Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

POTENTIAL OF ENDOPHYTIC FUNGI TO REDUCE *CALOTROPIS PROCERA* LEAVES TOXICITY IN JEDDAH, SAUDI ARABIA

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ARTICLE INFO

Received:
11 Dec 2020
Received in revised form:
18 Mar 2021
Accepted:
23 Mar 2021
Available online:
28 Apr 2021

Keywords: Endophytic Fungi, *Calotropisprocera*, Leaves toxicity, Medicinal plants

ABSTRACT

Calotropis procera (Ushar) leaves are frequently used in traditional medicine to treat several diseases. However, the plant contains toxic compounds rendering it unsafe material. Hence, the objective of this study was to treat *C. procera* dried leaves powder by bioprospecting for fungal endophytes to be a safe applications. Three selected fungal isolates including *Cladosporium sphaerospermum*, *Cochliobolus lunata* and *Cochliobolus* sp. were used for the reduction of *C. procera* dried leaves powder toxicity. Sporulation of each of these fungi was increased in the presence of 2 g more than 1 g of dried leaves powder in PDB media at 25°C within 4 weeks of incubation. Also, *C. sphaerospermum, Cochliobolus* sp., and *C. lunata* were tested for their ability to reduce the toxicity of *C. procera* dried leaves powder by using thin-layer chromatography (TLC). Only *C. sphaerospermum* showed the ability to reduce the Calactin toxin presented in powdered leaves Furthermore, the toxicity test of dried leaves powder treated by *C. sphaerospermum* showed significant (100%) survival of the adult earthworm *Lumbricusrubellus* compared to the negative control, which induced 100% mortality of worms. These results were also comparable with *C. sphaerospermum* powdered mycelium, which showed no signs of toxicity. It can be concluded that fungal endophytes such as *C. sphaerospermum* are promising candidates for eliminating leaves toxicity.

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To Cite This Article: Najjar AA, Alharbi DS, Bohkari FM, Bafeel SO, El-Zohri MH, Shafi ME, et al. Potential of Endophytic Fungi to Reduce *Calotropis Procera* Leaves Toxicity in Jeddah, Saudi Arabia. Pharmacophore. 2021;12(2):71-8. https://doi.org/10.51847/6mqdq8ntK2

Introduction

Calotropis procera is a toxic plant with remarkable traditional medicinal effects to treat several diseases and infections. It frequently exists in America, Africa, and Asia [1]. It is naturally found in the northern, central, and southern regions of Saudi Arabia and is known in Arabic as Ushar [2, 3]. The toxic compounds of *C. procera* present in flowers, fruits, leaves, stems, and roots. However, leaves contain the major toxicity activities, specifically in latex [4]. Several studies indicated the ability of endophytic fungi to reduce plant toxicity for safe medical, industrial, and agricultural applications.

Endophytic fungi are asymptomatically intercellular community residents in medicinal plants, mostly do not cause diseases to their host [5]. These beneficial fungi protect their host from harmful agents and support plant adaptations in adverse environmental conditions [6]. Also, they are attracting considerable attention due to their bioactive secondary metabolite properties which are relatively unstudied. It is promising to use fungal endophytes treatment to eliminate plant toxicity which mainly depends on the species of fungi, substrates used, and incubation period.

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There are nearly one million endophytic species in the plant kingdom [7]. Various endophytes exist as colonizing the plant parts, which remain symptomless, and the plant tissue remains functional and unaffected, In the past two decades, endophytes have been extensively investigated and found producing multiple biomolecules, biocatalysts, and biological enzyme applied in medicine, agriculture, and industry. It has been observed that metabolites produced by endophytes inhibit the growth of pathogens, Endophytes make up the bulk of undiscovered fungal diversity. Natural products derived especially from medicinal plants have been subjugated for human use for thousands of years to make human life easier [8, 9]. They are great targets to discover medicine. The establishment of fungal repositories from different ecological niches is an essential step in exploiting their potential value to discover new drugs. Fungal secondary metabolites are defined as low molecular weight compounds not required for growth but are produced as adaptations for specific functions in nature [10]. Many endophytes have the ability to produce various biologically active metabolites that may be used directly or indirectly as therapeutic agents against various diseases. There are exciting possibilities to exploit endophytic fungi to mass-produce new and known bioactive secondary metabolites [11]. The KSA has a varied range of plants, including various medicinal trees, shrubs, and plants [12-14]. *Calotropis procera* is a medicinal toxic shrub spread widely in different areas of Saudi Arabia. It grows commonly in agricultural areas, around farms, and especially in the Tehama plain, its sandy warm parts [15]. Previous reports mentioned that this plant species has antimicrobial, anticancer, anti-inflammatory, antidiabetic and antioxidant that are used traditionally to treat several diseases, the medicinal properties of *C. procera* may refer to the presence of the endophytic fungi and their biological activities inside plant tissues [16].
The objective of this work was to evaluate the ability of selected fungal endophytes to eliminate the toxicity from *C. procera* leaves. Also, toxicity activity was carried out to provide the evidence for safe applications.

