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Antifungal potential of eukaryotic microalgae against the fungus *Colletotrichum gloeosporioides*

Potencial antifúngico de microalgas eucarióticas contra o fungo *Colletotrichum gloeosporioides*

Potencial antifúngico de microalgas eucariotas frente al hongo *Colletotrichum gloeosporioides*

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Abstract
As an alternative to the use synthetic fungicides in the control of agricultural pests, research is being directed to natural compounds, called biopesticides. Microalgae produce a wide variety of bioactive molecules, with several biological activities already recorded, including antifungal against phytopathogens. According to that, the objective of this study was to evaluate *in vitro* antifungal activity of the microalgae *Conticribra weissflogii* and *Tetraselmis suecica* against the phytopathogen *Colletotrichum gloeosporioides.* To obtain the microalgal biomass, cultures were carried out in 10 L reactors, in triplicates. The biomass was subsequently freeze-dried and then went through the extraction process using as solvent absolute ethanol (99,99%). The antifungal activity of the extracts was evaluated by the broth microdilution methodology, with a concentration range of 0.115 to 6 mg mL^{-1}. The Minimum Inhibitory Concentration (MIC) was determined visually, by the absence of fungal growth. The microalgae *C. weissflogii* showed fungistatic activity, with a MIC of 1.5 mg mL^{-1} and *T. suecica* did not register inhibition of the fungus evaluated. This study highlights the potencial of eukaryotic microalgae as sources of alternative antifungal compounds to synthetic pesticides, a more environmentally sustainable option. In addition, this is a pioneering work on the antifungal activity of these microalgae species against the phytopathogen *C. gloeosporioides.* Further research may be carried out aiming the isolation and identification of active biomolecules of *C. weissflogii* with antifungal property, as well as, in the future, new biopesticides may be formulated from this extract.
Keywords: Microalgae; Chlorophycea; Diatom; Phytopathogens; Anthracnose.

Resumo
Como alternativa ao uso de fungicidas sintéticos no controle de pragas da agricultura, pesquisas estão sendo direcionadas aos compostos naturais, denominados biopesticidas. As microalgas produzem uma grande variedade de compostos bioativos, com diversas atividades biológicas já registradas, incluindo a antifúngica frente a fitopatógenos. Dessa forma, o objetivo deste trabalho foi avaliar a atividade antifúngica *in vitro* das microalgas *Conticribra weissflogii* e *Tetraselmis suecica* frente ao fitopatógeno *Colletotrichum gloeosporioides*. Para obtenção da biomassa microalgal, os cultivos foram realizados em garrafões de 10 L, em triplicatas. A biomassa foi posteriormente liofilizada e, em seguida, passou pelo processo de extração utilizando o solvente etanol absoluto (99.99%). A atividade antifúngica dos extratos foi avaliada pela metodologia de microdiluição em caldo, com intervalo de

concentração de 0.115 a 6 mg mL^{-1}. A Concentração Inibitória Mínima (CIM) foi determinada visualmente, pela ausência de crescimento do fungo. A microalga *C. weissflogii* apresentou atividade do tipo fungistática, com CIM de 1.5 mg mL^{-1} e *T. suecica* não registrou inibição do fungo avaliado. Este estudo destaca o potencial das microalgas eucarióticas como fontes de compostos antifúngicos alternativos aos pesticidas sintéticos, uma opção mais sustentável para o meio ambiente. Além disso, trata-se de um trabalho pioneiro acerca da atividade antifúngica destas espécies de microalgas frente ao fitopatógeno *C. gloeosporioides*. Novas pesquisas poderão ser realizadas direcionadas ao isolamento e identificação das biomoléculas ativas de *C. weissflogii* com propriedade antifúngica, assim como, no futuro, novos biopesticidas possam ser formulados a partir deste extrato.
Palavras-chave: Microalgas; Clorofícea; Diatomácea; Fitopatógenos; Antracnose.

