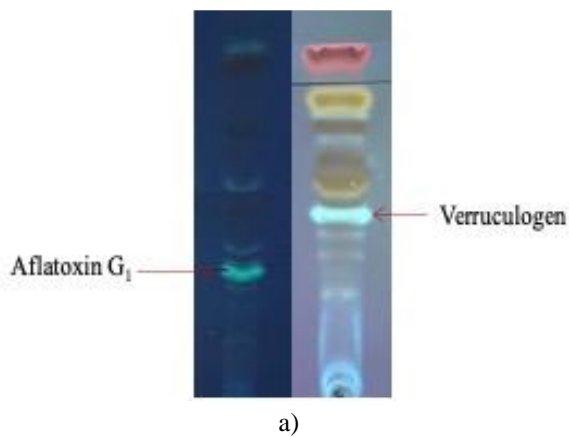
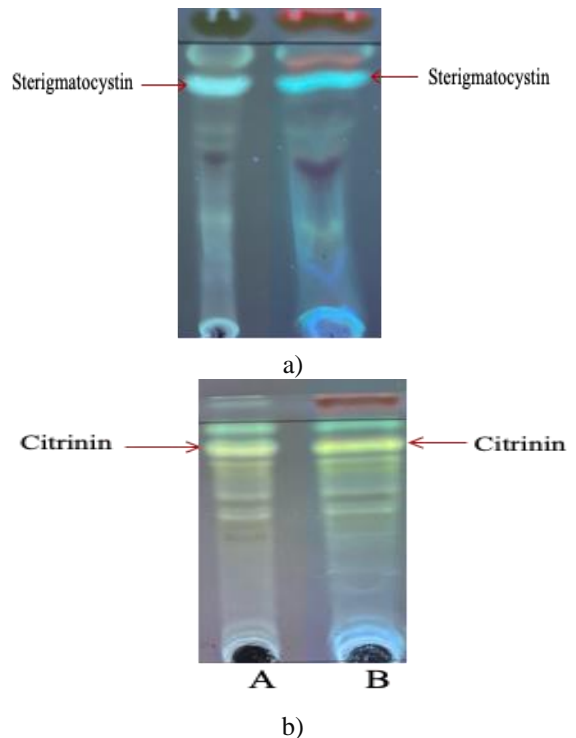


Figure 3. TLC plates were identified (a) aflatoxin G₁ on the control PDA medium (left) and verruculogen on the supplemented PDA medium with *C. roseus* leaf extract (right) from *A. caespitosus*, (b) aflatoxin B₁ on the control PDA medium (left) and aflatoxin B₁ and cyclopiazonic acid on supplemented PDA medium with *C. roseus* leaf extract (right) from *A. flavus* and (c) aflatoxin B₂, ochratoxin A and sterigmatocystin from *A. elegans*.



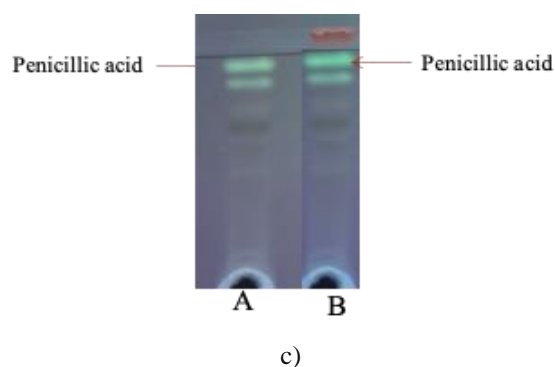


Figure 4. TLC plates were identified same mycotoxins extracted from (a) *A. nidulans*, (b) *P. commune*, and (c) *P. chrysogenum* on control PDA medium (left) and supplemented PDA medium with *C. roseus* leaf extract (right).