Materials and Methods

Plant Leaf Collection and Preparation of Dried Leaves Powder
A healthy and mature *C. procera* shrub naturally spread in Jeddah, Saudi Arabia from October to September 2017 at an average temperature of 30°C. *Calotropis procera* leaves were randomly collected, washed by using sterilized water, and kept in laboratory bags, immediately, transferring leaf samples to the microbiology lab to start further processes. The plant was identified by Dr. Manal H. El-Zohri Plant Taxonomist at Faculty of Science, King Abdulaziz University. 2000 g of fresh leaves were dried in shade to avoid loss of essential oils for 7 days. Dried leaves (128 g) were powdered in a mixer grinder (Waring Commercial, USA).

Fungal Inoculum Preparation
Three endophytic fungal isolates including *C. sphaerospermum*, *Cochliobolus lunata,* and *Cochliobolus* sp.were provided by Dr. Azhar Najjar, Faculty of Science, King Abdulaziz University. Each fungal isolate was transferred from the stock culture on PDA plates to obtain pure cultures and incubated at 28°C. Based on the growth rates, the incubation days varied between 7 days for *C. lunata* and *Cochliobolus* sp. and 14 days for *C. sphaerospermum*.

Fungal Sporulation Analysis
The sporulation of the three selected fungal isolates *C. sphaerospermum*, *Cochliobolus lunata,* and *Cochliobolus* sp. were measured by using a hemocytometer method. A single 5-mm block of fresh culture was cut out from the fungal colony near the margin using sterilized cork borer and transferred into a test tube containing 5 ml sterile distilled water. These tubes were mixed vigorously by using a vortex mixture (VELP Scientifica, New York) for 5 min to make a uniform spore suspension. Then, 10 µl of fungal spores suspension was transferred into a hemocytometer and then the spores were counted in five microscopic fields. The average of spores was recorded using the following formula:

$$\text{Total cell/ml} = \text{Total cells counted} \times \text{dilution factor/number of squares} \times 10{,}000 \text{ cells/ml} \quad (1)$$

Under low power of the objective lens (10X) of the microscope. The sporulation for 5 mm of each fungal block was estimated to contain 5×10^{6}, $4.22\times10^{4,}$ and 6×10^{5} spores/ml, respectively [17]. These preparations were used in further experiments.

Effect of Different Leaves Amounts on Fungal Sporulation
Thirty ml of potato dextrose broth (PDB) medium was placed in loosely cap Schott Bottles with a capacity of 100 ml and different amounts of dried leaves powder (1, 2, and 3 g, individually) were added. The initial pH of the culture medium was set at 5.5 and autoclaved (121°C for 15 min). Treated samples contained the media were mixed with blocks (5 mm) of the selected fungal species individually. Control samples were only inoculated with each fungus in PDB media. All samples were incubated in an orbital shaker incubator (28°C, 150 rpm) for one week [18]. The fungal sporulation was counted in both treated and control samples.

Evaluation of the Reduction of C. procera Leaves Toxicity by using Endophytic Fungi

Thirty ml of PDB medium was placed in three loosely cap Schott Bottles with a capacity of 100 ml and mixed with 2 g of *C. procera* dried leaves powder. The pH of the medium was set at 5.5 and autoclaved (121°C for 30 min), three replicates were taken. Treated dried leave powder were inoculated by one block (5 mm) of three selected fungal species including *C. sphaerospermum*, *Cochliobolus lunata,* and *Cochliobolus* sp., individually. All samples were incubated in an orbital shaker incubator (28°C, 150 rpm) for 7, 14, 21, and 30 days [17]. After every incubation period, three samples of each untreated and treated cultures were evaluated for fungal sporulation then analyzed with thin-layer chromatography (TLC) during 30 days.

