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Isolation of Alternaria alternata Mycotoxins and Biological Evaluation of Alternaria Extracts in Caenorhabditis elegans

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List of abbreviations

^{1}H COSY	Correlation Spectroscopy
^{13}C-APT	Attached Proton Test
ACN	Acetonitrile
ALP	Alterperylenol
ATX-I	Altertoxin I
ATX-II	Altertoxin II
ATXOE	*Alternaria alternata* extracts
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
EtOAc	Ethyl acetate
f.c.	Final concentration
FA	Formic acid
FUdR	5-Fluoro-2'-deoxyuridine
HMBC	Heteronuclear Multiple Bond Coherence
HSQC	Heteronuclear Single Quantum Coherence
m/z	Mass to charge ratio
mAU	Milli-Absorbance Units
MeOH	Methanol
NOESY	Nuclear Overhauser Effect Spectroscopy
Rf	Retention factor
STTX-II	Stemphyltoxin III
TCA E	Tricycloalternarene E

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1. ABSTRACT

Alternaria species are widespread filamentous fungi known for their diverse range of secondary metabolites, including biologically active compounds and mycotoxins. The perylene quinones altertoxin I (ATX-I) and altertoxin II (ATX-II) have attracted high interest due to their biological activity and potential health and agricultural risks. The aim of this study was to investigate secondary metabolites from *Alternaria* species by using both chemical and biological approaches.

The first part of this master thesis focuses on the chemical characterization of two *Alternaria alternata* extracts (ATXOE_9 and ATXOE_11) obtained from fungal cultures grown in mCDB medium for one week. The extracts originated from mycelium or spores and were cultivated at 24 °C or 28 °C before extraction. Following LC-MS-based dereplication and chromatographic fractionation, ATX-I (15.97 mg) and ATX-II (12.44 mg) could be successfully isolated with purities of 99% and 97%, respectively. Their identities were confirmed with NMR spectroscopy.

In the second part, the impact of extracts derived from 5 *Alternaria alternata* strains and 6 *Alternaria infectoria* strains on lifespan and oxidative stress response was investigated in the model organism *Caenorhabditis elegans*. While extracts tested at concentrations of 50 and 200 µg/mL exerted only minor (mostly insignificant) effects on the nematocidal survival, the oxidative stress response assay showed species-dependent differences. In particular, *A. alternata* extracts significantly and dose dependently lowered fluorescence intensity suggesting pronounced protective effects against oxidative stress.

The obtained chemical and biological data provide a valuable foundation for future projects to identify the metabolites responsible for the observed biological effects.

1.1. ZUSAMMENFASSUNG

Alternaria Arten sind weit verbreitete filamentöse Pilze, die für die Produktion einer Vielzahl unterschiedlicher Sekundärmetabolite bekannt sind, darunter biologisch aktive Verbindungen und Mykotoxine. Besonders die Perylenchinone Altertoxin I (ATX-I) und Altertoxin II (ATX-II) haben aufgrund ihrer biologischen Aktivität sowie ihrer potenziellen gesundheitlichen und agrarwirtschaftlichen Risiken großes Interesse geweckt. Ziel dieser Arbeit war die Untersuchung von Sekundärmetaboliten aus *Alternaria* Arten und Stämmen unter Anwendung sowohl chemischer als auch biologischer Methoden.

Der erste Teil der Arbeit konzentriert sich auf die chemische Charakterisierung der *Alternaria alternata*-Extrakte ATXOE_9 und ATXOE_11, die aus Pilzkulturen gewonnen wurden, die eine Woche lang in mCDB-Medium kultiviert wurden. Die Extrakte stammten aus Myzel oder Sporen und wurden vor der Extraktion bei 24 °C bzw. 28 °C kultiviert. Nach LC-MS-basierter Dereplikation und chromatographischer Fraktionierung konnten ATX-I (15,97 mg) und ATX-II (12,44 mg) mit Reinheiten von 99% bzw. 97% isoliert werden. Die Identität der isolierten Altertoxine wurde mittels NMR-Spektroskopie bestätigt.

Im zweiten Teil wurde der Einfluss von Extrakten, die von fünf *Alternaria alternata*-Stämmen und sechs *Alternaria infectoria*-Stämmen stammten auf Lebensdauer und oxidative Stressantwort im Modellorganismus *Caenorhabditis elegans* untersucht. Während die bei 50 und 200 µg/mL getesteten Extrakte nur geringe (überwiegend nicht signifikante) Effekte auf das Überleben der Nematoden zeigten, wies das Testmodell zur oxidativen Stressantwort artspezifische Unterschiede auf. Insbesondere reduzierten *A. alternata*-Extrakte die Fluoreszenzintensität signifikant und konzentrationsabhängig, was auf eine ausgeprägte Schutzwirkung gegenüber oxidativem Stress hindeutet.

Die gewonnenen chemischen und biologischen Daten stellen eine wertvolle Grundlage für zukünftige Projekte zur Identifizierung der für die beobachteten biologischen Effekte verantwortlichen Metaboliten dar.

2. AIM OF THE WORK

This thesis was divided into two main projects. The first project focused on the mycochemical characterization of two fungal extracts from *Alternaria* species, including LC-MS-based dereplication, chromatographic fractionation, targeted isolation and structural elucidation of selected altertoxins.

The second project aimed to investigate the biological activity of fungal extracts of 11 different strains of two different *Alternaria* species (*A. alternata* and *A. infectoria*) using both survival and oxidative stress response assays employing the model organism *Caenorhabditis elegans*. The aim of this project branch was to generate biological data that can be used to support identify those secondary metabolites contributing to any biological effect.

3. INTRODUCTION

3.1. Fungal Secondary Metabolites and Mycotoxins

Fungi are well known producers of many structurally diverse, low-molecular-weight secondary metabolites with a broad range of biological activities. These compounds, unlike primary metabolites, are not directly required for the fungal development, growth or reproduction, but often contribute to ecological adaptation, defence mechanisms and interspecies competition. Because of their strong structural diversity and biological activity, fungal secondary metabolites represent an important source of pharmacologically relevant compounds, including antibiotics like penicillin, immunosuppressants and anti-cancer agents (Keller, 2019; Keller et al., 2005).

On the other hand, certain fungal metabolites may have toxic properties and are therefore referred to as mycotoxins. Mycotoxins are toxic low-molecular-weight secondary metabolites, produced by many filamentous fungi, including species of *Alternaria*, *Fusarium*, *Aspergillus*, *Claviceps,* and *Penicillium*. These compounds frequently contaminate food products and agriculture, posing potential risks to human and animal health. Depending on their chemical structure and mechanism, mycotoxins may have hepatotoxic, mutagenic, teratogenic, carcinogenic, nephrotoxic, or cytotoxic effects (Bennett & Klich, 2003).

Therefore, fungal secondary metabolites and mycotoxins have become highly relevant research topics regarding toxicology, food safety, natural product chemistry and drug discovery.

3.2. *Alternaria* Species and Altertoxins

Species of the genus *Alternaria* are widespread filamentous fungi that occur ubiquitously from soil and plants to indoor environments and agricultural produce. Many *Alternaria* species are well known plant pathogens and are frequently associated with the contamination of fruits, vegetables, cereal and oil seeds, leading to post-harvest decay or plant disease. Because of their widespread occurrence and ability to produce many different toxic secondary metabolites, *Alternaria* species have rapidly gained high

attention in toxicological and food safety research (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011).

Among the various metabolites produced by *Alternaria* species, perylene quinone derivates such as altertoxin I (ATX-I) and altertoxin II (ATX-II) are of particular interest for both chemical characterization and biological evaluation, due to their high biological activity. These altertoxins have been reported to exhibit a higher mutagenic and genotoxic effect in comparison to other *Alternaria* mycotoxins, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT) and tenuazonic acid (TeA). ATX-II is considered one of the most genotoxic *Alternaria* secondary metabolites due to the presence of its reactive epoxide group, which is assumed to contribute significantly to its DNA-damaging properties (Fleck et al., 2012; Schwarz et al., 2012).

As already stated, *Alternaria* species produce numerous structurally related secondary metabolites. The structural diversity and complexity of fungal extract compositions therefore require comprehensive analytical approaches for metabolite characterization, dereplication and targeted isolation. The chemical structure of the most common *Alternaria alternata* secondary metabolites and their molecular weight are shown in Figure 1.

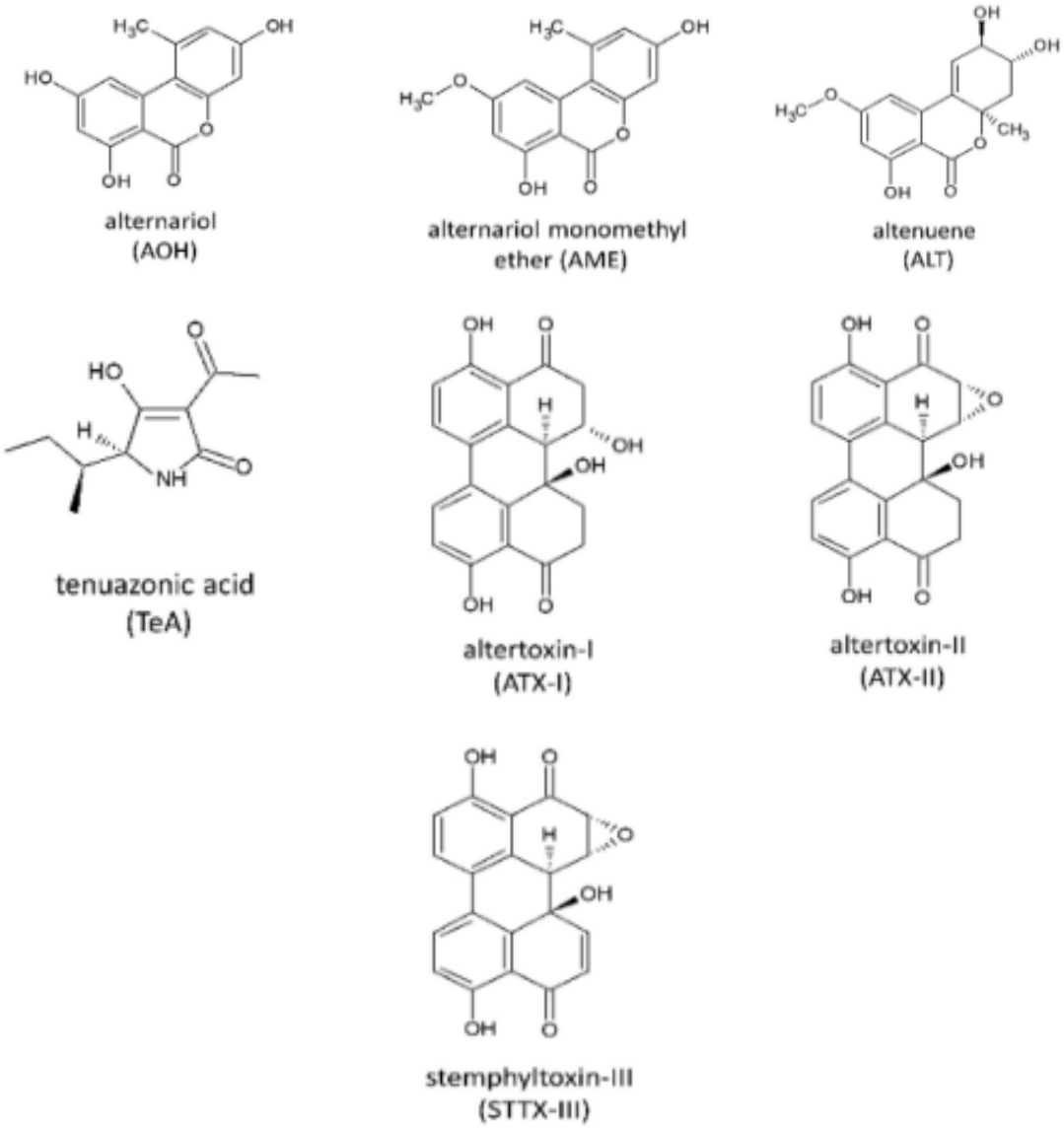

Figure 1: Chemical structures of common Alternaria species secondary metabolites alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA), altertoxin I (ATX-I), altertoxin II (ATX-II) and stemphyltoxin III (STTX-III) (Louro et al., 2024)

3.3. Mycochemical Analysis and Targeted Isolation of Fungal Metabolites

The analysis of fungal secondary metabolites commonly involves chromatographic and spectroscopic techniques for compound profiling, dereplication, purification, and structural elucidation. Because of the structural complexity of fungal extracts, analytical methods capable of separating and characterizing chemically diverse metabolites are essential in natural product research. In particular, UHPLC coupled with spectroscopic and

mass spectrometric detection methods enables rapid analysis of fungal metabolites and their physicochemical properties, as well as the characterization of secondary metabolites based on chromatographic behaviour, UV absorption and molecular mass information (Wolfender et al., 2015).