Brine Shrimp Larvae Bioassay

Artemia salina larvae were examined for sensitivity to 6 fungal extracts, including exophytic isolates *A. caespitosus*, *A. flavus*, *A. nidulans*, *P. commune*, and *T. purpurogenus*, and endophytic isolates *A. caespitosus*, *A. elegans*, and *P. chrysogenum*. In this investigation, it was discovered that all the larvae shrimp survived in the nontoxic control DMSO. After 6 hours, all fungal isolates died at high rates even at the maximum concentration of 10 µg/ml. Furthermore, at a concentration of 6 µg/ml, these exophytic and endophytic fungal isolates were hazardous. Among all the fungal isolates, the lowest concentration of 2 µg/ml showed the least amount of harmful and the lowest mortality. At 2 µg/ml, the mild toxicity of *A. nidulans* (exophytic) and *A. elegans*

(endophytic) extracts was 57% and 55%, respectively. The LC₅₀ for all fungal extracts was found to be 2 µg/ml, as reported in **Table 2**. The larvae of brine shrimp are susceptible to poisonous chemicals, and the brine shrimp lethal test is an excellent method of determining the toxicity of biological poisons.

The approach has the advantages of being simple to use, quick and affordable, and sensitive to a wide range of chemicals. Several researchers [41] have described bioassays for harmful chemical identification employing *A. salina* larvae. The current study assessed the susceptibility of brine shrimp larvae to six distinct fungal extracts at three different doses (2, 6, and 10 µg/ml). There is a positive link between fungal concentration extracts of 2-10 µg/ml and the death rate of 49-100%, while the LC₅₀ for all fungal extracts was found to be 2 µg/ml. Meyer's toxicity index determined that the extract is poisonous when the LC₅₀ is less than 1000 µg/ml, resulting in the death of the larvae due to the bioactive chemical found in the extract. When the LC₅₀ value exceeds 1000 µg/ml, the extract is non-toxic [42]. As previously stated, mycotoxin production was discovered in exophytic isolates *A. caespitosus*, *A. flavus*, *A. nidulans*, *P. commune*, and endophytic isolates *A. elegans* and *P. chrysogenum*. These findings were substantiated by shrimp larvae sensitivity data, which revealed that the six toxigenic isolates caused a high number of deaths, particularly *A. nidulans* (exophytic), which was shown to be the most toxigenic to brine shrimp larvae. These findings corresponded with those of Zainudin and Perumal (2015) [29], who discovered that all mycotoxins isolated from the *Aspergillus* genus were toxic to brine shrimp larvae regardless of concentration.

Table 2. Mortality (%) of the brine shrimp larvae after treatment with fungal extracts.

SI no.	Fungal type	Fungal species	Mortality after 6 h.			
			Control	2 µg/ml	6 µg/ml	10 µg/ml
1	Exo	<i>A. caespitosus</i>	0	49%	73%	98%
2		<i>A. flavus</i>	0	51%	77%	100%
3		<i>A. nidulans</i>	0	57%	77%	100%
4		<i>P. commune</i>	0	49%	70%	100%
7	Endo	<i>A. elegans</i>	0	55%	75%	100%
8		<i>P. chrysogenum</i>	0	50%	76%	98%
Toxicity			NT	ST	T	VT

The brine shrimp *A. salina* larvae bioassay was also used to determine the toxicity of *C. roseus* leaf extracts. In this investigation, it was discovered that all the larvae shrimp survived in the nontoxic DMSO control. The doses (0.7, 1.5, and 3.125 µg/ml) demonstrated modest toxicity after 6 h, although the lowest mortality was recorded at the lowest concentration of 0.7 µg/ml. Furthermore, the concentrations (6.25, 12.5, 25, and 50 µg/ml) were hazardous. At the highest doses (100 and 200 µg/ml), substantial rates of mortality-very

toxic were recorded. Furthermore, as demonstrated in **Table 3**, the LC₅₀ was identified at a concentration of 6.25 µg/ml.

Table 3. Mortality (%) of the brine shrimp larvae after treatment with *C. roseus* leaf extracts.

