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Biotransformation of nitazoxanide by filamentous fungi - a microbial model to mammalian metabolism

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Filamentous fungi exhibit complex morphology and display various shapes depending on the species and environmental conditions, characteristics that provide advantages in biotransformation studies. These characteristics serve as advantages in biotransformation studies by allowing substances to undergo processes that lead to the production of both existing metabolites and new compounds with potential pharmacological activity. In this regard, the biotransformation of drugs using microorganisms emerges as an economical and ecologically viable strategy for modifying the structures of biologically active compounds, studying the metabolism of molecules, and eliminating or reducing their toxicity. Therefore, the objective of this study was to investigate the biotransformation capacity of the drug nitazoxanide (NTZ) by the endophytic fungi *Aspergillus niger* ATCC 9029 and *Cunninghamella elegans* ATCC 9245. High-performance liquid chromatography was employed to monitor metabolite formation, while ultra-high-performance liquid chromatography coupled with sequential mass spectrometry (UHPLC-QTOF/MS) was utilized to identify these metabolites. After an incubation period of 240 hours, NTZ was transformed into two metabolites by *C. elegans*, while *A. niger* demonstrated a consumption rate of 94.17% for NTZ, with two additional metabolites identified. This study highlights the potential of using fungi both as a model for metabolism and as a means of producing metabolites on a larger scale, while also identifying a new and significant area of application for the biotransformation approaches involving filamentous fungi.

Keywords: nitazoxanide; biotransformation; *Aspergillus niger*; *Cunninghamella elegans*; metabolites.

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Introduction

Drug biotransformation is a critical process in modern pharmaceutical development, driving advancements in understanding metabolic pathways, identifying active metabolites, and optimizing the efficacy, toxicity, and bioavailability of bioactive compounds (1,2). Nitazoxanide (NTZ, Figure 1) is a nitrothiazole benzamide compound with a wide range of antimicrobial activity that was first synthesized by Rossignol and Cavier in 1975 (3). Since its discovery, NTZ has been widely applied for the treatment of various infections caused mainly by intestinal parasites and pathogenic aerobic and anaerobic bacteria in humans and animals. NTZ and its metabolite, tizoxanide (Figure 2), are related to the broad spectrum of *in vivo* activity against intestinal parasites and aerobic and anaerobic bacterial enteric pathogens infecting animals and humans (4). In 2015, *in vitro* studies presented that NTZ was documented as an anti-HIV activity, being the first thiazolidic compound to this application (5).
Despite the well-established metabolic pathway of NTZ in humans, there remains a need to explore alternative models for studying its biotransformation, particularly for identifying novel metabolites and understanding the underlying enzymatic mechanisms (1).

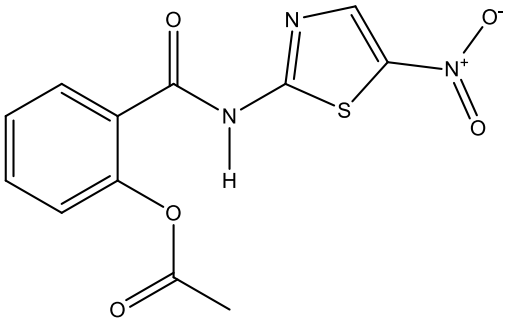


Figure 1. Chemical structure of nitazoxanide (NTZ).

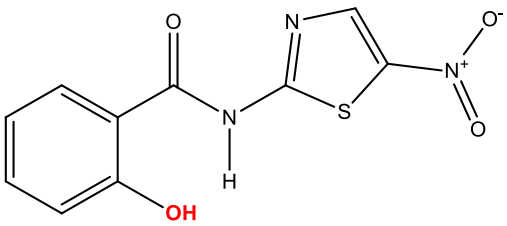


Figure 2. Chemical structure of tizoxanide.

Drug metabolism studies are carried out in animal models, such as pigs, rats, and mice. However, these *in vivo* studies have suffered several limitations regarding ethical, financial, and study time issues (6). As a result, alternatives for *in vitro* studies were developed using cell lines, fragments of the human liver, and other tissues. These studies provide useful information at the cellular level. However, *in vitro* studies also have disadvantages: cellular genotype variability, incomplete identification of metabolites, and reduced attainment of metabolites of interest (7,8).

Given the disadvantages of *in vivo* and *in vitro* studies in research into drug biotransformation, endophytic fungi provide several advantages over chemical synthesis. Therefore, biotransformation is a useful method for producing novel compounds; enhancing the productivity of the desired compound; overcoming the problems associated with chemical analysis and leading to basic information to elucidate the biosynthetic pathway (9). Microbial factories pose advantages such as rapid growth, low cost, ecologically viable technique, convenient genetic manipulations, and with optimization of the method, can produce a high level of product biotransformation, through a process known as "green chemistry" (1,2,10).

