universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

"Cytoprotective effect of Quercetin on Caco-2 cells exposed to Alternariol and Alternariol-monomethyl-ether"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 838
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften UG2002/ Master's Programm NUTRITIONAL SCIENCES UG2002
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Index

Index of illustrations and tables

1. Introduction

Mycotoxins are biologically active products formed as secondary metabolites by a few species of fungi, which easily colonize field crops and inevitably contaminate them with their toxins. Contamination can also take place after the harvest and storage, wherefore a wide variety of foods can be affected. This represents a threat to food safety and risks for disease in humans and animals consuming these foods (Chiesi et al. 2015; Fernandez-Blanco et al. 2014; EFSA, 2011; Turnera et al. 2009).

The main producers of mycotoxins are the genera *Fusarium, Penecillium, Aspergillus, Alternaria* and *Claviceps.* The same mycotoxin can be produced by different species and one species may produce many different mycotoxins, which explains that foods can be contaminated simultaneously with several mycotoxins (Chiesi et al. 2015; Fernandez-Blanco et al. 2014; Speijers et al. 2004).

The conditions of mycotoxins production in fungi depend on various environmental parameters such as humidity, temperature, substrate composition, light, pH and the availability of oxygen. Mycotoxins mostly occur in hot, humid and light places with substrate in abundance to a level of optimum pH and oxygen (Schwarz et al. 2012; EFSA, 2011; Turnera et al. 2009; Speijers et al. 2004).

In regards to the risks for the health of consumers, mycotoxins are substances that primarily cause chronic toxicity. Their effects depend on the mycotoxin, the type of food and the consumer. Due to their great diversity of chemical structures, mycotoxins have plenty of biological properties and toxicological effects. Although the acute toxicity of mycotoxins is not very high, the greater risk for the consumer is caused by chronic exposure. About possible long term effects through chronic exposition of especially small amounts, is still relatively little known. The most important effects are: carcinogenic, immunotoxic, teratogenic, mutagenic, and pathological effects on metabolism and various organs (Schwarz et al. 2012; EFSA, 2011; Turnera et al. 2009; Speijers et al. 2004).

An additional important effect is the acute metabolic response to an exposure of mycotoxins. It is important to consider that mycotoxins lead to the production of reactive oxygen species (ROS), which can result in oxidative stress and oxidation of macromolecules, mainly lipids (Tiessen et al. 2013; Pfeiffer et al. 2007). However, the human body contains two different defense systems, an enzymatic and a non-enzymatic, in order to protect the cells against a toxic environment. They reduce the production of ROS to a minimum, or degrade those generated, to reduce the toxic effect. Oxidative stress can lead to an increase of the antioxidant enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the antioxidant cellular component glutathione (GSH), which protects the cells against the consequences of oxidative damage (Fernandez-Blanco et al. 2014).

1.1. Alternariol and Alternariol-monomethyl-ether: Properties and toxicity

Alternariol (AOH) and Alternariol-monomethyl-ether (AME) are the main toxic metabolites produced by the fungi of the genus *Alternaria* and can provoke serious health problems for humans and animals. They are found in a wide variety of cereal products, fresh and refrigerated vegetables and fruits and stored feedstuffs (Fernandez-Blanco et al. 2014; Schwarz et al. 2012; Bensassi et al. 2011, EFSA, 2011). According to the scientific opinion of the EFSA, especially *"Legumes, nuts, oilseeds and in particular sunflower seeds"* are contaminated with AOH and AME. Although they are commonly found in a wide variety of food and feed, there are no specific regulations in Europe or in other regions of the world for any of the Alternaria toxins (EFSA, 2011).

Alternariol

The chemical structure of AOH ($C_{14}H_{10}O_5$) is shown in Fig.1; structurally AOH is characterized as a dibenzo-α-pyranone, which molecular weight is 258.2 (Fernandez-Blanco et al. 2014).

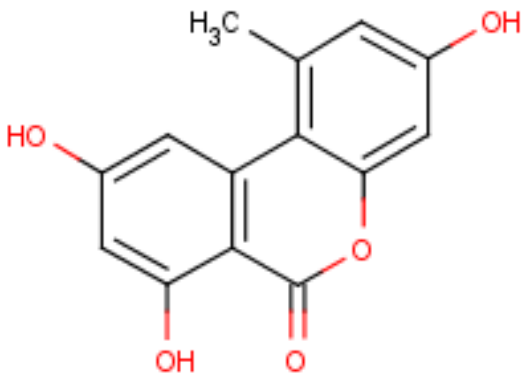

Fig.1 Chemical structure of AOH

(http://www.fermentek.co.il/Alternariol.htm)

The toxic effects of AOH are diverse. It has been shown that AOH leads to cytotoxic effects in mammalian cells by causing ROS generation and LPO production, which results in impaired cellular viability. Moreover, AOH induces the antioxidant enzymatic defense system as well as the GSH activity in cell cultures (Fernandez-Blanco et al. 2015; Juan-García et al. 2015; Fernandez-Blanco et al. 2014; Juan-García et al. 2013; Tiessen et al. 2013). Besides, AOH can provoke cell cycle arrest, apoptosis of cells and DNA damaging effects (Fernandez-Blanco et al. 2015; Pfeiffer et al. 2007; Lehmann et al. 2006).

AOH, as well as AME, have shown fetotoxic and teratogenic effects in animals as well as mutagenic and clastogenic effects in various *in vitro* systems (Juan-García et al. 2013; EFSA, 2011). Furthermore, the genotoxic activity of AOH in mammalian systems has been demonstrated (Pfeiffer et al. 2007; Marko 2007; Lehmann et al. 2006; Shan et al. 2000), as well as its estrogenic potential (Lehmann et al. 2006).

Alternariol-monomethyl-ether

The chemical structure of AME ($C_{15}H_{12}O_5$) is shown in Fig.2; structurally also AME is characterized as a dibenzo-α-pyranone, with a molecular weight of 272.2 (Bensassi et al. 2011).

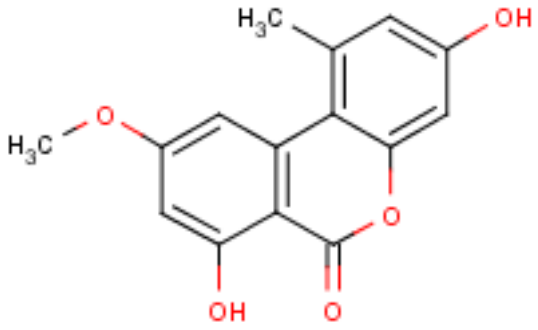

Fig.2 Chemical structure of AME

(http://www.fermentek.co.il/Alternariol-methyl-ether.htm)

Although AME is considered less toxic than AOH, the toxic effects are quite similar. AME is able to induce DNA strand breaks in human carcinoma cell lines (Fehr et al. 2009). Furthermore, it has been demonstrated that, by activating the mitochondrial pathway of apoptosis, AME induces cell death in human colon carcinoma cells (Bensassi et al. 2011). Moreover, both AOH and AME, have been associated with the etiology of esophageal cancer (Liu et al. 1991, Liu et al. 1992).

1.2. Quercetin

Quercetin, is a bioactive phytochemical that belongs to a large group of polyphenolic flavonoid substances, which are divided in several subgroups. Characteristic for flavonoids is a phenyl benzo(c) pyrone-derived structure, which consist of two benzene rings and is linked by a hetercyclic pyrone or pyran ring. The chemical structure of quercetin ($C_{15}H_{10}O_7$) is demonstrated in Fig.3; the molecular weight is 338.26 (Xi et al. 2012; Harwood et al. 2007).

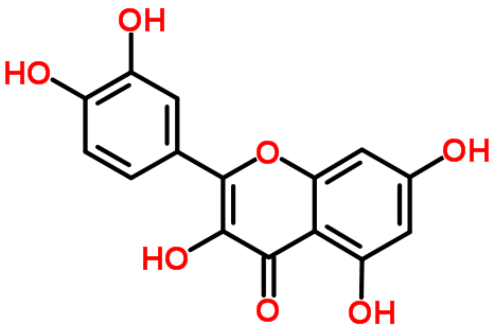

Fig. 3 Chemical structure of Quercetin

(http://www.chemspider.com/Chemical-Structure.4444051.html)

Quercetin belongs to the subgroup of flavonol. In plants, quercetin is mainly found conjugated to sugars at the 3-position of the unsaturated C-ring. The most commonly present O-β-glycosides are quercitrin and rutin (Fig. 4 and Fig. 5) (Harwood et al. 2007).

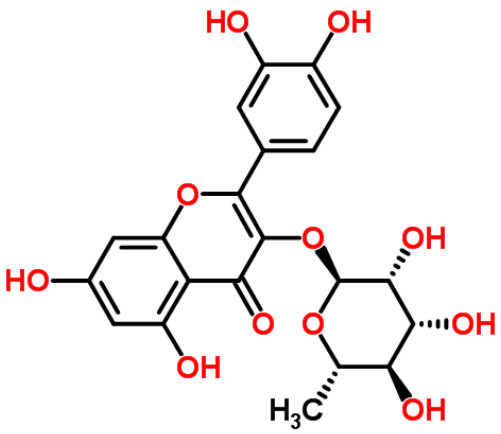

Fig. 4. Chemical structure of Quercitrin

(http://www.chemspider.com/Chemical-Structure.4444112.html)

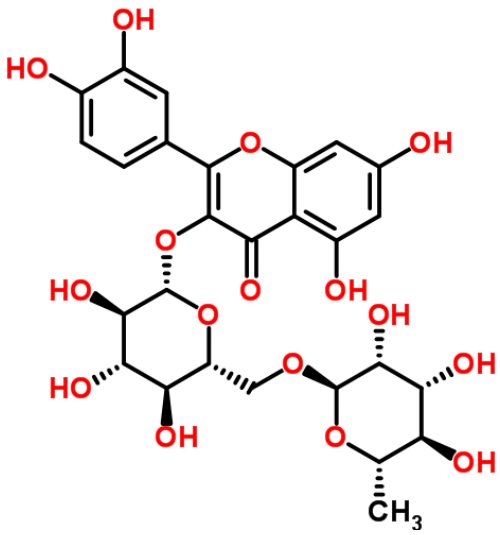

Fig. 5. Chemical structure of Rutin

(http://www.chemspider.com/Chemical-Structure.4444362.html)

Quercetin is found in several dietary sources, mainly in onions, capers, kale, broccoli, apples, cherries, grapes, berries, red wine, black and green tea (Harwood et al. 2007; Harborne et al. 2000). In Western countries the average intake ranges from about 25 to 30 mg per day (approximately 75 to 90 µM) (Russo 2007)

The regular dietary intake of quercetin is associated with numerous potential health benefits, due to its various biological and pharmacological effects: antioxidant (in vitro and in vivo), anticarcinogenic (in vitro and in vivo), antiinflammatory (in vitro and ex vivo), bacteriostatic (in vitro), chelation (in vitro), cardioprotective (in vivo) and secretory properties (in vivo) (Xi et al. 2012; Jan Øivind Moskaug et al. 2004; Harwood et al. 2007; Harborne et al. 2000). It is also associated with cytoprotective effects and can inhibit oxidative stress (Xi et al. 2012; Barcelos et al. 2011).

