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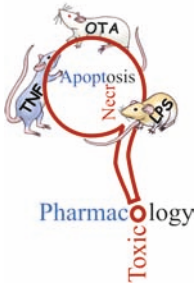

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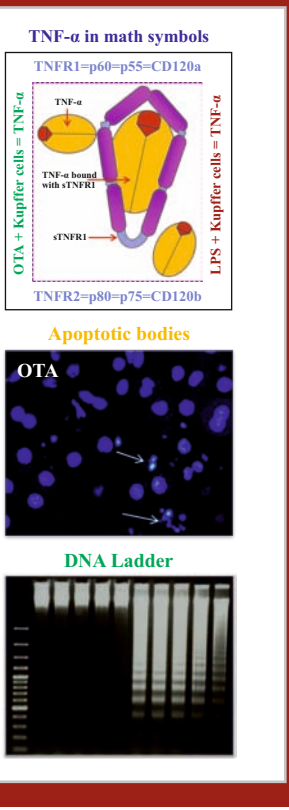

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Betreuer: Prof. Dr. Ernst Petzinger

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eingereicht von

Ebtisam Essid
Tierärztin aus Tripolis-Libyen

Gießen 2013

Mit Genehmigung des Fachbereichs Veterinärmedizin
der Justus-Liebig-Universität Gießen

Dekan: Prof. Dr. Dr. h.c. Martin Kramer

Gutachter:

Prof. Dr. Ernst Petzinger
Prof. Dr. Reto Neiger

Tag der Disputation: 16. 05. 2013

Dedicated to my dear

parents, my beloved husband Yousef,

my daughter Aya, and soul of Mr. Ahmed Almomani

Erklärung

Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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Some results of this work had been published:

Essid, E., Dernawi, Y., Petzinger, E., 2012. Apoptosis induction by OTA and TNF-α in cultured primary rat hepatocytes and prevention by silibinin. Toxins 4, no. 11: 1139-1156.

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Essid, E., Petzinger, E., 2011. Silibinin pretreatment protects against Ochratoxin A mediated apoptosis in primary rat hepatocytes. Mycotox Res 27, 167-176.

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Al-Anati, L., Essid, E., Reinehr, R., Petzinger, E., 2009. Silibinin protects OTA-mediated TNF-alpha release from perfused rat livers and isolated rat Kupffer cells. Mol Nutr Food Res 53, 460-466.

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Table of contents

List of abbreviations

°C	Degree Celsius
3-Ac-DON	3-Acetoxydeoxynivalenol
ActD	Actinomycin D
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
ATP	Adenosintriphosphate
Bcl-2	B-cell lymphoma 2
bp	Base pair
C	Cytosine
C in figures	Solvent control
Ca^{2+}	Calcium ion
CIT	Citrinin
CO_2	Carbon dioxide
CPT	Camptothecin
DD	Death domain
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbeccos modified Eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECD	Extracellular domain
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alii (and others)
Ex/Em	Excitation/Emission
FasL	cluster of differentiation 95 ligand (CD95L)
FCS	Fetal calf serum

FDA	Food and drug administration
Fig.	Figure
g	Gram
GGT	Gamma-glutamyltransferase
GMCSF	Granulocyte macrophage colony stimulating factor
GSH	Glutathione
GSH-Px	Glutathione peroxidase
h	Hour
H_2O_2	Hydrogen peroxide
Hak	Hamster kidney
HCC	Hepatocellular carcinoma
HepG2	Human hepatocellular carcinoma cell line G2
HPCT-1E3	Rat hepatocytoma cells subclone 1E3
IARC	International Agency for Research on Cancer
ICD	Intracellular domain
IFN-α	Interferon alpha
IHKE	Immortalized human kidney epithelial cells
IL2	Interleukin 2
IL5	Interleukin 5
IL6	Interleukin 6
IU	International unit
K^+	Potassium ion
Kb	Kilobase
kDa	Kilodalton
kg	Kilogram
l	Liter
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

M	Molecular weight in gram
mA	Milliampere
MDA	Malonedialdehyde
MDCK-C7	Madin-Darby canine kidney cells subclone C7
mg	Milligram
mJ/cm^2	Millijoule per square centimeter
min	Minute
mm	Millimeter
mM	Millimole
MMP	Mitochondrial membrane potential
NER	Nucleotide excision repair
NF-kB	Nuclear transcription factor kappa B
OTA	Ochratoxin A
PBS	Phosphate buffered salt solution
PCR	Polymerase chain reaction
pmol	Picomole
PTWI	Provisional tolerable weekly intake
RAW264.7	Macrophage cell line
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
rpm	Revolutions per minute
s	Second
SDS	Sodium dodecyl sulfate
SB	Silibinin
sTNFR1	Soluble tumour necrosis factor receptor 1
sTNFR2	Soluble tumour necrosis factor receptor 2
Tab.	Table

TAE	Tris acetate EDTA buffer
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBH	Tertiary butyl hydroperoxide
TE	Tris EDTA buffer
TLR4	Toll like receptor 4
TNF-α	Tumour necrosis factor-alpha
TNF-α antagonist I	(QEKQNTVATAHAGFFLRENEG) is an analog of the amino acid sequence (159-178) of the human 55 kD TNF receptor.
TNF-α antagonist II	(WP9QY) a synthetic peptide (YCWSQYLCY) that binds to TNF-α receptor ligand binding site.
TRADD	Tumour necrosis factor receptor type 1-associated death domain protein
TTE	Tris Triton EDTA lysis buffer
TET	Tris EDTA Triton lysis buffer
U937	Human histiocytic lymphoma
UVC	Ultraviolet C (λ 100-295 nm)
V	Volt
V/V	Volume to volume ratio
VIO	Viomellein (mycotoxin)
Vol.	Volume
XAN	Xanthomegnin (mycotoxin)
µg	Microgram
µl	Microliter
µM	Micromole
µm	Micrometer

List of figures

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I. Abstract

Rat primary hepatocytes were prepared by two different methods: the classical enzymatic perfusion method yielded hepatocytes, which were stably cultivated without DNA fragmentation for up to 96 h, whereas the collagenase-prepared hepatocytes showed apoptosis events as early as from the start of preparation even in the absence of ochratoxin A (OTA). Apoptosis occurred after application of 20 ng/ml tumour necrosis factor alpha (TNF-α), however, only in the presence of 200 ng/ml of the transcriptional inhibitor actinomycin D (ActD). This toxic effect is prevented completely in presence of 25 µg/ml soluble TNF-α receptor 1 (sTNFR1). The transcriptional inhibitor actinomycin D (ActD) alone caused no apoptosis. Furthermore, in the presence of sTNFR2 up to 375 µg/ml in hepatocyte cell cultures showed no preventive effect indicating that TNF-α-mediated apoptosis in cultured hepatocytes occurs only via TNFR1. Apoptosis also occurred after application of 12.5 µM ochratoxin A (OTA) in cultured hepatocytes and in HepG2 cells. However, that was not prevented by sTNFR1 up to 500 µg/ml and by sTNFR2 up to 375 µg/ml, indicating that TNFR1 and 2 are not involved in OTA mediated apoptosis in cultured hepatocytes. Moreover, treatment of cultured hepatocytes and HepG2 cells with lipopolysaccharide (LPS) of 0.1 up to 12.5 µg/ml exhibited no cytotoxic or apoptotic effects. The antioxidative flavanolignan silibinin in doses from 130 to 260 µM prevented chromatin condensation, caspase 3 activation, and apoptotic DNA fragmentation

that were induced by OTA, by 10 mM hydrogen peroxide (H_2O_2), and by ultraviolet (UVC) light (50 mJ/cm^2), respectively. To achieve protection by silibinin the drug was applied to hepatocyte cultures for 2 h in advance.

OTA stimulated lipid peroxidation on cultured immortalized rat liver HPCT-1E3 cells as was revealed by malondialdehyde (MDA) production and ROS generation. Lipid peroxidation and ROS generation occurred further by H_2O_2 and ActD/TNF-α incubation. These reactions were also suppressed by silibinin pre-treatment. We conclude that the anti-apoptotic activity of silibinin against OTA, H_2O_2, and ActD/TNF-α is caused by the antioxidative effects of the flavanolignan.

Furthermore, cytotoxicity of the pro-apoptotic toxins was revealed by MTT-test and Live/Dead kit. When applied separately, ActD and TNF-α showed no cytotoxic effects after 24 h, but were cytotoxic if applied in combination. The used concentrations of OTA, H_2O_2, and the dose of UVC caused a substantial decrease in cell viability within 36 h that was prevented mostly by silibinin. Taken together, that OTA mediated apoptosis in cultured rat primary hepatocytes not via TNFR1 or 2, but by oxygen radicals and lipid peroxidation. Silibinin is a potent protective compound against apoptosis and cytotoxicity caused by OTA and the investigated compounds.

II. Introduction

2.1. Apoptosis

The demise of cells is a biological process; it is for the survival of multicellular organisms as essential as cell division. In eukaryotic organisms, cell death can occur by either of two distinct mechanisms, necrosis or apoptosis.

Apoptosis or programmed cell death is ordered destruction of a cell and it is distinguishable from death by necrosis, which is considered as a random event. Apoptosis in contrast to necrosis is an active metabolic process. Cell suicide occurs due to intentional and unintentional reasons.

For example, during development many cells are produced in excess, which eventually undergo self-destruction and thereby, contribute to sculpturing many organs and tissues. Apoptosis is also required in the differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and harmful cells (Meier et al., 2000).

Unintentional cellular insults may also trigger cell suicide such as those caused by ultraviolet light or chemical agents (Schwartzman and Cidlowski, 1993). Thus, defects in the apoptotic process results in many human diseases involving too much apoptosis such as neurodegenerative disorders, AIDS and ischemic diseases or too little apoptosis such as cancer, autoimmune diseases and spreading of viral infections (Fadeel et al., 1999, Lowe and Lin, 2000, Lawen, 2003).

Apoptosis and necrosis can occur independently, sequentially, as well as simultaneously (Zeiss, 2003).

The morphological features of dead cells are the fundamental distinctions between these two processes **(Fig. 1)** (Walker et al., 1988, Elmore, 2007).

The necrosis is characterized by a loss of membrane integrity, which results in failure of calcium and sodium ion pumps leading to osmotic cell swelling, cell rupture and finally, release of cellular contents into the surrounding tissue thereby causing inflammation reaction (Zeiss, 2003).

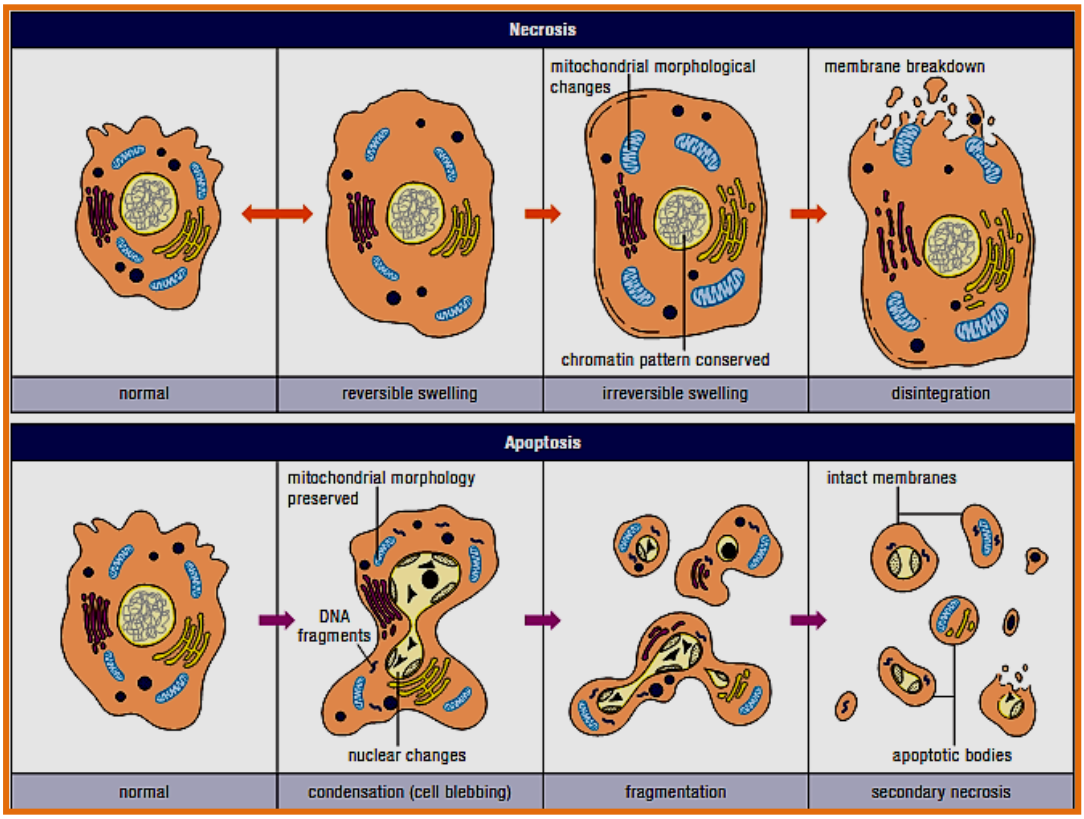

Fig. 1: The distinct physiological difference in necrotic versus apoptotic cell death on the individual cell scale. Adapted from A. Wyllie, V. Donahue, B. Fischer, D. Hill, J. Keesey, S. Manzow, Apoptosis and cell proliferation. Boehringer Mannheim GmbH, Biochemica, 1998. A guide to apoptosis identification methods and assays available through Boehringer Mannheim GmbH, Biochemica.

The early morphological feature of apoptosis is cytoplasmic condensation (Kerr, 1971) due to loss of cell water and chromatin condensation which has uniform texture in cells where it forms a teardrop shape.

The cell membrane forms protrusions and pinch off a portion of cellular contents to form apoptotic bodies. These bodies are subsequently engulfed by macrophages without causing inflammation (Elmore, 2007). At apoptosis pathways, a family of proteins called caspases are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine-aspartate-proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes followed by the induction of apoptosis (Thornberry and Lazebnik, 1998).