Leaves extracts	Mortality after 6 h.	Toxicity
Control	0%	NT
0.7 µg/ml	8%	ST
1.5 µg/ml	25%	

3.125 µg/ml	42%	
6.25 µg/ml	52%	
12.5 µg/ml	65%	T
25 µg/ml	72%	
50 µg/ml	88%	
100 µg/ml	97%	VT
200 µg/ml	100%	

The brine shrimp lethality assay is a quick, low-cost, and easy bioassay for assessing the bioactivity of plant extracts; the results usually coincide with the plant's cytotoxicity and anticancer capabilities [43]. As we explained in the last section, the toxicity of herbal extracts is generally valued as LC₅₀ values using Meyer's toxicity index. Extracts with LC₅₀ less than 1000 µg/ml are poisonous, while extracts with LC₅₀ greater than 1000 µg/ml are non-toxic [42]. Brine shrimp larvae were examined for sensitivity to *C. roseus* ethanolic leaf extracts at nine different doses (0.7, 1.5, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/ml). The current investigation found a link between leaf extract concentrations ranging from 0.7 to 200 µg/ml and a fatality rate ranging from 8 to 100%. At a concentration of 6.25 µg/ml, the LC₅₀ was determined. Matata *et al.* (2018) [44] discovered that *C. roseus* methanol leaf extract had a brine shrimp LC₅₀ of 6.7 µg/ml, which is similar to the current study's finding. Furthermore, Kabubii (2015) [45] discovered that *C. roseus* hexane leaf extract was the most cytotoxic, with an LC₅₀ value of 159.78 µg/ml, but *C. roseus* methanol-water leaf extract yielded a brine shrimp LC₅₀ of 3 µg/ml, which contradicted our findings. According to Fernández *et al.* (2013) [46], the cytotoxic property of *C. roseus* leaf extract may be attributable to the presence of anticancer chemicals in the leaf, such as vinblastine and vincristine. Depending on their polarity, different solvents extract variable amounts of components in crude plant material that may be hazardous to biological experiments [47].

CONCLUSION

Thin layer chromatography was used to investigate the ability of 18 chosen isolates from exophytic and endophytic fungus, including *Aspergillus*, *Penicillium*, and *Talaromyces* species, to create mycotoxins on PDA. Exophytic isolates were tested for mycotoxin generation by *Aspergillus* (9 species), *Penicillium* (2 species), and *Talaromyces* (1 species). *Aspergillus flavus* produced aflatoxin B₁, which fluoresced blue; *A. nidulans* created sterigmatocystin, which fluoresced light green; *A. caespitosus* produced aflatoxin G₁, which fluoresced green; and *P. commune* produced citrinin, which fluoresced yellow-green. Four of the twelve exophytic isolates were toxigenic, accounting for 33% of the exophytic fungus samples. Endophytic isolates were tested for mycotoxin generation by *Aspergillus* (4 species) and *Penicillium* (2 species). Aflatoxin B₂ was created by *A. elegans* with blue fluorescence, and penicillic acid was synthesized by *P. chrysogenum* with green fluorescence. Furthermore, two of the six endophyte isolates were toxic,

accounting for 33% of the endophytic fungus samples. To examine the influence of *C. roseus* leaf extract on fungal mycotoxin generation, toxic isolates were grown on an enriched PDA medium with *C. roseus* leaf extract and screened by TLC. Some toxicogenic isolates were found to produce the same mycotoxins as PDA, whilst others boosted their activity and produced more than one mycotoxin. On brine shrimp larvae, identified fungal extracts and *C. roseus* leaf extracts were tested for toxicity. At the greatest concentration, all fungal isolate extracts revealed high rates of mortality. Furthermore, large amounts of leaf extracts were linked to a high rate of mortality. *C. roseus* has been utilized as an attractive plant in public places despite its toxicity. The presence of this plant may expose you to potentially dangerous compounds including toxigenic exophytic fungus spores.

ACKNOWLEDGMENTS: This project was supported by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah. The authors, therefore, acknowledge with thanks DSR.

CONFLICT OF INTEREST: None

FINANCIAL SUPPORT: None

ETHICS STATEMENT: None

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