PhD Thesis

Cytotoxicity, cytoprotection and antioxidant capacity studies in biological samples

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List of abbreviations

7-AAD	7-aminoactinomycin D
ATCC	American Type Culture Collection
ATP	adenosine-5′-triphosphate
AUC	area under the curve
BCD	β-cyclodextrin
CAM	calcein-acetoxy-methylester
CBB	Comassie Brilliant Blue
CD	cyclodextrin
DAPI	4′,6-diamidino-2-phenylindole
DIMEB	2,6-di-O-methyl-β-cyclodextrin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	desoxyribonucleic acid
DPPH*	1,1-diphenyl-2picrylhydrazyl radical
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FDB	fluorescein-dibutyrate
FIS	fisetin
FITC	fluorescein-isothiocyanate
FL	Na-fluorescein
GER	geraldol
H_2O_2	hydrogen-peroxide
HepG2	human liver cancer-derived cells (cell culture)
HPBCD	(2-Hydroxypropyl)-beta-cyclodextrin
hs-CRP	high sensitivity-C-reactive protein
LOD	limit of detection
MDCK	Madine, Darby canine kidney (cell culture)
NaF	sodium-fluoride
NaN_3	sodium-azide
ORAC	Oxygen Radical Absorbance Capacity
OTA	ochratoxin A
PBS	phosphate-buffered saline
POD	proxidase (Horseradish roots)
SD	standard deviation
SEM	standard error of mean
TAC	total antioxidant capacity
TE	Trolox equivalent
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic-acid
ZEA	zearalenone

1. Introduction

It has been known for a long time, that the optimal amount of ingested carotinoids, flavonoids, other polyphenols and many other compounds primarily with antioxidant activity is a major factor in the prevention of chronic degenerative diseases (malignant tumors, autoimmune disorders, cardiovacular diseases, etc.). In spite of the large number of observations from epidemiologic studies, animal experiments and *in vitro* experimental models the mechanism of the protective effects of plant compounds is still not fully understood. It can be hypothesized and in fact, partially verified that the antioxidant characteristics is one of the most important factors in cytoprotection. The luminescence cell viability assays are widespread in studying of the cytoprotective and toxic effects of natural and synthetic molecules as well. Using *in vitro* cellular models, treatment of the tissue cultures with agents causing cell injury and monitoring of the toxic effects by viability tests are frequently used procedures. The measurement of viability – despite the availability of the large number of commercially available ready to use reagent kits – is in reality still proved to be a very difficult and complex procedure because all of the above mentioned mechanisms can play a role in the decrease of cell viability. One of the most frequently used viability tests is the bioluminescence detection of intracellular ATP. Luminescence cell viability assays can be used in both adherent and in suspension cell cultures. In spite of the fact that luminescence cell viability studies are widely used methods, one should be careful in correct interpretation of the obtained results. Quite often, interpretation of the viability data is difficult because the different cell lines do not behave the same way and also the mode of action of the underlying mechanisms are hard to understand.

For example, intracellular ATP is considered to be very constant in living cells and the amount of ATP in the sample is regarded as a measure of the number of live cells. However, the turnover of ATP is very fast and different treatments can affect ATP concentrations rapidly (increase, decrease). Therefore, ATP data alone do not always correlate with the number of living cells and especially with the intracellular energy supply reflecting viability. Hydrophobic fluorophores (mostly in ester form) are non-fluorescent probes that easily enter both live and dead cells. Calcein-acetoxy methylester (CAM) is proposed as a simple viability probe relying on the idea that only live cells can cleave the ester bond. Hydrolysis results highly fluorescent and charged products that stay inside the cells. In enzyme activity-based viability methods including esterase, oxido-reductase and protease dependent processes, it is important to know if the enzymatic reaction is really an energy-driven reaction, and truly reflects viability of the cells. Another difficulty might arise from spontaneous release of fluorescent products from the

cells or release due to specific transport mechanisms such as ATP-driven ABC transport proteins. In order to overcome these difficulties, one should obtain as many information as possible from the same sample. A reference parameter should always be included (total intracellular protein or DNA content), in addition to the quantification of fluorescence and ATP data for correct interpretation of the results.

My dissertation includes two main areas. The first part presents the cytotoxicity, cytoprotection assays in *in vitro* cellular models using adherent tissue cultures treated with metabolic inhibitors (NaF, NaN_3) and ochratoxin A (OTA) mycotoxin. Dose-response relationship was investigated with a multiparametric viability assay developed by us (intracellular ATP, esterase activity derived from fluorescence, total protein content and amount of DNA). To do this, I have developed a novel perchloric acid extraction procedure that has not been published in the literature before. Our method was capable for determining all of the above mentioned parameters from the same sample.

Furthermore, we wanted to test, whether CDs are able to affect the known antiproliferative effects of FIS and GER on HepG2 liver cancer cell line. The potential effect of the CDs on the uptake of the toxin was tested in *in vitro* HepG2 liver cell cultures.

In the other part of my dissertation I show our antioxidant capacity assays, which play a significant role in cytoprotection in several biological samples. The determination of the total, non-enzymatic antioxidant capacity (TAC) was performed with the modified enhanced chemiluminescence assay (ECL) developed by us, as well as with the well known ORAC (Oxygen Radical Absorbance Capacity) and DPPH (1,1-diphenyl-2-picrylhydrazyl) stable free radical assays known from the literature. The total antioxidant capacity tests were done in alcoholic extracts of so far less known and studied medicinal plant samples, and also in serum samples of septic patient groups.

2. Aims

2.1. Studies of cytotoxicity and cytoprotection

2.1.1. Multiparametric luminescent cell viability test in toxicological models

Based on literature data we hypothesized that a single parameter (for example ATP and/or calcein fluorescence) does not give precise information on the viability of the tissue cultures treated with metabolic poisons or mycotoxins. During our cellular experiments we tried to get answers to the questions listed below:

- Is the detected signal/parameter proportional with the number of living cells?
- Are there any parameters that reflect not only the number of cells in the sample but also their viability?
- Is it possible to separate living (untreated), injured (treated) and already dead cells?
- Are there such parameters, which reliably reflect true viability?
- Is the generally accepted calcein ester staining an ATP-dependent process and is it suitable for reflecting the viability of the cells?

2.1.2. Entrapment of flavonoids (fisetin and geraldol) with β-cyclodextrins

Fisetin is a commonly occuring flavonoid (flavonol) aglycone in the nature. One of the most important metabolites of FIS is its 3-O-methylated derivative called geraldol, which is formed during the methylation of FIS by catechol-O-methyl transferase. CDs are widely studied host molecules, they have a conical structure with a hydrophobic interior and a hydrophilic exterior space.

In this study, our aim was to test how CDs can influence the antiproliferative effects of FIS and GER on HepG2 liver cancer cell line.

2.1.3. Interactions of zearalenone with native and chemically modified cyclodextrins

Zearalenone is a widespread xenoestrogenic mycotoxin produced by several *Fusarium* species. Previous studies highlighted that β-CDs are able to form stable complexes with different mycotoxins such as citrinin, aflatoxin B1, ochratoxin A and zearalenone. Our aim was to test the potential impact of CDs on the uptake of ZEA using *in vitro* cellular experiments and HepG2 liver cell line.