Caspase activation plays a fundamental role in the execution of apoptosis by the extrinsic (cell surface death receptor) and the intrinsic (mitochondrial) pathway **(Fig. 2)**. Both pathways lead to activation of each specific initiator caspases and converge at the level of the executioner caspases. These two signalling pathways separate have shared intersections (Budihardjo et al., 1999).

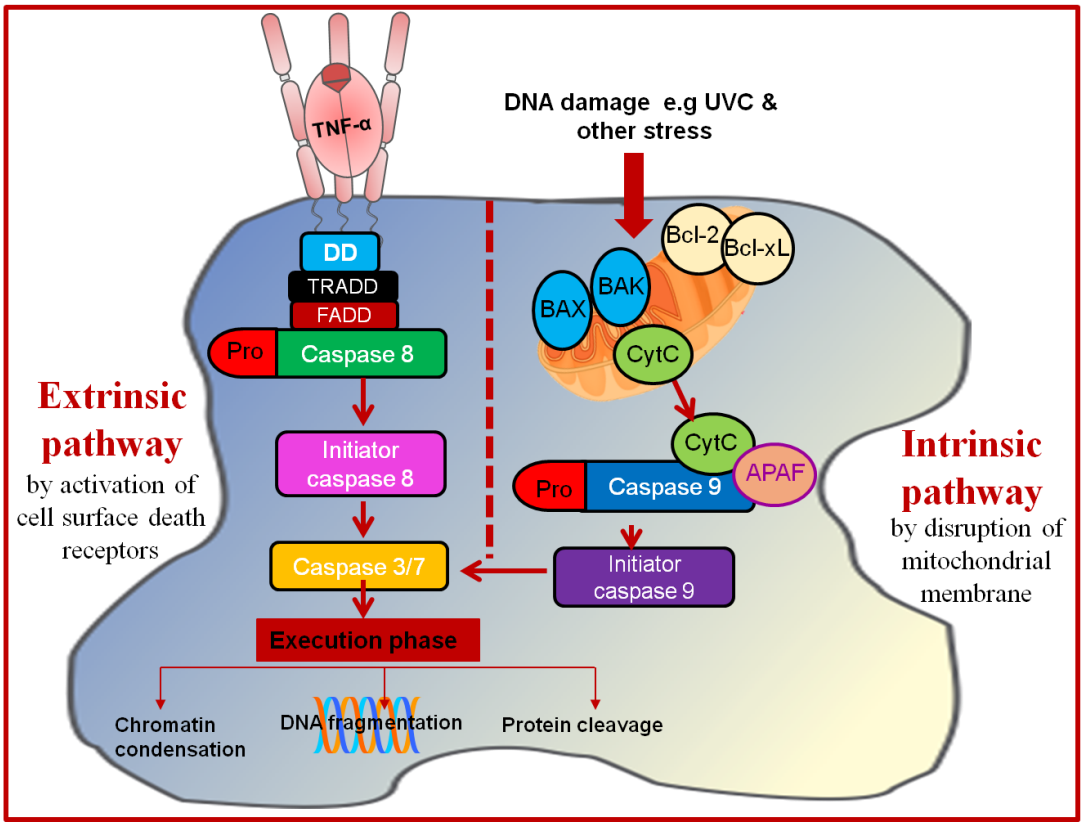

Fig. 2: Signalling pathways of apoptosis

The extrinsic pathway involves an activation of death receptors whereas the intrinsic pathway is by a disruption of mitochondrial membrane integrity off. Both pathways converge in the caspase cascade.

2.1.1. Intrinsic signal pathway

The intrinsic or mitochondrial pathway is frequently activated in response to signals resulting from DNA damage or other stress events. This pathway hinges on the balance of activities between pro- and anti-apoptotic signals of the Bcl-2 family. The proteins of the Bcl-2 family regulate the permeability of outer mitochondrial membrane and determine whether a pro- or anti-apoptotic signal will be released inside the cell. Normally, pro-apoptotic proteins such as cytochrome c are released from the mitochondria into the cytosol forming a complex of cytochrome c, adenosine triphosphate (ATP), pro-caspase 9 and

apoptosis protease-activating factor-1 (Apaf-1). This protein complex called apoptosome activates the inactive pro-caspase 9. Once activated, the initiator caspase 9 activates the executioner caspases 3 and 7 and finally triggers a cascade of events leading to apoptosis (Mayer and Oberbauer, 2003).

2.1.2. Extrinsic signal pathway

The extrinsic pathway begins outside the cell and relays on apoptotic messages via activation of pro-apoptotic receptors in the cell surface. These are activated by molecules known as pro-apoptotic ligands. The interaction of these ligands, such as tumour necrosis factor (TNF-α) or FasL activates their respective cell surface receptors. Tumour necrosis factor alpha (TNF-α) is a common extrinsic factor that initiates apoptosis. TNF-α binds to the TNF receptor 1 at the outer membrane of the cell, subsequently the so-called death domain at the cytoplasmic site of the receptor is activated.

As a result, cytosolic proteins with their own death domains bind and are activated. The first protein that binds to the cytosolic part of the receptor is the TNF receptor associated death domain or TRADD.

Then, the protein FAS (associated protein with death domain) binds, thus, recruiting pro-caspase 8. This protease is able to autocatalyse the hydrolysis of its inhibiting segment leading to active caspase 8, which dissociates from the receptor, and thus enabling the initiation of the effector caspase cascades (Andera, 2009).

2.1.3. Execution cleavage of DNA

The end result of either pathway is the activation of effector caspases and the cleavage of specific cellular substrates, i.e DNA and cytoskeletal proteins, resulting in the morphologic and biochemical changes associated with the apoptotic phenotype (Riedl and Shi, 2004). In a normal cell, DNase forms a complex with an inhibitor and is inactive to prevent DNA cleavage. After initiation and activation of the caspase cascade, the active caspase 3 is able to cleave this inhibitor. Activated DNase cleaves DNA at cleavage sites which located at regular intervals of 180 bp in between histone proteins of nucleosomes. Caspase 3 cleaves many other proteins such as proteins of the cytoskeleton, hereby losing structure of the cell. Next, other proteins cause the cells to collapse into vesicles forming the so called apoptotic blebs. Most blebs contain mitochondria, also portions of the nucleus including DNA.

These components allow energy to be maintained and new protein synthesized. The rapid breakup of the cell into the formed vesicles avoids an inflammatory reaction in the surrounding tissue. The various processes during the execution phase lead to significant modification of destruction and decomposition of the outer membranes of the cells. Based on the modified membrane structure such as loss of phospholipid asymmetry and the translocation of phosphatidylserine (PS) to the outer leaflet of the lipid bilayer, phagocytes like macrophages can recognize the blebs, and in the cytosol of the phagocytising cell, the blebs fuse

with lysosomes and these organelles contain enzymes that finally digest the blebs and their components.

2.1.4. Apoptosis in the liver

Liver is by mass the largest secretory gland in the body. It is a unique organ with many crucial roles sustaining life. It neutralizes harmful toxins, wastes, and drugs. Therefore, it is at great risk of contamination from environmental toxins and those contained in over-processed foods.

Hepatocytes are the major cells in the liver and constitute at least two-third of the liver mass and hence their susceptibility to TNF-α is of relevance in disease progression. The prominent feature of most liver diseases is apoptosis of hepatocytes. In fact, a wide range of injurious stresses can lead to apoptosis manifested as liver damage. The TNF-α administration in large doses to rat causes acute liver failure within hours due to the induction of apoptosis through the TNFR1 signalling pathway (Leist et al., 1997). TNF-α signalling in hepatocytes can lead to the activation of inflammatory pathways or pathways leading to cell death.

Until now, it is unclear to determine which type of signalling will predominate. Many of the details of TNF-α signalling have been established *in vitro*. The liver belongs to the postmitotic tissues, and the proliferation index of hepatocytes is low. Thus, the molecular mechanisms of TNF-α signalling and apoptotic events in hepatocytes are only partially understood.

2.2. Hepatotoxins

2.2.1. Ochratoxin A

Ochratoxin A (OTA) was isolated for the first time from *Aspergillus ochraceus* and identified as a toxic metabolite in 1965 by van der Merwe and his co-workers under an experimental survey (Van der Merwe et al., 1965a, van der Merwe et al., 1965b).

It is produced by secondary metabolism of many filamentous mold species belonging to the genera *Aspergillus* (Wicklow et al., 1996, Bennett and Klich, 2003) and *Penicillium* (Pitt, 1987) in different geographical areas under a wide variety of climate conditions.

Toxin production occurs under food storage conditions and is minimal in field surroundings. OTA has been found to be ubiquitously present in all kind of food and feed. Moreover, OTA is continuously and unavoidably ingested with food, and consequently is present in blood in most if not all tested people, in particular, in the USA and European populations at moderate climates (Jimenez et al., 1999).

OTA is a weak organic acid with a molar mass of 403.8 $g.mol^{-1}$. It consists of a chlorinated dihydroisocoumarin moiety linked through a 7-carboxyl group by an amide bond to one molecule of L-β-phenylalanine **(Fig. 3)**.

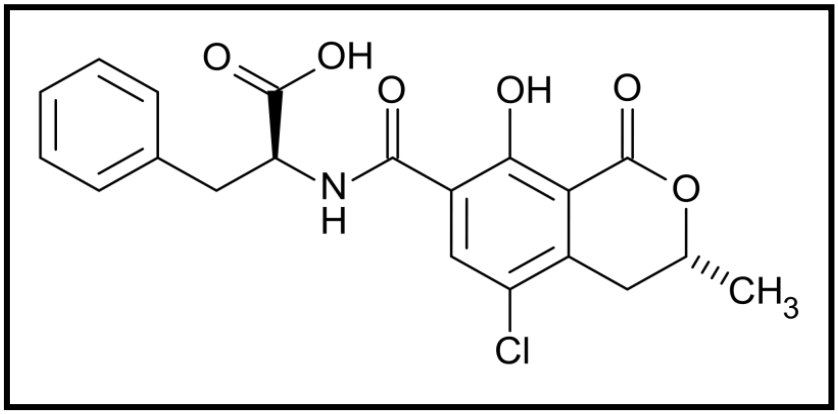

Fig. 3: Chemical structure of ochratoxin A

OTA structurally consists of a para-chlorophenolic group containing a dihydroisocoumarin moiety that is amide-linked to L-phenylalanine. Several metabolites related to OTA have been also identified, particularly, ochratoxin B (OTB) the dechloro analogue of OTA, ochratoxin C (OTC) its ethyl ester, the isocoumaric derivative of OTA, ochratoxin α (Otα), and its dechloro analogue, ochratoxin β (OTβ). Its chemical name is L-phenylalanine-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl]-(R)-isocoumarin.

OTA forms a crystalline structure which varies from colourless to white. This molecule emits green fluorescence in acid solution and blue fluorescence in alkaline solution under UV light (Bredenkamp et al., 1989).

OTA is stable and possesses resistance to acidity, high temperatures, and high pressure steam sterilization from 121°C to 250°C (Trivedi et al., 1992, Boudra et al., 1995). Moreover, this molecule is only partially degraded at normal conditions of cooking. Once foodstuffs are contaminated, it is very difficult to remove OTA totally. Following the discovery of human and animal spontaneous nephropathies, toxicological and pathological effects of OTA have been studied and examined many times by the International Agency for Research on Cancer (IARC) (1993).

These studies showed that OTA has nephrotoxic, hepatotoxic, neurotoxic, teratogenic, and immunotoxic effects on several species of animals and can cause kidney and liver tumours in mice and rats (Bennett and Klich, 2003, Gagliano et al., 2006). However, its toxicity varies depending on the sex, species and the cellular type of the tested animals (O'Brien et al., 2001).

The genotoxic effect of OTA is still controversial due to contradictory results obtained in various microbial and mammalian tests. Nevertheless, evidence of DNA-adducts formation was shown following chronic exposure of OTA to rat and sub-acute exposure to pig (Faucet et al., 2004).

A provisional tolerable weekly intake (PTWI) for humans of OTA at 112 ng/kg body weight (b.w.) was established after OTA evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1991. Four years later, OTA was re-evaluated by the JECFA and the PTWI was reconfirmed, rounding it to 100 ng/kg b.w. per week.

The Committee retained the previously established PTWI of 100 ng/kg body weight per week, pending the results of ongoing studies on the mechanisms of nephrotoxicity and carcinogenicity, and recommended a further review (Bakker and Pieters, 2002). However, the total daily intake of OTA from food in various European countries was much below and was e.g 0.9 ng/kg of b.w. in Italy (Brera et al., 2008). However, in North African countries the limits already were reached.

2.2.1.1. Ochratoxin A cytotoxicity

The primary target of OTA in all tested species is the urinary system, precisely the kidney. The toxicokinetics of the mycotoxin partially explains why the kidney damage is the strongest: the kidneys get over the high renal blood flow continuously delivered OTA, which is filtered but also secreted in the proximal tubule. Increasing the urine concentration increases the OTA concentration in the lumen of the proximal tubule strongly before the toxin is actively reabsorbed in the course of the nephron. In this way, OTA accumulates in the tissue of the kidney (Gekle et al., 2005). The cytotoxic effects of OTA are based on the inhibition and/or activation of enzymes, of which several use phenylalanine as a substrate because this amino acid is part of the OTA molecule. It is believed that the phenylalanine moiety in the OTA molecule interacts as a surrogate substrate of these enzymes. The main targeted enzyme is phenylalanine-tRNA synthetase which is inhibited in eukaryotic microorganisms (Creppy et al., 1979), prokaryotes (Konrad and Roschenthaler, 1977), mammalian cells (Creppy et al., 1983), and experimental animals *in vivo* (Creppy et al., 1984). This inhibition causes a reduction in protein synthesis, which is an important effect of acute and sub-acute OTA toxicity. In addition to inhibition of protein synthesis RNA-synthesis inhibition is another end point of OTA toxicity (Dirheimer and Creppy, 1991) and probably DNA synthesis may be inhibited too. An enzyme affected early by RNA inhibition is phosphoenolpyruvate carboxykinase, the key

enzyme in the gluconeogenic pathway which depletes indirectly due to specific degradation of the mRNA coding for this enzyme (Meisner et al., 1983). *In vitro,* the addition of 1.0×10^{-4} M OTA to isolated rat liver mitochondria led to inhibition of succinate-cytochrome c reductase, succinate dehydrogenase and succinate oxidase due to effects on the mitochondrial respiration and oxidative phosphorylation. OTA impair the mitochondrial membrane and inhibits the succinate- supported electron transfer activities of the respiratory chain (Wei et al., 1985). However, the concentrations required for these enzyme inhibitions are very high in the range of mM concentrations and may be unlikely to occur *in vivo*.