Biotransformation studies of biologically active compounds through microorganisms, mainly by filamentous fungi, and their possible glycosylated derivatives have been extensively investigated because biotransformation usually shows advantages such as one-step simple reaction and eco-friendly production (2,11,12). *Aspergillus* and *Cunninghamella* are genera of filamentous fungi that have frequently been proposed for glycosylation reactions, and it is an interesting tool in the production of molecules with improved pharmacological profiles (12,13). To address these knowledge gaps, this study proposes the utilization of filamentous fungi *Cunninghamella elegans* and *Aspergillus niger* (14) as microbial models to mimic mammalian NTZ metabolism. These fungi, known for their capacity to express cytochrome P450-like enzymes, can perform Phase I (oxidation, reduction, hydrolysis) and Phase II (conjugation) reactions, yielding metabolites that may aid in understanding NTZ metabolism and identifying novel compounds with pharmacological potential (15,16). To this end, sensitive analytical techniques for separating the analytes produced and identifying them are essential for the performance of the study. Among the main techniques applied, is High Performance Liquid Chromatography (HPLC) coupled to a mass detector (17–19).

Biotransformation provides an efficient and environmentally friendly way to produce large-scale metabolites of drugs and is considered an alternative approach to asymmetric synthesis and conventional *in vivo* and *in vitro* metabolism studies (20,21). This process is attractive for the synthesis of new drugs, which are also required for biological and toxicity tests and analytical standards. Filamentous fungi are also considered promising as new biocatalysts, especially when they involve chiral compounds since many authors report stereoselective reactions mediated by these microorganisms. These chemo and stereoselective processes are very important in the synthesis of various chemicals and pharmaceuticals (22).

Despite significant advancements in elucidating the metabolism of nitazoxanide in mammals, limited research has explored the potential of filamentous fungi as microbial models for this process. This study addresses this gap by systematically investigating the biotransformation capabilities of *A. niger* and *C. elegans*, two filamentous fungi with established enzymatic capacities analogous to mammalian systems. In this context, this study aimed to evaluate the biotransformation capacity of NTZ by the fungi *C. elegans* ATCC 9245 and *A. niger* ATCC 9029 as a microorganism model for mammalian metabolism. We employed UHPLC-MS/MS for the analysis and prediction of the chemical structure of the proposed metabolites. This study highlights the potential of using fungi both as a model for metabolism and as a means of producing metabolites on a larger scale, while also identifying a new and significant area of application for the biotransformation approaches involving filamentous fungi.

Experimental section

Materials

Chemicals

NTZ was donated by Althaia S/A Pharmaceutical Industry (Atibaia, São Paulo, Brazil). As culture media, Potato-Dextrose Broth and Agar were obtained from Kasvi (Paraná, Brazil). Water was purified by a Milli-Q system Millipore (Molshein, France). Acetonitrile (HPLC grade) and ethyl acetate were purchased from Merck (New Jersey, USA) and Vetec (São Paulo, Brazil), respectively.

Microorganisms

Cunninghamella elegans ATCC 9245 was generously donated by Laboratório de Bioconversão (LABICON), Universidade Federal de Goiás, Brazil) and *Aspergillus niger* ATCC 9029 was generously donated by Valério R. Aquino, Unit of Microbiology of Hospital de Clínicas, Porto Alegre, Rio Grande do Sul, Brazil. Stock cultures were cut in discs of 0.5 cm in diameter of Sabouraud-Dextrose Agar and maintained on sterile sodium chloride 0.9% (w/v) at 5 ºC.

Methods

Biotransformation assay

The biotransformation assay was divided into three stages. Stage I: individuals standards spore solution of *A. niger* and *C. elegans*. It was prepared by growing the fungi for 3 days at 27 ºC in tubes containing Potato dextrose agar. The

standardization technique was adapted from the Clinical and Laboratory Standards Institute (CLSI) M38 protocol for filamentous fungi (23) to obtain 10^8 spores/mL using a spectrophotometer (Analyser 800-M), and inoculated in sterile Potato-Dextrose Broth (19 ml) in Falcon tubes and cultivated for 48 hours at 27 ºC in an orbital shaker (New BrunswickTM Innova® 2300) at 120 rpm. Stage II: the substrate of NTZ was added into each vial containing Potato-Dextrose Broth (Drug Control, DC) and Potato-Dextrose Broth with standard inoculum (Biotransformation, BIO) to give the final NTZ concentration of 100 µg/mL. Control samples consisted of sterile Potato-Dextrose Broth (Blank: B) and with standard inoculum (Growth Control: GC). All control samples were submitted under the same conditions of incubation. Stage III: samples were collected under aseptic conditions every 48 hours (triplicate) for 10 days. Samples were stored at al low temperature (-20 ºC) until the extraction processes.

The biotransformation study of NTZ followed the flowchart shown in Figure 3. *A. niger* ATCC 9029 and *C. elegans* ATCC 9245 were chosen based on previous literature review which indicates this strain is promising for the biotransformation process, expressing enzymes necessary for carrying out the reaction in drugs. Also, previous studies conducted in our study group had shown the best pH and shaken conditions were chosen to conduct this study.