1.3. Alternative methods: cell cultures

To assess the toxicity of mycotoxins and receive adequate toxicological information to define toxicicological limits that can be considered safe for the consumer, animal experiments (*in vivo*) and alternative methods such as cell cultures (*in vitro*) are needed. For the use of alternative methods the rule of the 3R has to be considered (Schiffelersa et al. 2014; Russel and Burch 1959):

- Replacement: Methods which avoid or replace the use of animals
- Reduction: Methods which minimize the number of animals used per experiment, if they are required
- Refinement: Methods which minimize suffering and improve animal welfare.

In vitro assays using cell culture are among the alternative methods. They are precursors of whole animal studies and yield fundamental information. The big advantage of cell lines established to determine cytotoxicity and mechanisms of toxicity *in vitro* of contaminants and natural toxics in foods are: fast application, high reproducibility and the experimental conditions are controllable at every time. Therefore cell cultures are suitable for the assessment of natural toxics such as mycotoxins (Gomez-Lechon et al. 1993). However, subsequent *in vivo* assays are required.

The changes caused by mycotoxins are assessed by the indicators of toxicity (shown in table 1), which are parameters that are determined to quantify changes in the structure and physiology of the assay components substrate (Bouaziz et al. 2006).

Table 1. Indicators for cytotoxicity

Endpoint	Parameter
Cell Morphology	Cell size and shape Cell-cell contacts Differentiations of the membranes
Cell viability	Vital dye uptake Cell number Replacing efficiency
Cell adhesion	Attachment to culture surface Cell-cell adhesion
Cell proliferation	Proteins Increase in cell number
Membrane damage	Composition and stability Leakage across cellular membrane

(Manuel et al. 2009)

***In vitro* cytotoxicity assays**

There are distinct assays used to determine the different endpoints by *in vitro* methods. An *in vitro* cytotoxicity assay, which is commonly used and is relatively simple to perform, is the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The MTT is a yellow soluble tetrazolium dye, which is converted into an insoluble blue colored formazan by the mitochondrial succinate dehydrogenase. The succinate dehydrogenase, as part of the citric acid cycle and respiratory chain, is a particularly interesting enzyme to determine the degree of toxic effects of these two processes. Only the metabolically active cells within viable mitochondria retain the ability to this reaction. The MTT is an established assay to detect general cytotoxic compounds and agents which have the mitochondria as a specific target. Furthermore, it is rapid, sensitive, adaptable and compared to other methods relatively inexpensive (Ruiz et al. 2006).

2. Objectives and work plan

2.1. Objectives

The objectives of this investigation were, on the one hand, to study the cytotoxicity induced by AOH, AME and quercetin in Caco-2 cells and on the other hand, to determine the cytoprotective effect of quercetin against the cellular damage caused by the mycotoxins AOH and AME in Caco-2 cells.

The following objectives are proposed:

- Determine the viability of Caco-2 cells after single challenge the cells with AOH, AME and quercetin.
- Determine the viability of Caco-2 cells after combined challenge with AOH and AME
- Determine the cytoprotective effect of quercetin in Caco-2 cells exposed to AOH and AME, individually and in combination.

2.2. Work plan

To achieve the objectives the following work plan was proposed:

- Become familiar with culture techniques, as well as, the working conditions in a cell culture lab.
- Become familiar with the process of freezing/unfreezing and counting of cells.
- Individual AOH and AME exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.
- Individual quercetin exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.
- Combined AOH and AME exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.

- Combined AOH and quercetin exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.
- Combined AME and quercetin exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.
- Combined AOH, AME and quercetin exposure of Caco-2 cells for 24 and 48 hours. Determination of the cell viability by the MTT assay.

3. Materials and methods

3.1. Reagents and materials

- Dulbecco's Modified Eagle's Medium (DMEM)
- AOH (258.2 g/mol purity >96%, SIGMA)
- AME (272.2 g/mol purity >96, SIGMA)
- Quercetin (338.26 g/mol purity >98%, SIGMA)
- Fetal calf serum (FCS)
- Hepes
- Trypsin
- Isoton ®
- Antibiotics: Penicillin y Streptomycin
- Fungizone
- Non-essential aminoacids (NEAA)
- Deionized water (resistivity ≤18 MV cm) → Milli-Q water purification system.
- Sodium bicarbonate ($NaHCO_3$)
- Sodium hydroxide (NaOH)
- Sodium chloride (NaCl)
- Hydrogen chloride (HCl)
- Sodium pyruvate ($C_3H_3NaO_3$)
- Potassium chloride (KCl)
- Disodium phosphate (dihydrate) ($HNa_2PO_4(H_2O)_2$)

- Potassium dihydrogen phosphate (KH_2PO_4)
- Tetrazolium bromide (MTT)
- Dimethyl sulfoxide (DMSO)
- Glycine

Preparation of the culture medium

- 13.4 g/l DMEM, 3.4 g/l $NaHCO_3$, 10 g/l Hepes and 10 ml/l $C_3H_3NaO_3$
- Fill up to 1 l with deionized H_2O
- Shake and mix it with magnetic stirring
- Adjust pH to 7.4 with NaOH
- Filtrate to 2 sterile bottles of 500 ml
- After the filtration add:
    - FCS 10% (100 ml)
    - Antibiotics 1% (10 ml)
    - Fungizone 0.1% (1 ml)
    - NEAA 1% (10 ml)

Preparation of the stock solution of AOH

(Stored in the dark, in the freezer).

- Dissolve 5 mg of AOH (SIGMA) into 1.97 ml of DMSO. (10.000 µM)
- Stock solutions was maintained at -18 °C

Preparation of the stock solution of AME

(Stored in the dark, in the freezer).

- Dissolve 5 mg of AME (SIGMA) into 1.8 ml of DMSO. (10.000 µM)
- Stock solutions was maintained at -18 °C

Preparation of the stock solution of quercetin

(Stored in the dark, in the freezer).

- Dissolve 9.85 mg of quercetin (SIGMA) into 3 ml of DMSO. (10.000 µM)
- Stock solutions was maintained at -18 °C

Preparation of PBS

- Dissolve 4 g NaCl, 0.1 g KCl, 0.88 g $HNa_2PO_4(H_2O)_2$, 0.12 g KH_2PO_4 in 450 ml of deionized water.
- Adjust pH to 7.4 with NaOH
- Adjust the volume to 500 ml
- Transfer into a 500 ml bottle and autoclave it

Preparation of the trypsin solution

- Dissolve 5 ml trypsin in 45 ml PBS
- Divide this preparation in tubes of 10 ml

Preparation of MTT (always prepared freshly the day of use)

- Dissolve the required quantity according the following relation
  → 5 mg MTT: 1 ml PBS

Preparation of the Sorensen's glycine buffer

- 1.88 g Glycin and 1.46 g NaCl
- Make up to 250 ml with deionized H_2O
- Shake and mix it with a magnetic stirring
- Adjust pH to 10.5 with NaOH
- Transfer into a 250 ml bottle

3.2. Dilutions

Dilutions of quercetin

- Prepare dilution A (5.000 µM) by diluting 40 µl of the stock solution in 40 µl of DMSO
- Prepare dilution B (2.500 µM) by diluting 40 µl of solution A in 40 µl of DMSO
- Prepare dilution C (1.250 µM) by diluting 40 µl of solution B in 40 µl of DMSO
- Prepare dilution D (625 µM) by diluting 40 µl of solution C in 40 µl of DMSO
- Prepare dilution E (312,5 µM) by diluting 40 µl of solution D in 40 µl of DMSO

Dilutions of AOH and AME

- Prepare dilution A (5.000 µM) by diluting 40 µl of the stock solution in 40 µl of DMSO
- Prepare dilution B (3.000 µM) by diluting 24 µl of the stock solution in 56 µl of DMSO
- Prepare dilution C (2.500 µM) by diluting 40 µl of solution A in 40 µl of DMSO
- Prepare dilution D (1.500 µM) by diluting 40 µl of the solution B in 40 µl of DMSO
- Prepare dilution E (1.250 µM) by diluting 40 µl of solution C in 40 µl of DMSO
- Prepare dilution F (750 µM) by diluting 40 µl of the solution D in 40 µl of DMSO
- Prepare dilution G (625 µM) by diluting 40 µl of solution E in 40 µl of DMSO
- Prepare dilution H (312,5 µM) by diluting 40 µl of solution E in 40 µl of DMSO
- Prepare dilution I (156,25 µM) by diluting 40 µl of solution H in 40 µl of DMSO

The final concentrations of AOH, AME and quercetin tested were achieved by adding the culture medium. The final DMSO concentration in medium was ≤1% (v/v).