Resumen

Como alternativa al uso de fungicidas sintéticos en el control de plagas en la agricultura, las investigaciones se están orientando hacia los compuestos naturales, denominados biopesticidas. Las microalgas producen una gran variedad de compuestos bioactivos, con diversas actividades biológicas ya documentadas, incluyendo actividad antifúngica contra fitopatógenos. El objetivo de este trabajo fue evaluar la actividad antifúngica, *in vitro*, de las microalgas *Conticribra weissflogii* y *Tetraselmis suecica* contra el fitopatógeno *Colletotrichum gloeosporioides*. Para la obtención de la biomasa microalgal, los cultivos se llevaron a cabo en garrafas de 10 L, por triplicado. La biomasa fue posteriormente liofilizada y, a continuación, sometida a un proceso de extracción utilizando etanol absoluto (99,99%) como solvente. La actividad antifúngica de los extractos fue evaluada mediante la metodología de microdilución en caldo, con un rango de concentración de 0,115 a 6 mg mL^{-1}. La Concentración Inhibitoria Mínima (CIM) se determinó visualmente, observando la ausencia de crecimiento del hongo. La microalga *C. weissflogii* mostró una actividad de tipo fungistático, con una CIM de 1,5 mg mL^{-1}, mientras que *T. suecica* no presentó inhibición del hongo evaluado. Este estudio resalta el potencial de las microalgas eucariotas como fuentes de compuestos antifúngicos alternativos a los pesticidas sintéticos, constituyendo una opción más sostenible para el medio ambiente. Además, se trata de un trabajo pionero sobre la actividad antifúngica de estas especies de microalgas frente al fitopatógeno *C. gloeosporioides*. Futuras investigaciones podrían dirigirse al aislamiento e identificación de las biomoléculas activas de *C. weissflogii* con propiedades antifúngicas, así como a la formulación de nuevos biopesticidas basados en este extracto.
Palabras clave: Microalgas; Clorofíceas; Diatomeas; Fitopatógenos; Antracnosis.

1. Introduction

Anthracnose is a disease caused by phytopathogenic fungi that belong to the genus *Colletotrichum* (Ciofini et al., 2022). These pathogens mainly target flowers, young fruits, and plant branches, and can also manifest during the storage of ripe fruits (Udin et al., 2018). Among the main agents of anthracnose, the *C. gloeosporioides* complex stands out, known for its wide range of plant hosts, affecting more than 470 plant species (Peralta-Ruiz et al., 2023). As for the affected crops, papaya, mango, avocado, banana, guava and strawberry stand out (Udin et al., 2018). *Colletotrichum* spp. causes major losses in agricultural production and economic impacts, which is why it was listed among the 10 most relevant fungal pathogens in the world (Dean et al., 2012).

To solve the challenge of plant pathogen fungi, pesticides were developed to manage and protect crops against these harmful agents (Peng et al., 2021). However, the continuous and indiscriminate use of these chemical compounds causes adverse effects on human health and the environment, in addition to the emergence of resistant pathogenic strains (Lopes-Ferreira et al., 2022). As a promising alternative to reliance on fungicides and chemical additives, recent research is being conducted that explores the antifungal potential of natural compounds, called biopesticides, such as microalgae extracts (Costa et al., 2019; Essiedu, Adepoju & Ivantsova, 2020). This approach not only aims to control plant pathogens, but also represents a more ecologically sustainable option (Liu et al., 2019).

Microalgae are part of a diverse and heterogeneous group of photosynthesizing microorganisms, with more than 50,000 species described and elucidated to date (Guiry, 2024). Microalgae biomass is a rich source of biologically active secondary metabolites such as lipids, polysaccharides, carotenoids, vitamins, phenols, and phycobiliproteins (Eze et al., 2023). Due to this great diversity of bioactive compounds in microalgae, several studies have been conducted on their biotechnological potential (Barbosa et al., 2023). Among them, the biological control of microorganisms from extracts and

compounds isolated from microalgae stands out, with several applications already registered, such as antibacterial, antiviral, antiprotozoal, and antifungal (Falaise et al., 2016).

In agronomy, the biological control of pests from extracts and compounds isolated from microalgae is an area that has grown, with some *in vitro* and *in vivo* experiments already carried out, including susceptibility tests of phytopathogenic fungi (Kim et al., 2018; Perveen et al., 2022; Lage et al., 2024). However, in view of the great biodiversity of microalgae and their wide biotechnological potential, few studies have been carried out on the antifungal activity of this group against phytopathogens, and many species of microalgae have not yet been contemplated.