2.2. Antioxidant capacity assays in biological samples

2.2.1. Development and validation of the modified chemiluminescence assay

For the measurement of the non-enzymatic total antioxidant capacity several different methods exist in the literature. It was an important aim of our work to significantly modify and to validate

an enhanced chemiluminescence-based TAC method. Our further objective was to adapt our ECL technique to plate reader luminometer using various biological samples, including plant extracts and human serum samples. We wanted to demonstrate the potential usage of serum TAC analyses in severe systemic inflammation by comparing the results obtained for control and septic patient groups.

2.2.2. TAC assay of the seed and pericarp of three *Coffea* species

Another aim of our TAC analyzes was to investigate the antioxidant capacity of mature/immature seeds as well as mature/immature pericarps of two *Coffea* species, *Coffea benghalensis* and *Coffea liberica* and to compare the data with the already thoroughly studied *Coffea arabica*. A further aim was to find new sources of natural antioxidants as nutraceuticals, and a new utilization of wasted residues of coffee products. We used three different methods for the determination of the antioxidant capacity of the plant extracts: enhanced chemiluminescence (ECL), classical DPPH* method and ORAC assay.

3. Methods

3.1. Cytotoxicity, cytoprotection

We worked out a multiparametric vability assay to test the effect of various treatments. First, we monitored the entry of FDB (fluorescein - dibutyrate) and CAM (calcein-acetoxy-methylester) fluorophores into Jurkat cells (human peripherial T-lymphoblast cell line) by combined measurement of the fluorescence intensity and the fluorescence polarization. These measurements were performed in a Hitachi F4500 fluorescence spectrophotometer (37 ºC, 490 nm excitation and 520 nm emission wavelengths, respectively). The effects of OTA (5-50 μM), NaF (1-20 mM) and NaN_3 (1-10 mM) were tested in monolayer cell cultures. MDCK (Madine, Darby canine kidney; ATCC: CCL 34; immortalized tubular cells) and HepG2 (human liver cancer-derived cells; ATCC: HB-8065™, *homo sapiens*, epithelial) cell cultures were used. The adherent cells were cultured in 96-well plates and four different parameters were determined from the same sample (ATP, calcein fluorescence, nucleic-acid content and total protein). The cells were fixed/extracted with perchloric acid. In every case, the ATP content was measured by a bioluminescence method, while calcein fluorescence of the samples was determined after alkalizing the perchloric acid cell extracts in a Perkin Elmer EnSpire Multimode plate reader (λexc.=490 nm; λem.=520 nm). The nucleic acid content of the fixed cells (DAPI staining, λexc.=355 nm; λem.=460 nm) was determined by the Perkin Elmer EnSpire Multimode plate reader, in area scan mode. For intracellular protein determination, perchloric acid fixed cells were solubilized with 1 M sodium hydroxide. The determination of the intracellular total protein

content was done with the Bradford reaction on a BioTek Synergy HT plate reader. ATP/protein, ATP/nucleic acid, calcein fluorescence/ATP and DAPI/total protein ratios were also determined. The results were compared to the values obtained for control, untreated cells and were expressed as percentage of the control. The apoptosis/necrosis rate caused by the treating agents was measured with flow cytometric investigations (Beckman-Coulter FC500 type flow cytometer) with FITC-Annexin V/propidium iodide and 7-amino-actinomycin D staining. The data analysis was done using CXP software. For morphological studies, in addition to protein staining the actin cytoskeleton was also examined with fluorescently labeled phalloidin and fluorescence microscopy.

The investigation of the complex formations of FIS and GER flavonoids with β-cyclodextrins, as well as the interactions of ZEA with native and chemically modified CDs was done in adherent HepG2 cell cultures. The adherent, monolayer cell line was maintained in DMEM high glucose (4.5 g/l) medium with 10 % FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml), by standard protocol. Trypsinized cells (1 ml trypsin-EDTA/25 cm^2 flask) were pipetted into 30 ml medium and were cultured in 96-well (250 µl/well, 10^4 cells), 24-well (500 µl/well, $2x10^4$ cells) or 6-well (2 ml/well, $8x10^4$ cells) sterile plastic plates for 24 h. Then the medium was replaced with fresh medium or buffer, which contained the sufficient amount of toxic materials for the examination. The cells were washed (PBS) repeatedly after the treatments, then the samples were fixed in perchloric acid as in the multiparametric viability tests. During the multiparametric luminescence cell viability assays all treatments were done as 5 independent experiments (plates) with a technical repetition number of 16 for each concentration in each plate.

One-Way ANOVA analysis was performed when data for one cell line and for one type of treatment at a certain concentration were compared with those of the control (~100%). Student's t-test was applied when two cell lines' results with the same treatments were compared and also for the comparison of calcein measurements after PCA and detergent extraction. Spearman's rank correlation was done for comparison of data sets for DAPI and protein values within one cell line and one type of treatment. IBM SPSS Statistics, Version 20 program was used. The level of significance was set at $p < 0.05$.

For FIS and GER treated HepG2 cells, the obtained results were derived from three independent experiments. Statistical analyses were performed employing One-Way ANOVA test (IBM SPSS Statistics, Version 21) where the level of significance was set at $p < 0.05$.

Data reported in the presence or absence of CDs of ZEA-treated HepG2 cells (mean±SEM) were derived from at least three independent experiments. Statistical analyses were performed

by One-Way ANOVA test (IBM SPSS Statistics, Version 21). The level of significance was set at $p< 0.05$.

3.2. Antioxidant capacity assays

A Berthold Lumat LB9507 tube luminometer was used to optimize our antioxidant measurements based on enhanced chemiluminescence (ECL) and to follow the time course of the signal. The BioTek Synergy HT plate reader equipped with programmable injectors was used for the serial measurements. The Berthold LB 9507 tube luminometer with injectors was used in the rate meter mode. Our reagent contained albumin-stabilized peroxidase enzyme (POD) and hydrogen peroxide, the chemiluminescence signal came from luminol and 4-iodophenol in the presence of POD/H_2O_2. The measurements were standardized by Trolox (water-soluble E vitamin derivative). In case of the tube luminometer, ice-cold POD-ECL reagent was injected into the tubes already containing the blank/standard/sample/H_2O_2 solutions. The measuring interval was 2 s with a total measuring time of 400 s. For the Biotek Synergy HT plate reader measurements white, 96-well microplates were used. ECL-POD working solutions were premixed immediately before the measurements as described above and were kept at 0 °C protected from light. 20 µl blank/standard/sample was pipetted into the microplate wells in triplicates or more parallels. Then 270 µl of premixed POD-ECL solution was pipetted into the microplate using an 8-channel pipette and the plate was shaken for 10 s in a horizontal shaker. The ECL reaction was triggered by automatic injection of 20 µl of the diluted H_2O_2 solution using the programmable injector of the Biotek Synergy HT plate reader. Luminescence of the samples was monitored for 10 min at 64 s measuring intervals and 0.2 s detection time/well. The antioxidant capacity was calculated based on the area under curve (AUC) of the light signal or on the time delay of the signal. We calibrated our ECL method by the use of Trolox standard series (0-100 µM). Our ECL method was validated, whereby limit of detection (LOD), imprecision, linearity and recovery were defined.