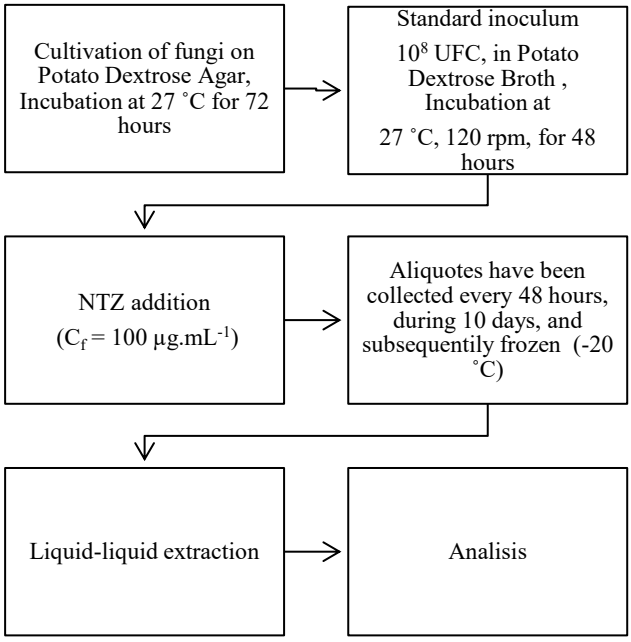


Figure 3. Flowchart of the experimental steps.

Sample preparation and analysis

Cultures and controls were submitted to a liquid-liquid extraction with three equal volumes of ethyl acetate. Samples obtained from the biotransformation reaction were also extracted with ethyl acetate. Aliquots of 2 mL were vigorously shaken three times with ethyl acetate (2 mL). The organic phase was separated, and the solvent was evaporated to dryness at room temperature. The residues were dissolved in 2 mL of acetonitrile, filtered in a 45 µm filter, and analyzed by HPLC.

High-Resolution Mass Spectrometer Analysis

The structural elucidation of metabolites formed was performed through the UHPLC Shimadzu (Model UFLCXR) equipped with Agilent Zorbax Eclipse Plus C18 column (2.1 x 50mm, 8µm). ESI-MS/MS analyses were performed on a microTOF-Q III (Bruker Daltonics, Bremen, Germany) equipped with an ESI interface operating in negative ion mode using a capillary voltage of 4.5 kV was used to identification of metabolites of NTZ. The other optimum values of the ESI-Q-TOF parameters were drying gas temperature, 200 °C; drying gas flow, 6.0 $L.min^{-1}$, and nebulizing gas pressure 2 bar. The detection was made considering a mass range of 20 – 1000 m/z acquired with a spectra rate of 1.00 Hz. Nitrogen was used as drying and nebulizing gas. The mass spectrometer was calibrated through a solution of sodium formate (10 mM) in negative mode, and the mass data were processed using Data Analysis 4.3 software (Bruker Daltonics, Bremen, Germany).

After 240 hours of experiment, aliquots of Biotransformation (BIO), (Drug Control) DC, (Microbiological Control) MC, and Blank were collected, extracted, and analyzed by UHPLC-ESI-MS/MS, at Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA).

Results and Discussion

The biotransformation of NTZ by *C. elegans* ATCC 9245 and *A. niger* ATCC 9029 was conducted over 10-day period. For sample preparation, culture fluids were subjected to liquid-liquid extraction using ethyl acetate was the extractor solvent in liquid-liquid extraction (three extractions), followed by combining the extracts, drying under airflow, and redissolving the residues in acetonitrile for HPLC analysis. The recovery rate of NTZ in the drug control (DC) extraction was 78%.

The extracts were analysed by UHPLC. The elution profiles of the extracts from *C. elegans* ATCC 9245 grown with NTZ for 10 days showed two products ($P1_{C.\ elegans}$ and $P2_{C.\ elegans}$) as well as residual NTZ (Figure 4). Blank and MC had no peaks corresponding to any of those products.

Cunninghamella is a genus frequently employed for glycosylation reactions and serves as a valuable tool in