Fungal Sporulation

Every week, three treated samples were mixed individually by laboratory blender for 5 minutes. Five ml of the mixture was transferred to a test tube for fungal sporulation analysis. Control samples were calculated as the fungal sporulation at 0 week for 2 g of dried leaves powder with the fungal disc in PDB media.

Detection of C. procera Dried Leaves Powder Toxicity by TLC Analysis

The samples were divided into two groups: Untreated dried leaves powder (Inocula-free) and treated dried leaves powder. Based onthe toxicity experiment the content of untreated and treated dried leaves powder of each bottle were freeze dried, followed by n-hexane extraction at 1:10 (w/v) in shaker incubator (PEMED 3525) at room temperature according to Dwivedi *et al*., [19]. Both extractions were filtered (pore size, 1µm) through a glass microfiber filter (Ahlstrom Munksjö, Helsinki, Finland) after 24 hours. The toxicity level was analyzed using TLC.

Ten µl of untreated and treated leave extracts were applied on Thin-layer chromatography (TLC) using silica gel pre-coated plate (Sigma-Aldrich).

The TLC plate was developed in a solvent system of toluene: ethyl acetate: formic acid (7.5:2.5:0.5 v/v) at room temperature. After development, the plate was examined in a chromate-Vue cabinet (model UVP Upland, CA, USA) and visualized under long-wavelength UV 366 nm [17].

Toxicity Activity

Lumbricus Rubellus earthworms at the mature stage were provided by Dr. Anas Faqeah and identified by Dr. Manal Shafi, Faculty of Science, King Abdulaziz University. They were acclimatized with the laboratory conditions for 1 week by maintaining their optimal condition such as temperature at 20±1ºC, dark for survival to rule out any probable influence or interference on the experimental set up by the laboratory conditions.

Three groups were prepared for toxicity activity as follows: *C. sphaerospermum* only, negative control only leaves (inocula free), and treated samples (leaves powder + fungus), in sequences.

Three Schott bottles (100 ml) of each group containing 30 ml of PDB medium were prepared for the three groups. The pH of the medium was set at 5.5. After sterilization, first and third group bottles were inoculated by 1 block from *C. sphaerospermum* stock. All group bottles were incubated at 28°C and 150 rpm for 30 days. After incubation, all samples were frozen and transferred to a freeze drier until dry.

In each group, the three different values (100, 300, and 500 mg) were weighed from dried samples and added into 1 ml of sterilized water to make different concentration suspensions (100, 300, and 500 mg/ml). Also, 1 ml of sterilized water was added to earthworms control with no addition [20].

The worms were washed and placed on top of the filter papers (Schleider and Schuell GF50, Dassel, Germany) in covered Petri-dish. One ml of each concentration suspensions (100, 300, and 500 mg/ml) for three groups and earthworms control were added into the dishes and incubated, individually, in the dark at 20±1ºC for 48 hours. Three replicates were taken. Survive and mortality of the earthworms were counted by comparing the mean surviving earthworms of the tested group and control set [18].

Statistical Analysis

One-way ANOVA, SPSS 0.22 was used to analyze the data for fungal count followed by a T-test to determine the significant difference of the data and to compare the means at $p \leq 0.05$.

Results and Discussion

Effect of Different Leaf Amounts on Fungal Sporulation

The three selected fungal isolates were grown in PDB media with different amounts of dried leaves powder of *C. procera* (1, 2, and 3 g) at 28°C after one-week incubation. As shown in **(Figure 1)**, the fungal sporulation for three fungal isolates was significantly ($p < 0.05$) higher in media containing 2 g and 3 g of dried leaves powder when compared to media containing 1 g of dried leaves powder. The difference in sporulation between media containing 2 g and 3 g of dried leaves powder was not significant ($p > 0.05$).

Interestingly, the presence of different amounts of dried leave powder (1, 2, and 3 g) in media (treated sample) significantly ($p < 0.05$) increased the fungal growth to the average values of (1.2×10^8, 1.6×10^9, 8.8×10^8) compared to the fungal sporulation without dried leave powder (control sample) with average values of (5.4×10^7, 5.7×10^7, 1.1×10^7), respectively.