To avoid the repeated isolation and investigation of already known compounds, dereplication strategies are commonly applied during mycochemical investigations. Dereplication refers to the early identification of previously described metabolites within complex extracts using chromatographic and spectroscopic data and literature-based data. Following analytical characterization, targeted isolation of selected metabolites can be achieved using chromatographic separation methods. The structural confirmation of isolated target compounds is commonly performed using nuclear magnetic resonance (NMR) spectroscopy, which represents one of the most important analytical techniques for structural elucidation of natural products (Gaudêncio et al., 2023).

3.4. *Caenorhabditis elegans* as a Model Organism for Bioactivity Assessment

The nematode *Caenorhabditis elegans* is a widely used model organism in biological, toxicological, and pharmacological research. Due to its short lifespan, transparent anatomy, small body size, simple and rapid cultivation, as well as its highly conserved cellular signalling pathways, *C. elegans* represents a valuable preclinical *in vivo* model for studying e.g. stress responses, aging, and the biological effects of natural products and environmental toxins. Furthermore, many genes and molecular pathways involved in stress responses, xenobiotic metabolism, and pathology are conserved between *C. elegans* and higher organisms, including humans (Kaletta & Hengartner, 2006).

Oxidative stress responses in *C. elegans* are mainly regulated by the SKN-1 transcription factor, which is functionally related to the mammalian Nrf2 signalling pathway. The activation of SKN-1 induces the expression of detoxification and antioxidant genes, including glutathione S-transferase-4 (gst-4). Transgenic gst-4p::GFP reporter strains, such as CL2166, enable the visualization and quantification of oxidative stress responses through fluorescence measurement and are therefore commonly used for toxicological and antioxidant screening assays (Huynh & Wu, 2022).

In addition to oxidative stress response assays, survival and lifespan assays in wildtype N2 worms are frequently conducted to evaluate the potential lifespan-extending effects of worms treated with natural products and biologically active compounds (Kampkötter et al., 2008).

3.5. ELINA and Bioactivity-Guided Compound Identification

Bioactivity-guided fractionation and compound identification are commonly used strategies in natural product research to link biological effects to specific chemical constituents within multicomponent mixtures. This approach combines biological screening with analytical chemistry to identify compounds responsible for the observed bioactivities of extracts. By correlating bioassays with chemical information, the biologically active metabolites can be prioritized for further investigation.

The ELINA ("Eliciting Nature's Activities") concept is a biochemometric workflow that combines bioactivity data with analytical and spectroscopic data to identify metabolites responsible for the observed biological effects. ELINA enables the prioritization of potentially bioactive compounds prior to isolation and supports the targeted natural product-based drug discovery process, by correlating bioactivity results with chemical data obtained from techniques such as NMR and LC-MS (Zwirchmayr et al., 2020).

In the present study, ELINA-guided compound identification represents a project step beyond the scope of this thesis and is performed in collaboration with other researchers.

4. MATERIALS AND METHODS

4.1. Instruments and lab ware

Instrument	Supplier
Acquity UPLC H-Class	Waters Corporation
Analytical balance	Sartorius Secura 225D-15
Balance	Sartorius Entris II Essential Line
Centrifuge	Zentrifuge Heraeus Megafuge 1.0R
Dissecting microscope	Zeiss
Flash Chromatography/Interchim®-puriFlash®	Advion Interchim scientific
Genevac EZ-2 centrifugal evaporator	Genevac UK
Incubator (16 °C)	Memmert GTR0214
Incubator (25 °C)	INCU-Line Tower, VWR
Laminar airflow	Steril Werkbank B10
Light microscope	Nikon Labophot-2, Reichert-Jung Neovar 2
Plate Reader	Tecan GENios microplate reader
Plate shaker	IKA MS3 digital
Sample concentrator	Techne
TLC spray cabinet	CAMAG® TLC Spray Cabinet II
UV lamp	CAMAG®
Vortex	Stuart variable speed Mini Vortex Mixer

Lab ware	Supplier
Cell culture plates	Sarstedt TC plate, 96 well, Standard, F. flat bottom, transparent, non-pyrogenic, non-cytotoxic; DNase/RNase/DNA-free; sterile
Chikane flask	SCHOTT DURAN®
Eppendorf tube	Safe-Lock Tubes
Falcon (15 mL)	CELLSTAR Tubes; Greiner bio-one
Falcon (50 mL)	Sarstedt, 50 mL Tube
Multi-channel pitpette	Eppendorf Research (30-300 µL)
Parafilm	Bemis Parafilm “M”
Petri dish	Gosselin, Round petri plate PS na 3 ventsH14
Pipette tips	Eppendorf, Sarstedt, StarLab
Reservoir	VWR North America Cat. No 89094-680
Serological pipettes	Sarstedt
Test tube	Assistent®; heavy wall, rimless
TLC capillary	Micropipettes, BLAUBRAND® intraMARK, BRAND
TLC plate	Merck, TLC silica gel 60 F254, Aluminium sheets 20x20cm
UHPLC vials	MACHERY-NAGEL, Vials N9 clear

4.2. Chemical reagents

Chemical	Supplier and Charge
Acetonitrile	VWR BHD 83639.320
Agar	BioScience; Art.-Nr. 6494.3
Danchlor	Klorin 2250FR122Q
ddH_2O	Purified using ion exchanger
DMSO	Emsure; Merk 1.02952.1000
Epigallocatechin gallate	TargetMol Chemicals, T2988
Ethyl acetate	Rectapur, distilled according to ÖAB
Formic acid	99-100% a.r., Chem-Lab NV
FUdR	Sigma-Aldrich; F0503-250MG
Methanol	VWR-83638.320
NaOH	Merck 1.064621000
Paraquat	Sigma-Aldrich; 856177-250MG
Quercetin	Sigma-Aldrich; Q4951
Sulfuric acid	Sigma-Aldrich; 258105

4.3. Composition of Media

NGM Agar:

Peptone	1.25 g
NaCl	1.5 g
Agar	8.5 g
ddH_2O	487.5 ml

All components were weighed into an Erlenmayer flask and homogenized using magnetic stirring. The mixture was subsequently sterilized by autoclaving at 121 °C and 2 bar. Afterwards, the medium was reheated in a water bath to 50-60 °C, and the following components were added under sterile conditions:

1M CaCl	0.5 mL
5 mg/mL Cholesterol in EtOH	1 mL
1M $MgSO_4$	1 mL
1M KPO_4	25 mL

The solution was mixed again using magnetic stirring, after which 25 mL aliquots of the warm NGM medium were poured into Petri dishes. Following cooling and solidification for ~1 hour, the plates were transferred into sterile plastic bags and stored inverted at 4 °C.

S-Basal:

NaCl	5.85 g
K_2HPO_4	1 g
KH_2PO_4	6 g
ddH_2O	to 1000 mL
5 mg/mL Cholesterol in EtOH	1 mL

The salts were dissolved in water, and the resulting solution was sterilized by autoclaving. Afterwards, 1 mL of a 5 mg/mL ethanolic solution was added under laminar air flow (LAF) conditions.

Trace metal solution:

Na_2EDTA	1860 mg
$FeSO_4$ x $7H_2O$	690 mg
$MnCl_2$ x $4H_2O$	200 mg
$ZnSO_4$ x $7H_2O$	290 mg
$CuSO_4$ x $5H_2O$	25 mg
ddH_2O	to 1000 mL

All components were dissolved, followed by autoclaving of the solution. Afterwards, it was stored in the dark.

S-Complete Medium:

S-Basal (w/o cholesterol)	1000 mL
5 mg/mL Cholesterol in EtOH	1 mL
1M KCitrate Buffer pH 6	10 mL
Trace metal solution	10 mL
1M $CaCl_2$	3 mL
1M $MgSO_4$	3 mL

All components were mixed and dissolved under aseptic conditions, followed by sterilization of the solution via filtration.

LB-Medium:

Bacto-Tryptone	10 g
Bacto-Yeast	5 g
NaCl	5 g
1M NaOH	to adjust pH
ddH_2O	to 1000 mL

All components were dissolved and the pH was adjusted to 7 using 1M NaOH prior to bringing the solution to the final volume. The completed solution was then sterilized by autoclaving.

OP50 *E. coli* stock:

E. coli cells (cell bank stock stored at -70 °C)	500 µL
LB-Medium	250 mL

S-Complete Medium	to adjust concentration
ddH_2O	30 mL

LB-Medium (250 mL) was inoculated with 500 µL of a standardized *E. coli* working cell bank stock and incubated on a shaker at room temperature for 24 hours. Following incubation, the culture was centrifuged at 3000 × g for 10 minutes and the supernatant was discarded. The resulting pellet was washed twice with double-distilled water (ddH_2O) and subsequently air-dried. After determination of the pellet weight. The suspension was adjusted to a final concentration of 100 mg/mL using S-complete medium.

M9-Buffer:

KH_2PO_4	3 g
K_2HPO_2	6 g
NaCl	5 g
1M $MgSO_4$	1 mL
ddH_2O	to 1000 mL

All components were mixed and sterilized via autoclaving.

X-reagent:

Danchlor (5% NaOCl)	1 mL
5M NaOH	0.5 mL

Directly before usage, both compounds were mixed.

4.4. Fungal material and extracts

The *Alternaria alternata* and *Alternaria infectoria* extracts were provided by the research group of Univ.-Prof. Dr. Doris Marko from the Department of Food Chemistry and Toxicology at the University of Vienna. The extracts were obtained from fungal cultures grown in mCDB medium for one week. Cultivation was carried out using different fungal strains which originated from either mycelium or spores, which were then incubated at 24 °C or 28 °C before extraction.

4.5. Chromatographic methods

4.5.1. Ultra-High-Performance Liquid Chromatography (UHPLC)

Ultra-high-performance liquid chromatography (UHPLC) analysis was performed on a Waters Acquity UPLC H-Class system comprising a quaternary solvent manager, sample manager, column manager, isocratic solvent manager, fraction collector, PDA detector, ELSD and QDa. UHPLC was used to analyse and dereplicate the different components of the provided fungal extracts using a standard gradient method. For sample preparation, the extracts were dissolved in MeOH to achieve a concentration of 5 mg/mL. The parameters are summarized in Table 1.

Table 1: Parameters of the UHPLC PDA-ELSD/QDa method for ATXOE_9 and ATXOE_11

Injection volume [µL]	2		
Flow rate [mL/min]	0.3		
Temperature [°C]	30		
PDA wavelength [nm]	205, 220, 254, 360		
ELSD [Gain]	500		
QDa	**Mode**	**Cone Voltage [V]**	**Capillary Voltage [kV]**
	Positive	15	0.8
	Negative	30	0.8
Mass range [Da]	100 – 1200		
Make-up Solvent	MeOH:H_2O (9:1) + 10 mM ammonium formate		
Make-up Solvent-flow rate [mL/min]	0.4		
Mobile Phase A	H_2O + 0.1 % FA		
Mobile Phase B	ACN + 0.1% FA		

Sample concentration	5 mg/mL	
Gradient		
time [min]	**% A**	**% B**
0	95	5
12	2	98
15	2	98
15.3	95	5
17	95	5

4.5.2. LC-MS-based Dereplication

The obtained data from the UHPLC-PDA-ELSD/QDa analysis was used to dereplicate and annotate the components of the *Alternaria alternata* (ATXOE) extracts.

ELSD (evaporative light scattering detector) retention time from the chromatograms was used to identify the main peaks. Retention times were then matched with PDA (photo diode array) spectra absorption patterns and mass spectrometry *m/z* values from QDa (quadrupole mass detector). With possible mass adducts, the molecular weight was then calculated. Finally, annotation of peaks was performed by comparing *m/z* values and their possible molecular weights with literature reports of *Alternaria alternata*. SciFinder® was used for literature research.