In this context, the objective of the present study was to evaluate the in vitro antifungal potential of the ethanolic extracts of the microalgae *Conticribra weissflogii* and *Tetraselmis suecica* against the phytopathogen *Colletotrichum gloeosporioides*.

2. Methodology

The present research is an experimental, lab study of qualitative and quantitative nature (Pereira et al., 2018).

Cultivation and production of microalgal biomass

The eukaryotic microalgae strains *Conticribra weissflogii* (ALCB137430) and *Tetraselmis suecica* (ALCB137434) were obtained from the Microalgae Collection of the Bioprospecting and Biotechnology Laboratory (LaBBiotec) of the Institute of Biology, an ex-situ collection located at the Institute of Biology of the Federal University of Bahia (UFBA) in Salvador, Bahia, accredited in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge as C945879. The use of the strains, which belong to the genetic heritage, is registered under No. C9458879 (SISGEN).

The cultivation of microalgae was conducted according to the guidelines established by Lourenço (2006). The cultivation conditions adopted included a temperature of 22 ± 1 °C, irradiance of 35 µmol of m^{-2} s^{-1} photons, a photoperiod of 12 h (light/dark cycles), pH 7.0 and constant aeration. For both microalgae species, the Conway culture medium (Richmond, 2004) was used, and for the diatom *C. weissflogii*, 2 mL L^{-1} of sodium silicate (Na_2SiO_3) was added at a concentration of 0.32 mM. The volume of the culture was gradually increased (Figure 1A and 1B) until it reached the final stage, in glass photobioreactors of 10 L, in triplicates (Figure 1C) of each strain. For following the growth, 3 mL of sample was collected daily over 15 days of cultivation and absorbance monitored at 680 nm, to determine the appropriate time to harvest and collect the biomass.

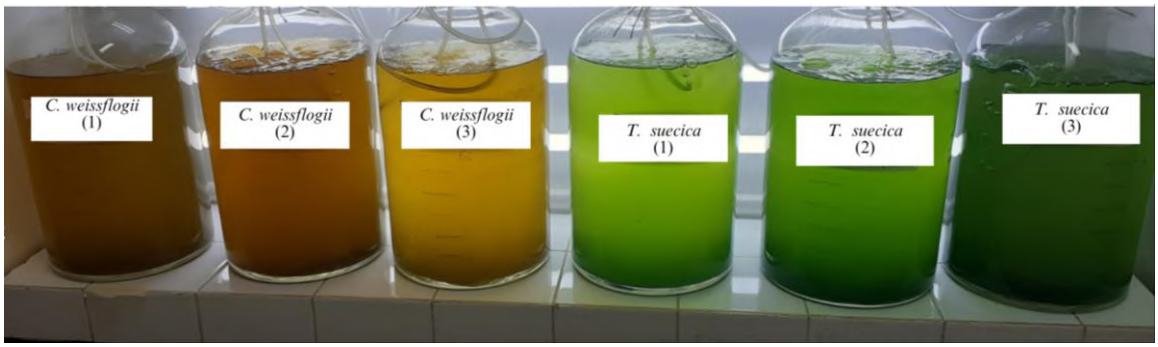
After the 15-day experiment, the precipitate (microalgal biomass) and the supernatant (culture medium) were separated by centrifugation (5,000 rpm for 20 min), using the MPW-351® centrifuge (Chu et al., 2004). The biomass resulting from the centrifugation was stored in a freezer at -20 ºC and the supernatant was discarded. To obtain the dry biomass, the frozen material was submitted to lyophilization process over 48 h, under a vacuum of 0137 µHg and a temperature of -30 °C, using the Terroni® Enterprise II Lyophilizer. The dry biomass obtained was weighed and stored in the freezer at -20 ºC.

Next, Figure 1 shows cultivation of the microalgae:

Figure 1 - Cultivation of the microalgae *C. weissflogii* and *T. suecica*. (a) Cultivation in photobioreactors with a capacity of 200 mL, in triplicates; (b) Cultivation in photobioreactors with a capacity of 1 L, in triplicates; (c) Cultivation in photobioreactors with a capacity of 10 L, in triplicates.

(a) **(b)**

(c)



Source: Authors.