For the DPPH assays the Perkin Elmer EnSpire Multimode reader equipped with monochromators was used in absorbance mode. For standardization of the assay Trolox series were applied (0-266,4 µM). Absorbance values were read at 517 nm after 30 min of incubation at 25 °C in the dark. Antioxidant capacities were calculated either by using the equation of the calibration line or by expressing the antioxidant activity of the extracts in % of the blank using the formula: $(A_{blank} - A_{sample})/A_{blank}$ x 100. TAC values were also referred to 1 g of dried plant and were given as Trolox equivalent (TE, µmol/g) or % TAC/g.

For the ORAC assay the Biotek Synergy HT plate reader was used in fluorescence mode at 37 °C with 490 nm excitation and 520 nm emission filter settings. The measurement was done

based on a generally accepted protocol, with slight modification. AAPH was used as an oxidant, Na-fluorescein (FL) was used as the riporter molecule and Trolox series (0-266,4 μM) were used as standards. Readings/wells were taken at every 150 s for 80 min. TE was calculated by subtraction of the fluorescence intensities of the corresponding blank (area under curve, AUC) values from those of the Trolox standards (net AUC) and in this way a calibration line was obtained based on net AUC vs Trolox concentrations. TE data for the examined plants were obtained from the regression equation of the standards and were also referred to 1 g of dry plant. The plant extracts used in our studies were as follows: licorice (*Glycyrrhiza glabra*), common yarrow (*Achillea millefolium*), common wolf straw (*Aristolochia clematitis*), ordinary shit (*Calluna vulgaris*), reddish honeycomb (*Helleborus purpurascens*), European sea buckthorn (*Hippophäe rhamnoides*), knotweed (*Polygonum lapathifolium*), apricot bean grass (*Polygonum maculosa*), silverweed cinquefoil (*Potentilla anserina*), sage sage (*Salvia glutinosa*) and sage sage (*Salvia nemorosa*). The aerial parts of the plants were collected in Romania and in Hungary (between 2015 and 2016) at their natural habitat. These plants were selected based on comparison of ethnomedicinal data collected in Romania and on those obtained from scientific databases. There were only a few records on the antioxidant capacity of the listed plants. The collected plants were dried at room temperature and were ground. For each plant 0.5 g of dried drug was extracted with 10.0 ml of 50 % ethanol by continuous shaking for 30 min on a horizontal shaker (200 rpm). The extracts were filtered and stored at −20 °C until analysis.

The mature and immmature fruits of *C. benghalensis, C. liberica*, and *C. arabica* were collected in the Botanical Garden of the University of Pécs, Hungary in the spring of 2014. For all three tested plants the immature and mature pericarps as well as the immature and mature seeds were used for our antioxidant assays. Samples were ground (0.25 g each) and extracted with 5 ml 50% ethanol. The extracts were shaken for 20 min, then after filtering were stored at -20 °C until analyses.

Regarding the use of human serum samples, our study protocol was authorized by the Regional Research Ethical Committee of the University of Pécs (4327.316-2900/KK15/2011) and it was performed according to the ethical guidelines of the 2003 Helsinki Declaration. Written informed consent was obtained from all enrolled patients or their appropriate surrogates after detailed information regarding the study design and blood sampling. The control group comprised ophthalmologic patients from the Department of Ophthalmology, University of Pécs, Hungary without acute inflammatory or infectious disease. The patient group included individuals with established diagnosis of sepsis or severe sepsis. The septic patients were treated

at our multidisciplinary university Intensive Care Unit (ICU). In case of septic patients, a follow-up study was performed during their ICU stay where serum samples were obtained at day 1, 2, 3 and 5 after establishing the clinical diagnosis. In the control group, only one blood sample was taken. Septic patients were further subdivided into survivor and non-survivor groups based on 7-day mortality at the ICU. Venous blood was obtained from every patient into plain tubes with accelerator gel using a closed blood sampling system (BD Vacutainer®). After 45 min, clotted blood samples were centrifuged at 1500 g for 10 min and sera were stored in Eppendorf tubes at −80 °C until further analyses.

For statistical testing IBM SPSS Statistics for Windows, Version 22 program was used during our experiments on different biological matrices. Distribution of data was evaluated by Shapiro-Wilk test. Since our data showed normal distribution, independent samples t-tests were performed for investigating differences between patient groups. One-way ANOVA test was used for performing follow-up comparisons. Possible correlations between quantitative parameters were determined by Pearson's rank correlation test. When comparing the two antioxidant capacity methods, Bland Altman and Passing Bablok analyses were used. Data were expressed as mean ± SEM, in the Bland and Altman analysis SD was applied. $p < 0.05$ was considered as being statistically significant.

4. Results and discussion

4.1. Viability assays in *in vitro* tissue cultures (cytotoxicity, cytoprotection)

4.1.1. Interpretation of the intracellular fluorescence data

The fluorescent signal derived from FDB showed good correlation with Jurkat cell number (R^2 = 0,959, FDB: 1,6 μM, $1x10^4$ – $1x10^6$ cells/ml, n=42). Due to the low background fluorescence of non-hydrolyzed dyes (FDB, CAM), in the case of cell suspensions, there is no need to lyse the cells. The distribution (inside/outside) of produced fluorescein and calcein molecules was different in Jurkat cells. Free Na-fluorescein (control sample) showed uniformly low fluorescence polarization (P) values while calcein polarization increased with time with a tendency to go to saturation. Fluorescein derived from FDB gave only a temporary increase in P with a consecutive decrease with time. The finding indicates that the FDB enters the cells but within a short time is released as well. These data suggest that the strongly charged calcein leaks out from the cells at a negligibly small extent while FDB-derived fluorescein with less negative charges readily leaves the cells. The optimal incubation time of CAM was 40 minutes.

4.1.2. Perchloric acid fixation/extraction method for ATP, calcein, DAPI fluorescence and total protein measurement

One of the major goals of our work was to apply our novel extraction method for obtaining as many as possible viability parameters for the same cell sample. ATP and calcein were simultaneously extracted from the washed cells with PCA without loss of calcein fluorescence. Intensities of alkalinized extracts were compared with those obtained after 0.1%Triton X 100–PBS lysis of the cells. Student's t- test did not show significant difference between the two extractions ($p = 0.374$). The PCA fixed cells could be easily stained with DAPI and the total fluorescence/well showed close correlation with total protein data (Fig. 1). In case of MDCK-NaN_3 and HepG2-NaF treatments we observed a significantly reduced protein content vs. cell number (DAPI fluorescence) compared with the other samples. Although the correlation estimated by Spearman's rank correlation test between intracellular protein and cell number was quite good (R^2 between 0.8–0.99), the number of cells measured by DAPI fluorescence was a more stable parameter than protein levels.