Table 2. Final concentration range

Substance	Range of concentration (µM)
AOH	3.125 – 100 + 3.125 – 30
AME	3.125 – 100 + 3.125 – 30
Quercetin	3.125 – 100
AOH+AME (1:1)	3.125 – 30 (both 1.56 – 15)
AOH+Quercetin (1:1)	3.125 – 100
AME+Quercetin (1:1)	3.125 – 100
AOH+AME+Quercetin (0,5:0,5:1)	3.125 – 50 (mycotoxins 1.56 – 25, quercetin 3.125 –50)

3.3. Equipments

- Analytical balance
- Autoclave
- Automatic pipettes (10, 100 and 1000 µl)
- Beakers (250, 500 and 1000 ml)
- Cold storage cell (4 °C)
- Eppendorf cuvettes®
- Filters and filtration pump
- Freezer (-18 °C)

- Latex disposable exam gloves
- Magnetic stirrer
- Multichannel pipettes (50 and 250 ml)
- Parafilm M sealing film
- pH meter
- Plate Shaker
- Pipettes (5, 10, 25 and 50 ml)
- Reagent reservoir
- Tubes (5 and 10 ml)
- Vial racks
- 500 ml bottles
- 500 ml cell culture bottles
- 96-well culture plates
- Beckmann Coulter Z1 particle counter, Germany
- Centrifuge 5810R eppendorfs AG, Hamburg, Germany
- Incubator THERMO SCIENTIFIC HEPA CLASS 100, model 371, USA
- Laminar Cabin: TELSTAR BIO-II-A, Valtek Nova, Spain
- Microtiter plate reader Wallace Victor, 1420 Multilaber Counter, Perkin Elmer, Turku, Finland
- Microscope NIKON eclipse TE2000-s, Japan
- Mycoplasma Stain Kit, Sigma–Aldrich, St Louis Mo. USA

3.4. Cell lines and maintenance

The human epithelial colorectal adenocarcinoma cell line (Caco-2) was obtained from American Type Culture Collection (ATCC HTB-37). Due to their diverse biological membrane properties, like enzymatic and transporter systems, Caco-2 cells are established *in vitro* tools. They are commonly used to study the absorption rate and the metabolism of drug compounds (Lakshmana et al. 2009). The Caco-2 cells were grown in DMEM medium in tissue culture flasks. They were supplemented with 25 mM HEPES buffer, 1% (v/v) of NEAA, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% (v/v) inactivated

FCS. The incubation conditions were strictly observed: pH 7.4, 37 °C under 5% CO_2 and 95% air atmosphere and at constant humidity. Every two days the culture medium was changed. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St Louis Mo. USA).

3.5. Cell viability by the MTT assay

Caco-2 cells were cultured in 96-well tissue culture plates by adding 200 µl/well of a suspension of 3 x 10^4 cells/well. After cells reached 90% confluence the culture medium was replaced and the cells were exposed to:

- 200 µl of fresh medium containing the dilutions of AOH ranging from 3.125 to 30 µM + a control during 24h and 48h; or
- 200 µl of fresh medium containing the dilutions of AME ranging from 3.125 to 30 µM + a control during 24h and 48h.
- 200 µl of fresh medium containing the dilutions of AOH ranging from 3.125 to 100 µM + a control during 24h and 48h; or
- 200 µl of fresh medium containing the dilutions of AME ranging from 3.125 to 100 µM + a control during 24h and 48h; or
- 200 µl of fresh medium containing the dilutions of quercetin ranging from 3.125 to 100 µM + a control during 24h and 48h;

To study the cytoprotective effect of quercetin, the cells were exposed to the following combinations:

- 200 µl of fresh medium containing the dilutions of AOH and AME (1:1), both ranging from 1.56 to 15 µM + a control during 24h and 48h.
- 200 µl of fresh medium containing the dilutions of AOH and quercetin (1:1), both ranging from 3.125 to 100 µM + a control during 24h and 48h.
- 200 µl of fresh medium containing the dilutions of AME and quercetin (1:1), both ranging from 3.125 to 100 µM + a control during 24h and 48h.
- 200 µl of fresh medium containing the dilutions of AOH, AME and quercetin (0.5:0.5:1). AOH and AME were ranging from 1.56 to 25 µM and Quercetin from 3.125 to 50 µM + a control during 24h and 48h.

  [Control = DMEM medium with 1% DMSO]

The plates were incubated for 24 and 48 h at 37 °C, and the cytotoxicity was detected by the MTT assay as described by Ruiz et al. (2006) with some modifications. After 24 and 48 h of exposure to AOH, AME, quercetin or their combinations, the medium was removed and each well received 200 µl of fresh medium and 50 µl of MTT. The plates were covered with aluminum foil (darkness) and incubated for 4 h at 37 °C. Then, the MTT containing medium was removed and the resulting formazan was solubilized in 200 µl of DMSO and 25 µl of Sorensen's glycine buffer. The absorbance was measured at 570 nm using an ELISA microtiter plate reader Wallace Victor (1420 Multilaber Counter, Perkin Elmer, Turku, Finland). The cell viability was expressed by the percentage relative to control cells (1% DMSO). The mean inhibition concentration (IC_{50}) was determined, which is defined as the concentration inducing 50% loss of cell viability and was calculated from full dose-response curve. The IC_{50} was taken as the criterion of cytotoxicity.

3.6. Statistical analysis of data

Statistical analysis of data was performed using a comparison of means with a Student's t-test to determine the differences between cytotoxicity by Caco-2 cells after incubation with AOH, AME or quercetin (SPSS version 22, Chicago, IL, USA). Data were expressed as means ± SD from at least three experiments. P values ≤ 0.05 were considered statistically significant.

4. Results and Discussion

The present study was performed to evaluate the cytoprotective effect of quercetin against the cellular damage caused by AOH and AME in Caco-2 cells. First, Caco-2 cells were exposed individually to several concentrations of AOH, AME and quercetin during several incubation times to determine their cytotoxic effects. Second, several combinations of each mycotoxin combined with quercetin were tested to determine the cytoprotective effect of the polyphenol.

4.1. Influence of Alternariol, Alternariol monomethyl ether and quercetin on cell viability of Caco-2 cells

Cellular viability was tested in Caco-2 using 96-well microtiter plates. The Caco-2 cells were exposed to mycotoxins and quercetin individually, at the same concentration range (3.125 to 100 µM + a control). The cytotoxic effect of AOH, AME and quercetin in Caco-2 cells was evaluated by the MTT assay at 24 and 48h of exposure. Figures 6 to 8 show the dose-response curves for AOH, AME and quercetin after 24 and 48h.

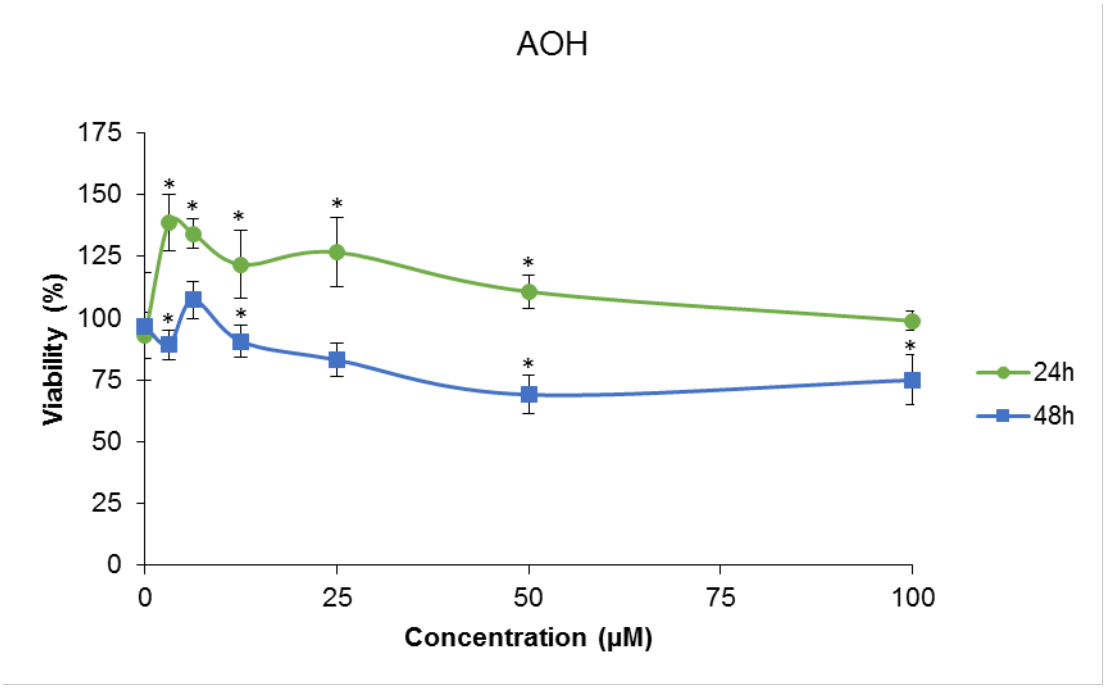

Fig. 6. Effects of the mycotoxin AOH on the viability of Caco-2 cells by MTT following exposure for 24h (●) and 48h (■) in the absence (control) or presence of different concentration of AOH (from 3.125 to 100 µM). Results are expressed as the mean ± SD of the three independent experiments. (*) p ≤ 0.05 indicates significantly different values from the control.

As shown in Fig. 6, AOH did not decrease the number of viable Caco-2 cells after 24h of exposure at any of the concentration tested (3.125 - 100 µM). In contrast, the number of viable Caco-2 cells is affected after 48h of exposure to AOH and shows a significant reduction ($p \leq 0.05$) of Caco-2 cells proliferation from 50 up to 100 µM. The highest inhibition in cell proliferation was 30% (50 µM), compared to its own control. No IC_{50} was obtained at any time tested. Moreover, cell proliferation, through stimulations of the mitochondrial function, raised up to 150% compared to the control after AOH exposure (3.125 - 50 µM) for 24h.

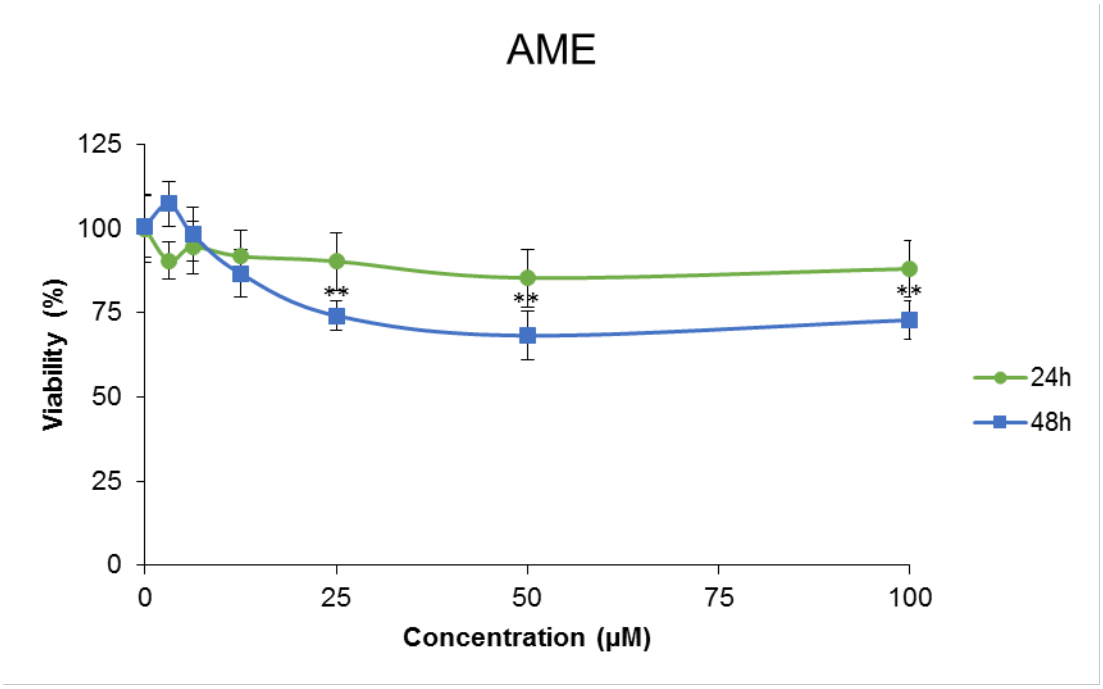

Fig. 7. Effects of the mycotoxin AME on the viability of Caco-2 cells by MTT following exposure for 24h (●) and 48h (■) in the absence (control) or presence of different concentration of AME (from 3.125 to 100 µM). Results are expressed as the mean ± SD of the three independent experiments. (**) p ≤ 0.01 indicates significantly different values from the control.