4.5.3. Flash Chromatography

For fractionation of the ATXOE extracts, an Interchim®-puriFlash® chromatography was used. Because of the large amount of the extracts, the sample was applied in a dry load.

For the preparation of the dry load, the sample was homogenized with the same amount of Celite using a mortar and pestle, due to thermolability of the altertoxins. The resulting mixture was transferred into a cartridge, which was then filled up to the top with additional Celite. The cartridge was tightly packed, and proper assembly was ensured by verifying

the integrity of the threads, the presence of frits at both ends and the overall seal. After conditioning the system and the column with the starting solvent composition, the cartridge was introduced into the system.

The flash chromatography was performed using a reversed-phase Puriflash C18 HQ column (15 µm, 20 g, 22 bar). As mobile phases, H_2O (solvent A) and ACN (solvent B) were used. Isolation was achieved using a gradient elution at a flow rate of 6 mL/min. The gradient started at 95% solvent A and 5% solvent B, which was gradually shifted to 2% solvent A and 98% solvent B over 120 min. During the elution of altertoxin I and II, the gradient was temporarily held constant by introducing plateau phases to improve compound separation and collection. The total run time was 185 min to ensure no further relevant peaks.

Eluting compounds were monitored using UV detection (scan range 200-400 nm with fixed wavelengths at 205, 254, 280 and 304 nm) and ELSD at 35 °C. Fractions were collected automatically throughout one analytical and two semi-preparative runs, resulting in a total of 560 test tubes. All parameters are summarized in Table 2.

Table 2: Parameters for Interchim®-puriFlash®, [1] was individually adjusted in between depending on the ELSD signal; [2]a plateau was individually built in depending on the ELSD signal

Applied amount	Run 1	Run 2	Run 3
	20 mg	100 mg	100 mg
Column	Puriflash column 15 C18 HQ (15 µm, 20g, 22 bar)		
Flow rate [mL/min]	6		
Run time [min]	185		
PDA wavelength [nm]	Range 200-400; fixed 205, 220, 254, 360		
ELSD	35 °C		
Solvent A	H_2O		
Solvent B	ACN		
Collection volume [mL]	6[1]		

Gradient[2]	Time (h:min:s)	% A	% B
	00:00:00	95	5
	02:00:00	2	98
	02:30:00	2	98

4.5.4. Thin Layer Chromatography (TLC)

An already established TLC system was used to analyse the generated flash chromatography fractions for the content of mycotoxins altertoxin I and altertoxin II. The obtained flash chromatography fractions were dried using a Genevac EZ-2 centrifugal evaporator (Genevac, UK), and the residual material was subsequently dissolved in ~ 500 µL MeOH. For each fraction, 5 µL were applied to the TLC plate using a capillary. The stationary phase consisted of a thin silica gel layer coated on a solid support and EtOAc was used as the mobile phase. For comparison of the chromatographic profiles, an ATXOE_9 reference sample was applied to each TLC plate alongside the analysed fractions. Following development, the TLC plates were examined under UV light (254 and 366 nm). Derivatization was performed by spraying the plates with a methanolic 1% vanillin, and 5% H_2SO_4 solution, followed by heating at 100 °C for 5 minutes to visualize the compounds. The parameters are summarized in Table 3.

Table 3: TLC parameters for ATXOE extracts

Application volume [µL]	5
Stationary phase	Silica gel
Mobile phase	EtOAc
UV light [nm]	254, 366
Spraying reagents	1% vanillin, 5% H_2SO_4in MeOH
Derivatization	5 min at 100 °C

4.5.5. Pooling of generated Flash Chromatography fractions

For the isolation of altertoxin I and II from the ATXOE extracts, fractions obtained from flash chromatography exhibiting identical compound profiles were combined to increase yield. The fraction composition was assessed by UHPLC, as described in 4.5.1, and TLC, as described in 4.5.4.

4.6. Structure Elucidation

4.6.1. Nuclear Magnetic Resonance Spectroscopy (NMR)

For NMR sample preparation, ~2 mg of the isolated compounds were dried in a desiccator and dissolved in deuterated methanol (MeOH-d_4) at a concentration of 3 mg/mL, which was added using a Hamilton syringe. Each 600 µL of the solutions were transferred into NMR tubes and immediately sealed.

NMR measurements of the isolated compounds from ATXOE extracts were performed on a Bruker Avance 500 MHz Ultrashield spectrometer. Samples were analysed using one-dimensional (^{1}H and ^{13}C-APT) as well as two-dimensional (HSQC, HMBC, ^{1}H COSY and NOESY) NMR experiments. Spectral analysis was carried out using MNova software and the identities of the isolated compounds were confirmed by comparing the spectral data with literature conducted by Mag. Pharm. Eva Waltenberger.

4.7. Bioactivity Assays in *Caenorhabditis elegans*

4.7.1. Cultivation and Chunking of *C. elegans*

N2 (wildtype) and CL2166 worms were maintained at 16 °C in an incubator on NGM agar plates seeded with *E. coli* OP50 solution. Upon depletion of the bacterial food source, the worms entered the *dauer* larval stage, which enables survival for up to four months under adverse conditions. To prevent starvation and maintain continuous cultures, worms were passaged by agar chunk transfer. Small agar sections (~1 cm²) containing worms were excised and transferred onto fresh NGM plates seeded with OP50. The plates were then again incubated at 16 °C for three days.

4.7.2. Synchronization

Gravid adult worms were collected from two to three NGM agar plates to generate an age-synchronized population by rinsing the plates with ~4 mL of ddH_2O and transferring the worm suspension into a 15 mL falcon tube, followed by centrifugation at 1600 × g for 5 minutes. The supernatant was carefully removed, leaving ~2 mL to avoid disturbing the pellet, which was then resuspended by vortexing and an addition of 7 mL of sterile water. To start the lysis of the adult worms and isolate the eggs, 1.5 mL of freshly prepared X-reagent was added to the suspension under constant shaking by hand. Lysis was closely monitored by placing 10 µL drops on the lid of a petri dish and observation under a dissection microscope. The reaction was stopped by adding 6.5 mL of M9 buffer as soon as all worms started to dissolve.

The released eggs were washed twice with 10 mL of M9 buffer and once with S-complete medium. Each washing step consisted of centrifugation at 2500 × g for 5 minutes, discarding the supernatant and vortexing. After the final washing step, the pellet was resuspended in 10 mL S-complete medium. The suspension was then transferred to a fresh 50 mL chicane flask. The 15 mL falcon tube was washed with another 10 mL of S-complete medium and added to the chicane flask. The flask was then gently shaken on a nutator at room temperature for two days.

4.7.3. Reseeding

After 48 hours, under a dissecting microscope, it is determined if all worms hatched. The concentration is then set to 80-100 worms/mL by counting each worm in 10 µL drops and diluting the suspension with S-complete medium to achieve the desired concentration. At least 10 drops must be counted for each sample, and the dilution is finished once there is ~1 worm counted per drop. 20 mL of the adjusted worm solution is then supplemented with 1.2 mL of OP50 bacteria stock solution to achieve a 5.66 mg/mL concentration of OP50.

The exterior wells of the 96-well plates were filled with 125 µL H_2O to establish a diffusion barrier. The rest of the wells were then filled with 90 µL worm/OP50 suspension and the final concentration should be between 8-18 worms per well. Finally, the plates were sealed

using parafilm to avoid contamination and evaporation, labelled and incubated at 25 °C for one day, until the animals reach L3 stage.

4.7.4. Sterilization

To stop the worms from reproducing and to maintain age synchronization, the animal suspension in each well was sterilized with 22.5 µL of a 0.6 mM 5-Fluoro-2'-deoxyuridine (FUdR) solution in S-complete medium. This final step brings the volume of each well to 112.5 µL and the OP50 concentration to 4.528 mg/mL. Afterwards, the plates were sealed using parafilm, gently shaken on a microtiter plate shaker for one minute and incubated again at 25 °C for 24 hours to establish an adult worm population.

4.8. Survival Assay

To conduct a survival assay, wildtype N2 worms were prepared as described in Section 4.7. The worm population was synchronized, reseeded into six 96-well plates and sterilized using FUdR solution. After reaching adulthood (day 0), the worms were treated with 12.5 µL of sample stock solution of the extracts (200 µg/mL and 50 µg/mL f.c.), Quercetin (200 µM and 100 µM f.c.) as positive control, and DMSO (0.7% f.c. in S-complete medium) as vehicle control. Quercetin was chosen as the positive control due to its reported lifespan-prolonging properties in *C. elegans* (Kampkötter et al., 2008). During this assay, five *Alternaria alternata* extracts (ATX-OE, VeA, WT, DSM_06 and DSM_10), as well as six *Alternaria infectoria* extracts (WSD_1-WSD_6) were analysed.

To optimize worm health, the plates were oxygenized every other day under the laminar air flow by removing the lid for one minute, then sealing it with parafilm and gently shaking on a microtiter plate shaker for 60 seconds before incubating again at 25 °C. To prevent starvation, 3.8 µL OP50 solution was added to each well on day 4.

The survival was closely monitored under a light microscope and the evaluation started once there were ~50-60% of worms left alive in the vehicle control. Survival rate was determined by counting all live, as well as dead animals in each well. Animals were evaluated as dead when they did not show any movement after exposure to intense light or shaking on the microplate-shaker.

Microsoft Excel was used to process the received raw data to evaluate the mean survival rate and standard deviation compared to the vehicle control for each sample per plate (n=3). GraphPad PRISM 6.0 was used for graphical presentation and statistical analysis of the received data from Microsoft Excel by conducting a standard One Way ANOVA and Dunnett's post hoc test with significance set at $p \leq 0.05$.

4.9. Oxidative Stress Response Assay

The oxidative stress response assay (ROS assay) was performed using the genetically modified *C. elegans* strain CL2166, carrying the inducible gst-4p::GFP reporter construct (*Strain: CL2166, Genotype: dvIs19 III. - Caenorhabditis Genetics Center (CGC) - College of Biological Sciences*, o. J.). The worms were prepared as described in Section 4.7. The animal population was synchronized, reseeded into six 96-well plates and sterilized using FUdR. After reaching adulthood (day 0), the worms were treated with 12.5 µL sample stock solution of the extracts (200 µg/mL and 50 µg/mL f.c.), paraquat (6 mM and 4 mM f.c.) as negative control, EGCG (200 µM and 100 µM f.c.) as positive control, and DMSO (0.7% f.c. in S-complete medium) as vehicle control. EGCG was chosen as the positive control due to its reported antioxidant properties, whereas paraquat was used as the negative control due to its oxidative stress-inducing properties (Capasso et al., 2025; Liu et al., 2020). With this assay, five *Alternaria alternata* extracts (ATX-OE, VeA, WT, DSM_06 and DSM_10), as well as six *Alternaria infectoria* extracts (WSD_1-WSD_6) were analysed.

To optimize worm health, the plates were oxygenized every other day under the laminar air flow by removing the lid for one minute, then sealing it with parafilm and gently shaking on a microtiter plate shaker for 60 seconds before incubating again at 25 °C.

The oxidative stress levels were measured using fluorescence right before and after sample treatment to observe potential autofluorescence and to establish a baseline (excitation 485 nm, emission 535 nm). After 4 and 6 days of incubation, GFP fluorescence was reassessed under the same conditions and normalized to the basal levels. Increased fluorescence relative to the control indicated higher gst-4 expression levels. For each of the three plates, three technical replicates were averaged to yield one value per condition; The mean values of the parallel replicates were presented as bar charts ±SD and statistical analysis was

performed in GraphPad Prism 6 using One-way ANOVA followed by Dunnett's post hoc test, with significance set at $p \leq 0.05$.

5. RESULTS AND DISCUSSION

This thesis comprises two independent research project parts. The first part focuses on the chemical characterization and targeted isolation of secondary metabolites from *Alternaria alternata* extracts using chromatographic and spectroscopic methods. The second part is dedicated to the investigation of the biological activity of selected *Alternaria alternata* and *Alternaria infectoria* extracts using the model organism *Caenorhabditis elegans.* The extracts were obtained from fungal cultures, originating from either mycelium or spores, grown in mCDB medium for one week at 24 °C or 28 °C before extraction. Survival, as well as oxidative stress response assays were performed to assess the biological effects of the fungal extracts. The results and discussion of both projects are presented separately in the following sections.