Extraction process

After the lyophilization, the dry biomass went through extraction process. In a 250 mL Erlenmeyer 1 g of dry biomass was added to 100 mL of absolute ethyl alcohol (Merck®). This material was homogenized in vortex and then sonicated in an ice bucket foe cell lysis, in two cycles of 6 min at 10% power, using the Sonopuls HD 2070 Sonicator model from Bandelin®. After removing the samples from the sonicator, they were placed on a pendulum agitator table for 72 h. Subsequently, the contents were transferred to 15 mL Falcon tubes and centrifuged at 4,500 rpm for 10 min. The supernatant was transferred to autoclaved glass vials to begin drying at room temperature until the solvent was completely evaporated, thus generating the extracts. Then, the extracts were stored in a freezer at -20 ºC until the antifungal testing stage.

Fungus Cultivation

The fungus *Colletotrichum gloeosporioides* (PPAM06) was obtained from the Phytopathology Laboratory of Embrapa Cassava and Fruits, located in Cruz das Almas, Bahia. It was isolated from the infected cassava (*Manihot* sp.) crop. The fungus was grown in sterile test tubes containing Merck® Potato Dextrose Agar (PDA) medium, prepared according to the manufacturer's recommendations. The established cultivation conditions were incubation in a heated greenhouse at 32 °C and replicated every five days.

Susceptibility test

The Broth Microdilution method was performed according to the methodology proposed by Hlima et al. (2019) and the M38-A/CLSI standard of the Clinical Laboratory Standard Institute (CLSI). In this experiment, the antifungal potential of

ethanolic extracts of the microalgae *T. suecica* and *C. weissflogii* was evaluated against the fungal isolate *C. gloeosporioides*. The assays were conducted under sterile conditions in triplicate.

The microalgae extracts were diluted in Synth®'s Dimethyl Sulfoxide (DMSO) and, from this dilution, a stock solution at a concentration of 100 mg mL^{-1} was prepared. The concentration range evaluated was from 0.0115 to 6 mg mL^{-1} (4% DMSO). The culture medium Sabouraud Dextrose (TM MEDIA®) was used and prepared according to the manufacturer's instructions. The microbial suspension was prepared in sterile saline solution (NaCl) at 0.9%, with turbidity adjusted to the McFarland standard of 0.5 barium sulfate ($BaSO_4$), equivalent to 1.5 x 10^8 CFU mL^{-1}.

In sterile plates of 96 wells, 50 µL of culture medium were pipetted into each well, and then 50 µL of the stock solution of the compound was added to the first column. The contents were mixed and 50 µL were transferred to well B, and so on until H, discarding the remaining 50 µL. Serial dilution resulted in a concentration range ranging from 0.0115 to 6 mg mL^{-1}. Subsequently, 50 µL of the microorganism suspension was added to each compartment. After this step, the samples were placed in an incubator oven at 32°C for 5 days. The lowest concentration of the compound that completely inhibited the visible growth of the fungus after 5 days of incubation at 32°C was considered as the Minimum Inhibitory Concentration (MIC).

The material from the wells without visible mycelial growth was transferred to Petri dishes containing PDA solid medium. These plates were placed in incubation under the standardized conditions (32°C for 5 days) and, at the end of the designated period, the Minimum Fungicide Concentration (CFM) and the type of activity (fungicide or fungistatic) could be calculated (Bona et al., 2014). The CFM/CIM ratio was used to evaluate the type of activity, with fungicide being < 4 and fungistatic ≥ 4 (Freires et al., 2016).

3. Results and Discussion

Growth curve

The microalgae growth was monitored daily by reading the absorbance in a spectrophotometer for 15 days in order to determine the most favorable time for the collection of microalgae biomass. According to literature, studies show that the secondary metabolites of marine microalgae are concentrated in the the stationary phase of growth, as well as a higher cell density (Ramos et al., 2017; Lage et al., 2023; Santos et al., 2024).

In the present study, the growth curve obtained for the microalgae *C. weissflogii* (Figure 2-A) obtained an exponential phase from day 0 to 8, followed by a stationary phase, not very well defined, from day 9 to 15. For this species of microalgae, the time determined for biomass collection was day 9, at the stationary growth phase where there was a higher density of microalgal biomass.

Regarding the growth curve of the chlorophycea *T. suecica* (Figure 2-B), an adaptation phase (lag) was observed from day 0 to 3, followed by an exponential phase from day 4 to 15. In this case, as there was no stationary phase of growth, the collection was settled for day 15, when there was a higher density of microalgal biomass.