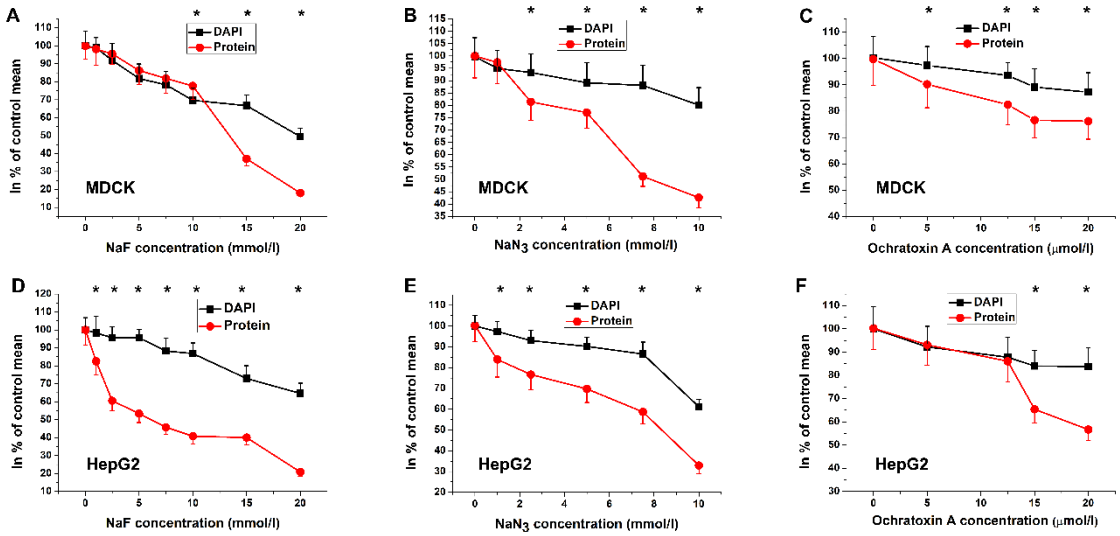

Figure 1: Dose dependent effects of NaF (A, D), NaN_3 (B, E) and OTA (C, F) on cell number measured as DAPI fluorescence and on total intracellular protein in MDCK and HepG2 cultures. DAPI nuclear staining data vs. total intracellular protein contents are expressed in % of the control cells (mean ± SD, 5 independent experiments, n = 5x16 replicates for each concentration). *: significant change between DAPI vs. total protein data (Spearman's rank correlation analysis, $p<0.05$).

4.1.3. Interpretation of viability parameters obtained after ATP depletion

4.1.3.1. NaF and NaN_3 treatments

4-h exposure with both agents dramatically decreased intracellular ATP and protein levels in both cell lines in a dose-dependent manner. The calcein fluorescence did not closely correlate with ATP and protein data. At the highest toxin concentration both samples lost practically all of their intracellular ATP contents however, the calcein fluorescence remained at 40-80% of the control value (100 %). The two cell lines did not respond uniformly to the treatments. We found an inverse relationship in ATP/protein vs. fluorescence/ATP ratios in both cell lines but HepG2 cells were more sensitive to NaF exposure than MDCK cells (Fig. 2. A,D). In MDCK cells calcein fluorescence/ATP values increased more prominently than in HepG2 cells after NaN_3 treatment (Fig. 2. B,E).

4.1.3.2. OTA treatment

Data obtained for kidney-derived (MDCK) and liver cancer cell cultures (HepG2) are seen in Fig. 2. C,F. OTA reduced the ATP content of both cell lines in a dose-dependent manner although in MDCK cells, ATP depletion was more pronounced than in HepG2 cultures. On the other hand, cells of liver origin lost more intracellular proteins compared with kidney cells. We observed a dose-dependent, but mild decrease in calcein fluorescence/protein levels in MDCK cells. As a result of different behavior between the two cell lines, ATP/protein decreased and calcein fluorescence/ATP increased in MDCK cells. In contrast, due to the higher protein loss

in HepG2 cultures at 15-20 μM OTA exposure, ATP/protein values slightly elevated and calcein fluorescence/ATP data remained constant (Fig. 2. C,F). When summarizing our data obtained for fluorescence/total intracellular protein levels the most striking difference was seen between MDCK and HepG2 cells with NaF and OTA exposure. Liver cells produced higher fluorescence/unit protein than kidney-derived cells.

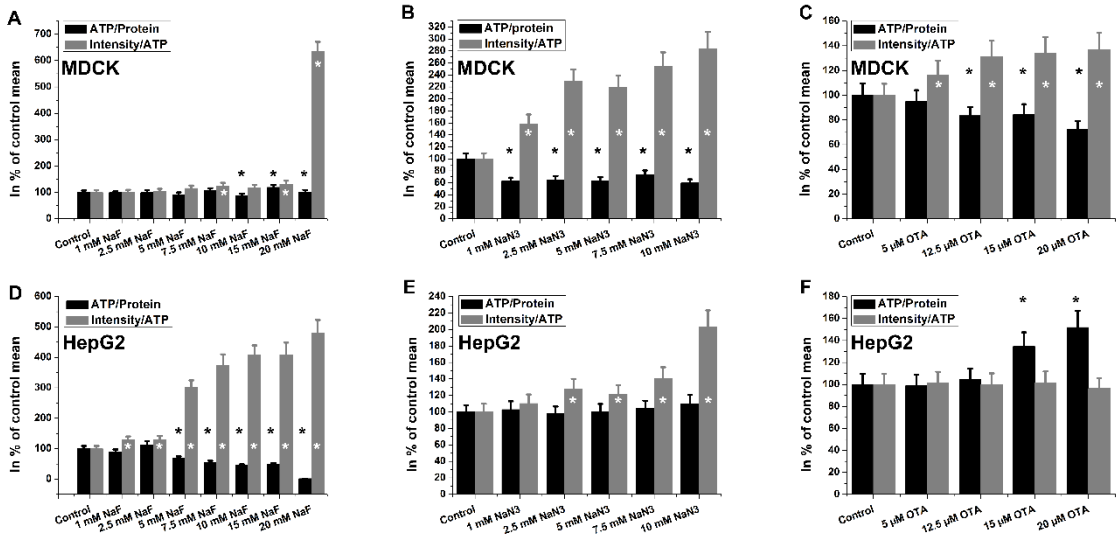

Figure 2: Effects of NaF (A, D), NaN_3 (B, E) and OTA (C, F) treatments in MDCK and HepG2 cell lines. Data are expressed as ATP/protein and calcein fluorescence/ATP in % of the control cells (mean ± SD, 5 independent experiments, n = 5x16 replicates for each concentration). *: significant change vs. control (one-way ANOVA test, $p < 0.05$).

4.1.3.3. Apoptosis assay

The apoptotic/necrotic cell ratios after NaF and OTA treatments were determined by flow cytometry using two different assays [FITC-Annexin-V/propidium iodide and 7-AAD (7-aminoactinomycin D) method]. The two types of assays did not give identical results, especially in case of the percentage of live, intact cells. 20 mM NaF exposure resulted in a dramatic increase of early apoptotic cells which could not be resolved by the 7-ADD method. Interestingly, in case of OTA treatment the 7-AAD assay indicated a much larger apoptosis rate than the FITC-Annexin V/PI measurement. The percentage of dead (necrotic) cells was similar in both assays. For the 7-ADD method the unstained small particles were considered to be cell debris. Cell debris was gated in the FITC-Annexin/PI measurement where two different-colored fluorescence intensities were analyzed.