2.2.1.2. Induction of apoptosis by OTA

With regard to cell death, the induction of apoptosis in several cell types of the urinary tract by OTA was described (Domijan et al., 2004, Sauvant et al., 2005). The urinary system is a known OTA-target and the most important one clinically. The administration of Wister rats with 120 microgram OTA/kg body weight daily, for 10, 30, or 60 days activated apoptotic processes and oxidative damage in both proximal and distal epithelial kidney cells (Petrik et al., 2003). Apoptotic cell alterations such as chromatin condensation, caspase 3 activation and DNA fragmentation were found if OTA was incubated at nanomolar concentrations with cultured human proximal tubule-derived cells (Schwerdt et al., 1999), and with dog renal collecting duct-derived cells (MDCK-C7) (Gekle

et al., 2000, Schwerdt et al., 2004) or at micromolar concentrations with immortalized human kidney epithelial (IHKE) cells (Rached et al., 2006). Whatever, these apoptotic changes were potentiated via inhibition and uncoupling of the mitochondrial respiratory chain (Schwerdt et al., 2004). Interestingly, OTA potentiated the pro-apoptotic action of TNF-α in MDCK-C7 cells (Gekle et al., 2000). Furthermore, apoptosis in human HeLa cells and hamster kidneys (HaK) was observed albeit at higher concentrations of OTA (Seegers et al., 1994).

Apoptosis induction by OTA is not limited to the urinary system, it can be found in the immune system and the liver. It has been shown that up to 10 µM of OTA induces apoptosis, evidenced by both DNA fragmentation and activation of caspase 3 and 9 detection, in human peripheral blood lymphocytes, and human lymphoid T cell line and Kit 225 cell (Assaf et al., 2004). It was also reported that OTA triggers inner mitochondrial transmembrane potential, resulting in changing of mitochondrial membrane permeabilization. Therefore, it was assumed that mitochondria are a central component in OTA-induced apoptosis in human lymphocytes (Assaf et al., 2004). Moreover, in bovine lymphocytes (Lioi et al., 2004), human hepatoma-derived cell line HepG2 (Renzulli et al., 2004) and liver of male mice (Atroshi et al., 2000) proceed to cell death through the apoptotic pathway. The induction of DNA adduct formation and DNA single strand breakdown by OTA is considered as an indicator for OTA-induced apoptosis (Creppy et al., 1985, Faucet et al., 2004).

Apart from apoptosis, necrosis also occurred under OTA burden. Necrotic changes were observed in rat liver (Aydin et al., 2003), rat myocytes (Okutan et al., 2004), and in germinal centres of the spleen and lymph nodes of Wistar rats (Kanisawa et al., 1977) and dogs (Kitchen et al., 1977). The parameters which determine the type of cell destruction are toxin dose and exposure time. For example, one week after OTA administration to male mice, only apoptotic without necrotic changes were observed in their liver, whereas, centrilobular necrosis and apoptosis were seen after two weeks (Atroshi et al., 2000). Gekle *et al.,* 2000, found that OTA at low dosage caused apoptosis and at higher dosage caused necrosis in MDCK-C11 cells (Gekle et al., 2000). Others found only apoptotic but not necrotic changes in rat kidneys, which occurred in a dose- and time-dependent manner (Domijan et al., 2004).

Furthermore, whereas OTA mainly causes nephrotoxicity it additionally also induces hepatotoxicity (Petzinger and Ziegler, 2000, Ringot et al., 2006). The perfusion of blood-free rat livers with OTA up to 12.5 µM mediated TNF-α release, which possibly triggers apoptosis via TNF/ Fas-signalling pathway in hepatocytes (Al-Anati et al., 2005). In primary rat hepatocytes and rat kidney cells, it has been shown that OTA induced oxidative DNA damage and apoptosis (Kamp et al., 2005, Cavin et al., 2009). The hepatotoxic effects of OTA are characterized by apoptotic changes in rat and mouse liver (Atroshi et al., 2000, Aydin et al., 2003, Ringot et al., 2006), but also in cultures of isolated rat hepatocytes (Chopra et al., 2010a, Essid and Petzinger, 2011). The apoptotic

effects of OTA in liver tissue were thought to be mediated by TNF-α (Chopra et al., 2010a) that is released from Kupffer cells (Al-Anati et al., 2010).

In tissue cultures of pure isolated rat hepatocytes OTA mediated apoptosis even in the absence of Kupffer cells and TNF-α (Essid and Petzinger, 2011). These cell damages by OTA likely occurs by oxidative stress via reactive oxygen species (Essid et al., 2012) and this thesis).

2.2.1.3. Ochratoxin A hepatotoxicity

The liver is among the OTA-target organs because of its food-borne exposure via the portal vein to OTA after mycotoxin absorption from the gut and of an enterohepatic circulation of OTA reported in mice (Roth et al., 1988). Subsequently, liver cells will be frequently in exposure to internally circulating OTA. Hepatotoxicity of OTA was observed previously in different species, especially in poultry.

In a histopathological study, the liver lesions in geese were caused by multifocal liver necrosis containing inflammatory cells and sometimes bacterial colonies. While in livers of broilers were enlarged and congested or, more often, shrunken. Fibrotic livers were covered by a sheet of fibrin (Schlosberg et al., 1997). In rats treated with OTA, the histopathological changes that are found in the liver tissue included granular or vacuolated degeneration and necrosis of the liver cells, sinusoidal and central vein dilatation, bile duct proliferation, enlargement of periportal areas with mononuclear cell inflammatory infiltration

and mild degrees of fibrous tissue proliferation (Aydin et al., 2003). Administration of OTA via gavage causes multifocal haemorrhages with fibrin thrombi in the livers of male Long-Evans and Sprague-Dawley (Albassam et al., 1987) and also decrease in blood coagulation factors II, VI, X, and plasma fibrinogen in Wister rats (Galtier et al., 1979).

Long-term studies of toxicity and carcinogenicity of OTA with diets containing OTA in different doses and time exposures induced hepatic-cell tumours in mice. It was not clearly indicated whether the liver tumours were benign or malignant (Kanisawa and Suzuki, 1978).

OTA is direct genotoxic i*n vivo*, and DNA single-strand breaks were observed in liver cells of mice after interperitoneal injection of OTA (Creppy et al., 1985).

It was also reported that *in vivo* OTA causes DNA adducts supporting a direct reaction with DNA (Pfohl-Leszkowicz et al., 1991). However, it was never shown that OTA derived radio-activities occurred in DNA nor were such adducts ever documented by physiochemical analysis (Mally et al., 2005). DNA damage, manifested as single-strand breaks *in vitro* in most studies with primary cultures of rat and mouse hepatocytes (Joint FAO/WHO, 2001). Therefore, direct genotoxicity of OTA was recently questioned (Turesky, 2005) but the debate on this topic is not definitely resolved (Pfohl-Leszkowicz and Manderville, 2012).

2.2.1.4. Cytokines and OTA

The immune system is composed of various types of cells, all of which are capable of autonomous regulation. The production of cytokines by these cells is influenced by several mycotoxins including OTA. It causes a significant release of pro-inflammatory cytokines TNF-α and IL-6 from blood-free perfused rat livers at micromolar concentration range and this release was comparable to that produced in rat livers by low concentration of LPS (Weidenbach et al., 2000, Al-Anati et al., 2005). An antagonistic effect on OTA-mediated TNF-α release from rat livers was seen if other mycotoxins such as 3-acetoxydeoxynivalenol (3-Ac-DON), xanthomegnin (XAN), citrinin (CIT), and viomellein (VIO) were simultaneously co-applied (Petzinger and Weidenbach, 2002). Others reported TNF-α release from mouse RAW264.7 macrophage cell line upon OTA treatment (Huttunen et al., 2004). In the thymoma cell line EL4 which was stimulated by phorbol 12-myristate 12-acetate (PMA) exposure to OTA showed a marked increase of IL-2 production, while IL-5 production was significantly decreased (Marin et al., 1996). Under certain conditions, OTA presents a powerful immunosuppressive effect, which is observed at high doses (Dirheimer and Creppy, 1991). Humoral and cellular immunity affections by OTA were also described (Holmberg et al., 1988). OTA is clearly taken as an important immunosuppressive agent (Al-Anati and Petzinger, 2006). OTA seems to play a role in the inhibition of the peripherals T- and B- lymphocytes proliferation and

stops the production of interleukin 2 (IL-2) and its receptors (Lea et al., 1989). Moreover, it blocks the activity of killer cells as well as the production of interferon (Luster et al., 1987).

2.2.2. Tumour necrosis factor alpha

The proinflammatory cytokine tumour necrosis factor alpha (TNF-α) plays a fundamental role in immune defense. It was isolated in 1975, based on its ability to kill tumour cells *in vitro* and to cause haemorrhagic necrosis of transplantable tumours in mice (Carswell et al., 1975). TNF-α is produced by a wide variety of cell types in response to various inflammatory stimuli, such as lipopolysaccharides (LPS), phorbol esters, zymosan, ultraviolet light, viral infections, protozoa, TNF itself, cytokines such as interleukin (IL-1, IL-2), interferon (IFN)-γ, IFN-α, the Granulocyte-Macrophage Colony-Stimulating Factor (GMCSF), and the Transforming Growth Factor (TGF)-β. Most later studies demonstrated the powerful pro-inflammatory effects of TNF-α (Dayer et al., 1985) and revealed its role as a central endogenous mediator of endotoxic shock (Tracey et al., 1986, Beutler et al., 2008). Furthermore, the pro-apoptotic cytokine activity of TNF-α was regarded as a double-edged sword: on one hand, it mediates physiological processes and on the other hand promotes pathogenesis of several health disorders. Meanwhile, TNF-α research has been implemented in studies with other mycotoxins e.g. rubratoxin B (Nagashima et al., 2001) and also fumonisins (He et al., 2002).

2.2.2.1. TNF-alpha receptors

Now it is clear that TNF-α binds to two distinct receptors referred to as p60 (also called p55 or type 1 or CD120a) and p80 (also called p75 or type 2 or CD120b), with an approximate molecular mass of 60 kDa and 80 kDa, respectively (Aggarwal, 2000). The two receptors bind TNF-α with almost equal affinity. The receptors exhibit in their ECD four cysteine-rich regions, each consisting of six cysteine residues. These cysteines are conserved within the two receptors. In contrast, the structure of the ICD of the two receptors is quite distinct and lacks enzymatic activity. The ICD of the p60 receptor contains a homophilic interaction region of approximately 80 amino acid residues towards its carboxyl terminal, called the death domain (DD) (Tartaglia et al., 1993) which is absent in the p80 receptor. This region was found to be required for TNF-α induced apoptosis, antiviral activity and nitric oxide synthase induction. Within the past decade, major advances have been made in understanding how TNF-α receptors transduce their signals.

A series of signalling molecules have been discovered that play a critical part in the TNF-α induced cellular responses. Some of the major TNF-α induced cellular responses were reviewed by Bazzoni and Beutler, 1996, and Aggarwal, 2000. Hepatocytes have, as many other cell types, TNF-receptors (Libert et al., 1991) and can synthesize TNF-α upon viral infection (Gonzalez-Amaro et al., 1994).

2.2.2.2. Induction of apoptosis by TNF-alpha

The major hot topic of TNF-α research is the pro-apoptotic effect of the cytokine in primary cells or cultured cell lines. Apoptotic effects of TNF-α were demonstrated in T-lymphocytes (Bonetti et al., 2003), HeLa cells (Cozzi et al., 2003), endothelial cells of rat coronary artery (Csiszar and Ungvari, 2004), U937 cells (Misasi et al., 2004), neuronal cells in the rat cerebral cortex but not in hippocampus (Montes-Rodriguez et al., 2004), endometrial cells (Okazaki et al., 2005) and vascular smooth muscle cells of rat and human (Tang et al., 2005). Moreover, TNF-α induced apoptosis in type 2 pneumocytes (T2-cells) and this apoptotic effects were eliminated *in vivo* by anti-TNF-α antibody treatment (Guthmann et al., 2005).

Pre-treatment with TNF-α was reported to sensitize several tumour cells to apoptosis, such as Hodgkin cell line HD-MyZ cells which were subjected to apoptotic cell death induced by antineoplastic agents and by ceramide (Schmelz et al., 2004). Moreover, pre-treatment with aspirin sensitizes HeLa cells to TNF-α-induced apoptosis as well (Kutuk and Basaga, 2004). Also luteolin greatly sensitized TNF-α-induced apoptotic cell death in a number of human cancer cell lines, including colorectal cancer COLO205, HCT116 cells and cervical cancer HeLa cells (Shi et al., 2004). However, these effects that evolved by the TNF-α receptors are not yet completely elucidated. In the intact liver model, TNF-α originates only from Kupffer cells (Al-Anati et al., 2005), and provokes a

detrimental effect on hepatocytes by activating a variety of cellular signal transduction pathways which ultimately could lead to programmed cell death (Kresse et al., 2005, Osawa et al., 2005). The main apoptotic effects of TNF-α are mediated by its receptor TNFR1 (Nagata, 1997, Micheau and Tschopp, 2003, Ding and Yin, 2004). In addition, TNF-α has been reported to induce apoptosis in rat and mouse hepatocytes *in vitro* and *in vivo* only in the presence of a transcriptional inhibitor such as ActD (Leist et al., 1994).