5.1. Mycochemical Characterization of *ATXOE* Extracts and Targeted Isolation of Altertoxins I and II

5.1.1. LC-MS-based Dereplication of ATXOE extracts

To gain insights into the constituent profile of the two *Alternaria alternata* extracts (=ATXOE_9 and _11; corresponding sample amounts are summarized in Table 4) that were provided by the research group of Doris Marko at the Department of Food Chemistry, University of Vienna, an LC-MS-based dereplication was conducted.

Table 4: Amount of obtained Alternaria alternata extracts

Extract	Amount [mg]
ATXOE_9	541.89
ATXOE_11	177.11

The UHPLC parameters for the mycochemical analysis of the ATXOE extracts are summarized in Table 1 in Section 4.5.1. LC-MS-based dereplication was performed as described in Section 4.5.2. Briefly, ELSD peaks were assigned to UV absorption maxima and *m/z* ions. Scifinder® (scifinder-n.cas.org) was used to compare the putative molecular weight with reported constituents of *A. alternata.* As the chromatograms indicated an identical composition for both extracts, dereplication was conducted using ATXOE_9.

Analysis of the UHPLC-ELSD-PDA/QDa chromatograms showed eight relevant peaks that could be tentatively annotated to constituents previously reported in literature (Table 5).

Compounds that were annotated in ATXOE_9 that are relevant for subsequent targeted isolation detected in the extracts were altertoxin I (ATX-I), altertoxin II (ATX-II), alterperylenol (ALP) and stemphyltoxin III (STTX-II). These compounds belong to the group of perylene quinones which are commonly reported in *A. alternata*. Univ.-Prof. Dr. Marko's research group was mainly interested in ATX-I and ATX-II, which is why the main focus lied on those two compounds for the targeted isolation. The corresponding retention times, UV absorption maxima, *m/z* values with possible mass adducts and proposed annotations are summarized in Table 5, while representative UHPLC chromatograms are shown in Figure 2.

UHPLC-ELSD-PDA/QDa analysis of the ATXOE extracts led to the annotation of several secondary metabolites, including alterperylenol (ALP), tricycloalternarene derivates (TCA 6a and TCA E), altertoxin I (ATX-I), altertoxin II (ATX-II), stemphyltoxin III (STTX-III) and altersolanol C. According to the ELSD signal intensities, ATX-I and ATX-II were the main constituents of the extracts, showing substantially higher peak intensities than all other detected compounds. Their high abundance made both metabolites particularly suitable targets for subsequent isolation and structural characterization. In addition, the chromatographic profile showed generally good separation of the major constituents, which could be an indication for favourable conditions for the chromatographic fractionation. However, the ATX-I and -II regions had closely eluting neighbouring signals. ATX-I was accompanied by a nearby peak which seemed to correspond to an ATX-I isomer, while ATX-II eluted near the annotated STTX-III. Such closely eluting signals may complicate purification and might require additional chromatographic separation steps to obtain pure target compounds. Nevertheless, the chromatographic profile suggested that targeted isolation of ATX-I and -II from the ATXOE extracts was feasible.

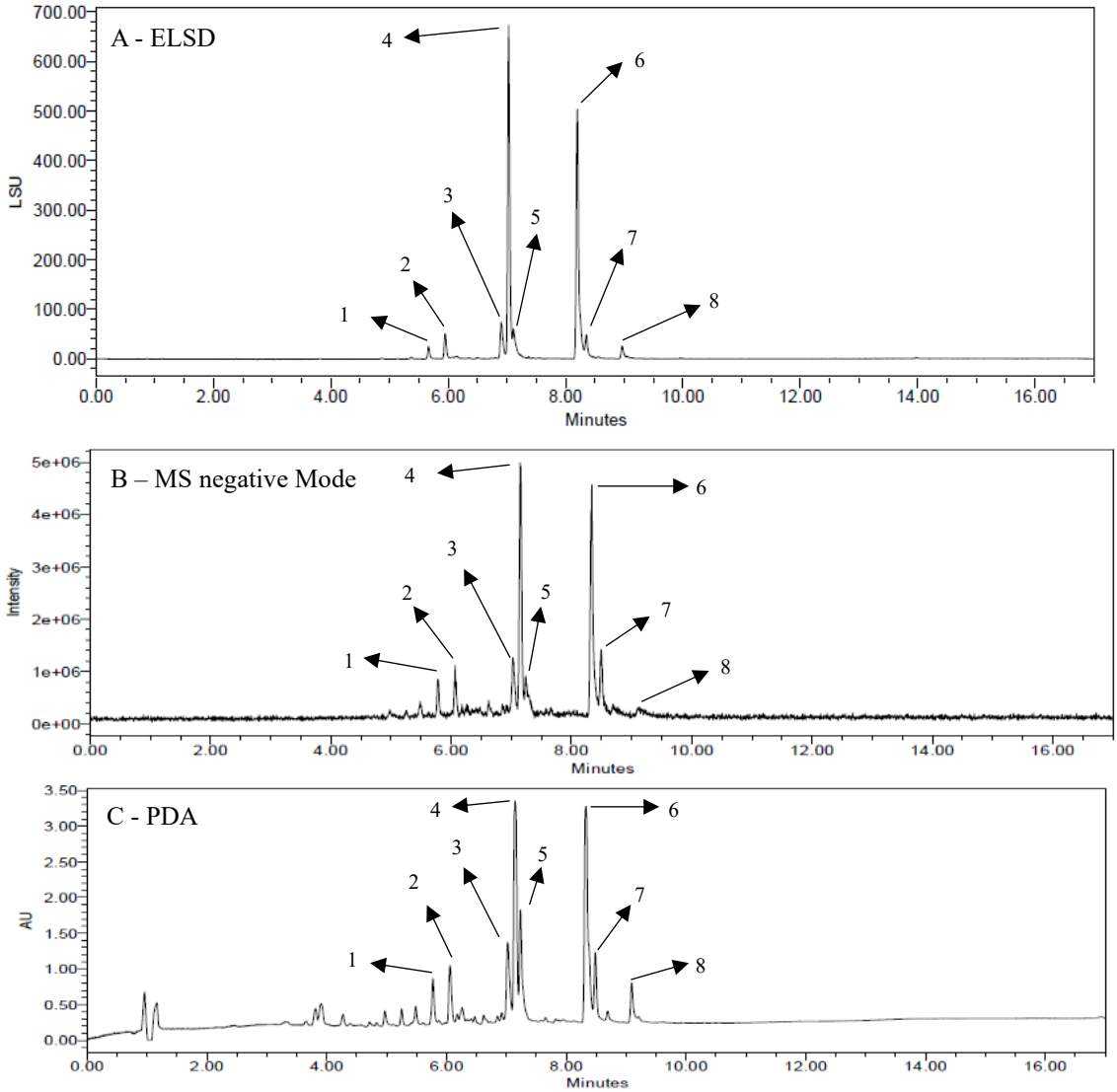

Figure 2: UHPLC-ELSD-PDA/QDa analysis of ATXOE_09 (A) Evaporative light scattering detection (ELSD) chromatogram, (B) mass spectrometric detection in negative ion mode, (C) photodiode array (PDA) chromatogram. Chromatograms were recorded under the analytical conditions described in Section 4.5.1. Peak numbering corresponds to the dereplication table (Table 5). Positive ion mode chromatogram is not shown, as no assignable peaks were detected in this mode.

Table 5: Annotation of UHPLC-ELSD-PDA/QDa data from ATXOE extracts showing retention times (Rt), UV absorption maxima (Amax), mass spectral data (m/z in negative and positive ion modes), tentative molecular weights and compound annotations.

Peak no.	Rt [min]	A_{max} [nm]	*m/z*		Tentative MW [g/mol]	Annotated substance
			Negative	***Positive***		
1	5,7	218, 258, 291, 369	349, 471, 513, 943 331, 303	-	350	ALP (or isomer)
2	5,9	220, 259, 289, 388	339, 349, 321, 383, 469, 853	-	340, 350	ALP (or isomer)
3	6,9	226, 260, 394, 478	301, 363, 291,	365	364	TCA 6a or TCA E
4	7,0	215, 258, 355	315 $[M\text{-}H\text{-}2H_2O]^-$, 333 $[M\text{-}H\text{-}H_2O]^-$, 351	317, 463	352	ATX-I
5	7,1	220, 258, 351, 395	293, 365	365	294	ATX-I Isomer
6	8,2	220, 259, 361	331 $[M\text{-}H\text{-}H_2O]^-$	-	350	ATX-II
7	8,3	219, 271, 376	329 $[M\text{-}H\text{-}H_2O]^-$, 347	333	348	STTX-III
8	8,9	220, 266, 350	277, 319	-	320	Altersolanol (C)

5.1.2. Fractionation of ATXOE with Flash Chromatography

Since the UHPLC analysis of ATXOE already showed an adequate separation of the peaks, it was decided to fractionate the extracts by translating the UHPLC method to flash chromatography by using a reverse phase C18 column and a flat gradient starting at 95%

H_2O and 5% ACN, which was gradually shifted to 2% H_2O and 98% ACN over the course of 120 min. Elution was continuously monitored via ELSD, and isocratic plateau phases were introduced individually to achieve optimal separation of the target perylene quinones ATX-I and ATX-II from co-eluting compounds. Detailed parameters for the flash chromatography are described in Section 4.5.3.

In a first small-scale analytical run, using 20 mg of ATXOE_11, the resulting chromatogram showed a comparable profile as in the previous UHPLC analysis with two main peaks that were annotated as ATX-I and ATX-II (Figure 3). At later retention times during the ACN washing phase, numerous unassignable peaks were detected exclusively by ELSD (red trace), with no corresponding signals observed in the UV (200-400 nm; orange trace). This indicates that residual compounds of no analytical relevance eluted during this phase and were therefore not considered for further analysis.

Due to the analytical run showing a sufficient separation of the desired peaks, two more semi preparative flash chromatography runs were conducted with 100 mg of ATXOE_11 and 100 mg of ATXOE_9, resulting in a total of 158 tubes for run 2 and 203 tubes for run 3.

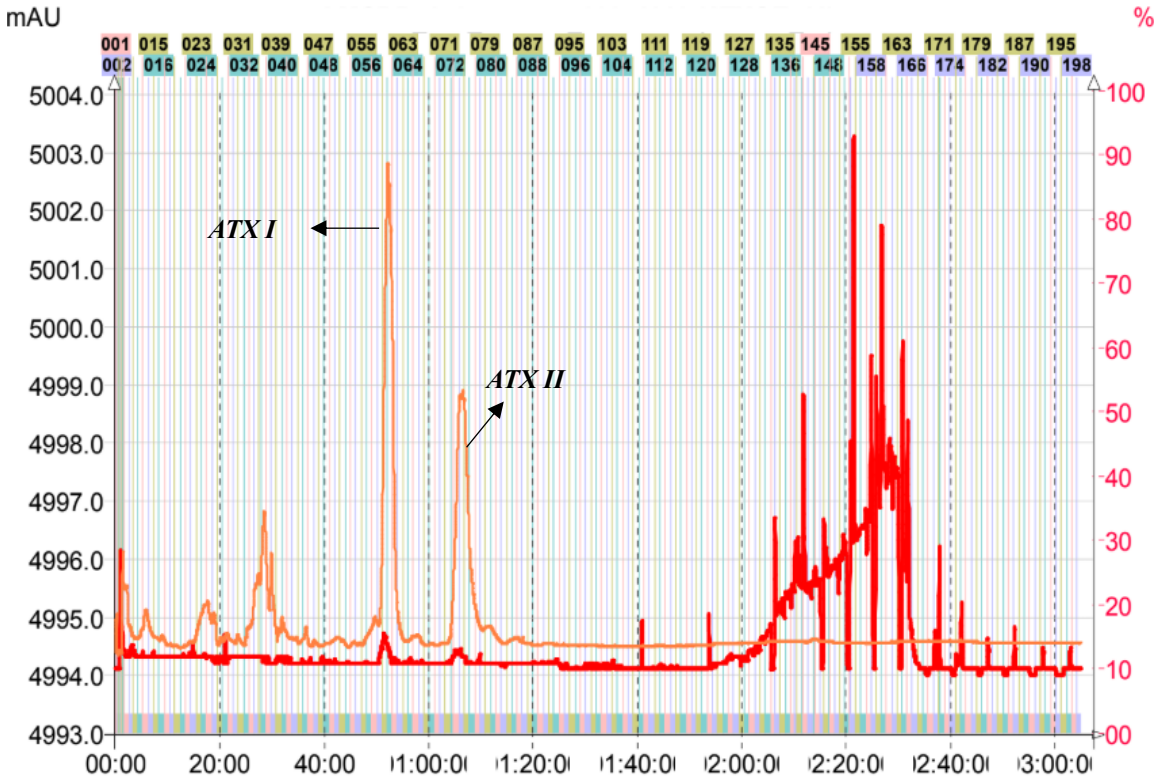

Figure 3: Flash chromatography collection chromatogram of the analytical run with ATXOE_11. ELSD (red trace) and UV detection (orange trace). Separation was performed on a RP C18 column using a water/acetonitrile gradient, as described in Section 4.5.3.