Also AME showed no significant decrease in the number of viable Caco-2 cells after 24h of exposure at any of the concentration tested (3.125 - 100 µM; Fig.7). However, AME affects the number of viable Caco-2 cells after 48h of exposure in a similar way as AOH and shows a significant dose-dependent inhibition ($p \leq 0.01$) of cell viability at concentrations ranging from 25 up to 100 µM. The highest inhibition in cell proliferation was 30% (50 µM) compared to its control. No IC_{50} was obtained either for AME at any time tested. On the other hand, AME did not stimulate cell proliferation compared to AOH after exposure for 24h.

In Fig. 8 the dose-response curve of quercetin is shown. As expected, quercetin had no influence on the number of viable Caco-2 cells after 24h/48h of exposure, except for the highest concentration tested (24h, 100 µM), which decreased cell viability significantly ($p \leq 0.01$). This is due to the fact that antioxidants, at high concentrations, can evolve pro-oxidant effects.

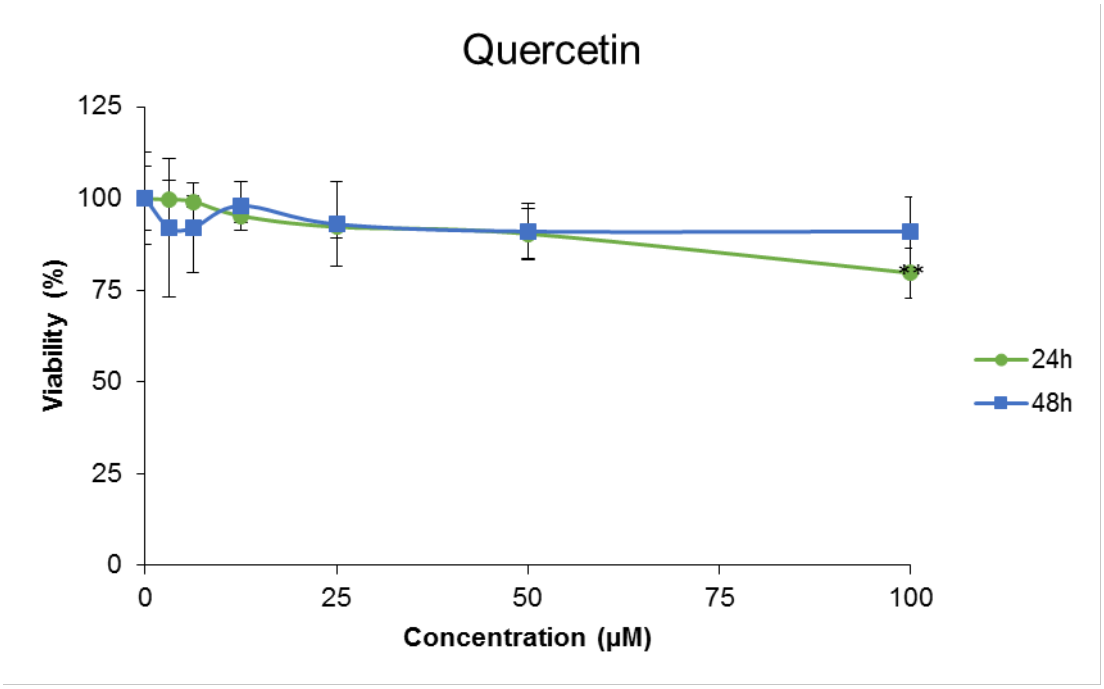

Fig. 8. Effects of quercetin on the viability of Caco-2 cells by MTT following exposure for 24h (●) and 48h (■) in the absence (control) or presence of different concentration of quercetin (from 3.125 to 100 µM). Results are expressed as the mean ± SD of the three independent experiments. (**) $p \leq 0.01$ indicate significantly different values from the control.

4.2. Influence of Alternariol + Alternariol monomethyl ether on cell viability of Caco-2 cells

To determine if the mycotoxins AOH and AME have synergistic properties, Caco-2 cells were exposed to the mycotoxins individually and to a combination of them, at the same concentration range (3.125 to 30 µM + a control). The cytotoxic effects of the combination were evaluated by the MTT assay at 24 and 48h of exposure. Figure 9 shows the dose-response curves for AOH + AME after 24 and 48h of exposure.

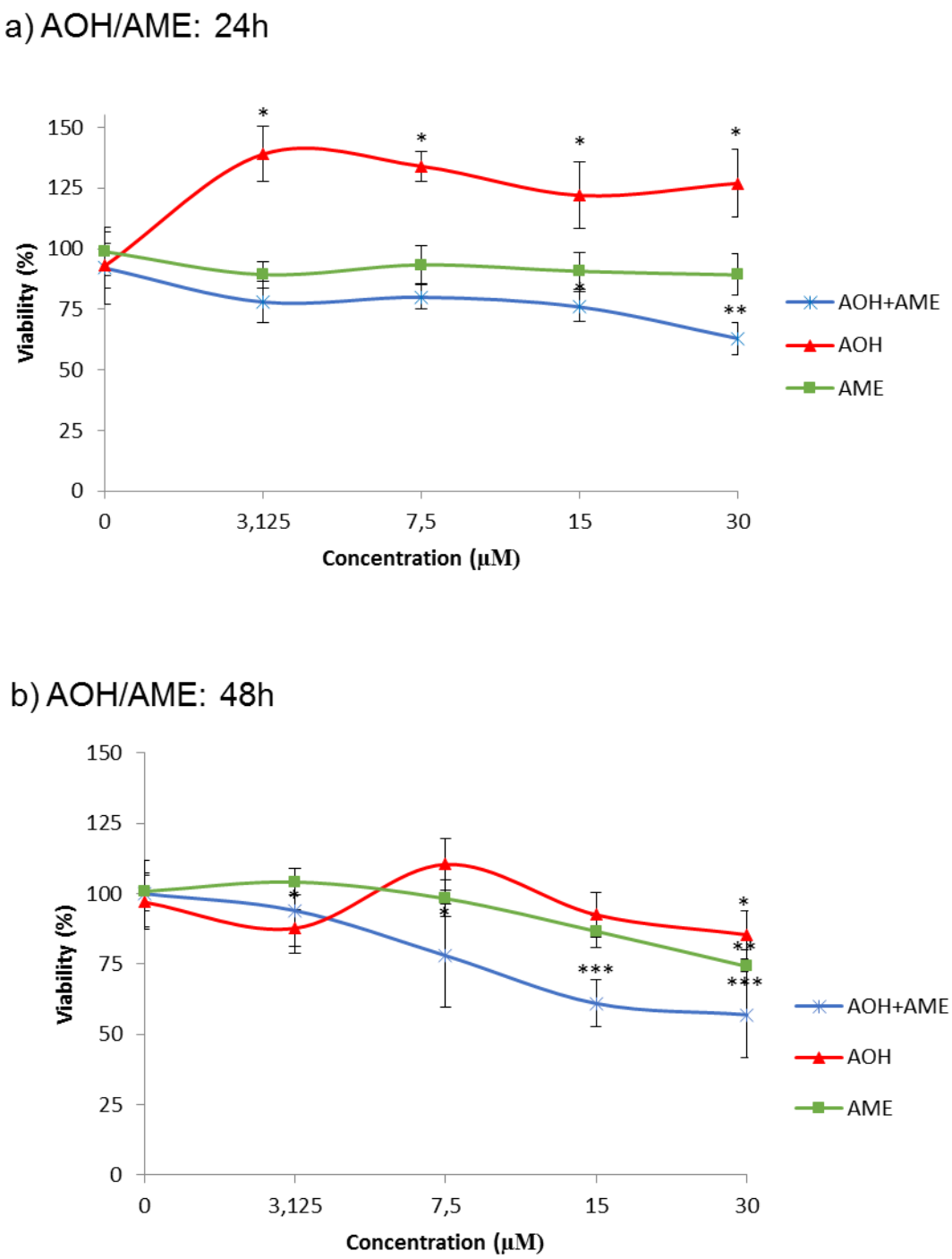

Fig. 9. Effects of the combination of the mycotoxins AOH + AME on the viability of Caco-2 cells by MTT-test. Exposure for 24h (a) and 48h (b) in the absence (control) or presence of different concentration of AOH+AME (from 3.125 to 30 µM). AOH + AME at molar ratio of 1:1. Results are expressed as the mean ± SD of the three independent experiments. (*) $p \leq 0.05$, (**) $p \leq 0.01$ and (***) $p \leq 0.001$ indicate significantly different values from the control.

The dose-response curves of AOH + AME are shown in Fig. 9. In contrast to the individual incubation of each mycotoxin, the combination of both showed significant influence on the number of viable Caco-2 cells after 24h of exposure (Fig. 9a). The combination shows a significantly reduced cell proliferation from 15 µM ($p \leq 0.05$) up to 30 µM ($p \leq 0.01$) compared to its own control. The highest reduction was 61% at 30 µM. However, when the combination was compared with AME tested alone, higher cytotoxic effects were observed, at all concentrations tested (from 3.125 to 30 µM). In contrast, the highest reduction (26% compared to AOH tested alone) was observed at 30 µM.