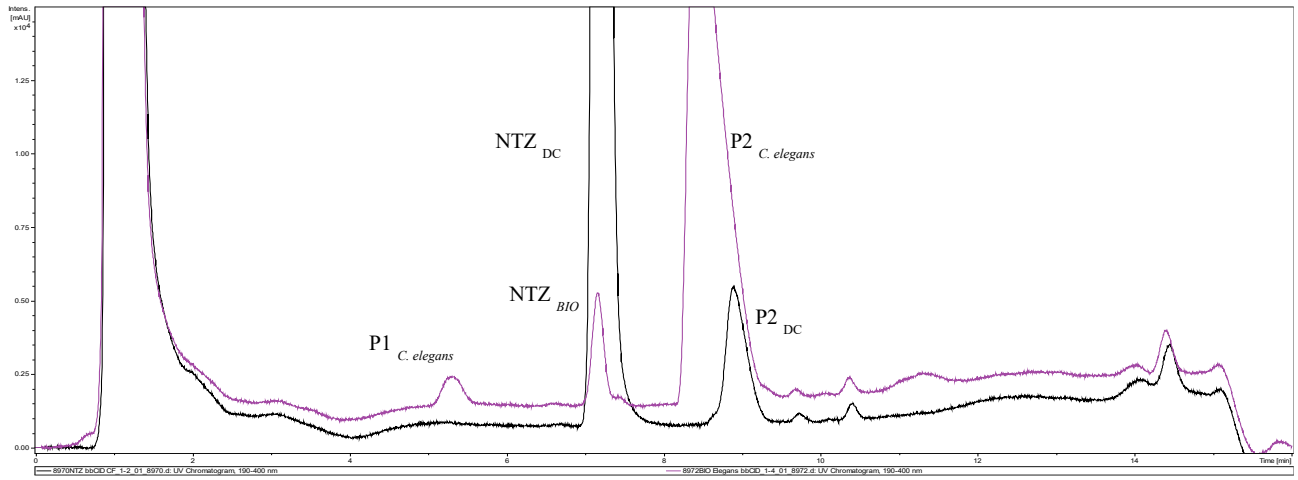


Figure 4. HPLC chromatograms. The black line expresses the extract obtained from the Drug Control flask and, in purple, the extract from the biotransformation of *C. elegans* ATCC 9245.

producing molecules with enhanced pharmacological profiles. Interestingly, our results revealed a different performance pattern for this genus in the biotransformation of nitazoxanide. As a result of this biotransformation study, BIO and drug control (DC) had the same retention time of NTZ in (7.2 min), UV spectrum (λ_{max} = 345 nm), and mass spectra as well. Extract from BIO showed peaks at 5.3 min ($P1_{C.\ elegans}$), 7.2 min (NTZ_{BIO}) and 8.7 min ($P2_{C.\ elegans}$) and the extract from DC showed NTZ and P2 peaks.

The first step in the analysis of NTZ metabolites was to evaluate the product ions and fragmentation products of the parent compound. According to the mass spectra, NTZ resulted in a deprotonated molecule $[M-H]^-$ at m/z 306 (Figure 5). The mass spectra obtained by LC/MS/MS for $P1_{C.elegans}$ had ionized molecule at m/z 410.744 [M-H] (Figure 6B, A) and $P2_{C.\ elegans}$ had ionized molecules at m/z 264.019 $[M-H]^-$ (Figure 6, B). The fragment ion at m/z 264 in the stage by the loss of $-CH_2CO$, indicative of NTZ deacetylation. The spectra of NTZ revealed the same dissociation pathway as $P2_{C.elegans}$. The $P2_{C.elegans}$ suggested that had been formed by hydrolysis to NTZ. Therefore, $P2_{C.elegans}$ was identified as tizoxanide, a known NTZ metabolite. Despite the DC showing $P2_{C.elegans}$ peak, the peak area in BIO was more major than DC, demonstrating the capability to NTZ biotransformation by *C. elegans* ATCC 9245.

Considering the mass difference between the $P1_{C.\ elegans}$ (m/z 264 $[M-H]^-$) and $P2_{C.elegans}$ (m/z 410 $[M-H]^-$) we observed a fragmentation related to glutamic acid conjugated (147 Da). A proposed biotransformation route is shown in Figure 9. Despite the reported glutamic acid conjugation bioreaction in a microorganism, this amino acid is mentioned as a molecule involved in conjugating xenobiotics as a pathway in the biotransformation of several compounds in other organisms. Glutamic acid is an amino acid associated, among other metabolic activities, in the process of detoxification of drugs in humans (24,25)

The amino acid conjugation is classified as phase II. Phase II reactions as synthetic reactions involving the conjugation of the drug or phase I metabolite with endogenous substances such as glucuronic acid, methyl groups, and glycosylation. Those molecules are involved in a large reaction catalysed by CYP 450 (9,12,26).

The elution profiles of the extracts from cultures of *A. niger* ATCC 9029 grown with NTZ for 10 days showed two metabolites ($P1_{A.niger}$ and $P2_{A.niger}$) as well as residual NTZ. In the chromatograms of microbiological control, there was no interference from endogenous or secondary metabolites from *A. niger* for the analysis of NTZ and its metabolites (Figure 7).