5.1.3. Targeted Isolation and Pooling of Altertoxin-containing Fractions

All tubes, which were collected during the two semi-preparative flash chromatography runs were analysed by TLC as described in Section 4.5.4 to identify fractions containing ATX-I and ATX-II.

Fractions, which exhibited similar chromatographic profiles, were pooled to collective fractions as described in Section 4.5.5. This resulted in a total of 23 collective fractions for run 2 and 22 collective fractions for run 3. The table summarizing the pooled fractions and corresponding test tube numbers is provided in Table 8 for run 2 and Table 9 for run 3 in the Appendix. A representative TLC plate depicting the collective fractions of flash chromatography run 2 is depicted in Figure 4. The pooling decisions were based on the combined evaluation of TLC and UHPLC data. Fractions, which were collected during the initial loading phase of the flash chromatography, were excluded from pooling, as they mainly contained solvent and non-retained material.

For some fractions containing ATX-II, additional UHPLC analyses were necessary, as ATX-II and STTX-III showed very similar TLC patterns and could therefore not be reliably distinguished by TLC alone. Since both metabolites also eluted very close to each other during flash chromatography, the affected fractions were analysed individually by UHPLC to evaluate their composition and purity before pooling. Additionally, UHPLC analysis showed the presence of another perylene quinone eluting shortly before ATX-I, which was tentatively annotated as STTX-IV (data not shown).

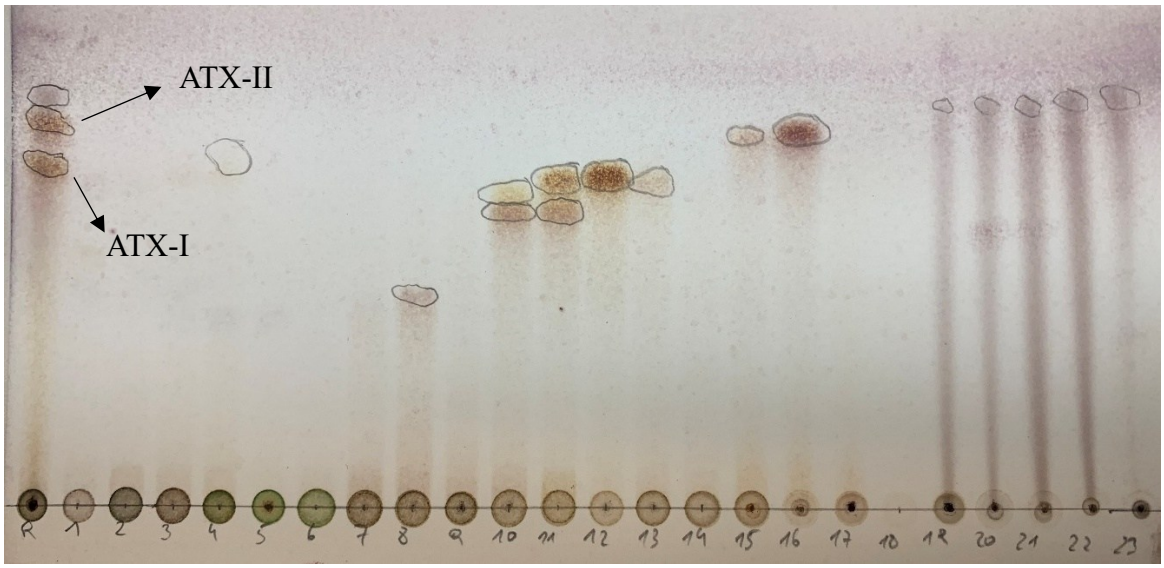

Figure 4: 23 Pooled Fractions of Flash Run 2 after derivatization as described in Section 4.5.4, R: Reference (ATXOE_9 whole extract)

An overview of the fractions from both semi-preparative flash chromatography runs that were combined to obtain ATX-I and ATX-II, together with the corresponding yields is summarized in Table 6. The pooling of ATX-containing fractions resulted in the successful isolation of 15.97 mg ATX-I and 12.44 mg ATX-II.

A comparable study used several chromatographic purification steps, including vacuum liquid chromatography and preparative reversed-phase C18 HPLC and isolated 84.0 mg of ATX-II and 8.0 mg of ATX-I from a large scale *Alternaria* culture extract. In the present study, a simpler purification procedure was used, however, similar mg-scale amounts of both altertoxins were obtained, showing that the used flash chromatography method enabled effective separation and isolation of the target compounds (Robles et al., 2021).

Table 6: Pooled fractions from the semi-preparative flash chromatography runs 2 and 3 containing ATX-I and ATX-II

	Altertoxin I	**Altertoxin II**
Test Tubes	Run 2: 74 - 76 Run 3: 86 - 89	Run 2: 99 - 102 Run 3: 120 - 134
Total		
Extract weight [mg]	**Altertoxin I [mg]**	**Altertoxin II [mg]**
719.9	15.97	12.44

5.1.4. Structure Elucidation of ATX-I and ATX-II

Structural elucidation of the targeted compounds was performed by Mag. pharm. Eva Waltenberger using NMR spectroscopy as described in Section 4.6.1. The identities of altertoxin I and altertoxin II were confirmed by comparison with NMR spectra found in literature (Mahmoud et al., 2022).

In addition to the NMR measurements, UHPLC-ELSD chromatograms of the isolated compounds showed single dominant peaks, confirming a high degree of purity after the purification process. The UHPLC chromatograms of ATX-I (99% purity) and ATX-II (97% purity) are shown in Figure 5Figure 5: UHPLC-ELSD chromatograms of ATXOE_9 and the isolated ATX-I and ATX-II with their corresponding chemical structures

The successful isolation and structural confirmation of both altertoxins showed that the used dereplication and flash chromatographic separation workflow was suitable for the targeted isolation of fungal secondary metabolites from the ATXOE extracts.

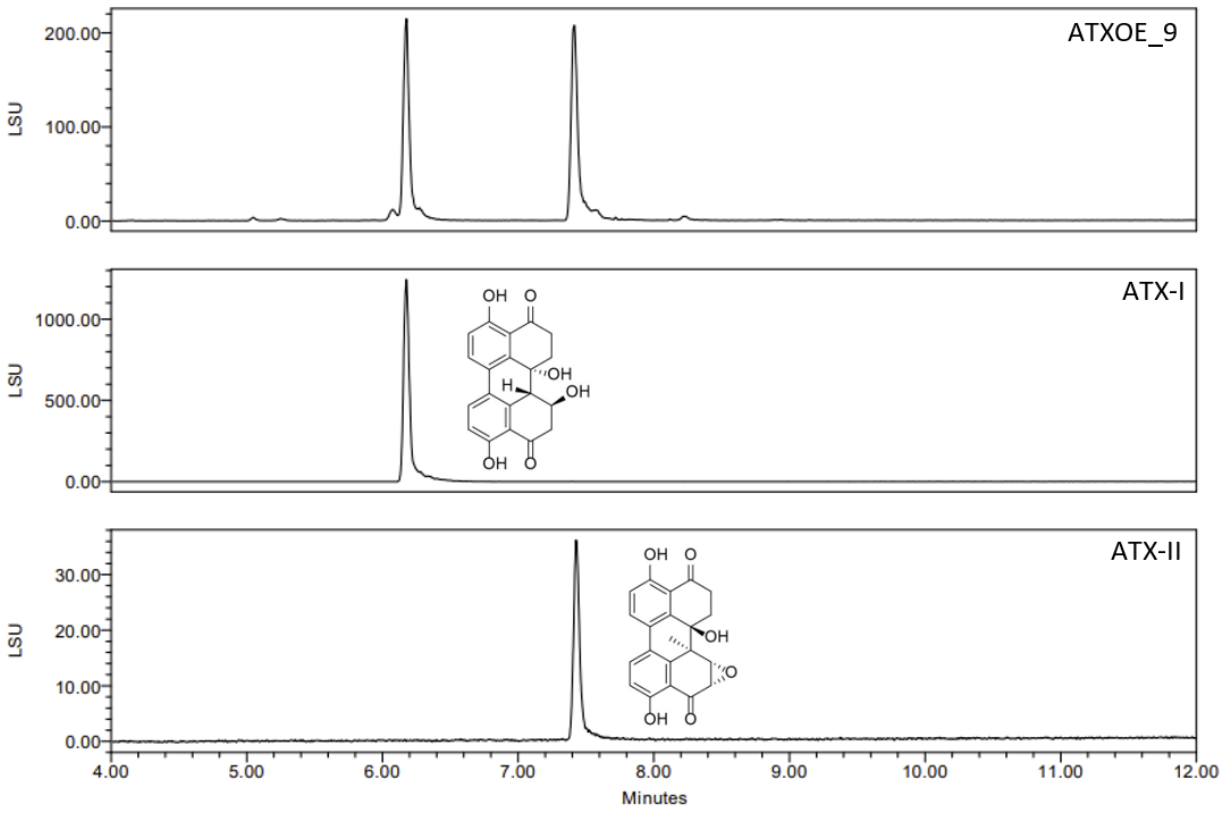

Figure 5: UHPLC-ELSD chromatograms of ATXOE_9 and the isolated ATX-I and ATX-II with their corresponding chemical structures

5.2. Bioactivity Assessment in *Caenorhabditis elegans*

For the second part of this project, extracts of different strains of *Alternaria alternata* and *Alternaria infectoria* species that were provided by the research group of Univ.-Prof. Dr. Marko were used for biological evaluation. An overview of the genus, strain, the respective parts of the fungi and abbreviations of all extracts used are summarized in Table 7. The aim was to generate bioactivity data from complex fungal extracts to support future integration within a biochemometric workflow following the ELINA (Eliciting Nature's Activities) concept. In this context, two assays were performed using the *C. elegans* model: a survival assay using wildtype N2 worms and an oxidative stress response assay using the gst-4p::GFP reporter strain CL2166. Together, these assays provide information on general toxicity and oxidative stress-related responses induced by the fungal extracts.

Table 7: Extract Abbreviations, Genus, Strain and used parts of the provided Alternaria alternata and Alternaria infectoria extracts for the C. elegans assays

Extract Abbreviation	Genus	Strain	Used part
ATX-OE	*A. alternata*	ATCC66981 ATX-OE	Mycelium
VeA	*A. alternata*	ATCC66981 VeA	Mycelium
WT	*A. alternata*	ATCC66981 WT	Mycelium
DSM 06	*A. alternata*	DSM 62006	Spores
DSM 10	*A. alternata*	DSM 62010	Spores
WSD 1	*A. infectoria*	WSD 17.1.2	Mycelium
WSD 2	*A. infectoria*	WSD 23.1.4	Mycelium
WSD 3	*A. infectoria*	WSD 3.1.1 (2)	Mycelium
WSD 4	*A. infectoria*	WSD 17.4.1 (2)	Mycelium
WSD 5	*A. infectoria*	WSD 40.2.6 (2)	Mycelium
WSD 6	*A. infectoria*	WSD 27.1.1 (2)	Mycelium

5.2.1. Survival Assay

The first assay that was performed to gather data for the biochemetric workflow was the *C. elegans* survival assay for comparing the worms' survival rates upon extract treatment to the untreated control group to observe potential life prolonging and/or toxic effects of the investigated material.