2.2.3. Hydrogen peroxide and cell death

Reactive oxygen species (ROS) are oxygen-containing molecules including hydrogen peroxide, hydroxyl radicals and superoxide radicals that are highly reactive on other complex molecules in the cells, such as DNA, lipids, and protein. ROS have been confirmed to be mutagenic because of DNA damage (Labaj et al., 2007, Horvathova et al., 2008), and lipid peroxidation leads to membrane destruction. ROS can also alter or inactivate enzymes through binding to several amino acid residues. Oxidant reactive species at physiological levels are important to life, being involved in some biological functions such as phagocytosis. Nevertheless, when overproduced due to exogenous stimulation, they cause irreversible cellular damage (Mari et al., 2012).

In the liver, Kupffer cells are the main sources of vascular reactive oxygen formation and the effects indicate that ROS-mediated liver cell damage is caused by activated Kupffer cells (Bilzer et al., 1999). Hydrogen peroxide (H_2O_2) is a

cytotoxin that mediates toxicity in primary rat hepatocytes in a dose- and time-dependent manner (Borle and Stanko, 1996, Horvathova et al., 2009) by provoking oxidative stress and apoptosis (Qiao et al., 2005). In HepG2 cells, also the treatment with H_2O_2 significantly induced death as evident by MTT (Ghaffari et al., 2012).

2.2.4. Ultra violet light and cell death

About 45% of the radiation from the sun is emitted in the form of UV light. Depending on its wavelength, it is divided into three types: UVA (λ 320-400 nm), UVB (λ 295-320 nm) and UVC (λ 100-295 nm). The ozone layer of the atmosphere completely absorbs UVC and largely absorbs UVB preventing them from reaching the earth's surface. The fact that the maximum absorption of the DNA is 260 nm (in the area of UVC radiation), made it a suitable instrument for studying cellular response to DNA damage (Batista et al., 2009).

UV radiation induced in different cell types a dose-dependent apoptosis by different signal pathways. UV radiation causes the formation of specific DNA losses, i.e cyclobutane pyrimidine dimers, and 6,4-photoproducts. In addition to oxidative damage, DNA single strand breaks and intrastrand crosslinks were generated. Due to this damage, the tumour suppressor p53 is activated, and cell cycle arrest is induced. The DNA damage is subjected to the nucleotide excision repair (NER). If this fails, it drives the p53 cell in the apoptosis, for example, via the induction of the pro-apoptotic Bax.

This leads to activation of the intrinsic apoptosis pathway. Next to the p53-dependent, classical pathway that results from both apoptotic pathways, the extrinsic and intrinsic pathway, is a direct UV light induced apoptosis: UV radiation causes direct activation of death receptors (FAS, TNFR).

In the intrinsic pathway activation of reactive oxygen species (ROS) occurs. These react with lipids and damage intracellular membranes, including the outer mitochondrial membrane. From mitochnodria cytochrome c is released and starts the caspase cascade through caspase 9 (Chigancas et al., 2000, Dunkern et al., 2001, Batista et al., 2009). Moreover, in hamster cell lines, UV at higher doses causes a prolonged G1 arrest and apoptosis without entry into the S-phase (Proietti De Santis et al., 2002). Further, UVC irradiation of primary rat hepatocytes has been shown to facilitate activation of apoptotic caspase cascades, DNA fragmentation, and chromatin condensation (Chopra et al., 2010a).

2.3. Silibinin

The polyphenolic flavonoid silibinin is extracted from the milk thistle *Silybum marianum* **(Fig. 4)**. It is also known as silybin that constitutes between 60%-70% of the silymarin mixture. Therefore, it is the main active biologic ingredient of the milk thistle (Wagner et al., 1974). Silymarin has been used for over 2000 years as a general medical herb. Since the 16th century it has been used mainly as a remedy to treat different ailments and illnesses, almost exclusively for

hepatoprotection in humans, because of its strong anti-hepatotoxic activity against various forms of liver damage and toxicity (Dehmlow et al., 1996, Schuppan et al., 1999, Wellington and Jarvis, 2001, Tripathi et al., 2008).

It was approved as Legalon® by the German Federal Drug Administration in 1984 for the treatment of Amanita phalloides poisoning. The extent of therapeutic silibinin applications has been widened for the treatment of human liver carcinoma due to its beneficial effects to interfere with many molecular events involved in cancer cell growth (Varghese et al., 2005, Kauntz et al., 2011). It was applied as additional chemopreventive agent in a variety of *in vitro* and *in vivo* cancer models from skin (Bhatia et al., 2001), lung (Singh et al., 2006), bladder (Tyagi et al., 2007, Singh et al., 2008, Zeng et al., 2011), breast, colon, prostate, and kidney (Cheung et al., 2010, Ozten-Kandas and Bosland, 2011, Kim et al., 2011).

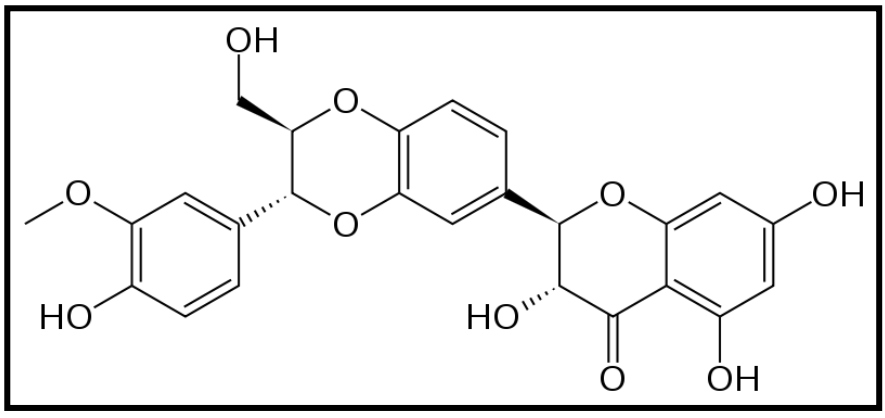

Fig. 4: Chemical structure of silibinin

2.3.1. Silibinin and the liver

Since ancient times silibinin has been used to promote liver health, in western society it is one of the top 10 most popular consumed natural products and is one of the oldest and thoroughly researched plants in the treatment of liver diseases.

Rats with partial hepatectomized liver, when subjected to silibinin pre-treatment showed increased synthesis of DNA, RNA, protein and cholesterol suggesting liver regeneration in the early stages (Srivastava et al., 1994). Furthermore, liver damage induced by CCl_4 in rats, was treated with oral silibinin 50 mg/kg for 5 days. The increased collagen content by the CCl_2-metabolite was reduced to 55 percent by silibinin treatment (Favari and Perez-Alvarez, 1997). Silibinin normalized the elevated biochemical parameters of liver and serum, mediated by acetaminophen (Ramellini and Meldolesi, 1976) and protects against oxidative stress induced by acetaminophen on rat liver (Campos et al., 1989). Acute and chronic administration of ethanol produces a drastic decrease in the hepatic content of reduced glutathione leading to liver damage that was prevented by silibinin treatment (Das and Vasudevan, 2006).

Iron overload is associated with liver damage, characterized by massive iron deposition in hepatic parenchymal cells, leading to fibrosis and eventually to hepatic cirrhosis. Pre-treatment in rats with silibinin reduced iron-induced increase in lipid peroxidation and levels of serum enzymes (Bhattacharya et al., 2000).

In animals (dogs, rabbits, rats, mice) intoxicated with phalloidin is evident, silibinin protects intoxicated animals when given either 60 min before or 10 min after phalloidin treated (Desplaces et al., 1975).

In long-term treatment study with silibinin (420 mg daily) increased survival rates significantly in a randomized, double-blind, placebo-controlled study of 105 people with liver cirrhosis. Moreover, in this study were no side effects reported (Ferenci et al., 1989). Silibinin has been used to protect liver from injury caused by ischemia (Wu et al., 1993), radiation (Kropacova et al., 1998) and virus hepatitis (Strader et al., 2002, Seeff et al., 2008).

2.3.2. Silibinin and the immune system

Silibinin is both immunosuppressive and immunomodulatory besides its antioxidant property (Min et al., 2007). It thus seems to possess anti-inflammatory properties by acting through different mechanisms such as its antioxidant action, membrane-stabilizing effect and inhibition of the production or release of inflammatory mediators such as arachidonic acid metabolites (Breschi et al., 2002).

Silibinin exhibits anti-inflammatory effects due to suppression of the pro-inflammatory cytokine TNF-α in cell cultures of human histiocytic lymphoma U-937 cells (Manna et al., 1999), in perfused rat livers (Al-Anati et al., 2009), and in mice (Schumann et al., 2003). It also restores impaired liver functions following partial hepatectomy (Horvath et al., 2001) and inhibits histamine

release from human basophil leucocytes (Miadonna et al., 1987). Protective effects of silibinin have been described in different models of experimental liver intoxication and were related to modulation of signalling cascades in hepatocytes and Kupffer cells causing inhibition of nitric oxide production, TNF-α release and lipid peroxidation (Dehmlow et al., 1996).

Silibinin exerts an inhibitory effect on the expression of TNF-α (Zi et al., 1997, Bannwart et al., 2010) and other proinflammatory cytokines such as interleukin-1b (IL-1b) and prostaglandin E2 (PGE2) induced by LPS in isolated mouse peritoneal macrophages and RAW 264.7 cells (Kang et al., 2004).

2.3.3. Silibinin effect on lipid peroxidation and oxidative stress

An antioxidant property of silibinin has been shown against various oxidative stress-causing agents in different cell models and *in vivo*. Silibinin has been said to be at least ten times more potent in antioxidant activity than vitamin E (Bindoli et al., 1977). Thus, silibinin shows strong antioxidant and anti-tumour activities (Johnson et al., 2002, Singh and Agarwal, 2009).

The ability of silibinin to protect a cell membrane against xenobiotic injury is attributed principally to its antioxidant potential to eliminate reactive oxygen species (ROS) (Basaga et al., 1997, Detaille et al., 2008). It links free radical scavenging in the liver via increasing reduced glutathione (GSH) (Mira et al., 1994, Basaga et al., 1997) and the level of the important antioxidant enzyme superoxide dismutase (Letteron et al., 1990, Muzes et al., 1991, Wang et al.,

2010) which causes a reduction in superoxide anion radical levels (Katiyar, 2002). It has been shown that silibinin inhibits linoleic acid peroxidation catalysed by lipoxygenase (Fiebrich and Koch, 1979) and that silibinin protects rat liver mitochondria and microsomes *in vitro* against the formation of lipid peroxides induced by various agents such as Cumene hydroperoxide (Bindoli et al., 1977). In a large number of studies, the cytoprotective activity of silibinin has been shown in hepatocytes from rats subjected to osmotic stress produced by hypotonic saccharose solutions (Ramellini and Meldolesi, 1976).

The perfused liver was used as experimental model in order to evaluate the effect of substances that induce oxidative stress or provide protection by scavenge. By this experimental model, it has been reported that phenyl hydrazine produces an increase in oxygen consumption in rat liver *in vitro* and in the release of thiobarbituric acid reactive substances (TBARS) in the perfusate (Valenzuela and Guerra, 1985). This stress is associated with a reduction in the amount of reduced glutathione (GSH) in the liver (Videla and Valenzuela, 1982). Pre-treated rat liver *in vivo* with silibinin 50 mg/kg intravenously, showed a significant reduction in the oxygen consumption stimulated by phenylhydrazine and in the release of TBARS (Valenzuela and Guerra, 1985). The antioxidant effect of silibinin was furthermore observed in rats with acute intoxication induced by ethanol (Valenzuela et al., 1985) or acetaminophen (Campos et al., 1989), which are peroxidation inducers that produce marked GSH depletion in the liver.

The hepatoprotective activity of silibinin has also been studied in rats with liver cirrhosis mediated by long-term administration of carbon tetrachloride. Muriel and Mourelle have shown that silibinin preserves the functional and structural integrity of hepatocytes membranes by preventing alterations of their phospholipid's structure produced by carbon tetrachloride and by restoring alkaline phosphatase and GGT activities (Muriel and Mourelle, 1990).

In a study performed in rat hepatocytes treated with tertiary butyl hydroperoxide (TBH), silibinin inhibits lipid peroxidation, and modulates hepatocytes Ca^{2+} content produced by TBH (Farghali et al., 2000). Silibinin has been shown to decrease oxidative injury from a variety of sources, inhibiting lipid peroxidation and membrane damage (Manna et al., 1999). It inhibits enzymatic peroxidation in rats through the lipoxygenase pathway, avoiding leukotriene synthesis (Alarcon de la Lastra et al., 1992). Finally, silibinin has been found to be a potent antioxidant (Bindoli et al., 1977, Kiruthiga et al., 2007, Asghar and Masood, 2008) in cases of exposure to UV radiation (Saliou et al., 1999, Dhanalakshmi et al., 2004a, Dhanalakshmi et al., 2004b).

2.3.4. Silibinin as anti-apoptotic agent

Silibinin provides anti-apoptotic effects in different cell culture models. Silibinin protects ECV-304 cells against H_2O_2-induced injury by increasing the NO content, the activity of GSH-Px and inhibiting signalling pathways mediated by caspase 3 (Wang et al., 2005).

It protects against apoptosis induced by isoproterenol in rat cardiac myocytes, by mitomycin C in human melanoma cells, by ochratoxin A in primary rat hepatocytes, and by UV light in the human HaCaT melanoma cell line (Zhou et al., 2006, Li et al., 2006, Jiang et al., 2009, Essid and Petzinger, 2011). Silibinin blocked the activation of caspase 3 in the HepG2 cell line and in primary rat hepatocytes (Pook et al., 2006, Essid and Petzinger, 2011). Silibinin inhibited UV-induced sunburn cell formation and apoptosis (Katiyar et al., 1997).