In addition, AOH + AME affected the number of viable Caco-2 cells after 48h of exposure and showed a significant dose-dependent inhibition of cell viability at concentrations ranging from 7.5 ($p \leq 0.05$) to 30 µM ($p \leq 0.001$; Fig. 9b). Similar inhibition in cell proliferation was observed for the combination in respect to AME and AOH tested alone. The range of reduction in cell proliferation was 18% to 25% compared to AME alone and 29% to 32% in comparison to AOH tested alone. No IC_{50} was obtained for AOH + AME at any concentration and time tested.

As shown in fig. 9 AOH + AME lead to a higher cytotoxicity compared to the individual incubation of AOH and AME. These results correspond to the expectation that AOH and AME have synergistic effects, when present at the same time.

4.3. Effects of quercetin on Caco-2 cells exposed to Alternariol

To study the cytoprotective effect of quercetin, Caco-2 cells were exposed to AOH and quercetin simultaneously, at the same concentration range (3.125 to 100 µM + a control) and incubation times. Figure 10 shows the concentration-response curves of the combination after 24 and 48h.

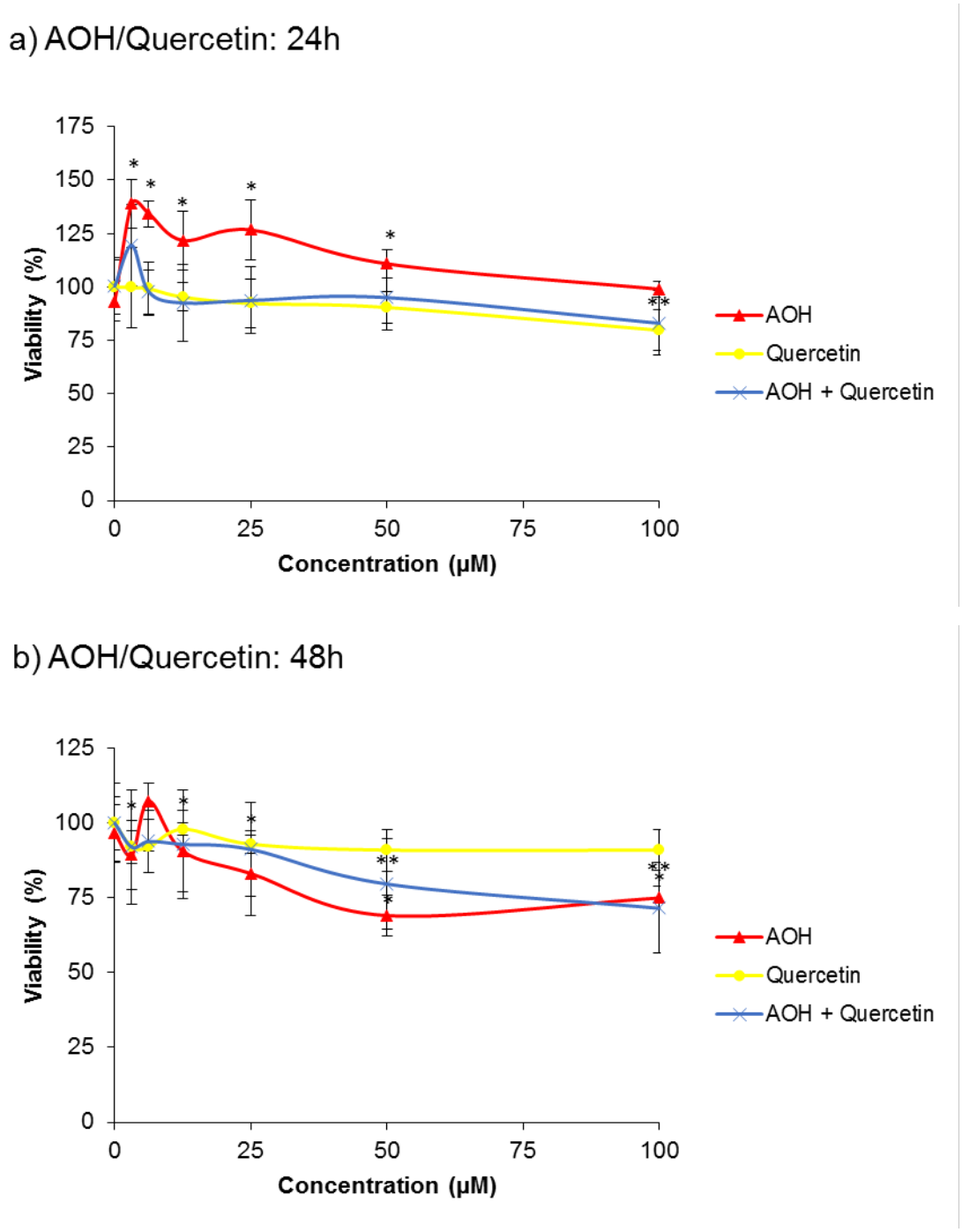

Fig. 10. Effects of quercetin on the viability of Caco-2 cells exposed to AOH by MTT-test. Exposure for 24h (a) and 48h (b) in the absence (control) or presence of different concentration of AOH (from 3.125 to 100 µM). Results are expressed as the mean ± SD of the three independent experiments. (*) $p \leq 0.05$ and (**) $p \leq 0.01$ indicate significantly different values from the control.

As shown in Fig. 10a, the combination of AOH + quercetin had not significant influence on the number of viable Caco-2 cells after 24h of exposure at any of the concentration tested (3.125 - 100 µM) compared to quercetin tested alone. However, the combination of AOH + quercetin decreased cell viability in respect to AOH tested alone (from 3.125 to 100 µM).

After 48h of exposure (Fig. 10b) the number of viable Caco-2 cells was affected by the combination and shows a significant reduction ($p < 0.01$) of Caco-2 cells proliferation from 50 up to 100 µM in respect to quercetin tested alone. However, similar cytotoxic effects were observed compared to AOH tested alone. No IC_{50} was obtained at any time tested. In comparison with the individual incubation of AOH, quercetin did show cytoprotectiv effects against the mycotoxin, when administrated simultaneously with AOH. Nevertheless, the combination of AOH + quercetin did not show any protective effect on Caco-2 cells.

4.4. Effects of quercetin on Caco-2 cells exposed to Alternariol-monomethyl-ether

The Caco-2 cells were exposed simultaneously to AME and quercetin. Again, they were tested at the same concentration range (3.125 to 100 µM + a control) and incubation times. In Figure 11 the concentration-response curves after 24 and 48h are shown.

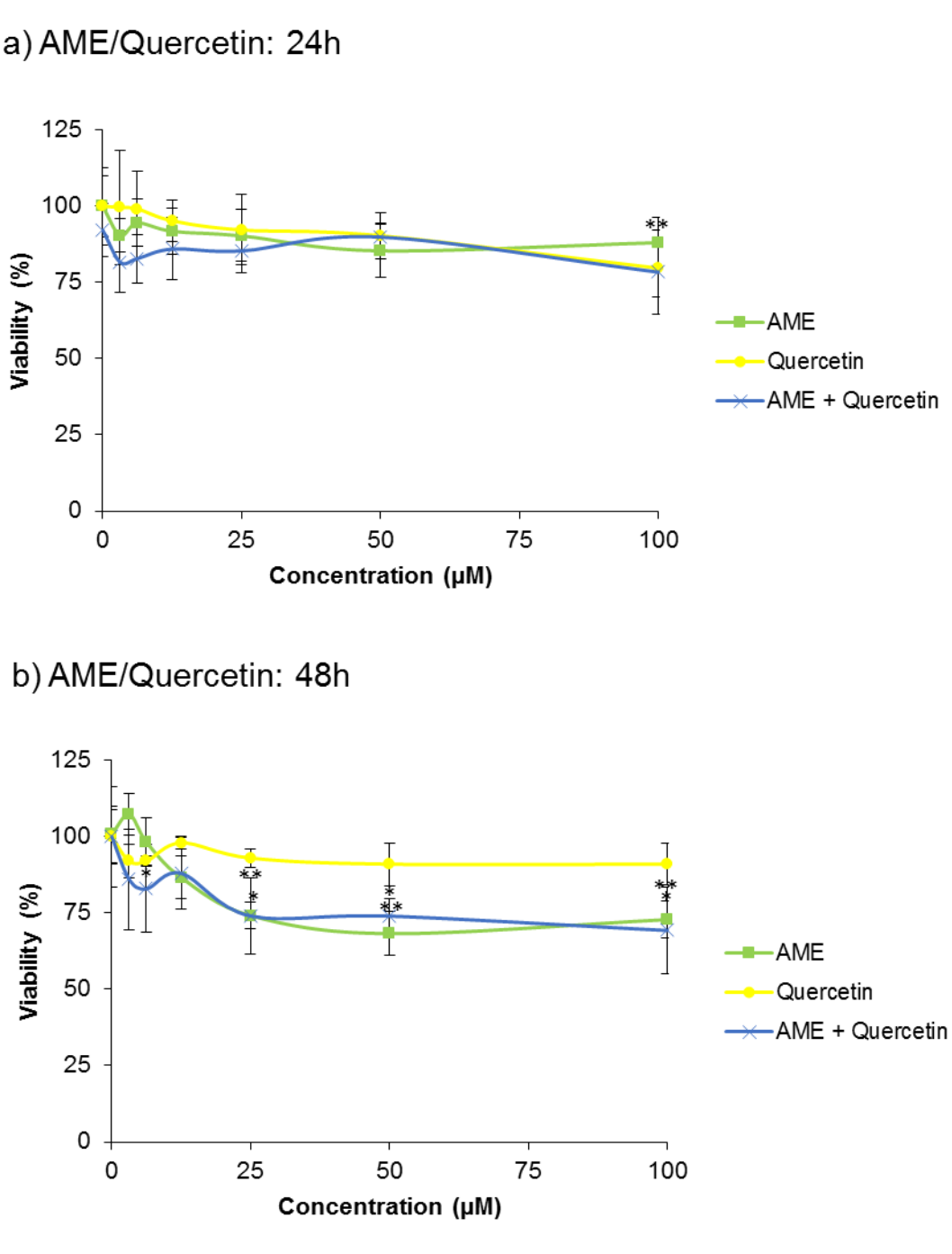

Fig. 11. Effects of quercetin on the viability of Caco-2 cells exposed to AME by MTT-test. Exposure for 24h (a) and 48h (b) in the absence (control) or presence of different concentration of AOH (from 3.125 to 100 µM). Results are expressed as the mean ± SD of the three independent experiments. (*) $p \leq 0.05$ and (**) $p \leq 0.01$ indicate significantly different values from the control.