The survival assay was carried out as described in Section 4.8 using a synchronized wildtype N2 worm culture treated with *Alternaria alternata* and *Alternaria infectoria* extracts at a concentration of 50 µg/mL and 200 µg/mL. Each condition was tested in three technical replicates in three parallel experiments. The worms' survival was assessed on day 17 of the experiment, when approximately 50-60% of worms were left alive in the 0.7% DMSO vehicle control group.

The survival rate of worms treated with the different fungal extracts was evaluated using Microsoft Excel and statistical analysis was performed with one-way ANOVA followed by Dunnett's post-test in GraphPad PRISM 6.0. The results for worms treated with *Alternaria alternata* are visualized in Figure 6 and *Alternaria infectoria* in Figure 7.

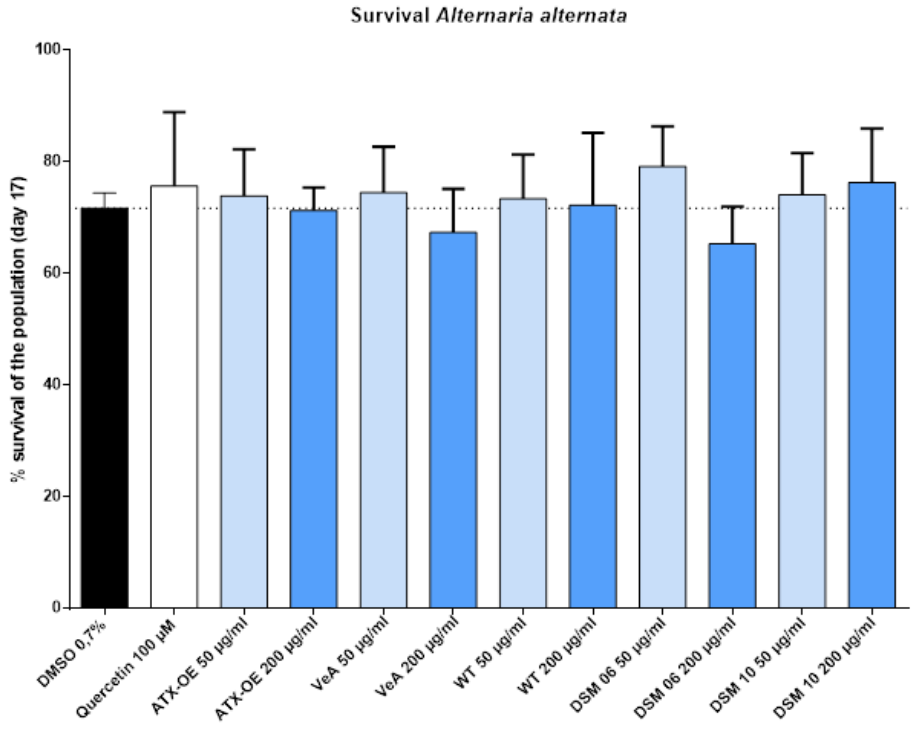

Figure 6: Survival rates for N2-wildtype worms treated with Alternaria alternata extracts at 50 µg/mL (light blue) *and 200 µg/mL* (dark blue) *compared to 0.7% DMSO vehicle control group, Quercetin (100 µM) was used as positive control; Data is shown as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-test; complete survival data are provided in Table 10 and Table 11 in the Appendix*

Treatment with *Alternaria alternata* extracts showed a slight improvement in survival rates for most treatment groups compared to the DMSO control. The exception to these findings were the 200 µg/mL ATX-OE, VeA and DSM 06 extracts, which resulted in either the same

or a lower survival rate than the control group. The lower concentration of 50 µg/mL generally resulted in higher survival rates compared to the corresponding 200 µg/mL treatment groups, therefore suggesting a slight concentration-dependent toxic effect. DSM 10 was an exception to this trend, as the 200 µg/mL treatment group showed a higher survival rate compared to the lower concentration.

It should also be noted that on the day of counting (day 17), approximately 70% of the DMSO treated worms were still alive, which is an unusually high survival rate at this late stage of their life cycle, making interpretation of these data difficult.

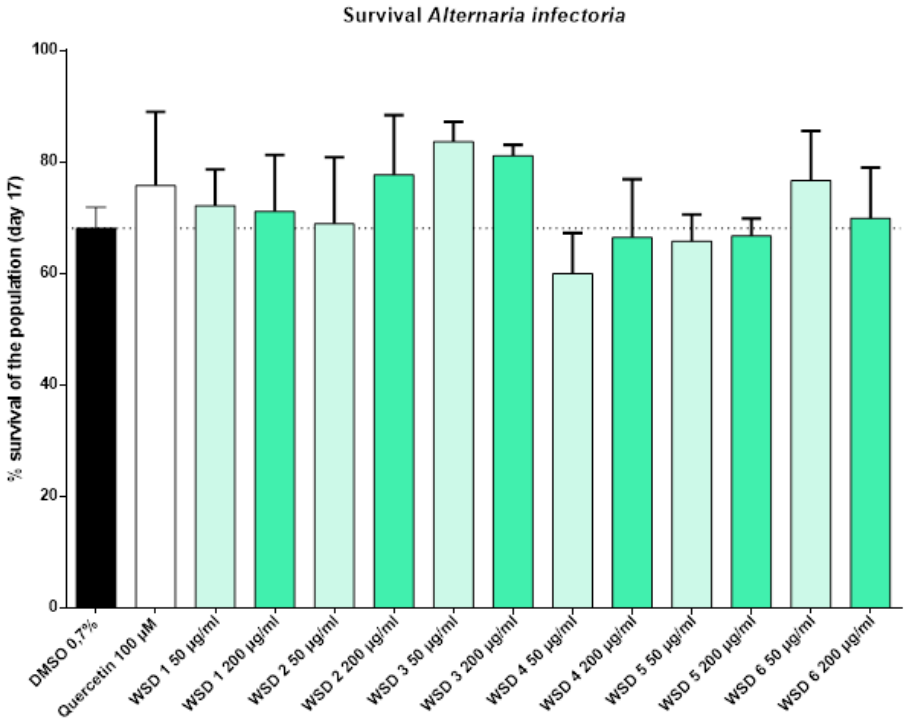

Figure 7: Survival rates for N2-wildtype worms treated with Alternaria infectoria extracts at 50 µg/mL (light green) *and 200 µg/mL* (dark green) *compared to the 0.7% DMSO vehicle control group, Quercetin (100 µM) was used as positive control; Data is shown as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-test; complete survival data are provided in Table 10 and Table 11 in the Appendix*

Treatment with *Alternaria infectoria* extracts generally resulted in slightly higher survival rates compared to the 0.7% DMSO vehicle control group. Exceptions were observed for WSD 4 and WSD 5 extracts, which resulted in lower survival for both tested concentrations. The reduced survival rates might be due to differences in secondary metabolite composition compared to other *A. infectoria* samples. In contrast to the *A.*

alternata extracts, there was no clear concentration-dependent effect shown for the *A. infectoria* treatment groups, as survival trends varied between individual extracts. WSD 1, WSD 3 and WSD 6 had slightly higher survival rates at 50 µg/mL, whereas WSD 2, WSD 4 and WSD 5 resulted in higher worm survival at 200 µg/mL.

Overall, both *Alternaria alternata* and *Alternaria infectoria* extracts showed only small effects on the survival rate of wild-type *C. elegans* with the applied assay conditions. While most treatment groups showed a slight increase in survival rates compared to the 0,7% DMSO control group, *A. infectoria* samples generally tended to exhibit somewhat higher survival rates (in particular WSD 3) than the *A. alternata* extracts. In contrast to the concentration-dependent trend observed in most *A. alternata* treatment groups, no consistent dose-dependent effect was detected for the *A. infectoria* extracts. Worms treated with the positive control quercetin resulted in a moderate increase in survival rate in both assay sets, consistent with its reported life-prolonging effects in *C. elegans* (Kampkötter et al., 2008). Although certain trends for a slight increase in life span were observable (except for a slight decrease for WSD 4), the high standard deviations between replicates limited the statistical power and prevented the detection of statistically significant differences among the fungal extracts. Additionally, the positive control exhibited only a slight increase in survival compared to the vehicle control, which may indicate a generally weak assay response under the applied conditions. Extending the assay duration or repeating late-stage measurements would not necessarily improve interpretability, due to the worms nearing their natural lifespan which could mask the effects of the extracts. For more reliable conclusions, the survival assay should be repeated under optimised and more tightly controlled conditions. Therefore, the results of this assay should be interpreted with caution. Most strikingly, however, is that no nematocidal effect was determined for all tested extracts (at least for concentrations up to 200 µg/mL).

5.2.2. Oxidative Stress Response Assay

The second assay that was carried out was the oxidative stress response assay (ROS assay). The ROS assay was performed as described in Section 4.9 using the transgenic *C. elegans* strain CL2166, which carries a gst-4p::GFP construct. Exhibition to oxidative stress results in the activation of the SKN-1/Nrf2 transcription factor, which induces the expression of

the glutathione S-transferase (gst-4) gene, thus resulting in increased green fluorescent protein (GFP) expression regulated by the same promotor in this strain. The measured fluorescence intensity is therefore an indicator for oxidative stress response activation in the treated worms (Huynh & Wu, 2022).

On day 6 of the experiment, evaluation of the fluorescence intensity revealed distinct differences between the tested *A. alternata* and *A. infectoria* extracts, the vehicle, positive, and negative controls (Figure 8).

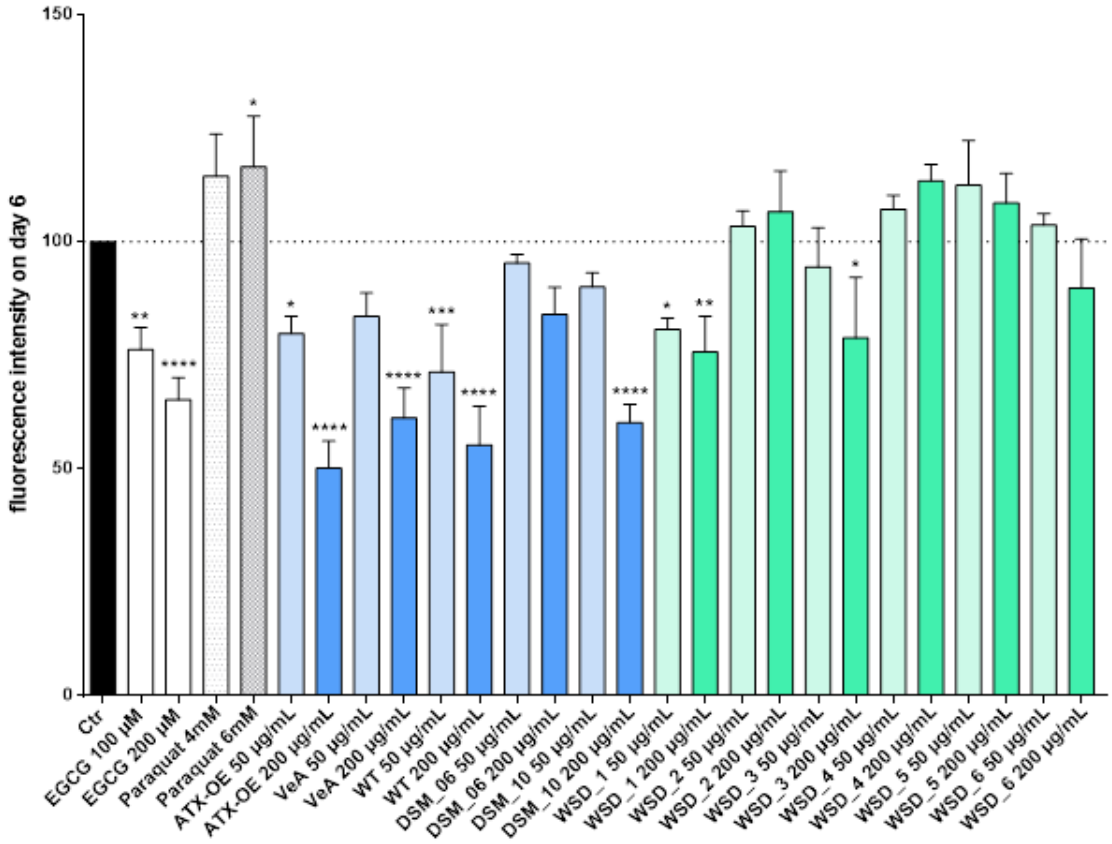

*Figure 8: Oxidative stress response in C. elegans (strain CL2166) exposed to fungal extracts. Fluorescence intensity (exc.: 485 nm; em.: 535 nm) of gst-4p::GFP reporter construct was measured 6 days after treatment with Alternaria alternata (blue) and Alternaria infectoria (green) extracts at 50 µg/mL (light blue/green) and 200 µg/mL (dark blue/green). EGCG (antioxidant positive control, 100 µM and 200 µM) and paraquat (pro-oxidative negative control, 4 mM and 6 mM). Data is shown as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001); complete data is provided in Table 12 and Table 13 in the Appendix*

All tested *A. alternata* extracts led to lower fluorescence intensities than the vehicle control group, suggesting reduced oxidative stress levels under the applied assay conditions. The most active extracts were ATX-OE (200 µg/mL) with 52% relative fluorescence intensity, VeA (200 µg/mL) with 56%, WT (200 µg/mL) with 55% and DSM_10 (200 µg/mL) with 56% ($p < 0.0001$).