The biotransformation reaction of the filamentous fungus *A. niger* ATCC 9029 led to the formation of two compounds (8.2 and 8.9 min retention times), which were not present in the controls. In the chromatograms of microbiological controls, no interference from *A. niger* for the analyses was observed, and no significant decrease in drug control of NTZ was observed. The presence of peak

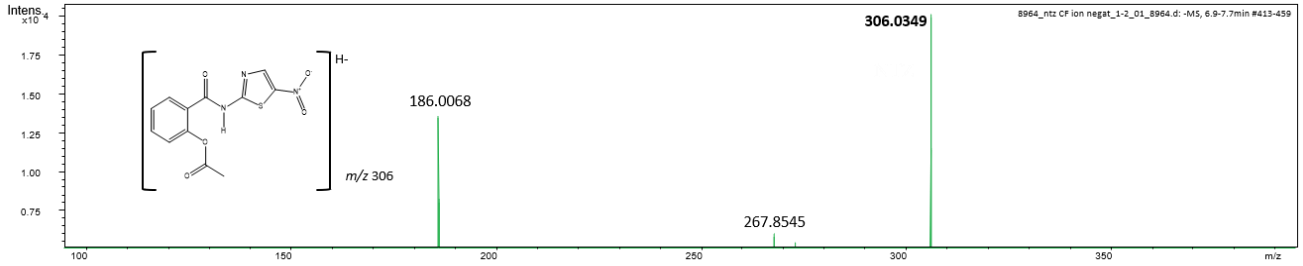


Figure 5. Fragmentation for NTZ standard.

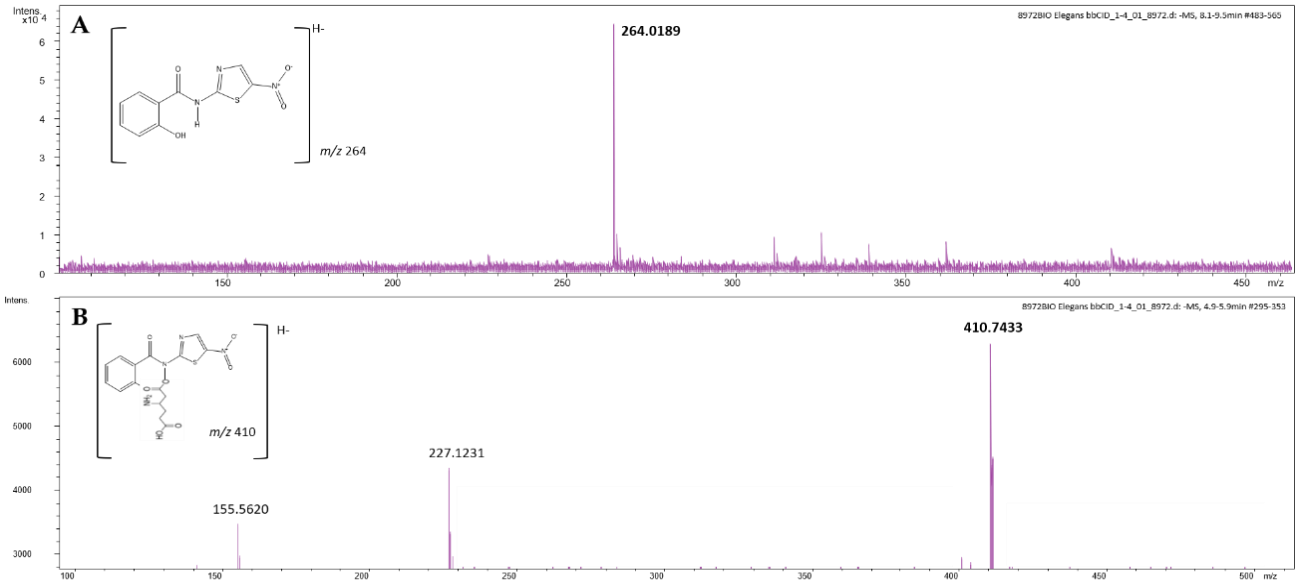


Figure 6. MS/MS spectra showing the fragments for P2$_{C.elegans}$ (A) and P1$_{C.elegans}$ (B) by the fungus *C. elegans* ATCC 9245.

P2$_{A.niger}$ in the drug control suggests that the drug is undergoing drug degradation. This way, they can be considered a degradation product of the drug. The presence of two peaks in BIO shows the fungi in the study can be biocatalyst compounds of industrial interest, but under the conditions submitted, there were signs of possible metabolites. In the chromatograms of culture controls, there was no interference from endogenous or secondary metabolites *A. niger* for the analysis. The peak area obtained from NTZ in BIO was only 5.84% of the NTZ peak area in DC, suggestive of the metabolism of the drug.

Using UHPLC-QTOF/MS it was possible the identification of NTZ. It was confirmed by comparison of the retention time and mass spectra with the authentic reference. Precursor ion can be observed in the mass spectrum with MS *m/z* 306 (M^-) and major fragments there of MS *m/z* 186 and 267 (Figure 5).

The mass spectrum in negative mode to the P2$_{A.niger}$ presents a molecular ion at MS *m/z* 264 (M^-), corresponding to the $(M-H)^-$ ion. Considering the mass difference between the standard and the molecule (*m/z* 42) we observed fragmentation related to deacetylation ($-CH_2CO$) in the molecule of NTZ. Therefore, P2$_{A.niger}$ was identified as tizoxanide (Figure 8, A) (4,27). This result was expected for this molecule in the experimental conditions according to previous assays.