2.4. Research objectives

In this thesis work, I have broadened and deepened my studies on apoptosis and its protection by silibinin on three cell culture models in the presence of several pro-apoptotic agents and stimuli. The reason is that the liver is a major organ for systemic release of inflammatory cytokines (e.g. TNF-α), due to its exposure to gut-derived bacterial toxins (e.g. lipopolysaccharides) or mycotoxins (e.g. OTA). There remains a gap in our understanding of the molecular mechanisms that determine which type of signalling will predominate in OTA toxicity. The previous meaning was that TNF-α is an important player in liver regeneration, and promotes the pathogenesis of chronic liver disorders. Meanwhile, the molecular mechanisms of TNF-α signalling in hepatocytes have been postulated. Therefore, the objective of this thesis is shedding the light on the hepatotoxicity of OTA *in vitro*, namely apoptotic pathway by comparing it with well known hepatotoxin apoptosis inducers (H_2O_2, TNF-α and UVC) and the ability of

silibinin to either reduce or prevent these hepatotoxic effects caused by all the studied toxins.

III. Materials

3.1. Chemicals and reagents

All standard chemicals and cell culture reagents utilized in experiments were listed in **table 1**.

Tab. 1: Chemicals and reagents used for the experiments

Chemicals	Distributor
Agarosa NEEO Ultra-Quality	Carl Roth GmbH & Co. Karlsruhe, Germany
Actinomycin D from *Streptomyces* sp.	Sigma-Aldrich Co. Steinheim, Germany
Calcium chloride dihydrate ($CaCl_2$•$2H_2O$)	E. Merck Darmstadt, Germany
Camptothecin ($C_{20}H_{16}N_2O_{4)}$	Sigma-Aldrich Co. Steinheim, Germany
Chloroform ($CHCl_3$)	Carl Roth GmbH & Co. Karlsruhe, Germany
Collagen R from rat tail	SERVA Electrophoresis GmbH, Heidelberg, Germany
Collagenase Type CLS 2	Leonorenstr. 2-6. D-12247 Berlin
Collagenase Type NB4	SERVA Electrophoresis GmbH, Heidelberg, Germany
Copper sulphate pentahydrate ($CuSO_4$•$5H_2O$)	E. Merck Darmstadt, Germany
D(+)-Glucose monohydrate ($C_6H_{12}O_6$•H_2O)	Carl Roth GmbH & Co. Karlsruhe, Germany

DDT ($C_{14}H_9Cl_5$)	Sigma-Aldrich Co. Steinheim, Germany
Dehydrocholic acid sodium salt ($C_{24}H_{33}O_5Na$)	Sigma-Aldrich Co. Steinheim, Germany
Dexamethasone ($C_{22}H_{29}FO_5$)	Sigma-Aldrich Co. Steinheim, Germany
Dextran (Leuconostoc mesenteroides, strain No. B-512)	Sigma-Aldrich Co. Steinheim, Germany
Dimethylsulfoxid (C_2H_6SO)	Carl Roth GmbH & Co. Karlsruhe, Germany
DNA ladder markers	Fermentas, St. Leon-Rot, Germany
Dulbeccos modified Egales medium (DEME) +4.5g/L Glucose +L-Glutamine +Pyruvate	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
Dulbeccos modified Egales medium (DEME) +1g/L without L-glutamine	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
EDTA ($C_{10}H_{12}N_2O_8Na_4$)	Sigma-Aldrich Co. Steinheim, Germany
EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$)	SERVA Electrophoresis GmbH, Heidelberg, Germany
Ethanol (C_2H_6O)	Carl Roth GmbH & Co. Karlsruhe, Germany
Ethidium bromide	Carl Roth GmbH & Co. Karlsruhe, Germany
Fetal calf serum	Sigma-Aldrich Co. Steinheim, Germany
Glucose anhydrous ($C_6H_{12}O_6$)	E. Merck Darmstadt, Germany
Glycin ($C_2H_5NO_2$)	SERVA Electrophoresis GmbH,

	Heidelberg, Germany
Heparin-Natrium-25.000-ratiopharm®	Ratiopharm GmbH
HEPES ($C_8H_{18}N_2O_4S$)	SERVA Electrophoresis GmbH, Heidelberg, Germany
Hoechst Stain (33342)	Sigma-Aldrich Co. Steinheim, Germany
Hydrochloric acid (HCl) 10N	Carl Roth GmbH & Co. Karlsruhe, Germany
Inosine ($C_{10}H_{12}N_4O_5$)	Sigma-Aldrich Co. Steinheim, Germany
Isoamyl alcohol ($C_5H_{12}O$)	Carl Roth GmbH & Co. Karlsruhe, Germany
L-Glutamine	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
Lipopolysaccharide	Sigma-Aldrich Co. Steinheim, Germany
Loading dye 6×	Fermentas, St. Leon-Rot, Germany
Magnesium chloride hexahydrate ($MgCl_2$•$6H_2O$)	E. Merck Darmstadt, Germany
Magnesium sulphate heptahydrate ($MgSO_4$•$7H_2O$)	E. Merck Darmstadt, Germany
Mangansum sulphate monohydrate ($MgSO_4$•H_2O)	Sigma-Aldrich Co. Steinheim, Germany
Ochratoxin A (MT-I-161A) ($C_{20}H_{18}CINO_6$)	CSIR, Food Science and Technology, Pretoria, South Africa
Paraformaldehyde	Carl Roth GmbH & Co. Karlsruhe, Germany
Pefabloc SC-Protease Inhibitor	Carl Roth GmbH & Co. Karlsruhe,

($C_8H_{10}NSO_2F \times HCl$)	Germany
Protease inhibitor cocktail	Sigma-Aldrich Co. Steinheim, Germany
Proteinase K	Fermentas, St. Leon-Rot, Germany
Penicillin-Streptomycin	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
Percoll™	GE Healthcare Europe Freiburg Germany
Phenol (C_6H_5OH)	Carl Roth GmbH & Co. Karlsruhe, Germany
Potassium chloride (KCl)	E. Merck Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO4)	E. Merck Darmstadt, Germany
Potassium hydroxide (KOH)	E. Merck Darmstadt, Germany
Purified bovine albumin	E. Merck Darmstadt, Germany
RNase A	Fermentas, St. Leon-Rot, Germany
Recombinant human insulin	SERVA Electrophoresis GmbH, Heidelberg, Germany
Recombinant rat tumour necrosis factor alpha	Sigma-Aldrich Co. Steinheim, Germany
SDS ($NaC_{12}H_{25}SO_4$) ultra pure	Carl Roth GmbH & Co. Karlsruhe, Germany
Silibinin	Sigma-Aldrich Co. Steinheim, Germany
Soluble tumour necrosis factor receptor 1 (sTNFR1)	Obtained from Prof. Dr. Joachim Roth, Institute of Physiology, Justus-Liebig-University Gießen, Germany

Soluble tumour necrosis factor receptor 2 (sTNFR2) Etancercept	Obtained from Dr. Markus Donner, Hepatology department, Heinrich-Heine- university, Düsseldorf, Germany
Sodium acetate ($C_2H_3O_2Na•3H_2O$)	Carl Roth GmbH & Co. Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. Karlsruhe, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Carl Roth GmbH & Co. Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co. Karlsruhe, Germany
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4•2H_2O$)	E. Merck Darmstadt, Germany
Sodium DL-lactate solution (60%)	Sigma-Aldrich Co. Steinheim, Germany
Sodium pyruvate ($C_3H_3NaO_3$)	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
Sucrose ($C_{12}H_{22}O_{11}$)	Carl Roth GmbH & Co. Karlsruhe, Germany
Thiazoly blue ($C_{18}H_{16}BrN_5S$)	Carl Roth GmbH & Co. Karlsruhe, Germany
TNF-α antagonist I (H-3736)	Bachem AG, Bubendorf, Switzerland
TNF-α antagonist II (N-1685)	Bachem AG, Bubendorf, Switzerland
Tris ($C_4H_{11}NO_{3)}$	E. Merck Darmstadt, Germany
Triton ×-100 ($C_{14}H_{22}O(C_2H_4O)_n$)	Sigma-Aldrich Co. Steinheim,

	Germany
Trypan blue	Sigma-Aldrich Co. Steinheim, Germany
Trypsin EDTA	PAA Laboratories GmbH, Haidmannweg 9. A-4061 Pasching
Urethane ($C_3H_7NO_2$)	Fluka Chemie AG Buchs, Germany

3.2. Kits

For the assessment of cell viability, caspase 3 activation of cultured primary rat hepatocytes and lipid peroxidation, reactive oxygen species of HPCT-1E3, the kits in **table 2** were used.

Tab. 2: Kits implemented in the investigations with primary rat hepatocytes or with HPCT-1E3 cells

Assay kits	**Distributor**
LIVE/DEAD®Reduced Biohazard Viability/Cytotoxicity Kit #1 (L-7013)	Invitrogen Molecular Probes, Karlsruhe, Germany
Caspase-3 Colorimetric Assay Kit Cat.-No. (PK-CA577-K106-200)	PromoCell GmbH, Heidelberg, Germany
BCA protein assay kit	Novagen (Merck KGaA), Darmstadt, Germany
Lipid Peroxidation (MDA) Assay Kit Cat.-No. (ab118970)	Abcam, Cambridge, United Kingdom
Reactive Oxygen Species (ROS), Cat.-No. (ABIN511831)	Antikoerper-online.de, Aachen, Germany

3.3. Equipment

The following standard laboratory equipment and apparatus were used for the experiments listed in **table 3**.

Tab. 3: Used equipment and apparatus and their specifications

Equipment	Model and distributor
Autoclave	SANOClav, Lam-201, Geislineen, Germany
Balance	CAHN Microbalance C-30. INC. Cerritos, California, USA
Balance	METTLER AE 260, DeltaRange®, Gießen, Germany
Benchmark microplate reader	BIO-RAD laboratories GmbH, Munch, Germany
Biohazard Laminar flow cabinet	danLAF® VFR 1806, Denmark
Bio photometer	Eppendorf AG, Hamburg, Germany
Centrifuge	Type 5415 Eppendorf-Nether-Hinz GmbH Hamburg, Germany
Centrifuge	Type 5417R Eppendorf AG, Hamburg, Germany
Centrifuge	BHG HERMLE Z2364, Gosheim, Germany
Fluorescence microscope	Leica DM IRE2, Wetzlar, Germany
Fluorescence microscope	Nikon Eclipse 80i, Japan

Freezer (-20°C)	LIEBHERR Premium, -20
Freezer (-80°C)	Nap Coil UF 400
Gradient centrifuge	Sigma 4k15, sigma international
Heating magnetic stirrer	Heidolph MR82
Heidolph Titramax 100	MAGV Laborbedarf Rabenau-Londorf, Germany
Incubator	New Brunswick Scientific co., INC. Edison, New Jersey, U.S.A
Light microscope	ORTHOMAT™ Leitz Fluovert, Leitz Wetzlar, Germany
Light microscope	Olympus IX70-S8F, Olympus Optical CO. LTD, Japan
Microliter pipettes (10 µl – 1000 µl)	Eppendorf-Nether-Hinz GmbH, Hamburg, Germany
Microplate reader	GloMax®-Multi Detection System, Promeg, USA
Perfusion system	House made, Giessen, Germany
pH-meter	CG 820, Schott-Geräte GmbH, D6238 Hofheim, Germany
PowerPac 3000	BIO-RAD
Refrigerator	LIEBHERR Premium
Spectrophotometer	Type DU® 640, Beckman Coulter GMBH, USA
Surgical and anatomical set	Hebu GmbH Weilheim, Germany

Thermomixer comfort	Eppendorf AG, Hamburg, Germany
UV irradiator	Stratalinker® UV Crosslinker 1800, Stratagene, USA
Vortex mixer	Heidolph, REAX IDR, Germany
Water bath	Gesellschaft für Labortechnik GmbH, D3006, Burgwedel, Germany

3.4. Disposable materials

All the following disposable materials were obtained from Sarstedt, Aktiengesellschaft, Co., Nümbrecht, Germany and Corning B.V., Amsterdam, Netherlands.

- Syringes (1ml, 5ml, 10ml and 20ml)
- Non pyogenic microcentrifuge tubes (1.5, 2, 15, 50 ml)
- Tissue culture plates (different sizes)
- Blue, yellow and white pipette tips
- Cell Lifter, 18 cm, S IND, 1/100.
- Absorbent paper.

3.5. Animals

Male Wistar rats (200-280 g) were used in all experiments under the approvement of Regierungspräsidium Gießen, (V54- 19 c 20-15 (1) GI 18/11-Nr.31/2007).The animals were fed ad libitum with Altromin® standard diet and received water ad libitum. They were kept under a 12-h light-dark cycle at 22°C temperature and ventilation under standard conditions. The health of rats was routinely tested by sentinel animals and the animals were found to be free of chronic infections and parasites.

3.6. Cell types

We used in this thesis three different liver cells **(Tab. 4)**:

1) Primary hepatocytes from Wister rat **(Fig. 5a)**.

2) HPCT-1E3 (hepatocytoma) cells are of rat origin and were obtained by fusion of rat hepatoma cells with rat hepatocytes (Petzinger et al., 1994, Blumrich et al., 1994). They were regarded as proliferating cells with characteristic properties of hepatocytes that combine the capacity for proliferation with the maintenance of important liver-specific enzyme activities and metabolic functions (Katz et al., 1992). Moreover, HPCT-1E3 cells are a good model for studying cytotoxicity that expresses several hepatocytes specific properties in contrast to other immortal cell lines **(Fig. 5b)**. Their suitability as an *in vitro* model has to been proven in order to replace *in vivo*

experiments (Kneuer et al., 2007).

3) HepG2 cells, they were originally derived in 1979 from an 11-year old Argentine boy (Aden et al., 1979), and were in culture with the intention of replacing fresh human hepatocytes in early toxicology screening. HepG2 cells are considered to be suitable *in vitro* models of the HCC since they have been shown to possess in part characteristics typical for primary hepatocytes **(Fig. 5c)**. Under cell culture conditions, they secrete most of the plasma proteins, such as fibrinogen, albumin and apolipoproteins (Knowles et al., 1980). The successful application of HepG2 cells in toxicology is mainly based on the capability of the cells to synthesize enzymes for xenobiotic metabolism comparable with normal human hepatocytes (Wilkening and Bader, 2003, Wilkening et al., 2003, Hewitt and Hewitt, 2004).