4.1.3.4. Morphological studies

The CBB (Comassie Brilliant Blue R250) stained control cells expressed normal morphology and the fine protein and nucleolar structures could also be visualized. 5 μM OTA did not cause major changes however, in many cells of both cell lines the staining intensity decreased while the number of condensed cells increased. 12.5 μM OTA caused a major decrease in cell number with both swollen and condensed cells together. Staining intensity was reduced, indicating intracellular protein loss (or decreased synthesis) in the cells. A similar picture was obtained at 20 μM OTA but a further decrease in staining intensity could be detected. In this way, with increasing concentration of OTA, the cell number decreased in both cell lines but MDCK cells expressed a more pronounced disorganization of the intracellular structures. The actin cytoskeleton showed normal morphology in untreated cells. The condensed cells appeared in both cell lines with increasing OTA concentration, but disorganization of the actin filaments was more pronounced in MDCK cells. In the MDCK culture the condensed cells showed small actin spikes protruding from the cells (hedgehog-like formation), while HepG2 cells were less affected.

4.1.3.5. Interpretation of ATP/DAPI data

ATP values and DNA contents (DAPI fluorescence) were expressed as % of the control. The two cell lines showed marked differences when ATP was referred to nucleic acid content. The extent of ATP depletion was the highest in NaF and NaN_3 treated cells when referred to DNA contents. We also observed, that HepG2 cultures were more sensitive to NaF and less sensitive to NaN_3 exposure than MDCK cells (Fig. 3. A,B, NaF and NaN_3). OTA caused a proportional decrease in both parameters for HepG2 cells while in MDCK cultures ATP depletion exceeded cell number loss (Fig. 3. A,B, OTA).

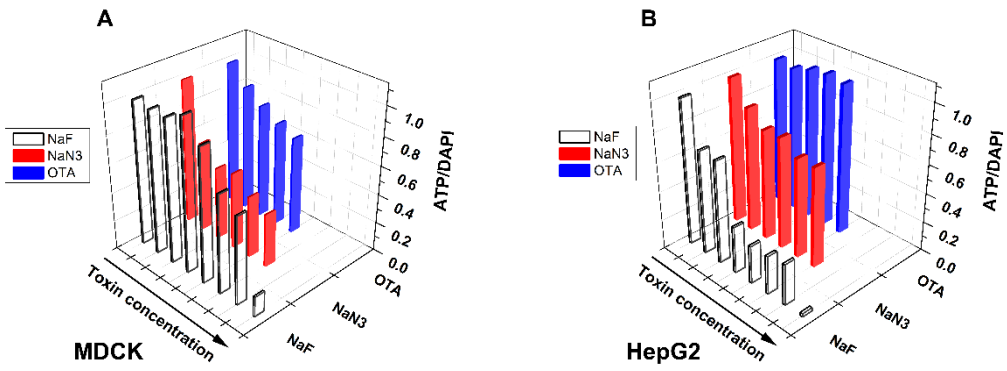

Figure 3: ATP values for NaF, NaN_3 and OTA treated MDCK and HepG2 cell lines referred to nucleic acid content (DAPI)

4.2. Complex formation of fisetin and geraldol flavonoids with β-cyclodextrin

4.2.1. Significance of CDs in regard of cellular actions of fisetin and geraldol

Since binding constants of flavonoid-CD complexes showed significant differences, we were curious how the stabilities of FIS-CD and GER-CD complexes affect the cellular action of these flavonoids. Therefore, antiproliferative effects of FIS and GER were investigated in the presence of CDs, applying HepG2 liver tumor cell line. The influence of BCD, HPBCD, and DIMEB was also examined on the cellular effects of FIS and GER. First, the influence of FIS and GER (0–100 μM) was tested on HepG2 cell line. After 24-h incubation, cell viability loss was evaluated based on ATP and total protein levels. Under the applied circumstances, very high IC50 values of flavonoids were determined (GER ~ 80 μM, FIS > 100 μM). Because we tried to induce significant cell viability loss but did not want to use extremely high flavonoid concentrations, during the following experiments HepG2 cells were treated with 25 and 50 μM flavonoid concentrations in the absence and presence of 1 mM CD. After 24-h incubation, ATP and total protein levels of HepG2 cells were quantified. These mechanisms resulted in the decrease of cellular ATP production. Recent investigations highlighted that CDs are able to interact with D-luciferin and interfere with bioluminescence imaging. Therefore, total protein levels of HepG2 cells were also evaluated. As Fig. 4. demonstrates, changes of ATP and total protein levels showed good correlation. CD and HPBCD alone significantly but relatively slightly decreased ATP and total protein levels while DIMEB caused approximately 30% decrease of both cellular parameters, in agreement with our previous observation (Fig. 4. A). Both FIS and GER resulted in the significant decrease of ATP and total protein levels ($p < 0.01$). During the co-treatment of HepG2 cells with FIS and CDs, only minor changes of ATP and protein concentrations were observed (Fig. 4. B,C). Only the co-treatment of cells with 25 μM FIS and 1 mM DIMEB resulted in the significant decrease of both ATP and protein levels; however, DIMEB alone led to the significant decrease of these parameters as well. Therefore, it is reasonable to hypothesize that this effect was due to the additive impacts of FIS and DIMEB and not to the formation of FIS-DIMEB complex. Furthermore, simultaneous presence of BCD and HPBCD during the treatment with GER did not cause significant effects compared with the effect of GER alone (Fig. 4. D,E).