The combination of AME + quercetin (Fig. 11a), showed similar effects as AME individually. There was no significant impact on cell viability observed after 24h of exposure at any of the concentration tested (3.125 - 100 µM).
When exposed for 48h, viability of Caco-2 cells showed a significant reduction ($p < 0.05$) of Caco-2 cells proliferation from 25 up to 100 µM compared to quercetin tested alone (Fig. 11b). No IC_{50} was obtained at any time tested.
Compared to the individual administration of AME, after 24 and 48h of incubation quercetin did not show any cytoprotective effect when given simultaneously with AME at any of the concentration (3.125 - 100 µM).

4.5. Effects of quercetin on Caco-2 cells exposed to Alternariol + Alternariol-monomethyl-ether

To study the interactions between the mycotoxins themselves and the cytoprotective effect of quercetin on this combination, the cells were exposed to different concentrations of the combination of AOH, AME and quercetin (ratio=0.5:0.5:1). In this combination AOH and AME ranged from 1.56 to 25 µM (together 50 µM) and quercetin from 3.125 to 50 µM. Figure 12 shows the concentration-response curves after 24 and 48h.

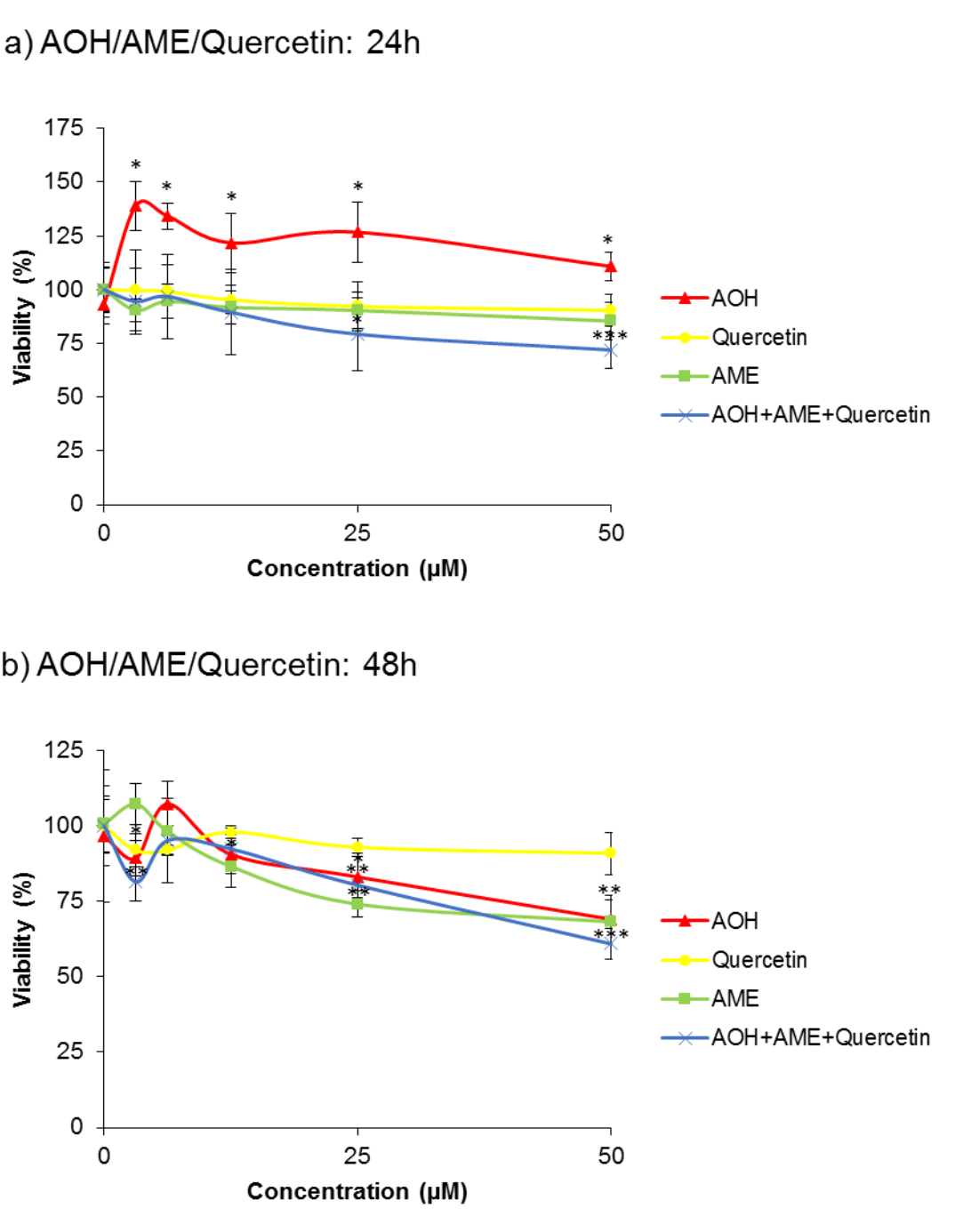

Fig. 12. Effects of quercetin on the viability of Caco-2 cells exposed to AOH + AME by MTT-test. Exposure for 24h (a) and 48h (b) in the absence (control) or presence of different concentration of AOH + AME (from 3.125 to 50 µM). AOH + AME + quercetin at molar ration of 0.5:0.5:1. Results are expressed as the mean ± SD of the three independent experiments. (*) p ≤ 0.05, (**) p ≤ 0.01 and (***) p ≤ 0.001 indicate significantly different values from the control.

The dose-response curves of the combined administration of AME, AOH and quercetin tested are shown in Fig. 12. The combination tested shows significant impact on cell viability after 24 and 48h of exposure. The highest reduction in cell proliferation after 24h ($p < 0.001$) was 45% for the combination compared to AOH tested alone. On the contrary, no differences were observed neither for quercetin nor for AME, when compared to the combination tested. No IC_{50} was obtained at any time tested.

In comparison to the individual incubation of AOH and AME after 24h, quercetin did not show any cytoprotective effects when given simultaneously with AOH + AME at any of the concentrations (3.125 - 50 µM). Moreover, also after 48h no cytoprotective effect was observed at any concentration tested (Fig. 12b).

As shown in fig. 9, the highest inhibition of cell proliferation of AOH + AME after 24h and 48h were 29% ($p<0.01$) and 43% ($p < 0.001$) respectively, at highest concentration tested (30 µM). Compared to that, the combination of AOH + AME + quercetin showed lower inhibition of the viability of Caco-2 cells, even at a higher concentration tested (50 µM). In comparison with the incubation of AOH + AME, quercetin did show cytoprotectiv effects against the mycotoxins, when given simultaneously. Nevertheless, the combination of AOH + AME + quercetin did not show any cytoprotective effect on Caco-2 cells (Fig. 11b).

Discussion

AOH and AME are the two main mycotoxins formed by the genus *Alternaria*. They are found in a wide variety of food and feed and, according to the scientific opinion of the EFSA, they can present a health hazard for humans and animals (EFSA, 2011). This study evaluated the cytotoxic effects of AOH and AME, and the cytoprotective effect of quercetin against the two mycotoxins on Caco-2 cells.

The study demonstrated that both AOH and AME inhibited Caco-2 cells growth in a time-dependent manner. According to the results obtained, individual toxicity of both mycotoxins is not high enough to have an impact after 24h of exposure at any of the concentrations tested (3.125 – 100 µM). These results correspond to observations obtained by Chiesi et al. (2015), Fernández-Blanco et al. (2014) and Tiemann et al. (2009). Similar to the here presented results in Caco-2 cells, they showed that AOH reduced cell viability in a dose-dependent way. Furthermore, none of them obtained an IC_{50} value for AOH in the concentration range tested (0.8 – 100 µM). Also Tiessen et al. (2013) found similar results in HT29 cells (human colon adenocarcinoma cell line). They neither obtained an IC_{50} for AOH (from 0.1 to 50 µM) after 24h of incubation by performing the WST-1 assay and SRB assay. On the contrary, Bensassi et al. (2012) demonstrated an IC_{50} value of about 65 µM (24h) for AOH in HCT116 cells (human colon cancer cell line), which seem to be more sensible to AOH, by using the FDA assay.

With respect to the stimulation of cell growth at low mycotoxin concentrations, Lombardi el al. (2012) and Prosperini et al. (2014) showed similar behavior in other cell lines exposed to mycotoxins than used in this work. At low-concentrations cell proliferation was stimulated, whereas at high concentrations it was inhibited.

Furthermore, the results of AME correspond to observations obtained by Tiessen et al. (2013). Similar to results here shown in Caco-2 cells, they showed a slight cytotoxic impact of about 10-15% in HT29 cells after 24h of incubation. Cytotoxic effects were determined by the SRB assay.

In addition, Tiessen et al. (2013) also tested the effects of AOH and AME on the mitochondrial activity of HT29 cells by the WST-1 assay. The mitochondrial activity, which is a pivotal parameter for cell viability, was not affected, neither by AOH nor by AME. Moreover, the results of AME are in accordance with observations obtained by Bensassi et al. (2011). They demonstrated a dose-dependent inhibition of cell viability and obtained an IC_{50} value of about 120 µM (24h) in HCT116 cells by using the FDA assay.

Numerous previous researches performed with AOH and AME in different cell lines showed that both mycotoxins have the ability to induce oxidative stress and to reduce cell viability in a time- and dose-dependent manner (Chiesi et al., 2015; Fernández-Blanco et al., 2014; Tiessen et al., 2013; Bensassi et al., 2012 and Bensassi et al. 2011). In contrary, there is a lack of knowledge about the possible synergistic properties of AOH and AME. Therefore, one objective of this investigation was to determine the combined viability assay of AOH and AME in Caco-2 cells. The results obtained demonstrated that AOH and AME have synergistic effects, when present at the same time. They induced significantly a dose-dependent inhibition of Caco-2 cells proliferation, which was higher compared to the individual incubation of the mycotoxins. They showed significant influence on the number of viable Caco-2 cells already after 24h of exposure. These results correspond to observations obtained by da Motta and Valente Soares (2000), who also observed the synergistic effects of AOH + AME.