In contrast, the *A. infectoria* extracts generally showed slightly higher fluorescence signals compared to the control group, indicating an increased oxidative stress response in the treated *C. elegans* worms. The samples with the highest fluorescence signals were WSD_4 (200 µg/mL) with 109% relative fluorescence and WSD_5 (50 µg/mL) with 108%. However, in comparison to the strong fluorescence-decreasing effects exhibited by *A. alternaria* extracts, the fluorescence-increasing effects after treatment with *A. infectoria* extracts was only mild. Additionally, some *A. infectoria* extracts resulted in a strong suppression of fluorescence intensities, which may reflect differences in the secondary metabolite composition and consequently varying effects on the oxidative stress response activation, including weaker pro-oxidative or potentially antioxidant effects.

In general, worms treated with lower sample concentrations tended to show weaker bioacitivities than worms that were treated with higher amounts of the respective extracts/compounds, indicating a concentration-dependent de- or increase in oxidative stress response for the tested samples, with WSD _5 being the only exception to this trend.

The EGCG positive control group produced lower fluorescence signals than the vehicle control group, consistent with its known antioxidant properties (Capasso et al., 2025). In contrast, the paraquat negative control group, which was used as an oxidative stress inducing agent, showed higher fluorescence intensities, reflecting the successful activation of the gst-4p::GFP oxidative stress response pathway (Liu et al., 2020). In addition, the 6 mM paraquat concentration showed slightly higher fluorescent levels than the 4 mM concentration, suggesting a concentration-dependent increase in oxidative stress response.

Overall, the results show clear concentration-dependent differences in the biological activity of the two tested fungal species, with *A. alternata* extracts demonstrating a clear fluorescence decreasing effect, whereas *A. infectoria* extracts tend to slightly increase the oxidative stress response in *C. elegans.*

6. CONCLUSION AND OUTLOOK

In this study, secondary metabolites from *Alternaria* species were investigated using both chemical and biological methods.

The first part of this thesis focused on the characterization of the fungal extracts by using LC-MS-based dereplication with subsequent chromatographic fractionation and targeted isolation of perylene quinone-type metabolites. The major constituents of the investigated extracts were found to be altertoxin I and altertoxin II. Flash chromatography was applied to achieve separation of the extracts including successful isolation of the target metabolites. However, the complexity of the extracts, as well as the challenges associated with obtaining highly pure target compounds was highlighted by the presence of structurally related perylene quinones and closely eluting metabolites such as STTX-III and a tentatively annotated STTX-IV, which could be sufficiently separated from the isolated compounds ATX-I (15.97 mg) and ATX-II (12.44 mg) to achieve high purities of 99% and 97%, respectively.

The second part aimed to assess the biological activity of *Alternaria alternata* and *Alternaria infectoria* extracts by using *Caenorhabditis elegans* as an *in vivo* model. The model was applied with N2 wild-type worms for the survival assay, as well as with the transgenic CL2166 (gst-4p::GFP) reporter strain in the oxidative stress response assay. Overall, only minor effects on worm survival were detectable in the survival assay, however the high variability and standard deviation between replicates limited the statistical significance of these results. Therefore, the survival data should be interpreted with caution. In contrast, the oxidative stress response assay showed pronounced and statistically significant effects for the investigated *A. alternata* and *A. infectoria* extracts, indicating a species-dependent difference for the modulation of oxidative stress response pathways in the worms.

The obtained chemical and biological data provide a valuable foundation for future analytical projects within the ELINA biochemometric workflow. The combination of bioactivity data with analytical information that will be generated from high resolution MS and/or NMR analysis may enable future studies to better identify the metabolite classes responsible for the observed biological effects.

7. REFERENCES

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8. APPENDIX

Table 8: Fraction pooling table for run 2 with corresponding test tubes and yield in mg

RUN 2		
Fraction	**Test Tubes**	**Yield**
F1	9-28	8.06 mg
F2	29-34	2.22 mg
F3	35-37	0.51 mg
F4	38	0.38 mg
F5	39-40	0.78 mg
F6	41-56	3.94 mg
F7	57-62	1.24 mg
F8	63-64	0.55 mg
F9	65-69	1.15 mg
F10	70-71	0.72 mg
F11	72	0.35 mg
F12	73-78	1.93 mg
F13	79-81	0.74 mg
F14	82-95	1.32 mg
F15	96-101	0.88 mg
F16	102-108	5.15 mg
F17	109-114	1.24 mg
F18	115-120	0.88 mg
F19	121-136	2.67 mg
F20	137-139	1.0 mg
F21	140-144	4.87 mg
F22	145-147	1.87 mg
F23	148-158	3.22 mg

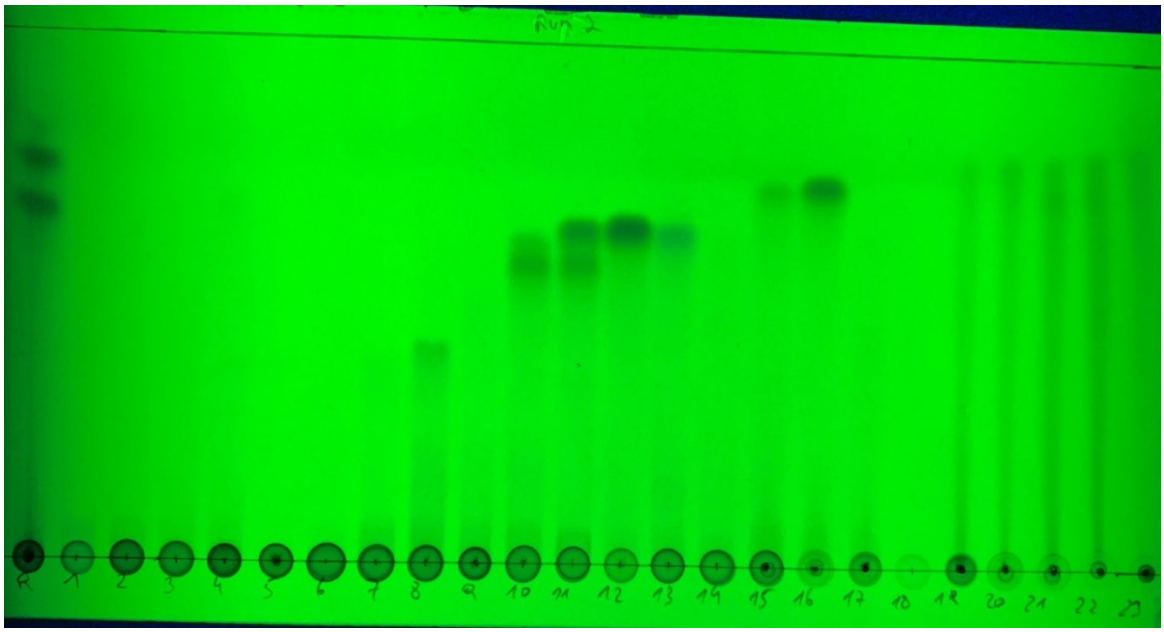

Figure 9: 23 Pooled Fractions of Flash Run 2 under 254 nm UV light, R: Reference (ATXOE_9 whole extract)

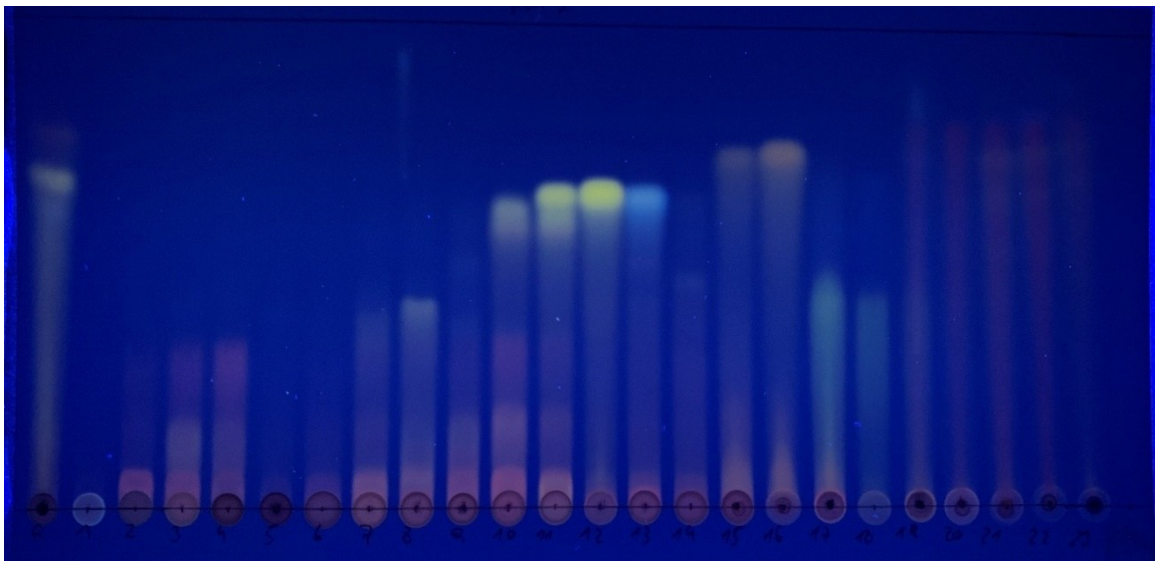

Figure 10: 23 Pooled Fractions of Flash Run 2 under 366 nm UV light, R: Reference (ATXOE_9 whole extract)

Table 9: Fraction pooling table for run 3 with corresponding test tubes and yield in mg

RUN 3		
Fraction	**Test Tubes**	**Yield**
F24	12-33	15.28 mg
F25	34-36	1.77 mg
F26	37-51	4.26 mg
F27	52-64	4.45 mg
F28	65-69	1.55 mg
F29	70-75	1.54 mg
F30	76-79	0.87 mg
F31	80-104	7.0 mg
F32	105-115	2.77 mg
F33	116-135	1.34 mg
F34	136-142	4.91 mg
F35	143-145	1.69 mg
F36	146-151	1.53 mg
F37	152-154	0.5 mg
F38	155-162	1.32 mg
F39	163-178	3.49 mg
F40	179-183	1.6 mg
F41	184	1.38 mg
F42	185-190	3.5 mg
F43	191-193	4.25 mg
F44	194-202	1.1 mg
F45	203	0.5 mg