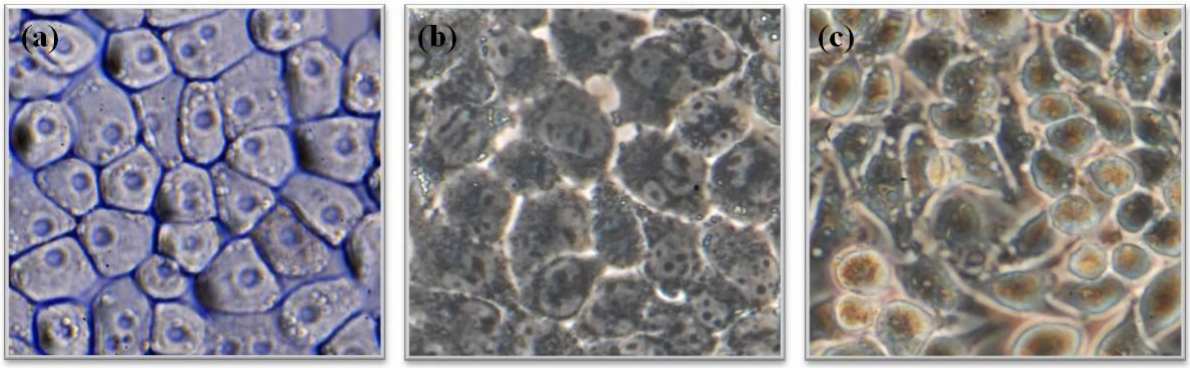

Fig. 5: Morphology of cultivated liver cells

Primary rat hepatocytes (a), HPCT-1E3 cells (b), and (c) HepG2 cells in monolayer cultures.

Tab. 4: Used cells types and their specifications

Cell name	Identification	Source
Hepatocytes	Rat primary cells	Prepared in Pharmacology & Toxicology Institute, Justus-Liebig-University Gießen
HPCT-1E3	rat hepatocytoma cell line	Pharmacology and Toxicology Institute, Justus-Liebig-University Gießen
HepG2	Human hepatoma cell line	Obtained from Dr. Ayub Darji, Institute of Medical Microbiology, Justus-Liebig-University Gießen

3.7. Solutions and buffers

3.7.1. Primary rat hepatocytes isolation buffers

3.7.1.1. Collagenase perfusion method buffers

The buffers used in the isolation of primary rat hepatocytes by the collagenase perfusion method are listed in **table 5**.

Tab. 5: Buffers and their chemical composition for the collagenase perfusion method

Buffer/Solution	Chemical/Solution	Weighted sample/Volume	Final concentration
Urethane 20%	$C_3H_7NO_2$	20 g	20%
	NaCl	0.9 g	0.9%

	$C_6H_{12}O_6$ Dissolve in 100 ml deionised water Storage: RT	5 g	5%
HCl solution 2N	HCl (10N) Deionised water Storage: RT	10 ml 40 ml	2N -
Krebs-Henseleit buffer Ca^{2+} free pH 7.4	$NaHCO_3$	2.12 g	25.2 mM
	NaCl	6.9 g	118.5 mM
	KCl	353 mg	4.73 mM
	$MgSO_4.7H_2O$	284 mg	1.15 mM
	KH_2PO_4	140.2 mg	1.03 mM
	Complete to 1000 ml by deionised water, gassing with O_2:CO_2, 95:5%; pH adjusted by HCl (2N) & store at RT.		
Tyrode Buffer pH 7.4	$C_6H_{12}O_6$	1.0 g	5.55 mM
	$NaHCO_3$	1.0 g	11.9 mM
	NaCl	8.0 g	137 mM
	KCl	0.2 g	2.7 mM

	$MgCl_2$	0.214 g	1.05 mM
	$NaH_2PO_4.H_2O$	58 mg	0.42 mM
	$CaCl_2.2H_2O$	264.7 mg	1.8 mM
	Complete to 1000 ml by deionised water, gassing with O_2:CO_2, 95:5%; pH adjusted by HCl (2N) & store at RT.		

3.7.1.2. EDTA perfusion method buffers

The buffers used in the isolation of primary rat hepatocytes by EDTA perfusion method are listed in **table 6**.

Tab. 6: Buffers and their chemical composition for EDTA perfusion method

Buffer/Solution	Chemical/Solution	Weighted sample/Volume	Final concentration
Perfusion Buffer pH 7.4	NaCl	8.2 g	140 mM
	KCl	190 mg	2.6 mM
	$MgCl_2.6H_2O$	163 mg	0.8 mM
	$Na_2HPO_4.2H_2O$	285 mg	1.6 mM
	KH_2PO_4	54.4 mg	0.4 mM
	$NaHCO_3$	2.1 g	25 mM
	EDTA (0.5 M)	4 ml	2 mM

	$C_6H_{12}O_6.H_2O$	3 g	15 mM
	Lactate solution (0.5 M)	4 ml	2 mM
	Sodium pyruvate	2 ml	0.2 mM
	Complete to 1000 ml by deionised water, gassing with O_2:CO_2, 95:5%; pH adjusted by HCl (2N) & store at +4°C.		

Washing Buffer pH 7.4	NaCl	4.1 g	140 mM
	KCl	95 mg	2.6 mM
	$MgCl_2.6H_2O$	81.5 mg	0.8 mM
	$Na_2HPO_4.2H_2O$	142.5 mg	1.6 mM
	KH_2PO_4	27.2 mg	0.4 mM
	$CaCl_2.2H_2O$	73.5 mg	1 mM
	Complete the volume to 500 ml. Adjust pH by HCl (2N) & store at +4°C.		

Percoll gradient centrifugation solution.	NaCl	344 mg	184 mM
	KCl	8 mg	3.4 mM
	$MgCl_2.6H_2O$	7 mg	1.1 mM
	$Na_2HPO_4.2H_2O$	12 mg	0.21 mM
	KH_2PO_4	2.3 mg	0.53 mM

	Percoll Complete the volume to 32 ml by deionised water. Storage : +4°C	27.8 ml	67%

3.7.2. MTT assay solutions

The solutions used in MTT test are listed in **table 7**.

Tab. 7: Solutions and their chemical composition for MTT test

Buffer/Solution	**Chemical/Solution**	**Weighted sample/Volume**	**Final concentration**
PBS pH 7.4	NaCl	8.0 g	137 mM
	KCl	0.2 g	2.68 mM
	KH_2PO_4	0.2 g	1.47 mM
	$Na_2HPO_4 \cdot 2H_2O$	1.3 g	9.4 mM
	Complete volume to 1000 ml by deionised water, adjust pH by HCl (2N) & store at RT.		
Thiazoly blue Solution	$C_{18}H_{16}BrN_5S$ Add 10 ml PBS to dissolve it & store at +4°C, in light protected bottle.	50 mg	12.068 mM

Isopropanol solution	C_3H_8O HCl (10 N) Storage: RT	98.86 ml 1.14 ml	98.86% 1.14%

3.7.3. Live/Dead kit buffers

The solutions used with Live/Dead kit, are listed in **table 8**.

Tab. 8: Solutions and their chemical composition for Live/Dead kit

Buffer/Solution	Chemical/Solution	Weighted sample/Volume	Final concentration
KOH 1M	KOH Deionised water Storage: RT	2.81 g 50 ml	1 M -
HBSS pH 7.4	HEPES Dissolve in 100 ml of deionised water. Adjust pH by KOH (1M) & store at RT.	23.83 g	1 M
Dye solution	HBSS SYTO®10 green DEAD red Mix thoroughly by vortexing. Store at +4°C in light protected bottle & use it directly.	1000 µl 2 µl 1 µl	99.7% 0.2% 0.1%

3.7.4. Chromatin staining solutions

The solutions used with chromatin staining are listed in **table 9**.

Tab. 9: Solutions and their chemical composition for chromatin staining

Buffer/Solution	Chemical/Solution	Weighted sample/Volume	Final concentration
PBS pH 7.4	NaCl	8.0 g	137 mM
	KCl	0.2 g	2.68 mM
	KH_2PO_4	0.2 g	1.47 mM
	$Na_2HPO_4 \bullet 2H_2O$	1.3 g	9.4 mM
	Complete volume to 1000 ml by deionised water, adjust pH by HCl (2N) & store at RT.		
PFA pH 7.4	$(CH_2O)N$	2 g	2%
	NaOH (1N)	15 drops	-
	Add 20 ml deionised water and heat at +50°C to dissolve it; complete volume to 100 ml by deionised water. Adjust pH by HCl (2N) & store at -20°C.		
Hoechst stain solution	Hoechst 33342	1.686 mg	2 mM
	Add 1.5 ml deionised		

	water to dissolve it. Store at +4°C in light protected bottle.		

3.7.5. DNA laddering buffers

The buffers required for the DNA laddering technique are listed in **table 10**.

Tab. 10: Buffers and their chemical composition for DNA laddering

Buffer/Solution	**Chemical/Solution**	**Weighted sample/Volume**	**Final concentration**
TTE-Buffer pH 8.0	Tris (1M)	2.5 ml	10 mM
	EDTA (0.5M)	5.0 ml	10 mM
	NaCl	365.25 mg	25 mM
	Triton (20%)	2.5 ml	0.2%
	SDS (20%)	12.5 ml	1%
	Complete volume to 250 ml by deionised water, adjust pH by HCl (2N) & store at RT.		
TET Buffer pH 8.0	Tris (1M)	2.5 ml	10 mM
	EDTA (0.5M)	0.5 ml	1 mM
	Triton (20%)	6.25 ml	0.5%

	Complete volume to 250 ml by deionised water, adjust pH by HCl (2N) & store at RT.		
Phenol sol.	Phenol	25 ml	50%
	Chloroform	24 ml	48%
	Isoamyl alcohol	1 ml	2%
	Fresh prepared Storage: RT		
Na acetate pH 5.2	($C_2H_3O_2Na•3H_2O$)	40.81 g	3 M
	Dissolve in 100 ml deionised water, adjust pH by glacial acetic acid & store at RT.		
TE-Buffer pH 8.0	Tris (1M)	2.5 ml	10 mM
	EDTA (0.5M)	5.0 ml	10 mM
	NaCl	365.25 mg	25 mM
	Complete volume to 250 ml by deionised water, adjust pH by HCl (2N) & store at RT.		

TAE-Buffer pH 8.0	Tris	4.844 g	40 mM
	EDTA (0.5M)	2.0 ml	1 mM
	Complete volume to 1000 ml by deionised water, adjust pH by glacial acetic acid & store at RT.		

IV. Methods

4.1. Preparation of tested compounds and positive controls

All compounds were weighted using a µg-sensitive balance (CAHN Microbalance C-30. INC. Cerritos, California, USA), then dissolved according to their chemical properties either in DMSO (OTA, SB, CPT, and ActD) or sterile distilled water (LPS and TNF-α), and stored at +4°C no longer than one month.

- Dimethylsulfoxide (DMSO)

As solvent control either dimethyl sulfoxide (DMSO) or distilled water were used. Both were sterile filtered before use. Since DMSO has been shown to be cytotoxic to complex mammalian cell systems, the effect of the solvent, especially, on apoptosis induction and the mitochondrial membrane potential (MMP) of rat primary hepatocytes, HepG2, and HPCT-1E3 cells, was tested in preliminary experiments. No alteration of the test parameters could be detected at a concentration of 0.1% DMSO, which was then used in all further experiments. Noticeably, in concentrations exceeding 0.3%, alterations in ssDNA formation and an influence on the MMP occurred in the treated cultures.

- Hydrogen peroxide (H_2O_2)

The measurement of oxidative stress in HPCT-1E3 and HepG2 cells was done using 10 mM H_2O_2, incubated for 6 h, as positive control in these experiments.

H_2O_2 has been successfully used earlier in studies investigating the oxidative stress *in vitro* (Guo et al., 2010, Kumar and Gupta, 2011).

- Camptothecin (CPT)

Exposure of HepG2 cells to 6 µM CPT for 24 h was used as positive control in the apoptosis assays. This plant alkaloid indirectly causes apoptosis by binding to the enzyme topoisomerase I rendering cells to apoptosis induction by the formation of DNA single strand breaks (Capranico et al., 2007).

- Lipopolysaccharides

LPS act as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of proinflammatory cytokines e.g TNF-α in many cell types, but especially in macrophages and B-cells. LPS was used in this study as control positive for TNF-α release (Yuan et al., 1998).

4.2. Isolation of hepatocytes

The hepatocytes were prepared by either the collagenase ex corpora perfusion method as described by (Petzinger et al., 1989) or the EDTA in situ perfusion method as described by (Wang et al., 1985).

4.2.1. Rat anaesthesia

The rats were anesthetized by an intra-peritoneal injection of 1-1.5 ml 20% urethane solution (1 g/ kg of b.w.) to produce deep degree of analgesia with minimal physiological changes. The anesthetized rats were placed in a

polystyrene box. The completeness of anaesthesia was verified by compressing the upper area of the tail with tweezers to test the abdominal muscle reflex (the twitching of the abdominal muscles together means that, the animal was not stunned enough). After full anaesthesia, the rats were placed and fixed on the operation table after which the abdominal region was disinfected with 70% (v/v) ethanol. Then they were heparinised with 0.3 ml/kg of b.w. Liquemin® 5000 IU/ ml in 0.9% NaCl i.v into the femoral vein.

4.2.2. Collagenase perfusion method

After the heparinisation of the rats in order to prevent blood clotting and to maximize blood clearance from the liver, laparotomy was performed by the removal of the abdominal skin. The abdomen was incised longitudinally along the linea alba up to xiphoid process (Sternum). Then the liver hilum was exposed by displacing the viscera.