On the other hand, quercetin is a flavonoid found in several dietary sources and associated with numerous potential health benefits (Xi et al. 2012; Barcelos et al. 2011; Harwood et al. 2007; Harborne et al. 2000). Because of its antioxidant capacity it is believed that quercetin is involved in the protection of cells against oxidative stress. Antioxidants were commonly associated with cytoprotective effects (Xi et al. 2012, Lombardi et al 2012; Barcelos et al 2011).
In this study, the highest concentration of quercetin (100 µM), which correspond approximately to the average daily intake of quercetin (75 – 90 µM), showed a significant decrease of the cell viability. This result corresponds to observations obtained by Lombardi el al. (2012). Similar to the results here shown in Caco-2

cells, they showed a significant decrease of the cell viability at 100 µM quercetin tested in CHO-K1 cells (Chinese hamster ovary cell line). Lower doses than 100 µM didn't cause any cytotoxic effect. However, the results obtained revealed that quercetin did not present cytoprotective effects on Caco-2 cells when exposed simultaneously to AOH, AME or AOH + AME.

Similar to the results here presented in Caco-2 cells, Barcelos et al. (2011) proved the cytotoxic and cytoprotective effect of quercetin and rutin in HepG2 cells (human hepatocellular liver carcinoma cell line), when exposed individually or together with aflatoxin B_1 (AFB_1). HepG2 cells treated with quercetin or rutin (pre-, simultaneous or post-treatment) showed a decrease in the number of AFB_1 – induced DNA strand breaks. Nevertheless, they observed that quercetin and rutin at higher concentrations (100 µg/mL) act as a prooxidant by increasing the intracellular ROS production. Matsuo et al. (2005) showed similar cytotoxic effects for quercetin (0 - 500 µM) and rutin (0 – 300 µM) in TIG-1 (human lung embryonic fibroblast cell line) and HUVE (umbilical vein endothelial cell line) cells.

Barcelos et al. (2011) also showed that at lower concentrations, quercetin and rutin inhibit ROS generation, due to their ability as efficient ROS scavenger. In addition, Choi et al. (2010) proved the impact of quercetin derivatives on HepG2 cells exposed to AFb_1, showing a decrease of oxidative stress, lipid peroxidation and GSH depletion. Furthermore, Xi et al. (2012) showed that quercetin could decrease cell damage induced by H_2O_2 and reduce apoptosis in SH-SY5Y cells (human neuroblastoma cell line).
On the other hand, Barcelos et al. (2011) proved that quercetin and rutin could modulate CYP isoenzymes, which are known to be involved in mycotoxin activation. Similar effects were found by Lombardi el al. (2012) in CHO-K1 cells exposed to Enniatins (ENs). Therefore, the protective effects of quercetin and its metabolites could be attributed to the inhibition of CYP isoenzymes and to their ROS scavenger properties.

Furthermore, the effect of quercetin on HepG2 and Caco-2 cells exposed to Ochratoxin A (OTA) was observed by Hundhausen et al. (2005), and Sergent et al. (2005). OTA is known to produce significant cytotoxic effects, through inhibition of various mitochondrial enzymes (cytochrome C oxidase, succinate dehydrogenase and ATPase), inhibition of protein synthesis, formation of DNA adducts and diminution of GSH. Both authors proved that quercetin shows no cytoprotective effect, when cell lines were exposed to quercetin and OTA.

Summarizing, we can conclude that AOH and AME have a slight cytotoxic effect on Caco-2 cells at the concentrations and the incubation times tested. Quercetin did not show stimulation of cell proliferation at any of the concentrations tested. When quercetin was simultaneously exposed to Caco-2 cells with AOH, AME or AOH+AME, no cytoprotective effect of the polyphenol was observed.

5. Conclusion

In conclusion, cell viability depends, among others, on a balance between prooxidant and antioxidant compounds. High levels of antioxidants, may correspond to polyphenols in food (as onions, capers, kale, broccoli, apples, cherries, grapes, berries etc.) and dietary supplements (quercetin – supplements and – rich diets, etc.) to protect against oxidative damage. According to the results obtained, there was no cytoprotective effect of quercetin against the mycotoxins AOH and AME. However, quercetin present cytoprotective effects in respect to other mycotoxins, with a higher toxicity than AOH and AME. The effect depends on the concentration of quercetin and the mycotoxins in food commodities, their simultaneous presence and interaction between the compounds. Therefore foodstuffs containing quercetin or its metabolites, could contribute to a reduced health risk of mycotoxins that are present in the diet. However, in order to understand better the mechanisms of the cytoprotective effects of quercetin, more research particularly on the bioavailability, bioaccessibility, and the mechanism of action of quercetin is required.

Abstract

Alternariol (AOH) and Alternariol-monomethyl-ether (AME) are the two main mycotoxins produced by the fungi of the genus *Alternaria.* They are found in a wide variety of food and feed, such as cereal products, vegetables and fruits, and can provoke serious health problems in humans and animals. Quercetin, is a bioactive phytochemical that belongs to the structural class of polyphenols and is found in several dietary sources. In Western countries the average daily intake ranges from about 25 to 30 mg (approximately 75 to 90 µM). The regular dietary intake of quercetin is associated with numerous potential health benefits, including cytoprotective effects and inhibition of oxidative stress. The aims of this investigation were to study the cytotoxicity produced by AOH and AME, individually as well as in a combination of the mycotoxins, and to determine the cytoprotective effect of quercetin against the cellular damage caused by the mycotoxins human colon adenocarcinoma (Caco-2) cells. The cytotoxic effects of the mycotoxins (3.125 to 100 µM) were evaluated by the MTT assay at 24h and 48h of exposure. The results demonstrated that both AOH and AME slightly inhibited Caco-2 cells growth in a time-dependent manner. However, no IC_{50} values were obtained at any time tested. Furthermore, the results demonstrate that AOH and AME have interactive effects, when present at the same time. They induced a significant dose-dependent inhibition of Caco-2 cells proliferation, which was higher compared to the individual incubation of the mycotoxins. To study the cytoprotective effect of quercetin, Caco-2 cells were exposed to the mycotoxins and quercetin simultaneously, at the same concentration range (ratio 1:1 and 0,5:0,5:1). In comparison with the individual incubation of the mycotoxins, quercetin showed a higher viability of Caco-2 cells, when given simultaneously with the mycotoxins. Nevertheless, there was no cytoprotective effect of quercetin against the mycotoxins AOH and AME observed, neither individually nor when present at the same time.

Zusammenfassung

Alternariol (AOH) und Alternariol-monomethyl-ether (AME) sind die beiden wichtigsten Mykotoxine, die von Schimmelpilzen der Gattung *Alternaria* gebildet werden. Man findet sie in einer Vielzahl von Lebens- und Futtermitteln, wie Getreide, Obst und Gemüse, und sie können schwere gesundheitliche Probleme bei Mensch und Tier verursachen. Quercetin ist ein bioaktiver Pflanzeninhaltsstoff aus der Gruppe der Polyphenole und kommt in einer Reihe von Nahrungsmitteln vor. In westlichen Ländern beträgt die durchschnittliche tägliche Aufnahme ca. 25 bis 30 mg (in etwa 75 bis 90 µM). Die regelmäßige Aufnahme von Quercetin ist mit zahlreichen positiven Eigenschaften für die Gesundheit assoziiert, einschließlich seiner zellschützenden Wirkung und der Hemmung von oxidativem Stress. Ziel dieser Studie war es, die von AOH und AME ausgehende Zytotoxizität, sowohl einzeln als auch in Kombination der beiden Mykotoxine, in menschlichen Kolon-Adenokarzinom-Zellen (Caco-2) zu bestimmen, und die zytoprotektive Wirkung von Quercetin auf die von den Mykotoxinen verursachte Zellschädigung zu untersuchen. Die zytotoxische Wirkung der Mykotoxine (3,125-100 µM) wurde nach 24h und 48h Exposition mit Hilfe des MTT-Assay bestimmt. Die Ergebnisse zeigen, dass sowohl AOH als auch AME das Wachstum der Caco-2-Zellen in einer zeitabhängigen Art und Weise leicht hemmen konnten. Jedoch wurde kein IC_{50}-Wert zu keinem getesteten Zeitpunkt erreicht. Des Weiteren zeigen die Ergebnisse, dass AOH und AME untereinander Interaktionseffekte aufweisen, wenn sie zur selben Zeit präsent sind. Sie induzierten eine signifikante dosisabhängige Hemmung der Zellproliferation der Caco-2-Zellen, welche höher war, verglichen mit der individuellen Inkubation der Mykotoxine. Zur Untersuchung der zytoprotektiven Wirkung von Quercetin, wurden die Caco-2 Zellen gleichzeitig Quercetin und den Mykotoxinen, in denselben Konzentrationsbereichen, ausgesetzt. Verglichen mit der individuellen Inkubation der Mykotoxine, bewirkte Quercetin eine geringere Hemmung der Zellviabilität der Caco-2-Zellen, wenn es zusammen mit Mykotoxinen verabreicht wurde. Nichtsdestotrotz konnte keine zellschützende Wirkung von Quercetin gegenüber den Mykotoxinen AOH und AME, weder einzeln noch in Kombination, beobachtet werden.

References

1. Turnera N. W., Subrahmanyamb S., Piletskyb S. A., 2009; Analytical methods for determination of mycotoxins. A review, *Analytica Chimica Acta*, Volume 632, Issue 2, Pages 168–180
2. EFSA (2011) Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on the risks for animal and public health related to the presence of Alternariatoxins in feed and food. *EFSA Journal* 9:2407
3. Speijers G.J.A., Speijers M.H.M., 2004; Combined toxic effects of mycotoxins. *Toxicol Letters* 153, 91–98
4. Chiesi C., Fernandez-Blanco C., Cossignani L., Font G., Ruiz M.J., 2015; Alternariol-induced cytotoxicity in Caco-2 cells. Protective effect of the phenolic fraction from virgin olive oil. *Toxicon* 93, 103-111
5. Fernandez-Blanco C., Font G., Ruiz M.J., 2014; Oxidative stress of alternariol in Caco-2 cells. Toxicol *letters* 229, 458-464
6. Bensassi F., Gallerne C., El Dein O.S., Hajlaoui M.R., Bacha H., Lemaire C. 2012; Cell death induced by the Alternaria mycotoxin Alternariol. *Toxicology in Vitro* 26, 915–923
7. Fernandez-Blanco C., Font G, Ruiz M.J., 2015; Oxidative DNA damage and disturbance of antioxidant capacity by alternariol in Caco-2 cells. *Toxicol Letters* 235, 61–66
8. Schwarz C., Kreutzer M., Marko D., 2012; Minor Contribution of alternariol, alternariol monomethyl ether and tenuazonic acid to the genotoxic properties of extracts from Alternaria alternata infested rice. *Toxicol Letters* 214, 46–52
9. Bensassi F., Gallerne C., El Dein O.S., Hajlaoui M.R., Bacha H., Lemaire C. 2012; Cell death induced by the Alternaria mycotoxin Alternariol. *Toxicology in Vitro* 26, 915–923
10. Solhaug A., Vines L.L., Ivanova L., Spilsberg B., Holme J.A., Pestka J., Collins A.,Eriksen G.S. 2012; Mechanisms involved in alternariol-induced cell cycle arrest. *Mutat. Res. Fundam*. Mol., 738–739, 1–11.