Among fungal isolates, *C. sphaerospermum* showed the best sporulation when treated with 2 g of dried leave powder (8.2×10^7). In contrast, the lowest fungal sporulation was observed for *Cochliobolus* sp. (5.8×10^6) in the same condition. In this study, 2 g of dried leaves powder was selected to carry out further experiments due to the greatest fungal sporulation values.

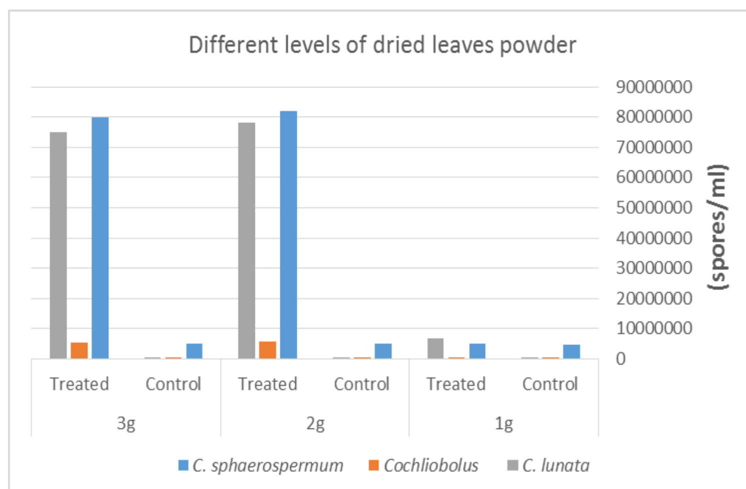

Figure 1. Fungal sporulation (spores/ml) of fungal isolates in different levels of dried leaves powder at 28°C after 7 days of incubation times.

Evaluation of the Reduction of C. procera Leaves Toxicity by using Endophytic Fungi

Fungal Sporulation

This study was prepared for evaluating the ability of the fungal species to use the dried leaves powder for growth as well as the ability of the fungal species to eliminate *C. procera* toxicity. **Figure 2** shows a representative of the fungal sporulation for three fungal isolates in treated samples were increased in PDB media containing 2 g of dried leaves powder started in 1 week gradually to 4 weeks compared to 0 week. Clearly, the results showed the ability of the three fungal isolates to utilize the nutrients present in the dried leaves powder and the non-toxicity of dried leaves powder to the fungal isolates. It was observed under a light microscope at 10X magnification after 4 weeks, the special ability of *C. sphaerospermum* to grow on dried leaves powder (3.7×10^8). On the other hand, the fungal growth for *Cochliobolus* sp. and *C. lunata* had less fungal sporulation (8.5×10^6 and 9.7×10^6, respectively) compared to *C.sphaerospermum* after 4 weeks.

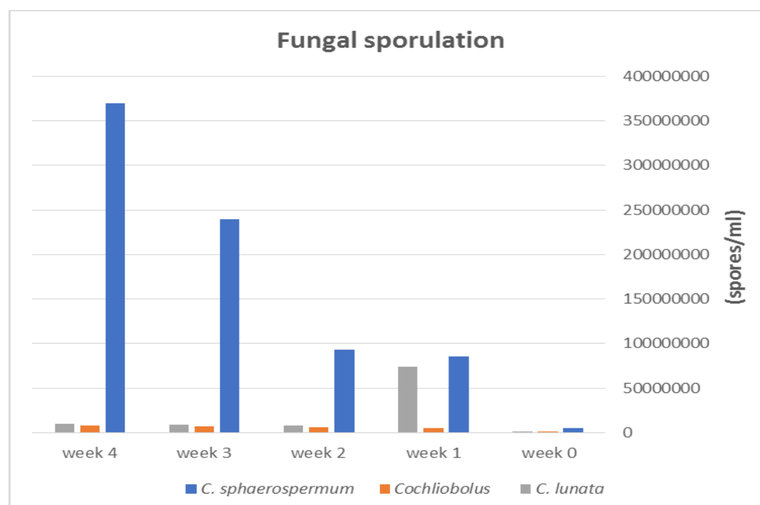

Figure 2. Fungal sporulation (spores/ml) of fungal isolates in treated samples (dried leaves powder with fungi) at 28°C during 30 days.