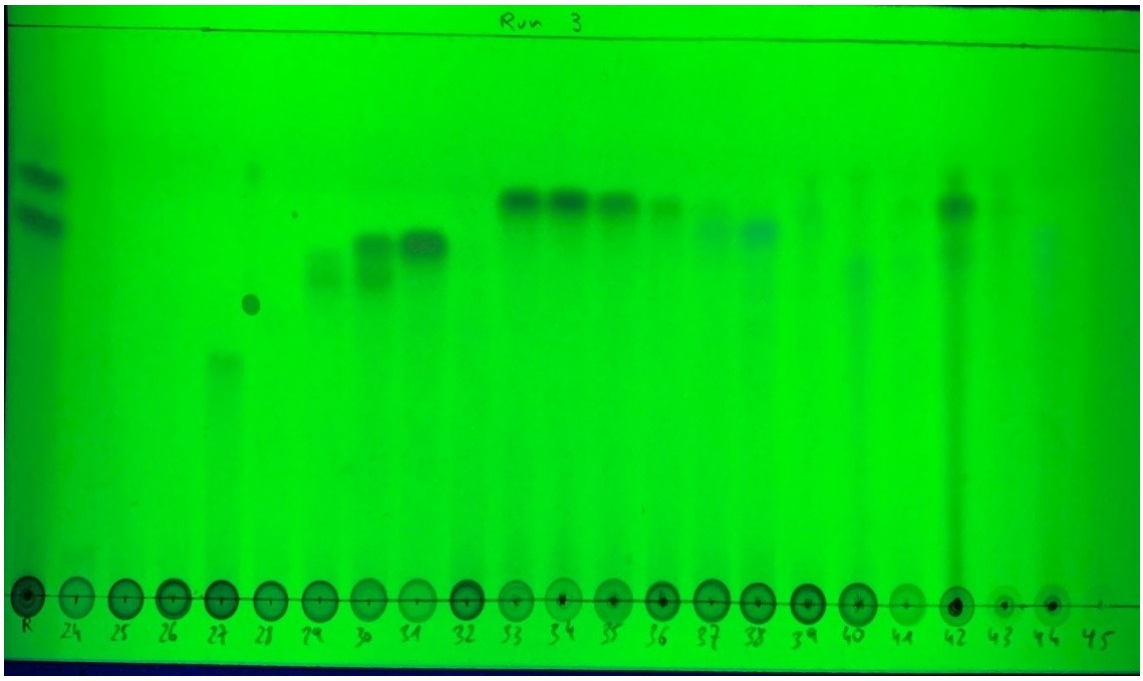

Figure 11: 22 Pooled Fractions of Flash Run 3 under 254 nm UV light, R: Reference (ATXOE_9 whole extract)

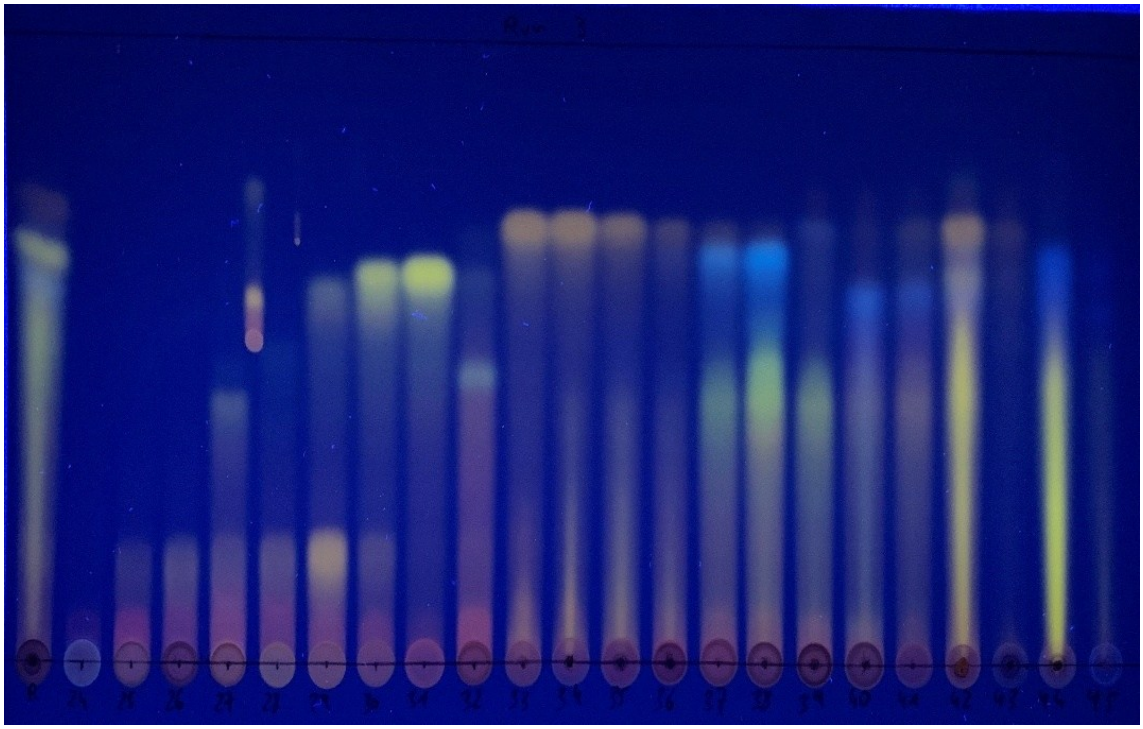

Figure 12: 22 Pooled Fractions of Flash Run 3 under 366 nm UV light, R: Reference (ATXOE_9 whole extract)

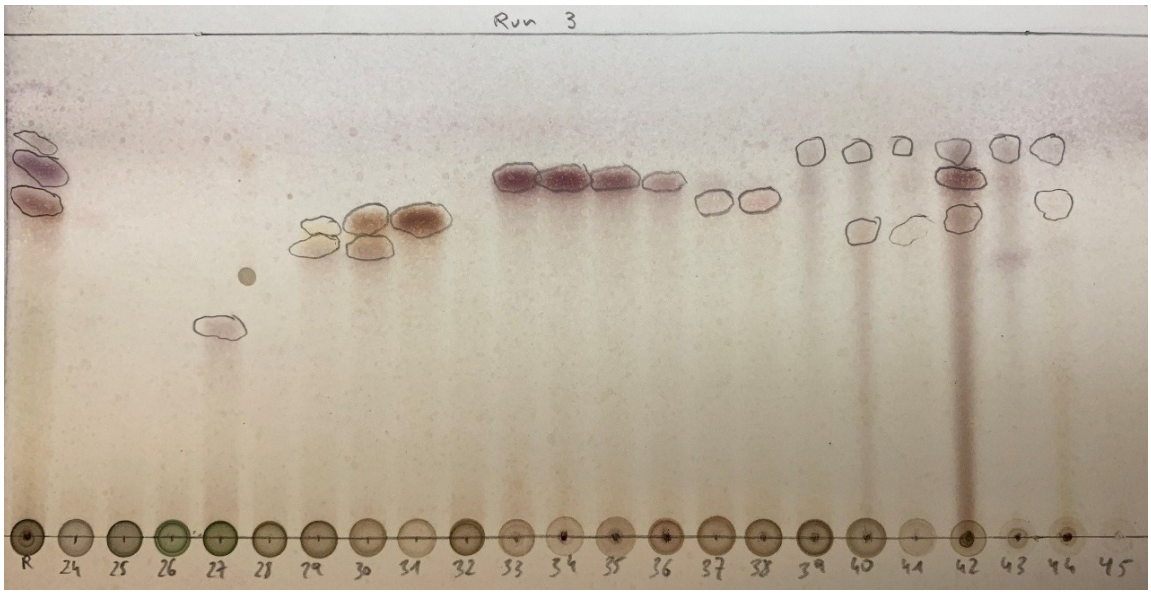

Figure 13: 22 Pooled Fractions of Flash Run 3 after derivatization with a methanolic 1% vanillin, and 5% H_2SO_4 solution, followed by heating at 100 °C for 5 minutes, R: Reference (ATXOE_9 whole extract)

Table 10: Survival assay plates 1-3; sample treatment with corresponding concentration, mean and standard deviation (SD); ρ values not shown due to lack of statistical significance

Sample	Concentration	Mean %	SD
DMSO	0.7%	71.75	2.235
Quercetin 100	100 µM	69.96	9.419
Quercetin 200	200 µM	79.24	8.890
WT 50	50 µg/mL	73.44	6.437
WT 200	200 µg/mL	72.32	10.558
ATX-OE 50	50 µg/mL	73.98	6.783
ATX-OE 200	200 µg/mL	71.36	3.348
VeA 50	50 µg/mL	75.54	6.687
VeA 200	200 µg/mL	67.44	6.339
DSM_06 50	50 µg/mL	79.24	5.846
DSM_06 200	200 µg/mL	65.37	5.412
DSM_10 50	50 µg/mL	74.17	6.064
DSM_10 200	200 µg/mL	76.36	7.860
WSD_1 50	50 µg/mL	72.14	5.298
WSD_1 200	200 µg/mL	71.12	8.229
WSD_2 50	50 µg/mL	68.90	9.730
WSD_2 200	200 µg/mL	77.69	8.740
WSD_3 50	50 µg/mL	83.64	2.903
WSD_3 200	200 µg/mL	81.09	1.588

Table 11: Survival assay plates 4-6; sample treatment with corresponding concentration, mean and standard deviation (SD); ρ values not shown due to lack of statistical significance

Sample	Concentration	Mean %	SD
DMSO	0.7%	68.11	3.079
Quercetin 100	100 µM	81.50	11.688
Quercetin 200	200 µM	68.46	5.451
WSD_4 50	50 µg/mL	59.97	5.932
WSD_4 200	200 µg/mL	66.43	8.536
WSD_5 50	50 µg/mL	65.73	3.952
WSD_5 200	200 µg/mL	66.73	2.542
WSD_6 50	50 µg/mL	76.63	7.289
WSD_6 200	200 µg/mL	69.88	7.409

Table 12: ROS assay plates 1-3; sample treatment with corresponding concentration, mean, standard deviation (SD) and ρ values compared to DMSO vehicle control group (= 100%)

Sample	Concentration	Mean %	SD	ρ value
Paraquat 4 mM	4 mM	119.065	8.247	$\rho > 0.05$
Paraquat 6 mM	6 mM	121.046	15.699	$\rho < 0.05$
EGCG 100	100 µM	76.234	4.900	$\rho < 0.01$
EGCG 200	200 µM	65.242	4.850	$\rho < 0.0001$
WT 50	50 µg/mL	71.275	10.503	$\rho < 0.001$
WT 200	200 µg/mL	55.252	8.573	$\rho < 0.0001$
ATX-OE 50	50 µg/mL	79.795	3.813	$\rho < 0.05$
ATX-OE 200	200 µg/mL	50.128	5.977	$\rho < 0.0001$
VeA 50	50 µg/mL	83.586	5.155	$\rho > 0.05$
VeA 200	200 µg/mL	61.226	6.517	$\rho < 0.0001$
DSM_06 50	50 µg/mL	95.276	1.907	$\rho > 0.05$
DSM_06 200	200 µg/mL	83.990	5.991	$\rho > 0.05$
DSM_10 50	50 µg/mL	90.071	3.075	$\rho > 0.05$
DSM_10 200	200 µg/mL	60.181	4.004	$\rho < 0.0001$
WSD_1 50	50 µg/mL	80.694	2.465	$\rho < 0.05$
WSD_1 200	200 µg/mL	75.702	7.863	$\rho < 0.01$
WSD_2 50	50 µg/mL	103.345	3.473	$\rho > 0.05$
WSD_2 200	200 µg/mL	106.616	8.965	$\rho > 0.05$

Table 13: ROS assay plates 4-6; sample treatment with corresponding concentration, mean, standard deviation (SD) and ρ values compared to DMSO vehicle control group (= 100%)

Sample	Concentration	Mean %	SD	ρ value
Paraquat 4 mM	4 mM	109.798	9.045	$\rho > 0.05$
Paraquat 6 mM	6 mM	112.053	0.683	$\rho < 0.05$
WSD_3 50	50 µg/mL	94.425	8.624	$\rho > 0.05$
WSD_3 200	200 µg/mL	78.824	13.265	$\rho < 0.05$
WSD_4 50	50 µg/mL	107.133	3.084	$\rho > 0.05$
WSD_4 200	200 µg/mL	113.445	3.511	$\rho > 0.05$
WSD_5 50	50 µg/mL	112.444	9.824	$\rho > 0.05$
WSD_5 200	200 µg/mL	108.528	6.490	$\rho > 0.05$
WSD_6 50	50 µg/mL	103.670	2.482	$\rho > 0.05$
WSD_6 200	200 µg/mL	89.834	10.786	$\rho > 0.05$