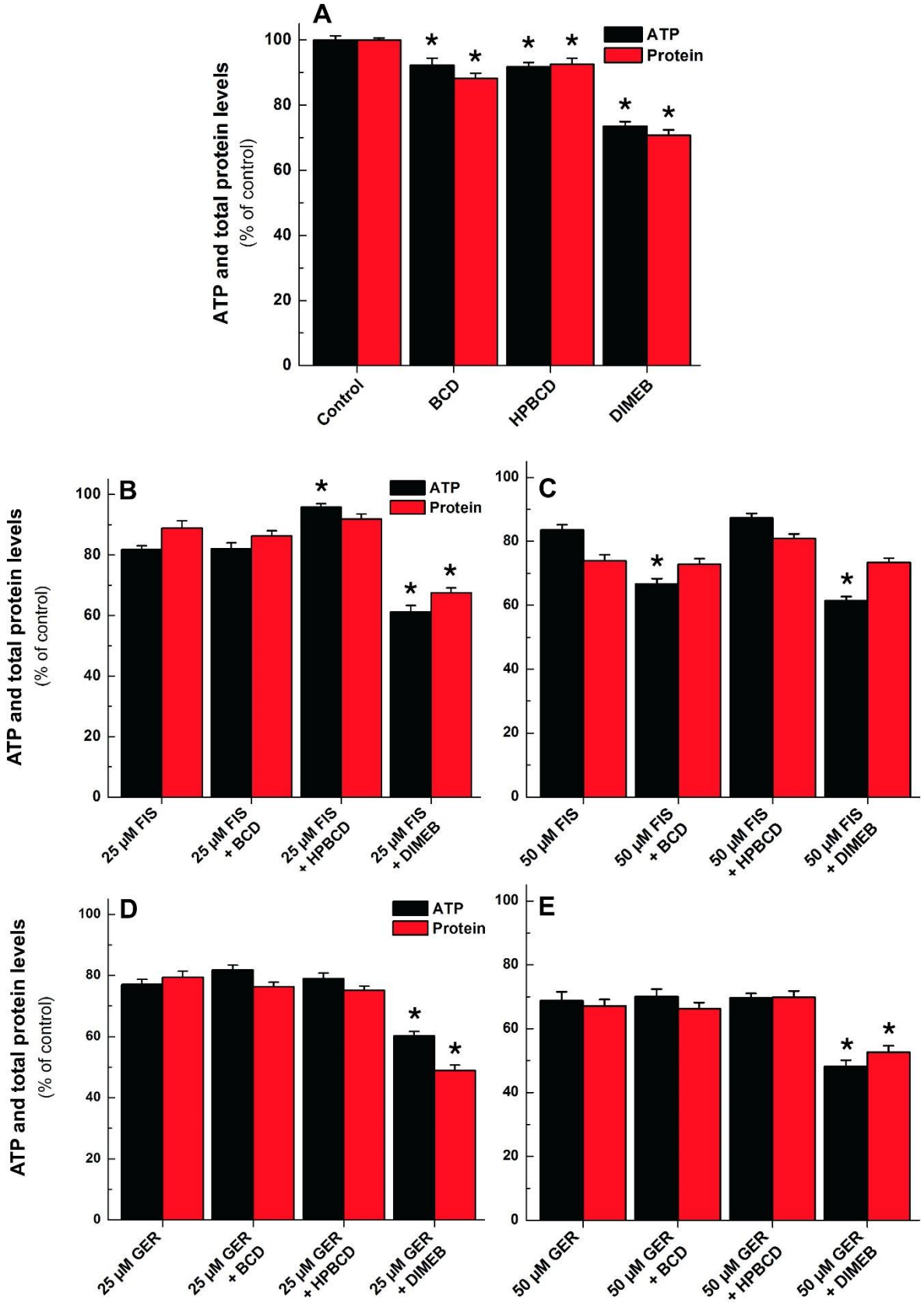

Figure 4: Intracellular ATP and total protein levels of HepG2 cells after 24-h treatment with cyclodextrins (1 mM; A), 25 and 50 µM fisetin + cyclodextrins (B and C, respectively), and 25 and 50 µM geraldol + cyclodextrins (D and E, respectively) (*$p<0.05$).

4.3. Interactions of zearalenone with native and chemically modified cyclodextrins

4.3.1. Impacts of BCD and DIMEB on the toxic effect of zearalenone *in vitro*

First, the dose-dependent effect of ZEA on HepG2 cells was investigated. Concentration dependent decrease of living HepG2 cells was observed after 24-h treatment with 20–100 μM ZEA (Fig. 5). Total DNA and protein values were in a very good correlation, indicating that the applied methods truly describe the number of living cells in the wells of the plates. Since 40 μM ZEA led to approximately 40–50% decrease of living cells in our preliminary studies, this concentration was selected in order to test the potential protective effects of CDs against ZEA-induced toxicity during the following experiments. Cells were treated with ZEA (40 μM) and BCD (1.0 or 2.5 mM) alone as well as in combination. As Fig. 6. demonstrates, the applied BCD concentrations alone did not have neither positive nor negative effect on HepG2 cells. The treatment with ZEA resulted in remarkable reduction of cell number, similarly to our previous observations. However, the co-treatment of ZEA-exposed cells with BCD significantly alleviated the toxic impact of ZEA, in a concentration-dependent fashion. Approximately 18% and 25% increase of the number of living cells was observed in the presence of 1.0 and 2.0 mM BCD, respectively (compared to that of control). Similar experiments were also performed with DIMEB, which forms more stable complexes with ZEA compared to the native BCD. Interestingly, even 1.0 mM DIMEB caused significant decrease of living cells (approximately 20–30%) therefore it did not have positive effect neither in its combination with ZEA (data not shown). Furthermore, 0.5 mM DIMEB did not have harmful impact on HepG2 cells under the applied circumstances; however, no significant positive or negative changes on ZEA-exposed cells were observed in the presence of 0.5 mM DIMEB (data not shown).

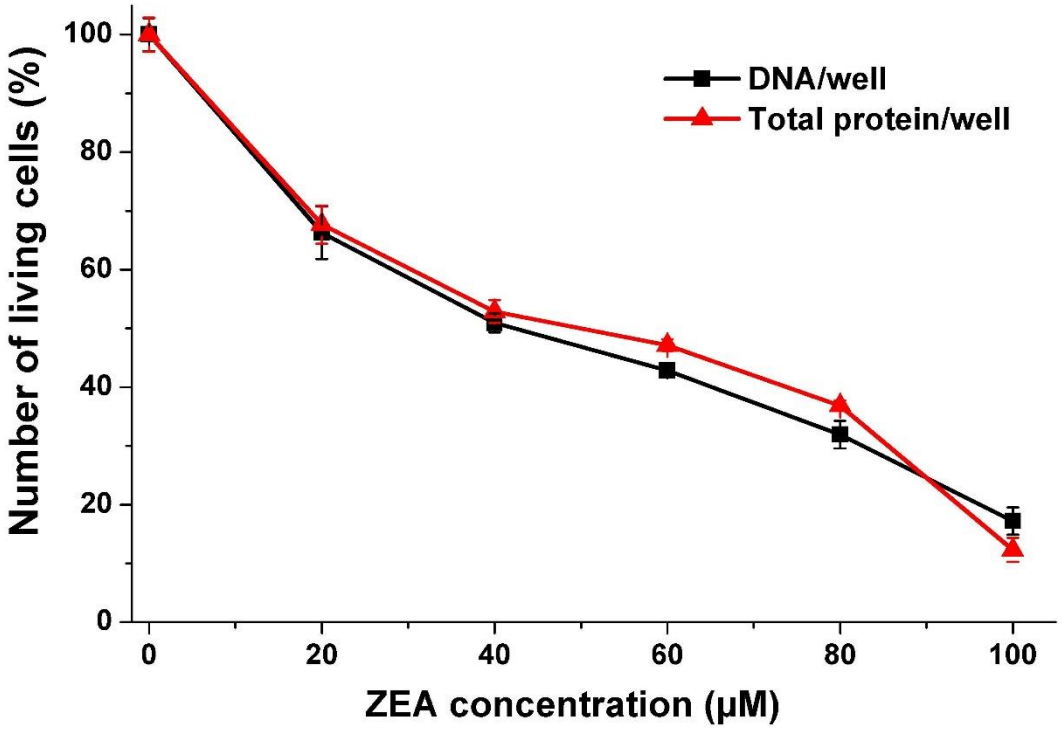

Figure 5: Decrease of the total DNA and protein level of HepG2 cells /well (based on DAPI staining and Bradford reaction) at 24 h after 0-100 μM ZEA treatment.