Besides the LC-MS/MS shown P1$_{A.niger}$ (Figure 8, A) and P2$_{A.niger}$ (Figure 8, B) similar spectra, precursor P1$_{A.niger}$ ion can be observed in the mass spectrum with MS *m/z* 339 $[M-H]^-$ and major fragments there of MS *m/z* 227, 325, 410 and the precursor ion observed in P2$_{A.niger}$ with MS *m/z* 339 and major fragments *m/z* 227 and 325. The optimization in the extraction process is essential to increase P2$_{A.niger}$ concentration, allowing accuracy in the elucidation of P2$_{A.niger}$. Despite this, other studies in our group suggest a conjugation reaction of tizoxanide and glutamic acid as shown in Figure 99.

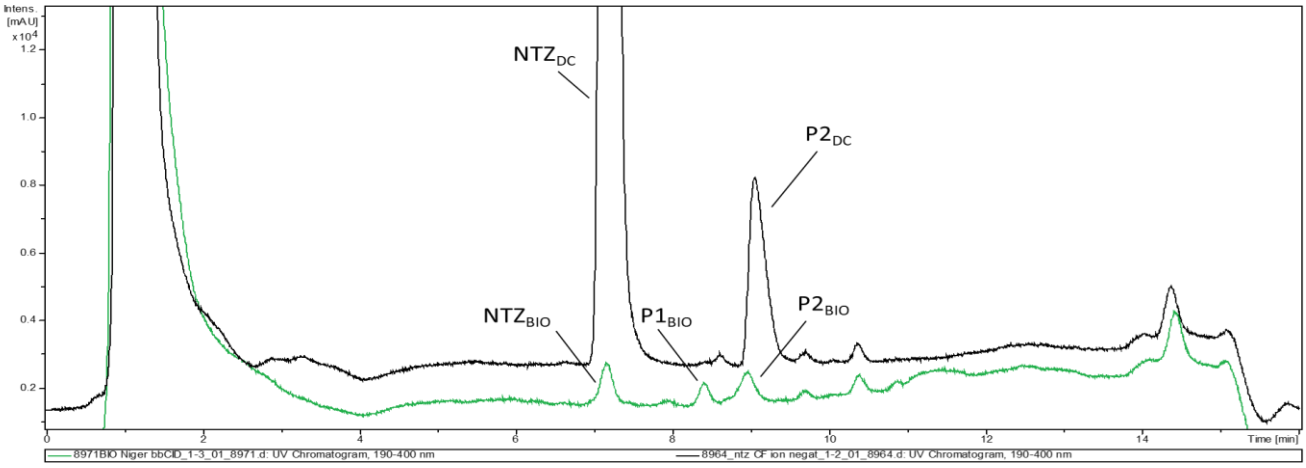


Figure 7. HPLC chromatograms: the black line represents the Drug Control (DC) flask, and the green line represents the Biotransformation (BIO) of NTZ by *A. niger* ATCC 9029 after the extraction process.