Detection of C. procera Leaves Toxicity by TLC Analysis

Untreated dried leaves powder (free) toxicity fingerprint was separated and identified from PDB media at 28°C within 4 weeks for five different sharp bands by TLC. The current study focused on one band that considers the main toxic compound in *C. procera* leaves, which is Calactin at Rf=0.493. The toxicity levels of untreated dried leaves powder were represented the sharp band densities with various Rf levels at all incubation times, indicating the stability of leaves toxicity with long incubation periods. In the case of treated samples by *C. sphaerospermum*, various sharp band densities were decreased

slightly with longer incubation periods. In contrast, no effectiveness was observed of treated samples by *Cochliobolus* sp. and *C. lunata* on band densities within 4 weeks. Due to these results, the current study focused on *C. sphaerospermum* species that has the ability to reduce the toxicity of *C. procera* dried leaves powder as shown in (**Figure 3**).

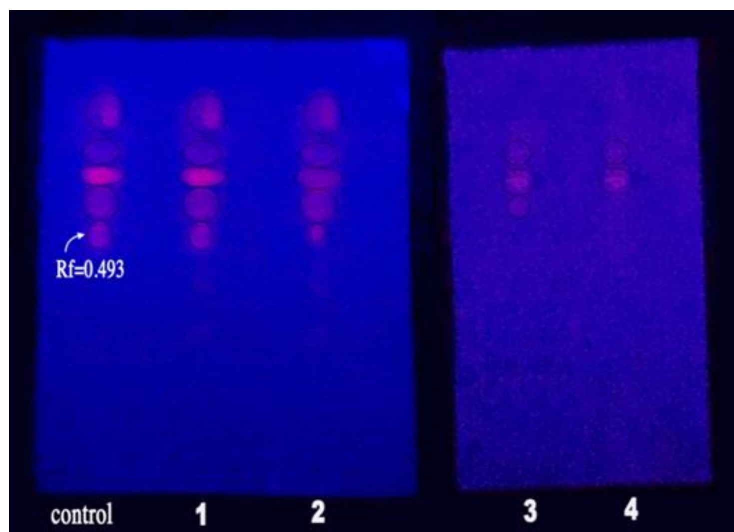

Figure 3. TLC analysis for *C. procera* untreated leaves powder (control) and treated samples by *C. sphaerospermum* within 4 weeks.

Toxicity Activity

Toxicity activity was demonstrated on earthworms survival as shown in (**Table 1**) for different concentrations (100, 300, and 500 mg/ml) of three tested groups including *C. sphaerospermum* growth, negative control only leaves (free), and treated leaves by *C. sphaerospermum* with sterilized water control for 48 hours. The result of the first group of *C. sphaerospermum* dried mycelium and the third group of treated leaves by *C. sphaerospermum* clearly showed no mortality without toxic effect at (100, 300, 500mg/ml) after 48 hours. While the second tested group of untreated dried leaves powder inflicted the worms after 24 to 48 hours of exposure with increasing magnitudes of mortality. The toxicity activity of *C. Procera* dried leaves powder was very high, an instance at the lowest concentration of 100 mg/ml of dried leaves powder, the earthworms did not survive whereas, at the same concentration, worms were survived with treated dried leaves powder by fungus *C. sphaerospermum*.

Table 1. Toxicity activity of different *Cladosporium sphaerospermum* concretions All tested samples were observed after 48 hours

Sample no.	Tested samples	Number of worms mg/ml			Survive
		100	**300**	**500**	
First	*C. sphaerospermum* growth	2	2	2	100%
Second	Negative control only leaves (free)	2	2	2	0%
Third	Treated samples (leaves powder + fungus)	2	2	2	100%

*Earthworms were added to 1 ml of sterilized water

The addition of dried leaves powder enhanced fungal sporulation of *C. sphaerospermum* showed dense fungal spores with black clumps in the culture containing 2 and 3 g of dried leaves powder, whereas the medium in the control bottles was normal fungal sporulation. The variation in sporulation among the fungal isolates indicates the fungal ability to consume the nutrients in the growth medium. Uppala *et al*. [21] reported that *C. sphaerospermum* produces enzymes such as benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase enzyme, which have high activity rates to break down the hydrocarbon components in different materials including plant leaves. In contrast, the production of these enzymes has not been reported yet from *C. lunata* and *Cochliobolus*sp.