Two ligatures were loosely placed around the portal vein and one around the caudal vena cava, just above the right renal vein. After that an infusion catheter was guided into the portal vein, which was fixed by tightening the ligatures. The caudal vena cava was cut just before the ligature, and immediately the liver was perfused by pre-warmed Krebs-Henseleit Ca^{2+} Free-buffer ($NaHCO_3$ 25.2 mM, NaCl 18.5 mM, KCl 4.73 mM, $MgSO_4$. $7H_2O$ 1.15 mM and KH_2PO_4 1.03 mM, pH 7.4 equilibrated with O_2:CO_2, 95:5%, 37°C) through the infusion catheter in the portal vein using a peristaltic pump to flush out the blood from the liver and

rats were killed by exsanguination. After ca. 5 min a blood-free liver was obtained. The chest was cut by opening the diaphragm. This leads to the collapse of the lungs. Immediately afterwards, the cranial vena cava was cannulated and secured by a ligature while the flow of the perfusate continued from the portal vein through the cranial vena cava after closing the caudal vena cava by tightening the loosely placed ligature.

Liver perfusion: the blood free liver was carefully dissected from the animal and placed in an experimental perfusion setup **(Fig. 6)**, installed in a temperature controlled hood.

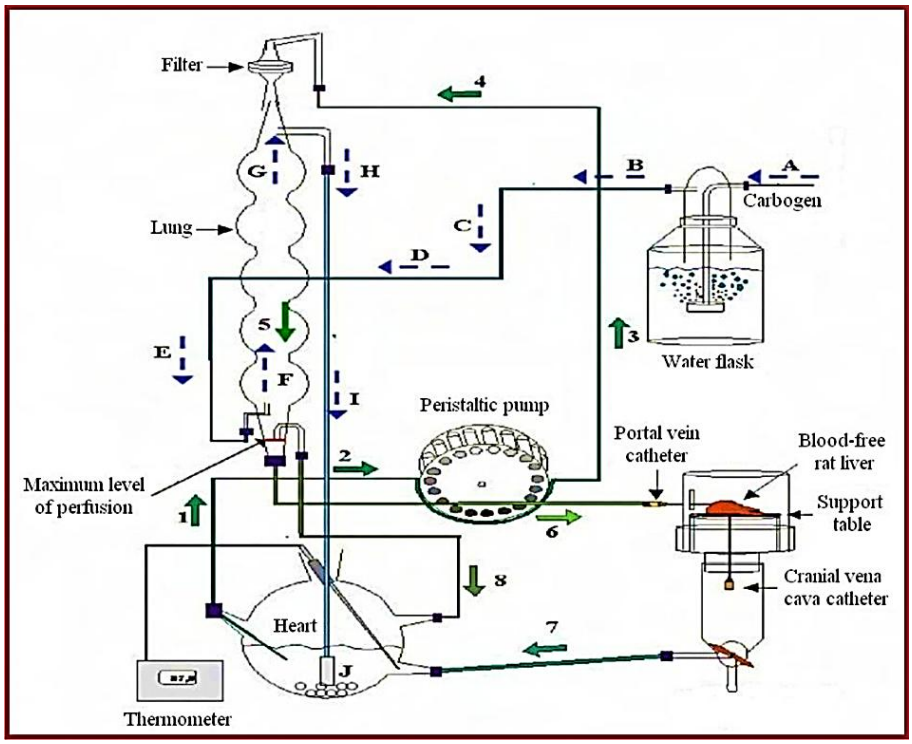

Fig. 6: The experimental perfusion setup

The figure shows the flow of gas and collagenase solution in the experimental perfusion setup. The flow of O_2\CO_2 gas comes from (A) a carbogen gas bottle through a water bottle, after that moves through a tube (B, C, D, and E) to enter the artificial lung (F and G) and leaves the lung through the tube (H and I) to reach the collagenase solution (J). The collagenase solution starts from the artificial heart (1 and 2) by the action of a peristaltic pump, and then passes (3 and 4) a filter on the top of the lung. The solution accumulates in the bottom of the lung and passes (6) from the maximum fluid level in the basal part of the lung to the liver via the portal vein catheter. The solution leaves the liver through the cranial vena cava catheter and circulates back to the heart (7). The extra pumped fluid returns to the heart as shown by label (8).

The liver then was perfused with 75 ml of Krebs-Henseleit Ca^{2+}-Free-buffer containing 60 mg collagenase type NB4 for 10-15 min in a temperature controlled hood at 37°C under 95% O_2-5% CO_2 gassing. The duration of the perfusion was determined by adspection of the liver; when the liver appeared

softened and the buffer was oozing through the capsule the perfusion was stopped **(Fig.7)**.

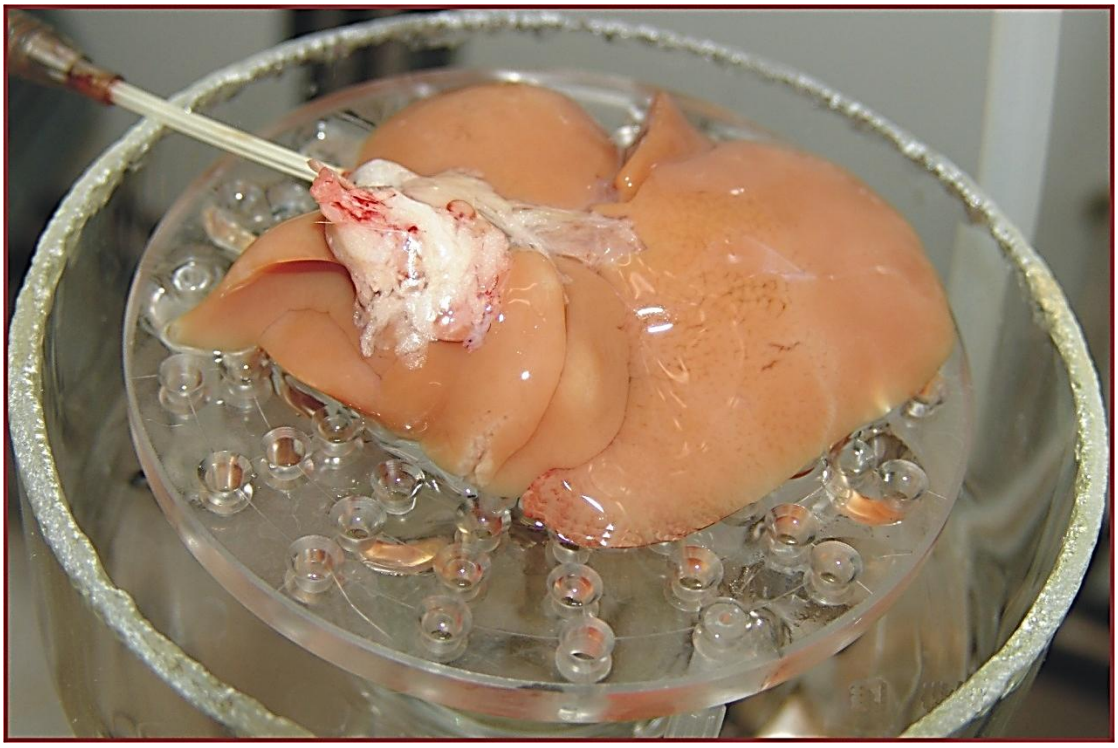

Fig. 7: Perfused blood-free rat liver

The liver of a male Wistar rat after perfusion by collagenase solution for ca. 15 min. The liver held together by its liver capsule shows no coherent tissue structure and perfusion solutions leaks from the bottom of the plastic plate.

Cell isolation: in this stage the liver carefully placed in a 100 ml beaker and minced with scissors. The liver pieces were digested for 3 min in perfusate solution by bubbling gas. The cell suspension was filtered through double layers of sterile gauze into 4 tubes. The volume was adjusted by Tyrode buffer (glucose 5.6 mM, $NaHCO_3$ 11.9 mM, NaCl 137 mM, KCl 2.7 mM and $MgCl_2$ 1.05, $NaH_2PO_4.H_2O$ 0.42 mM and $CaCl_2.2H_2O$ 1.8 mM, pH 7.4 equilibrated with O_2:CO_2, 95:5%, 37°C) and centrifuged at 350 rpm for 5 min (*BHG*

HERMLE Z2364). The pellet was re-suspended by Tyrode buffer then washed two times.

The cell suspension was filtered through 4-6 layers gauze in a 100 ml beaker and regenerated through incubation of the beaker in a shaker water bath (*Julabo SW1*) adjusted at 37^{o}C and provided by 95% O_2 -5% CO_2 gas for 15 min, then centrifuged again (*BHG HERMLE Z2364*).

Finally, the cell pellet was re-suspended in Dulbecco's modified Eagles medium (DMEM) with low glucose (1 g/l) containing 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml).

4.2.3. EDTA dissociation method

The hepatocytes were isolated by dissolving the cell bonds of the liver by perfusion with Ca^{2+}-free buffer containing- EDTA *in situ*. They were purified by low-speed centrifugation using a Percoll step-density gradient from non-parenchymal cells and debris.

Surgery: briefly, the liver perfusion was performed *in situ* by perfusing pre-warmed perfusion buffer (NaCl 140 mM, KCl 2.6 mM, $MgCl_2.6H_2O$ 0.8 mM, $Na_2HPO_4.2H_2O$ 1.6 mM, KH_2PO_4 0.4 mM, $NaHCO_3$ 25 mM, EDTA 2 mM, glucose 15 mM, lactate 2 mM and sodium pyruvate 0.2 mM, pH 7.4 equilibrated with O_2:CO_2, 95:5%, 37^{o}C) through the infusion catheter in the portal vein using a peristaltic pump to flush out the blood through caudal vena cava for ca. 5-10 min and rats were killed by exsanguination.

Liver perfusion: the direction of the perfusate flow was changed in this method from the portal vein through the cranial vena cava by ligating the caudal vena cava for ca. 30-45 min. The effluent perfusate was not re-circulated and the duration of perfusion was determined by the appearance of the liver. When the softening of the liver was evident and the buffer began to ooze through the liver capsule then the perfusion was stopped **(Fig. 8 A and B)**.

Cell isolation: the softened liver was excised and cut gently by scissor into small pieces; the pieces were put in a beaker containing 10 ml of pre-warmed washing buffer (NaCl 140 mM, KCl 2.6 mM, $MgCl_2.6H_2O$ 0.8 mM, $Na_2HPO_4.2H_2O$ 1.6 mM, KH_2PO_4 0.4 mM and $CaCl_2.2H_2O$ 1 mM, pH 7.4 equilibrated with O_2:CO_2, 95:5%, 37°C). An additional 10 ml of washing buffer was added, while gently swirling the beaker and the cell suspension was filtered through a nylon mesh (100 micron grid size).The cell suspension was then transferred to 50 ml Falcon tubes, centrifuged at 300 rpm/3 min, the supernatant was aspired, the cells were re-suspended by washing buffer and centrifuged at 450 rpm/5 min. After that, the pellet was re-suspended with 16 ml washing buffer and the volume was completed to 48 ml by adding 32 ml Percoll. The solution was swirled gently followed by centrifugation at 2100 rpm/5 min. After centrifugation, the intact cells pelleted, whereas damaged cells and cell debris floated at the top of the Percoll solution **(Fig. 8C and D)**. After discarding the upper layer and the supernatant, the cell pellet was re-suspended in Dulbecco's modified Eagles medium (DMEM) low glucose (1 g/l) containing 10% heat-

inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml).

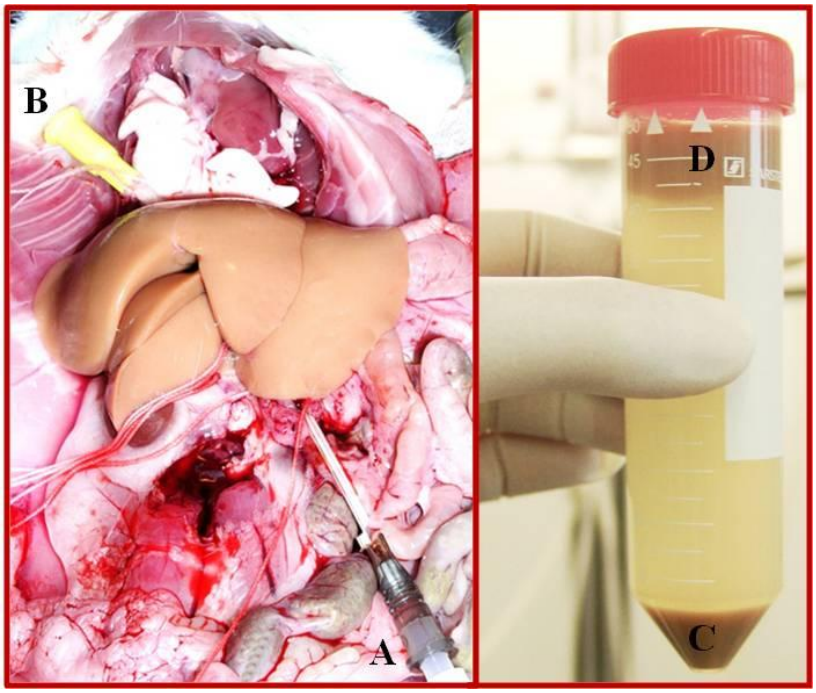

Fig. 8: perfused liver by EDTA buffer

The liver was perfused by EDTA buffer *in situ* in non re-circulated manner. The EDTA buffer passes to the liver via the portal vein catheter (A) and leaves the liver through the cranial vena cava (B) for ca.30-45 min. The intact hepatocytes (C) were purified from non-parenchymal cells, damaged cells and cell debris (D) by low-speed centrifugation using a Percoll step-density gradient.

4.3. Cell counting

1) 400 µl Tyrode buffer

2) 50 µl cell suspension

3) 50 µl trypan blue stain

Cell counting was achieved by filling a Bürker-Türk counting chamber with 10 µl obtained from a dissolute cell suspension that consisted of 400 µl Tyrode buffer, 50 µl cell suspension , and 50 µl trypan blue stain . The mean of live

cells in four large counted squares was taken and divided by 10 to get the number of cells in million per ml of original suspension. Then the cell number was adjusted to 1×10^6 cells/ml into the particular cell medium. The vitality always exceeded 80% by the collagenase perfusion method and reached up to 98% by the EDTA dissociation method.

4.4. Culture of hepatocytes

Isolated hepatocytes were seeded on rat tail collagen (0.5 mg/ml) coated Petri dishes or multiwell plates in Dulbecco's modified Eagle medium with low glucose (1 g/l) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin and left at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 h to attach. After that the medium was changed and the experiment was carried out.