In literature, other studies have already evaluated the growth curve of these microalgae species (Lane & Morel, 2000; Go et al., 2011; Garcia et al., 2012; Abiusi et al., 2013).

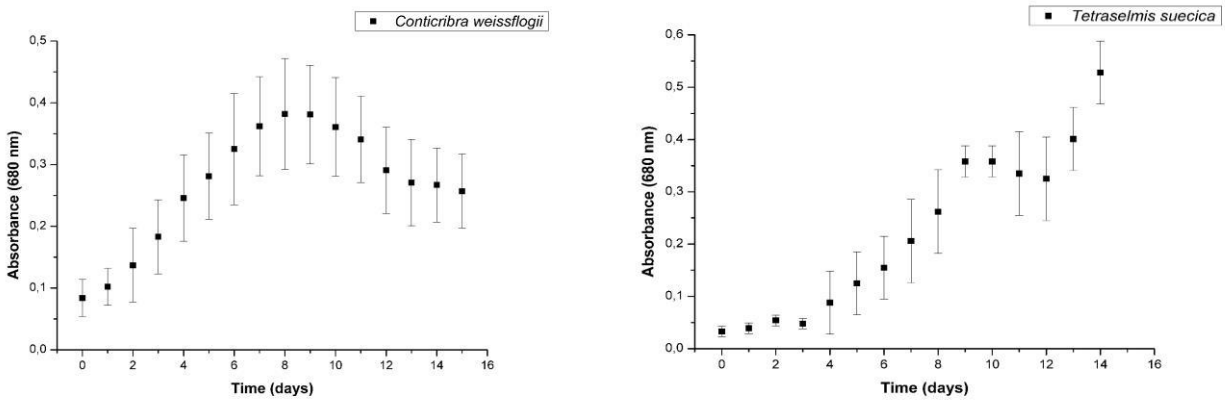
Lane e Morel (2000) evaluated the growth curve of the diatom *C. weissflogii* for six days, under different concentrations of heavy metals and carbon dioxide. In all the conditions evaluated, the microalgae presented exclusively the exponential phase from day zero to six, similar to the present study. In the study by García et al. (2012), the growth curve of the microalgae *C. weissflogii* was evaluated for nine days, under different salinity conditions. In all the conditions evaluated, the curve showed the exponential growth phase from day zero to seven, followed by the stationary phase from seven to nine. This result is relatively close to that obtained in the present study, since the stationary phase began on day eight.

In the study by Go et al. (2011), the growth curve of the microalgae *T. suecica* was followed for 8 days, under different intensities of white light (36.3 to 133.1 μmol m^{-2} s^{-1}). In all light concentrations evaluated, the microalgae showed the exponential growth phase from day zero to eight. Abiusi et al. (2013) analyzed the growth curve of the microalgae *T. suecica* under different light spectrums (white, red, blue, and green) for nine days. It was verified that with the white and red lights the curve remained in the exponential phase from day zero to nine and with the blue and green lights the exponential phase was from day zero to three, followed by a stationary phase from day four to nine. The present study used only white light in the cultivation of microalgae and obtained a result similar to that found by Abiusi et al. (2013).

The small differences observed among studies can be attributed to intrinsic factors of each species, as well as other factors, such as the volume and cultivation conditions, since they directly influence microalgae growth (Lourenço, 2006).

Figure 2 - Growth curve of the microalgae *Conticribra weissflogii* (a) and *Tetraselmis suecica* (b) according to absorbance at 680 nm.

(a) **(b)**



Source: Authors.

Susceptibility testing

In the present study, the broth microdilution method was used to evaluate antifungal activity ethanolic extracts. The diatom *C. weissflogii* visually inhibited 100% growth of the phytopathogen evaluated in the first three wells of the microdilution plate, thus recording the Minimum Inhibitory Concentration (MIC) of 1.5 mg mL^{-1} (Table 1). Chlorophycea *T. suecica* did not inhibit fungal growth at any of the concentrations evaluated, so it was not possible to determine the MIC (Table 1).