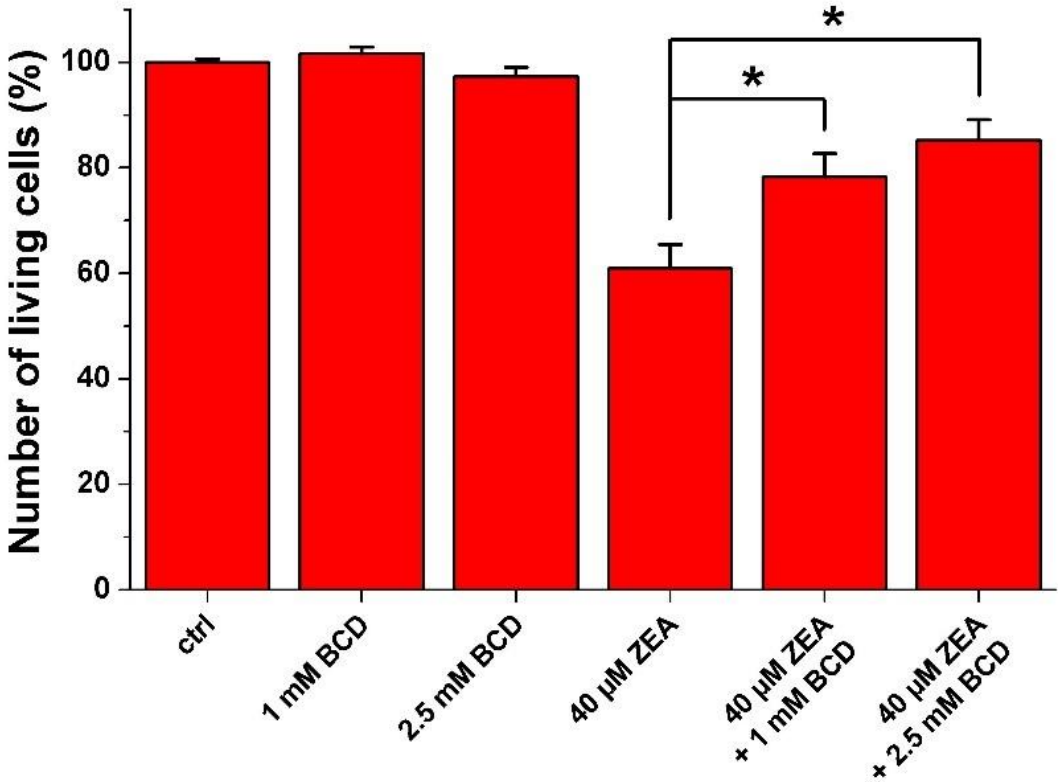

Figure 6: Number of living HepG2 cells in the absence and in the presence of ZEA (40 μM) and/or BCD (1.0 and 2.5 mM) after 24-h treatment based on total DNA content/well. Total protein measurements were in good agreement with the results suggested by DAPI staining. The applied concentrations of BCD significantly alleviated the viability loss induced by ZEA (*$p < 0.05$)

4.4. Antioxidant capacity tests (DPPH, ECL and ORAC)

4.4.1. Development and validation of enhanced chemiluminescence assay

4.4.1.1. Optimization of the working reagent

First, the optimal composition of the POD-driven chemiluminescence reaction had to be established. When varying the H_2O_2 levels and the POD activities the light output was followed for 700 s at 2 s resolution in a photon counting fluorimeter. Our results showed that high enzyme activities rapidly exhausted the substrate while 15 μU/ml POD activity gave an almost constant light emission with an intensity ideal for the measurements in both the tube luminometer and in the plate reader. When POD activity was kept constant (15 μU/ml) and H_2O_2 was varied, all hydrogen peroxide concentrations gave constant light signal but due to the upper detection limit of the tube luminometer (approximately 1,450,000 RLU/s) the 1.5 mmol/l H_2O_2 was chosen. In all subsequent measurements 15 μU/ml POD and 1.5 mmol/l H_2O_2 were used when preparing the working reagent. We also calculated the total light output/min and compared it with the substrate concentration. The H_2O_2 level was less than the Michaelis constant for horseradish peroxidase and a linear fit was found between reaction speed and substrate concentration which meant a 1st order kinetics ($y = 7\ 627\ 325x - 5\ 491\ 309$, $R^2 = 0.9877$ where y is the velocity and x is the substrate concentration). Another reason to keep H_2O_2 as low as possible was that the formed peroxyl radicals in the enzymatic reaction should be close to the concentration range of the antioxidants in the sample.

4.4.1.2. Analytical performance of the ECL assay

Using the optimized enzyme-substrate system the light output kinetics in regard of Trolox calibrators was studied by the tube luminometer, injecting the POD-ECL reagent with 5 parallel determinations. The time resolution was 2 s with a total measuring period of 400 s. In order to get larger number of replicates 12 independent calibrations all with 3 parallel measurements were performed on the plate reader by injecting the H_2O_2 substrate. LOD was defined as the mean light output (AUC) obtained for the blanks + 3SD of the blank mean. The LOD for the tube luminometer method was 8.0 μM TE, while for the plate reader assay it was 12.0 μM. The difference between the two measurements most probably lies in the much better time resolution of the tube assay. The best fit and the lowest LOD were obtained by the time shift method however, it is useful only when a fine time resolution is possible. In the literature, the most widely accepted method for the time shift evaluation is the time delay needed for the 10% increase of the light signal. In our view, the 1st derivative of the light emission curve is easier to deal with and it can also be used if the maximum light emission curves of the samples do not reach those of the standards. Imprecision data were obtained by plate reader assay performed

from the same liquorice extracts measured in three consecutive days at three Trolox equivalent levels. Using a biological sample matrix for imprecision testing, the ECL method showed good reproducibility. Even below 40 μmol/L TE the interassay imprecision did not exceed 8.0 % as CV. It means that the optimal measuring range of the assay is between 30 and 100 μM TE. Using liquorice extracts, linearity and recovery studies revealed an acceptable analytical performance. The method was linear in the range of 8–80 μM Trolox equivalents however, in the range between 8 and 40 μM Trolox equivalent a slight underestimation occurred which is reflected in the equation of the regression line. Recovery was also acceptable with an average underestimation of 6.22 % in the range of 20–90 μM Trolox equivalents. As it could be expected from the data of the linearity study, the highest underestimation was obtained below 40 μM TE.

4.4.1.3. Method comparison

Ethanolic extracts of 10 different plant species were compared using our ECL and the modified ORAC methods. The TAC values referred to 1 g of dried drug were both species and method dependent. For comparison of the ECL and the ORAC assays' data, Bland Altman and Passing Bablok analyses were performed. Bland and Altman analysis showed that the average of the differences of the data obtained by ORAC and ECL methods was found to be 941.6. It could be attributed to the fact that the ORAC method measures on the average 941.6 units (μmol/g) more than the ECL method. The Bland and Altman plot clearly shows, that at increasing analyte concentrations the differences in the data obtained by the two investigated measurements are also increasing. Based on the Passing and Bablok regression's equation, there is a constant and proportional difference between the ORAC and the ECL methods.