Nevertheless, the enhancement of fungal growth could be the additive effects of dried leaves powder and other nutrients present in this experiment. It is evident that the stimulation of the fungal sporulation rate might be due to the increasing carbon source from dried leaves power of *C. procera*.

In this study, the ability of three selected fungal isolates to grow on *C. procera* dried leaves powder at different incubation times (7, 14, 21, and 30 days) were remarkable values. Several studies have reported that *C. procera* has a significant fungicidal effect on *C. lunata* and other fungal isolates that was disagreeing with our results. No studies have indicated yet the ability of fungi to grow on *C. procera* to *Cochliobolus* sp. [22].

The greatest sporulation values of *C. sphaerospermum* on dried leaves powder were referred to its morphological characteristics including a high number of lemon shape spores, In contrast, *Cochliobolus* sp. and *C. lunata* have filamentous structures and less curved septate spores [23]. Raut *et al*. [24] showed that the application of *C. sphaerospermum* on rice and soybean improves their growth. This was similar to Tonukari *et al*. [25] who evaluated *Cochliobolus* sp. growing on maize. Paraszkiewicz *et al*. [26] showed that nickel induces enhanced lipid peroxidation in the mycelium of *C. lunata*. Also, Zhang *et al*. [27] showed that *C. lunata* enhances the level of lipid peroxidation products in mangrove plant seedlings.

Our result as explained by the TLC assay tends to be responsible for the activity of *C. sphaerospermum* to degrade toxicity of *C. procera*. Fungi degraded plant toxic was reported before by Najjar [17], who studied the detoxification of toxic phorbol esters from Malaysian *Jatropha curcas* kernel by other endophytic. The present study revealed in TLC profile, several bands at different R_f under UV 366. Five different bands were observed. The details of bands' color in leaves for treated and untreated samples were observed within 4 weeks. *Calotropis procera* fingerprint was identified by Dwivedi *et al*. [19] and used different techniques such as HPLC, LC-MS, and TLC. The most common toxic compound was quantified by HPLC including Calotoxin and Calactin at the retention time of 14.889 and 17.163, respectively in India [28]. According to previous studies, Calactin was observed as the highest cytotoxic effect and identified by TLC at Rf = 0.493.

The present study demonstrated that the increase in fungal sporulation in PDB media containing leaves powder clearly showed the ability of the *C. sphaerospermum* to utilize the nutrients present in the leaves and also showed the ability to degrade Calactin based on two g of leaves powder within 30 days of incubation. However, the toxicity level decreased markedly with an increasing incubation period. At 30days of incubation, Calactin band was no longer visible, indicating almost complete removal of Calactin in the culture medium. Agricultural and industrial applications need to remove the Calactin, which are the main toxic compounds within *C. procera* for safe utilization.

Sampedro *et al*. [29] demonstrated the efficacy of fungi (*F. oxysporum* and *F. lateritum*) for the phytotoxicity treatment of dry olive mill residue as they showed resistance to most abiotic factors like drought, stress, and salts. These fungi can degrade phytotoxic substances, mobilize nutrients, and promote more efficient use of the nutrients by the plants. Some plants with intrinsic fungi have been identified as useful tools for combating plant extract toxicity as most of those fungi have been reported to have inhibitory effects on the toxins. It has been reported that white-rot fungi can be used as a means of reducing plant toxicity by producing ligninolytic enzymes [30]. As reported by Yu and Keller [31], some saprophytes have been found to produce enzymes that play a significant role in the defense system of most fungi. Strategies for biological treatment with selected fungi are based on their ability to degrade a variety of natural lipids and compounds like phorbol esters as a defense mechanism or to provide nutrients for growth [32].

Some fungi have shown their ability in producing degradative enzymes [32]. In addition, some fungal strains survive and grow on different conditions and compounds because they adapt to efficiently extract nutrients and have the potential for biodegradation processes, Fungi as heterotrophic microorganisms can utilize carbon sources from various organic substrates [33]. *Cladosporium* sp. can grow on other toxic plant substrates such as *Jatropha* kernel [17].

Recently, Bezerra *et al.*, [34] reported the important toxic effects of latex and leaf extract of *C. procera* on animals with severe damages to testes and heart. The plant secretes poisonous milky latex. Even though it is toxic, the use of the plant in the treatment of many diseases has been known to be widespread since ancient times, *C. procera* plant has various biological activities including insecticidal, antifungal, and anticancer activities [35].