11. Shan J., Hou W., He X., An Y., Zhao Q., Li J., 2000; EGFr and Rb gene amplification in fetal esophageal epithelium treated by alternariol. *Henan Yike Daxue Xuebao* 35, 51–53.
12. Lehmann L., Wagner J., Metzler M., 2006; Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food Chem Toxicol* 44, 398–408
13. Marko D., 2007; Mechanisms of the genotoxic effect of Alternaria toxins. *Gesellschaft für Mykotoxin Forschung* (Ed.), Proceedings of the 29th Mycotoxin Workshop. Stuttgart-Fellbach, Germany.
14. Tiessen C., Fehr M., Schawarz C., Baechler S., Domnanich K., Böttler U., Pahlke G., Marko D., 2013; Modulation of the cellular redox status by the Alternaria toxins alternariol and alternariol monomethlyl ether. *Toxicol Lett* 216, 23–30.
15. Pfeiffer E., Eschbach S., Metzler M., 2007; Alternariatoxins: DNA strand-breaking activity in mammalian cells in vitro. *Mycotoxin Res* 23, 152–157
16. Fehr M., Pahlke G., Fritz J., Christensen M.O., Boege F., Altemoller M., Podlech J., Marko D., 2009; Alternariol acts as a topoisomerase poison, preferentially affecting the II alpha isoform. *Molecular Nutrition and Food Research* 53, 441–451
17. Liu G. T., Qian Y. Z., Zhang P., Dong Z. M., Shi Z. Y., Zhen Y. Z., Miao J., Xu Y. M., 1991; Relationships between Alternaria alternata and oesophageal cancer. *IARC Sci Publ*, 258-262.
18. Liu G. T., Qian Y. Z., Zhang P., Dong W. H., Qi Y. M., Guo H. T., 1992; Etiological role of Alternaria alternata in human esophageal cancer. *Chin Med J* 105, 394-400.
19. Russel W.M., Burch R.L., 1959; The principal of human experimental technique, Metheven, London, UK.
20. Schiffelersa M.J.W.A., Blaauboer B.J., Bakker W.E., Beken S., Hendriksen C.F.M., Koëter H.B.W.M., Krul C., 2014; Regulatory acceptance and use of 3R models for pharmaceuticals and chemicals: Expert opinions on the state of affairs and the way forward. *Regulatory Toxicology and Pharmacology* 69, 41–48

21. Gomez-Lechon M.J, Castell J.V., 1993; The use of in vitro methods for the education of the potential risk toxicity of xenobiotics, *Cell Biol. In Environ. Toxicol* 2, 259-277
22. Bouaziz C., Abid-Essefi S., Bouslimi A., El Golli E., Bacha H., 2006; Cytotoxicity and related effects of T-2 toxin on cultured Vero cells. *Toxicon* 48, 343–352
23. RUIZ M.J., FESTILA L.E., FERNANDEZ M., 2006; Comparison of basal cytotoxicity of seven carbamates in CHO-K1 cells. Toxicological & Environmental Chemistry, 88(2): 345–354
24. Gian Luigi Russo, 2007; Ins and outs of dietary phytochemicals in cancer chemoprevention. Biochemical pharmacology 74, 533–544
25. U.S.DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service National Institutes of Health 1992; TOXICOLOGY AND CARCINOGENESIS STUDIES OF QUERCETIN (CASNO.117-39-5) IN F344/NRATS (FEED STUDIES), NATIONAL TOXICOLOGY PROGRAM P.O. Box 12233 ResearchTrianglePark, NC27709
26. Harwood M., Danielewska-Nikiel B., Borzelleca J.F., Flamm G.W., Williams G.M., Lines T.C., 2007; A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*, Volume 45, Issue 11, 2179 – 2205
27. Harborne J.B., Williams C.A., 2000; Advances in flavonoid research since 1992. *Phytochemistry*, 55:481–504.
28. Jan Øivind Moskaug, Harald Carlsen, Mari Myhrstad, Rune Blomhoff, 2004; Molecular imaging of the biological effects of quercetin and quercetin-rich foods. *Mechanisms of Ageing and Development* Vol. 125, Issue 4, 315–324
29. LAKSHMANA RAO A., SANKAR G. G., 2009; CACO-2 CELLS: AN OVERVIEW, *JPRHC*, Vol.1, No.2, 260-275
30. Juan-García A., Juan C., König S., Ruiz M.J., 2015; Cytotoxic effects and degradation products of three mycotoxins: Alternariol, 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol in liver hepatocellular carcinoma cells. *Toxicol Letters* 235, 8–16

31. Juan-García A., Manyes L., Ruiz M.J., Font G., 2013; Applications of flow cytometry to toxicological mycotoxin effects in cultured mammalian cells: A review. *Food and Chemical Toxicology* 56, 40–59
32. Chiesi C., Fernandez-Blanco C., Cossignani L., Font G. and Ruiz M.J., 2015; Alternariol-induced cytotoxicity in Caco-2 cells. Protective effect of the phenolic fraction from virgin olive oil. *Toxicon* 93, 103-111.
33. Lombardi G., Prosperini A., Font G. and Ruiz M.J., 2012; Effect of polyphenols on enniatins-induced cytotoxic effects in mammalian cells. *Toxicol. Mech. Methods* 22, 687–695.
34. Tiemann U., Tomek W., Schneider F., Müller M., Pöhland R. and Vanselow J., 2009; The mycotoxins alternariol and alternariol methyl ether negatively affect progesterone synthesis in porcine granulosa cells in vitro. *Toxicol Lett* 186, 139–145.
35. Prosperini A., Font G. and Ruiz M.J., 2014; Interaction effects of Fusarium enniatins (A, A1, B and B1) combinations on in vitro cytotoxicity of Caco-2. *Toxicol In Vitro* 28, 88–94
36. Vila-Donat P., Fernandez-Blanco C., Sagratini G. and Font G., 2015; Effects of soyasaponin I and soyasaponins-rich extract on the Alternariol-induced cytotoxicity on Caco-2 cells. *Food and Chemical Toxicology* 77, 44-49
37. Da Motta S., Valente Soares L.M., 2000; A method for the determination of two Alternaria toxins, alternariol and alternariol monomethyl ether, in tomato products. *Brazilian Journal of Microbiology* 31, 315–320.
38. Barcelos G.R.M., Grotto D., Angeli J.P.F., Serpeleoni J.M., Rocha B.A., Bastos J.K., Barbosa F.Jr., 2011; Evaluation of antigenotoxic effects of plant flavonoids quercetin and rutin on HepG2 cells. *Pythother Res* 25: 1381-1388
39. Xi J., Zhang B., Luo F., Liu J. and Yang T., 2012; Quercetin protects neuroblastoma SH-SY5Y cells against oxidative stress by inhibiting expression of Krüppel-like factor 4. *Neuroscience Letters*, 527,115–120.
40. Seibert H., Maser E., Schweda K., Seibert S., Gülden M., 2011; Cytoprotective activity against peroxide-induced oxidative damage and cytotoxicity of flavonoids in C6 rat glioma cells. *Food Chem Toxicol* 49: 2398-2407

41. Matsuo M., Sasaki N., Saga K., Kaneko T., 2005; Cytotoxicity of flavonoids toward cultured normal human cells. *Biol Phar m Bull* 28: 253-259
42. Choi K.C., Chung W.T., Kwon J.K., Yu J.Y. Jang Y.S., Park S.M., Lee S.Y, Lee J.C., 2010; Inhibitory effect of quercetin on aflatoxin B1 - induced hepatic damage in mice. *Food Chem Toxicol* 48: 2747-2753
43. Hundhausen C., Bösch-Saadatmandi C., Augustin K., Blank R., Wolffram S., Rimbach G., 2005; Effect of vitamin E and polyphenols on ochratoxin A-induced cytotoxicity in liver (HepG2) cells. *J Plant Physiol* 162: 818-822
44. Sergent T., Garsou S., Schaut A., De Saeger S., Pussemier L., Van Peteghem C., Larondelle Y., Schneider Y.J., 2005. Differential modulation of ochratoxin A absorption across Caco-2 cells by dietary polyphenols used at realistic intestinal concentrations. *Toxicol Lett* 159: 60-70
45. Fermentek Biotechnology http://www.fermentek.co.il/Alternariol.htm (22.04.15 15:47)
46. Fermentek Biotechnology http://www.fermentek.co.il/Alternariol-methyl-ether.htm (22.04.15 15:55)
47. ChemSpider: Search an share chemistry: http://www.chemspider.com/Chemical-Structure.4444112.html (19.05.15 14:06)
48. ChemSpider: Search an share chemistry: http://www.chemspider.com/Chemical-Structure.4444362.html (19.05.15 14:15)
49. ChemSpider: Search an share chemistry: http://www.chemspider.com/Chemical-Structure.4444051.html (19.05.15 14:32)
50. Manuel Repetto Jiménez, Guillermo Repetto Kuhn; *TOXICOLOGÍA FUNDAMENTAL*, Cuarta edición, 2009

Acknowledgment

Maria-José Ruiz Leal, Professor of Toxicology (University of Valencia), for the opportunity to write my master's thesis under her guidance. It wouldn't have been possible without her support.

Celia Fernández-Blanco, PhD student (University of Valencia), for her time, help and patience in the laboratory.

Jordi Maňes Vinuesa, Professor of Food Science and Erasmus Coordinator (University of Valencia), for the chance to do my internship in his department.

My very special thanks go to Karl-Heinz Wagner, Vice Dean of the Faculty of Life Sciences & Deputy Head of the Department (University of Vienna), for the opportunity to do an exchange semester and to make this formative experience. Without his help as my supervisor in Vienna, it certainly wouldn't have been possible to realize all of this.

My very special thanks also to my family and friends who have always supported and believed in me during my education.

Declaration of interest

I hereby declare, that I have written this master thesis independently, did not use any other than the specified sources and aides and that I declared all mutatis mutandis acquired passages I have used.

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