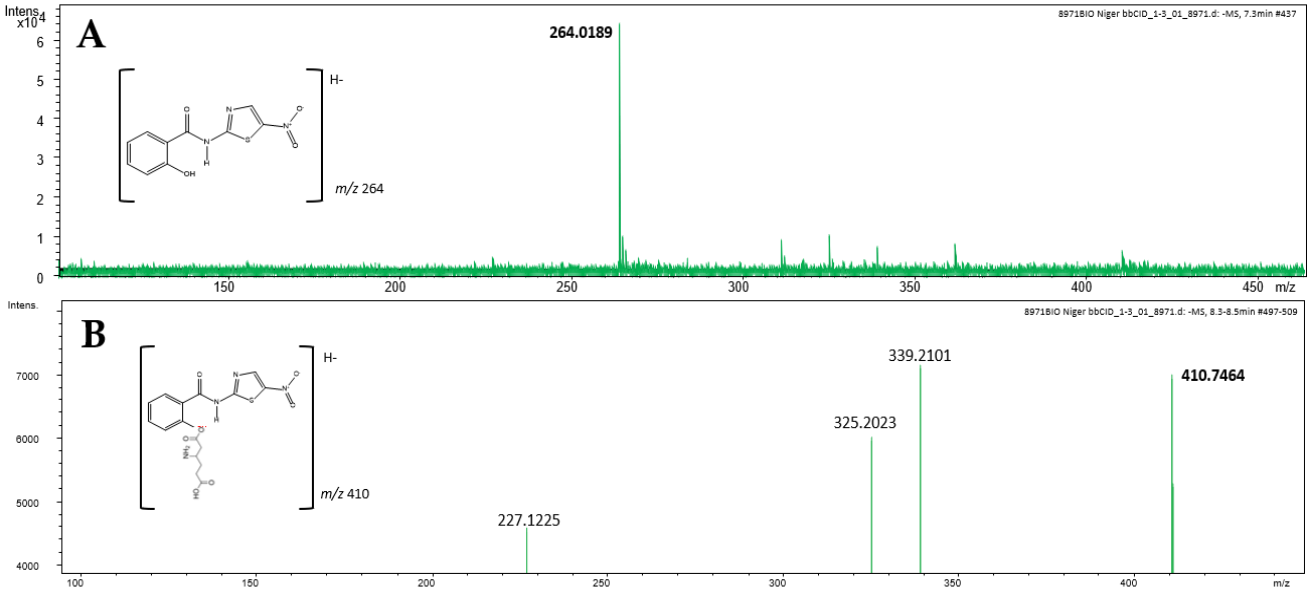


Figure 8. MS/MS of products obtained by NTZ biotransformation by *A. niger* ATCC 9029. **A**) P1$_{A.niger}$ and **B**) P2 $_{A.niger}$.

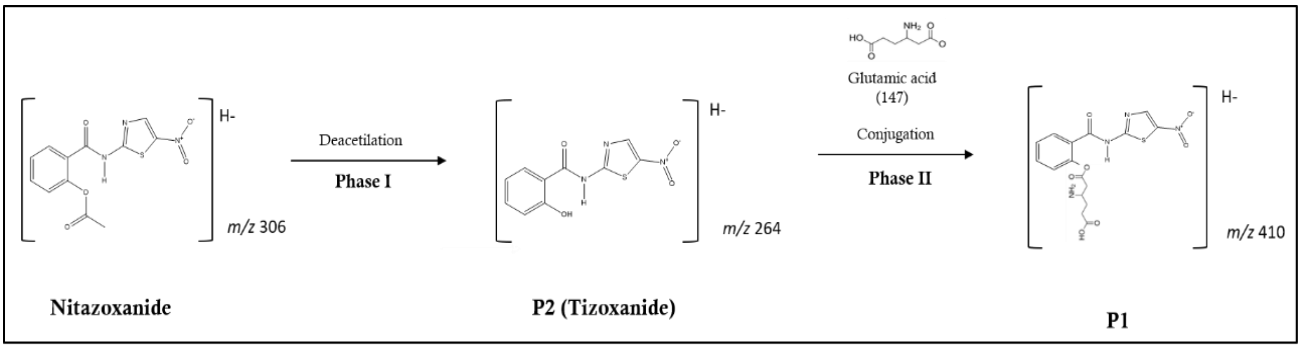


Figure 9. Pathway proposed metabolic of NTZ by *Cunninghamella elegans* ATCC9245 and *Aspergillus niger* ATCC 9029 biotransformation study.

The identification of tizoxanide, a known metabolite of NTZ, in our fungal biotransformation experiments suggests that *C. elegans* and *A. niger* can effectively mimic Phase I metabolic processes. Furthermore, the detection of a glutamic acid conjugate points to the involvement of Phase II conjugation reactions. While amino acid conjugation is less commonly reported in microorganisms compared to glucuronidation, its occurrence in our study highlights the metabolic versatility of these fungi. This is the first report about acid glutamic conjugation and *C. elegans* drug biotransformation.

The selection of *C. elegans* and *A. niger* as microbial models was based on their ability to mimic mammalian drug metabolism, particularly reactions mediated by cytochrome P450 (CYP) enzymes (20). Filamentous fungi, like mammals, are eukaryotic cells and possess a diverse range of enzymatic systems capable of performing phase I and phase II biotransformations (28). These fungi have been shown to catalyse reactions such as oxidation, reduction, hydrolysis, and conjugation, which are commonly observed in mammalian drug metabolism (1).

Nitazoxanide was chosen as a model drug due to its well-characterized metabolism in humans, primarily involving deacetylation to tizoxanide (4). This known metabolic pathway provides a benchmark for evaluating the ability of the fungal models to replicate mammalian metabolism. Furthermore, NTZ has a relatively simple chemical structure, making it easier to analyse and identify potential metabolites (3).

Filamentous fungi differ from yeasts in their morphology, physiology, and enzymatic capabilities. Filamentous fungi have a more complex cellular structure, with hyphae and mycelia, which provide a larger surface area for drug interaction and biotransformation (29). Additionally, filamentous fungi often possess a wider range of enzymes involved in drug metabolism compared to yeasts, making them more versatile models for studying mammalian metabolism (20).

The biotransformation of NTZ by *C. elegans* resulted in the formation of two metabolites, P1$_{C.elegans}$ and P2$_{C.elegans}$. P2$_{C.elegans}$ was identified as tizoxanide, the primary metabolite of NTZ in humans, based on its retention time

and mass spectra (4). This finding suggests that *C. elegans* is capable of replicating at least one aspect of mammalian NTZ metabolism.