*Cladosporium sphaerospermum*was isolated from *C. procera* leaves and identified as endophytic fungi. In agreement with our results, Bello *et al*. [16] indicated that *C. sphaerospermum* is a non-toxic endophyte. Furthermore, it has been reported that *C. sphaerospermum* can use its metabolic machinery to degrade 9 different volatile organic compounds (VOCs). For example, toluene compound isolated from *C. procera*, which is toxic for the CNS of humans and animals, can be degraded by this fungus and used as a single source of energy and carbon [36].

Calotropis procera is known to possess various medicinal properties, rarely known for its toxicity. All parts of *C.procera* are reputed to be toxic to humans, cattle, sheep, rabbits, and rats, may die from *C. procera* poisoning, bactericidal, larvicidal and cytotoxic effects [37, 38].

Although the latex of *C. procera* is known to possess various medicinal properties, it is rarely known for its toxicity. The fresh and aqueous extracts of latex have been reported to produce an anthelmintic effect on earthworms. Both fresh and dry latex had significant wormicidal activity. On the contrary, worms paralyzed by piperazine remained alive and gained motility when placed in freshwater, the appearance of hemorrhagic spots on the surface of the worms indicates that some functional impairment takes place. Moreover, numerous studies have indicated that fungal endophyte extracts did not affect normal human cells, *in vivo* as well as did not cause any disease [38].

Regarding the toxicity activity on the earthworms, this procedure has been used to establish the cytotoxic activity of earthworms when exposed to three tested groups with different concentrations in our laboratory. Many investigations have tested the toxicity activity on earthworms. Silva *et al*. [39] study showed in general, that active neonicotinoid substances in soil cause toxicity for earthworms that may already pose a risk to populations of soil invertebrates in the field after a single application. According to Cao *et al*. [40], the evaluation ecotoxicological study effect of microcystins on earthworms, no acute toxicity was recorded on earthworms. These results suggest that microcystins induce oxidative stress on earthworms could be bioaccumulated in the earthworm, which leads to disruption of the antioxidant system and lipid peroxidation, as well as alterations in lysosomal membrane stability. In the present study, earthworms survived with no negative effect in the first and third groups, which were in agreement as mentioned earlier in the discussion section about *C. sphaerospermum*

endophyte fungus and its harmless influence and acute toxicity of *C. procera*. The results of the present study also evaluated the effect of leaves on earthworms. The samples treated with *C. sphaerospermum* and distilled water remained alive even after 48 hours of exposition. On the other hand, for the second group, as the negative control, treatment of earthworms with only leaves without any inoculum was highly toxic. These results were similar to several reports, which discussed the acute toxicity of this plant leaves on earthworms [41].

Conclusion

Screening of different fungal isolates for their ability to reduce *C. procera* toxicity was conducted with different levels of dried leaves powder. In the preliminary evaluation, selected fungal isolates including *C. sphaerospermum*, *Cochliobolus* sp. and *C. lunata* grown in PDB containing 2 g of dried leaves powder after 30 days of incubation. The *C. sphaerospermum* had the special ability to grow on dried leaves powder with fungus sporulation (3.7×10^8) and reduce the Calactin toxin presented in *C. procera* dried leaves powder more than other fungal strains showed lower percentage values.
The ability of fungal isolates to eliminate Calactin, after adding 2 g of dried leaves powder in the PDB media within four weeks of incubation, clearly indicated their ability to produce fungal hydrolytic enzymes such as ligninolytic enzymes. These enzymes are regarded as important natural agents for reducing plant toxicity in *C. procer*a leaves.
In the toxicity activity using earthworms, the untreated dried leaves powder was highly toxic to these earthworms, where there was 100% mortality after 48 hours of exposure, but when treated with dried leaves powder and fungus *C. sphaerospermum* all worms survived even 48 hours.

Acknowledgments: This project was supported by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah. The authors, therefore, acknowledge with thanks DSR. Also, the authors are thankful to Prof. Ahmad Moharram for keeping a copy of fungal isolates in Assuit University Moubasher Mycological Center, Assiut, Egypt.

Conflict of interest: None

Financial support: None

Ethics statement: None

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