After microdilution, the contents of the wells in which no visible mycelial growth occurred were seeded on plates containing PDA medium under the standardized conditions (5 days, 32°C) to determine the Minimum Fungicide Concentration (CFM) and the nature of the activity (fungicide or fungistatic). After sowing, the CFM of the microalgae *C. weissflogii* was 6 mg mL^{-1}, thus registering a fungistatic activity (Table 1).

Table 1 - Values of Minimum Inhibitory Concentration (MIC) (mg mL^{-1}) and Minimum Fungicide Concentration (MFC) (mg mL^{-1}) of the extracts of the microalgae *C. weissflogii* and *T. suecica* against the phytopathogen *C. gloeosporioides*. The type of activity, fungicide (FC) or fungistatic (FT), was determined according to the CFM/MIC ratio.

Microalgae	CIM	CFM	Activity
Conticribra weissflogii	1.5	6	FT
Tetraselmis suecica	-	-	-

(-): No inhibition. Source: Authors.

In the literature, other studies have already reported the susceptibility of genus *Colletotrichum* to micro and macroalgae extracts, as well as their isolated compounds (Kim, 2006; Machado et al., 2011; Kim et al., 2018; Lage et al., 2024).

Lage et al. (2024) evaluated the antifungal potential of ethanolic extracts of the microalgae *Ankistrodesmus falcatus*, *Chaetoceros neogracilis*, *Desmodesmus brasiliensis*, *Dunaliella tertiolecta*, *Kirchneriella lunaris*, and *Tetraselmis gracilis* against the phytopathogens *C. gloeosporioides* and *C. fructicola*. The extracts were tested in the concentration range of 0.0115 to 6 mg mL^{-1}, by the broth microdilution method. Four of the evaluated extracts (*A. falcatus*, *C. neogracilis*, *D. brasiliensis* and *K. lunaris*) recorded antifungal activity. The lowest MIC values recorded were related to *K. lunaris* extract compared to *C. fructicola* (MIC 0.047 mg mL^{-1}) and *C. gloeosporioides* (MIC 0.75 mg mL^{-1}).

Kim et al. (2018) investigated the antifungal potential of chlorophycea *Chlorella fusca* in relation to *Colletotrichum orbiculare*. It was observed that the prior application of the *C. fusca* suspension to cucumber crops triggered the activation of defense responses in host cells, resulting in systemic acquired resistance (SAR) against *C. orbiculare*.

Kim (2006) evaluated 142 cyanobacterial strains against different phytopathogenic fungi, including *C. gloeosporioides*. Among these strains, six of them (*Dolichospermum solitarium*, *Calothrix brevissima*, *Nostoc commune*, *N. muscorum*, *Nodularia* sp. and *Oscillatoria angustissima*) demonstrated the ability to inhibit the growth of *C. gloeosporioides*, with emphasis on the species *N. muscorum* that exhibited the greatest antifungal potential.

Machado et al. (2011) investigated the impact of macroalgae extracts on the vegetative growth in vitro of *C. gloeosporioides*, through the analysis of the Mycelial Growth Velocity Index (MCVI). Their results indicated that extracts of *Hypnea musciformis*, *Laurencia dendroidea* and *Ochtodes secundiramea* exerted significant effect on growth inhibition of the fungus, especially the extracts of *O. secundiramea*, which at concentrations of 1 ppm and 2 ppm, reduced IVCM by 91% and 100%, respectively.

This was a pioneering study on the antifungal activity of the microalgae *C. weissflogii* and *T. suecica* against the phytopathogen *C. gloeosporioides*. Due to the great biodiversity and wide biotechnological potential of microalgae, there is a promising gap in the literature regarding antifungal activity against phytopathogenics and human or animal fungi (Falaise et al., 2016; Lage et al., 2022). Less than 1% of microalgae species has already been explored (Lage et al., 2024) so far. According to that, further work should be conducted in order to explore biotechnological potential of microalgae, leading to new inventions as sustainable biopesticides for pest control in agriculture.

4. Conclusions

The diatom *C. weissflogii* demonstrated antifungal potential against the phytopathogen *C. gloeosporioides* (MIC: 1.5 mg mL^{-1}), with fungistatic activity. Chlorophycea *T. suecica* did not show inhibition of fungal growth. This is a pioneering work on the antifungal activity of these microalgae species against phytopathogenic fungi. Further research can be carried out

aiming isolation and identification of active biomolecules with antifungal properties, as well as formulation of new biopesticides.

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