$P1_{C.elegans}$ was found to be a glutamic acid conjugate of tizoxanide, a novel metabolite not previously reported in human metabolism. This conjugation reaction is classified as phase II and involves the addition of glutamic acid to the tizoxanide molecule (24). Glutamic acid conjugation has been observed in other organisms, such as birds and pigs, as a detoxification mechanism for xenobiotics (27). The identification of this novel metabolite highlights the potential of *C. elegans* to generate unique metabolites not found in mammalian systems. This finding not only contributes to the understanding of biotransformation mechanisms in fungi but also suggests potential applications in the production of novel derivatives with distinct pharmacological properties.

The biotransformation of NTZ by *A. niger* also resulted in the formation of two metabolites, $P1_{A.niger}$ and $P2_{A.niger}$. $P2_{A.niger}$ was identified as tizoxanide similar to the findings with *C. elegans*. $P1_{A.niger}$ was found to have a similar mass spectrum to $P2_{A.niger}$, suggesting that it may be a derivative of tizoxanide. However, further studies are needed to fully elucidate the structure of $P1_{A.niger}$.

While both *C. elegans* and *A. niger* were able to produce tizoxanide, the primary metabolite of NTZ in humans, there were also differences in the metabolites formed. The identification of a glutamic acid conjugate of tizoxanide by *C. elegans* suggests that this fungus may possess unique enzymatic capabilities not found in mammalian systems. It is important to note that the absence of certain mammalian metabolites in the fungal study does not necessarily indicate that the fungi are not good models for mammalian metabolism. Rather, it may reflect differences in the specific enzymes expressed by the fungi and the reaction conditions used in the study (20).

This study has several limitations that should be considered. First, the concentrations of the metabolites formed were not quantified, making it difficult to assess the efficiency of the biotransformation process. Second, the structure of $P1_{A.niger}$ was not fully elucidated, requiring further investigation. Third, the study did not compare the biotransformation rates of NTZ by the fungi with those observed in mammalian systems.

Future studies should focus on quantifying the metabolites formed, elucidating the structure of $P1_{A.niger}$, and comparing the biotransformation rates with mammalian systems. Additionally, it would be valuable to investigate the enzymatic mechanisms involved in the formation of the novel glutamic acid conjugate of tizoxanide. Finally, it would be interesting to explore the potential of using other filamentous fungi as models for mammalian drug metabolism.

These results align with previous studies demonstrating the ability of *Cunninghamella* species to perform glycosylation and other biotransformation reactions (9,12,26). The biotransformation reaction of the filamentous fungus *A. niger* ATCC 9029 led to the formation of two compounds (8.2 and 8.9 min retention times), which were not present in the controls. In the chromatograms of microbiological controls, no interference from *A. niger* for the analyses was observed, and no significant decrease in drug control of NTZ was observed. The presence of peak $P2_{A.niger}$ in the drug control suggests that the drug is undergoing drug degradation. This way, they can be considered a degradation product of the drug. The presence of two peaks in BIO shows the fungi in the study can be biocatalyst compounds of industrial interest, but under the conditions submitted, there were signs of possible metabolites. The peak area obtained from NTZ in BIO was only 5.84% of the NTZ peak area in DC, suggestive of the metabolism of the drug.

The results obtained in this study pave the way for several avenues of future investigation. Firstly, the complete structural characterization of metabolite $P1_{A.niger}$ using techniques such as NMR will enable confirmation of its identity and potential pharmacological properties. Furthermore, scale-up studies of the biotransformation process could be conducted to produce sufficient quantities of the metabolites for more comprehensive pharmacological and toxicological assays. Investigation of the specific enzymatic mechanisms involved in the formation of the glutamic acid conjugate of tizoxanide also represents a promising area for future research, potentially revealing novel metabolic pathways in filamentous fungi. Finally, the application of this biotransformation approach to other structurally related drugs could expand the repertoire of microbial models for the study of drug metabolism.

Conclusions

We report the biotransformation of NTZ by *A. niger* ATCC 9029 and *C. elegans* ATCC 9245 furnished two derivatives. Microorganisms could be a reliable and efficient alternative to the employment of small animals or synthetic chemistry for obtaining sizable amounts of several drug derivatives. It demonstrates the importance and necessity of investment in these studies involving filamentous microorganisms. Thus, the models can be scaled up easily for the preparation of metabolites for structure confirmation by NMR and further pharmacological and toxicological studies. *Cunnighamella elegans* ATCC 9245 and *Aspergillus niger* ATCC 9029 fungi were able to biotransform NTZ in acid glutamic tizoxanide conjugated (P1) and tizoxanide (P2). This is the first report about acid glutamic conjugation and *C. elegans* drug biotransformation. Our study demonstrated that *C. elegans* and *A. niger* are capable of biotransforming NTZ, yielding metabolites such as tizoxanide and a glutamic acid conjugate, as identified by UHPLC-QTOF/MS. These findings highlight the potential of these fungi as models for mammalian drug metabolism.

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Conflict of interest

The authors declare no conflicts of interest.

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