4.4.1.4. TAC analyses of septic patients' sera

Total antioxidant capacity of the septic group was significantly ($p < 0.01$) higher than that of the control group. Also, we found significantly ($p < 0.01$) higher TAC data in the non-survivor group compared to survivors' values and in non-survivors vs. controls (Fig. 7.) Our data are in good agreement with those published in the literature. In spite of the significant difference found in the TAC values between control and septic patient groups and also the TAC's discriminating potential among survivors and non-survivors, we could not detect significant day by day TAC changes during the follow-up period. Our data indicate that TAC positively strongly correlated with serum uric acid and creatinine concentrations and a loose positive correlation was also found between serum TAC and hsCRP levels.

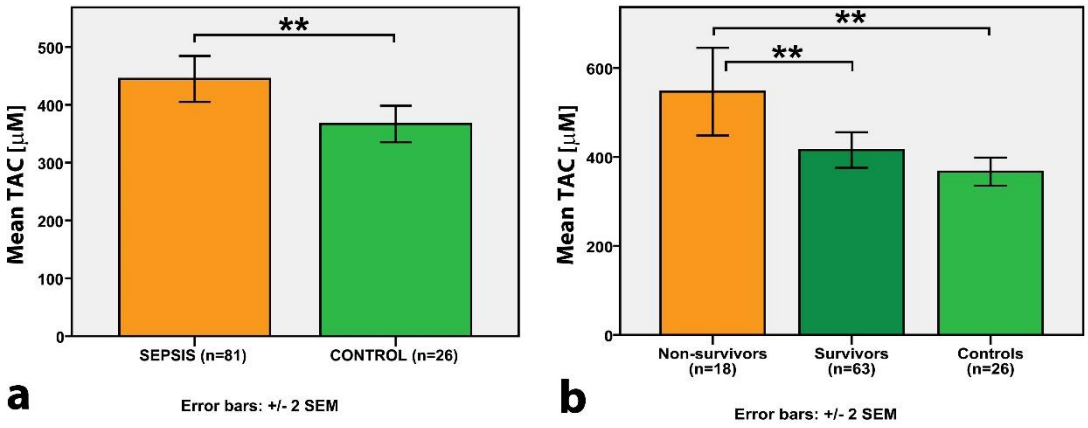

Figure 7: Statistical analysis of the TAC values in septic and in control patient groups (A) and in non-survivor, survivor and control subgroups (B). **p < 0.01

4.4.2. TAC analysis of the seeds and the pericarps of three *Coffea* species

The determination of the antioxidant capacity of the coffee extracts were done with three different methods (ECL, DPPH, ORAC). We could quantify the antioxidant activity by both three methods of all tested plants (*Coffea arabica*, *Coffea benghalensis*, *Coffea liberica*). Although *C. liberica* is less used commercially, the antioxidant effect of its green seeds is comparable to that of *C. arabica* and *C. robusta*. The ECL and DPPH TE/g values showed correlation (R^2=0.587, p=0.083 by Student's t-probe) while those obtained for the ORAC assay were considerably higher with a more uniform pattern and without correlation with the other two assays' data. The imprecision of the three assays was acceptable (ECL: ≤5%, DPPH: ≤10%, ORAC: ≤2%). The DPPH data were also calculated as % TAC using the equation described above. Our results showed closer correlation between the ECL method and the percentage antioxidant capacity obtained by the DPPH technique (R^2=0.6107, p=0.161 by Student's t-probe). The biggest difference was seen for the immature pericarp of *C. benghalensis* and for the mature pericarp of *C. liberica* where the DPPH method showed much higher antioxidant capacity than the ECL assay. In our experiments, the ORAC technique showed the highest values which did not correlate with the results of the other two assays. It is noteworthy, that in contrast to the previously published data, the antioxidant activity of mature fruit extracts of *C. benghalensis* (DPPH method) was lower than published by others however, that of immature pericarp was higher in our study. Among the tested plants, the mature pericarp of *C. liberica* showed the highest „scavenger" activity.

5. New observations

- Determination of a single cell viability parameter (e.g. intracellular ATP or esterase activity) can often be misleading.
- We proved that the esterase-dependent fluorogenic probe CAM is not a true viability parameter because formation of the intracellular fluorescence is not an ATP-driven process.
- The fluorescence intensity of calcein reflects the cell number rather than cellular viability.
- Simultaneous presence of BCD, HPBCD, or DIMEB did not modify considerably the effects of FIS or GER on HepG2 cells.
- The co-treatment of ZEA-exposed HepG2 cells with BCD significantly alleviated the toxic impact of ZEA.
- We can conclude that there is a significant systematic difference between measurement data obtained from ECL and ORAC assays.
- We found significantly higher TAC data in the non-survivor septic patient group compared with survivors' values and in non-survivors vs controls.
- Among the used antioxidant assays, the measured ECLand DPPH values indicated a loose correlation in contrast with the data of the ORAC assay, while a closer correlation was observed between the ECL technique and the expressed antioxidant potential studied by the DPPH method in each coffee species.
- The ORAC assay always showed a much higher Trolox equvivalent value than the other two methods.

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7. List of publications

Publications related to the present PhD thesis

Miklós Poór, Sándor Kunsági-Máté, **Nikolett Sali**, Tamás Kőszegi, Lajos Szente, Beáta Peles-Lemli: Interactions of zearalenone with native and chemically modified cyclodextrins and their potential utilization. *JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B-BIOLOGY*, 151 (2015): 63-68.
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[IF:0,925]

Tamás Kőszegi, **Nikolett Sali**, Maja Raknič, Zoltán Horváth-Szalai, Rita Csepregi, Marijana Zovko-Končić, Nóra Papp, Miklós Poór: A novel luminol-based enhanced chemiluminescence antioxidant capacity microplate assay for use in different biological matrices. *JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS*, 88:(2) (2017): 153-159.
[IF:2,238]

Nikolett Sali, Rita Csepregi, Tamás Kőszegi, Sándor Kunsági-Máté, Lajos Szente, Miklós Poór: Complex formation of flavonoids fisetin and geraldol with β-cyclodextrins. *JOURNAL OF LUMINESCENCE*, 194 (2018): 82-90.
[IF:2,686]

Book chapter related to the present PhD thesis

Nikolett Sali, Tamás Kőszegi: Luminescent Techniques in tissue culture models In: Tamás Kőszegi, Antonella Chesca (szerk.), LABORATORY TECHNIQUES WITH APPLICABILITY IN MEDICAL PRACTICE. Saarbrücken: LAP Lambert Academic Publishing, 2015. pp. 77-99

Presentations and posters related to the present PhD thesis

N. Sali, T. Kőszegi: Luminescent viability methods using tissue culture models. *Second International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences* (Brassó, Románia, 2012.05.24.-2012.05.27.) [előadás]

N. Sali, T. Kőszegi: Multiparametric Luminescent Viability Studies. ***The Third International Conference on Analytical and Nanoanalytical Methods for Biomedical and Environmental Sciences „ IC-ANMBES 2014”*** (Brassó, Románia, 2014.06.13-2014.06.15) [előadás]

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N. Sali, R. Csepregi, M. Tóth, T. Dénes, A. Kaszás, S. Bartha, T. Kőszegi, N. Papp: Antioxidant activity of plants used in the Transylvanian ethnomedicine. ***7th International Student Medical congress in Košice*** (Kassa, Szlovákia, 2015.06.24.-2015.06.26.) [poszter]

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