4.5. Culture of HepG2 and HPCT-1E3 cells

HepG2 cells were supplemented with fresh Dulbecco's modified Eagle medium with high glucose (4.5 g/l) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin, while HPCT-1E3 cells with fresh Dulbecco's modified Eagle medium with high glucose (4.5 g/l) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, 10 µg/ml insulin, 10 µg/ml inosine, 1.5 µM dexamethasone, 100 IU/ml penicillin, and 100 mg/ml streptomycin every second to third day. The cells were passaged about once a

week. All cell treatment steps described in this chapter were carried out with fluids pre-warmed to 37°C.

4.5.1. Cryopreservation and reactivation of the cell culture

HepG2 and HPCT-1E3 cells were stored at minus 196°C in liquid nitrogen until use. For cryopreservation, the cells were harvested by trypsination, re-suspended in culture medium and pelleted at 3000 rpm for 2 min at RT. The supernatant was discarded and 3×10^6 cells re-suspended in 1.5 ml of cryopreservation medium (FCS containing 10% DMSO). The cell suspension was transferred to a cryo tubes and stored at -20°C overnight then to minus 196°C. For defrosting, the cells were put into a water bath at 37°C and subsequently pelleted at 3000 rpm for 2 min at RT. The cells were washed twice with culture medium, re-suspended in 5 ml fresh culture medium and transferred to a T25 culture flask for cultivation. 24 h later, the medium was refreshed to remove dead cells.

4.5.2. Cell culture and passage

Sub-confluent cell cultures were harvested by trypsination: adherent HepG2 and HPCT-1E3 cells were washed twice with PBS and incubated with a 0.1% trypsin-EDTA-solution for 5 min at 37°C. The enzymatic reaction was stopped with culture medium, and the cell number/vitality determined by trypan blue exclusion as mentioned above. An appropriate amount was then transferred to a

new culture flask and incubated in a cell culture incubator at 37°C in a humidified atmosphere of 5% CO_2 in air.

4.6. Procedure of compound exposures

For chemical exposure, primary rat hepatocytes, HepG2, and HPCT-1E3 cells were seeded in different type plates at different densities, according to type of test. Exponential growing cells were then treated with the chemicals for the indicated time points.

4.7. Sensitization of cells by ActD

After the replacing culture medium from the adherent cells ActD (200 ng/ml) was added for 30 min, and then rat recombinant TNF-α was given to some dishes, at a concentration of 20 ng/ml unless otherwise indicated.

4.8. Cytotoxicity assay

Cytotoxicity of OTA, LPS, ActD, TNF-α, H_2O_2, and UVC was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983), detecting the cellular mitochondrial activity to convert MTT tetrazolium salt to water-insoluble formazan. 5×10^4 /100 µl DMEM hepatocytes seeded in 96 well tissue culture plates coated with collagen type I while HepG2 and HPCT-1E3 cells were seeded in non coated plates. The cells pre-incubated 3 h prior to the addition of OTA at concentrations 0, 0.5, 2.5 and 12.5 µM or LPS at 0.1, 0.5, 2.5 and 12.5 µg/ml or ActD at 0, 50, 100, 200, and

333 ng/ml or TNF-α at 0, 10, 15, 20, and 30 ng/ml or combination of 200 ng/ml ActD and of 10, 15, 20, and 30 ng/ml TNF-α or H_2O_2 at 0, 5, 10, and 15 mM into incubation media up 24, 36 h or radiation of UVC at 0, 25, and 50 mJ/cm^2. Another cell cultures pre-treated with and without silibinin 2 hours prior to the application of hepatotoxins. After that, the cells were incubated with 10 µl of MTT solution for 3 hours.

The formation of formazan crystals was pursued occasionally under the microscope. Subsequently, the medium was aspired and the formazan crystals were lysed by adding 100 µl isopropanol solution and mixed to ensure the dissolution of the crystals. Finally, the absorbance was measured by Benchmark microplate reader at dual wavelength (550 nm-655 nm). Plates were normally read within 1 h of adding the isopropanol. Cell viability rate was expressed as percentage of formazan absorbance of non-treated viable cells under each treatment regimen as following: % viability= (experimental absorbance/mean control absorbance) × 100.

4.9. Cell viability

The viability of isolated hepatocytes and HepG2 cells was evaluated using the LIVE/DEAD kit according to instructions. SYTO® 10, a green fluorescent nucleic acid stain, is a highly membrane permanent dye and labels all cells, including those with intact plasma membranes. DEAD Red (ethidium homodimer-2) is a cell membrane impermeable red fluorescent nucleic acid

stain that labels only cells with compromised membranes. Briefly, 0.5 ml cell suspensions containing (3×10^5 cells) were seeded on sterile glass cover slips. After 3 h incubation, the cells were pre-treated with and without silibinin at the given concentrations 2 h prior to the administration of OTA at 2.5 and 12.5 μM or LPS at 12.5 μg/ml for 24 h. After that, the cells were washed with HBSS before the dye mixture of LIVE/DEAD kit was added for 15 min at room temperature in darkness. Finally, cells were washed with HBSS again and were visualized under a fluorescence microscope. Fluorescence imaging was performed on a Leica DM IRE2 fluorescence microscope supplied with FITC filter (excitation 490 nm, emission 520 nm) and Texas Red filter (excitation 596 nm, emission 620 nm) from Leica, Wetzlar, Germany. The captured images were analysed with the Leica Fluorescence Workstation software (FW4000). Live cells appeared in green fluorescence and dead cells in red.

4.10. Techniques for the assessment of apoptosis induction

4.10.1. Analysis of hepatocyte nuclear morphology

In order to visually assess apoptosis induction by OTA, ActD/TNF-α, H_2O_2, and UVC, I stained the chromatin of fixed primary hepatocytes monolayer with DNA binding fluorochrome Hoechst stain 33342 and counted apoptotic nuclei using fluorescence microscopy. Briefly, 1 ml cell suspension containing 2×10^5 cells were seeded on collagen-coated two-well Lab-tek tissue culture chamber slides. After 3 h of incubation at 37°C, the cells were pre-treated with and

without silibinin at the given concentrations 2 h prior to the administration of 12.5 µM OTA, or ActD/TNF-α 200/20 ng/ml separately and in combination or 10 mM H_2O_2 or irradiated with UV light (254 nm; Stratalinker® UV crosslinker 1800, Stratagene, United States at a dose of 50 mJ/cm^2) for 24 h. After that, the cells were washed once with cold PBS and fixed with 2% paraformaldehyde for 10 min. Then, Hoechst 33342 solution was added at 1 µg/ml for 5 min at room temperature in darkness. Finally, stained cells were washed with PBS again, mounted and visualized under a fluorescence microscope. Fluorescence imaging was performed on Nikon Eclipse 80i fluorescence microscope supplied with a DAPI filter (excitation 360 nm, emission 460 nm) from Japan. The images were captured with the NIS elements software (V 3.10). The apoptotic and non apoptotic nuclei were counted in five fields randomly picked from each slide using Adobe Photoshop CS5 extended version 12.1.

The apoptotic nuclei per slide equal the mean of apoptotic nuclei of five fields, then the mean was converted to percentage by excel and given as apoptotic index. Finally, the standard deviation of three independent experiments was calculated and statistically compared using one-way ANOVA followed by Tukey's multiple comparison test with Graphpad Prism 5.03 software.

4.10.2. Caspase 3 activity

Caspase 3 activity was determined using Caspase-3/CPP32 Colorimetric Assay Kit according to the company instructions. The assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate DEVD-pNA. Briefly, isolated primary rat hepatocytes were seeded in 94 mm Petri dishes and incubated for 3 h. Then, the cells were incubated with different concentrations of OTA 0, 0.5, 2.5, and 12.5 µM or of LPS 0, 0.1, 0.5, 2.5, and 12.5 µg/ml or the cells were pre-treated with and without silibinin at 130 µM 2 h prior to the administration of 12.5 µM OTA or LPS or 10 mM H_2O_2 or irradiation with UV light at a dose of 50 mJ/cm^2 and at 260 µM 2 h prior to ActD/TNF-α 200/20 ng/ml for 12 h. The cells were harvested after 12 h without trypsinisation by a cell scraper from the incubation medium and centrifuged at 3000 rpm for 5 min at 25°C. The final pellet was re-suspended in 50 µl of chilled Cell Lysis Buffer (PromoCell) and incubated on ice for 10 minutes followed by centrifugation at 11000 rpm for 1 min. The supernatant (cytosolic extract) was transferred to a fresh tube and immediately, stored at -80°C. The protein content of the supernatant was assessed with BCA protein assay kit (Novagen). For colorimetric measurement of caspase 3 activity 150 µg protein of the cytosolic extract was diluted to 50 µl Cell Lysis Buffer for each sample, subsequently, 50 µl of 2x Reaction Buffer containing 10 mM DTT and 5 µl of the 4 mM DEVD-pNA substrate wXre added, then incubated at

37°C for 2 h. The enzyme activity (colour intensity from DEVD-pNA cleavage) was measured at 405 nm with a microplate reader.

4.10.3. DNA ladder fragmentation analysis and quantification

4.10.3.1. Isolation of genomic DNA from hepatocytes

Six million primary hepatocytes were seeded on rat-tail collagen coated 94×16 mm Petri dishes in 8 ml medium, treated for 24 and 36 h with and without tested hepatotoxins and preventive substances. After that, cells were harvested together with the incubation medium using a cell scraper since apoptotic cells tend to float in the medium. Cells were transferred into 15 ml Falcon tubes. The cell suspension was centrifuged at 2000 rpm for 5 min, 25°C. After discarding the supernatant cells were re-suspended in 1 ml cold PBS and transferred to 1.5 ml reaction tube. The washing by cold PBS was repeated. Cell suspension was centrifuged at 3000 rpm for 2 min, 25°C followed by discarding the supernatant and storing the pellet at -80°C. The genomic DNA was isolated according to Wörner and Schrenk (1996), by re-suspending the stored pellet in 400 µl TTE buffer containing 5 µl RNase A (10 mg/ml) and 10 µl proteinase K (10 mg/ml). The solution was vortexed and incubated at 55°C for 3 h. When the solution became clear the solution was extracted with 400 µl phenol (pH 8.6), chloroform, isoamyl alcohol (25:24:1) and vortexed for 10 seconds. Subsequently, the solution was centrifuged at 14000 rpm for 10 min 4°C. Then the supernatant (upper aqueous phase) was transferred to a new reaction tube

without disturbing interphase or lower organic phase. The extracted solution was extracted again by phenol. Then 400 µl chloroform and isoamyl alcohol (24:1) was added to it followed by vortexing for 10 sec. The solution was centrifuged at 14000 rpm for 10 min 4°C. The supernatant was transferred to a new reaction tube sequentially 1 ml -20°C cold 100% ethanol was added to the supernatant, and the tube was inverted 20 times. The DNA was pelleted by centrifugation at 14000 rpm for 15 min 4°C. The supernatant was aspired and the DNA pellet was washed with 500 µl 70% ethanol twice. The supernatant after centrifugation was discarded and the reaction tube was inverted on a tissue at 25°C for 5 min. Finally, the DNA pellet was completely dissolved by adding 30 µl TE buffer overnight at 4°C. The DNA content was measured by spectrophotometer.

4.10.3.2. Isolation of DNA from HepG2 cells

HepG2 cells were seeded at 13×10^6 on 94×16 mm Petri dishes in 10 ml medium, treated for 36 h, after that, cells were harvested together with the incubation medium using a cell scraper (since apoptotic cells tend to float in the medium). Cells were transferred into 15 ml falcon. The cell suspension was centrifuged at 3500 rpm for 5 min, 25°C. After discarding the supernatant cells were re-suspended in 1 ml cold PBS and transferred to 2 ml reaction tube. The washing by cold PBS was repeated. Cell suspension was centrifuged at 3000 rpm for 2 min, 25°C followed by discarding the supernatant and storing the

pellet at -80°C. The DNA was isolated by re-suspending the stored pellet in 1000 µl TET lysis buffer containing 1 µl proteinase K (10 mg/ml). The solution was vortexed and incubated for 20 min on ice. Next, the cell debris was spun down by centrifugation at 14000 rpm, 15 min, 4°C and the DNA- containing supernatant transferred to a 2 ml reaction tube. The solution was extracted with 1000 µl phenol (pH 8.6), chloroform, isoamyl alcohol (25:24:1) and vortexed for 10 seconds. Subsequently, the solution was centrifuged at 14000 rpm for 10 min 4°C. Then the supernatant (upper aqueous phase) was transferred to a new reaction. Next, the solution was treated with 15 µl RNase (10 mg/ml) for 30 min at 37°C and subsequently extracted with phenol twice. Then, the DNA was precipitated by addition of 100 µl of 3 M Na-acetate and 1000 µl of 100% Isopropanol. A DNA pellet was derived by another centrifugation step (14000 rpm for 30 min at 4°C), the supernatant decanted and the DNA washed with 70% ethanol. After decanting, the purified DNA pellet was dried for 10 min at RT and finally the DNA was dissolved in 50 µl TE Buffer. The DNA content was measured by spectrophotometer.

4.10.3.3. Agarose gel electrophoresis

The qualitative determination of DNA fragmentation was visualized by agarose gel electrophoresis analysis by preparing the agarose gel from 1.5 g agar (Biozym, Hessian-Oldendorf, Germany) in 100 ml of Tris-acetate electrophoresis buffer 1x (TAE, 4.0 mM Tris-HCl, 1 mM EDTA, 1.14 mM

acetic acid pH 8.0). For this purpose, 10 µl of the DNA suspension, mixed with 5 µl Loading Dye Solution 6x (Fermentas), was applied to the agarose gel at 130 V for 1.5 h separated by electrophoresis. TAE 1x buffer was used as a running buffer. As a length standard in the first lane of the gel were 3 µl of a marker (DNA Ladder GeneRulerTM, Size Standard 100-1000 or 100-10000 bp-Ladder and both Fermentas) applied.

4.10.3.4. Ethidium bromide staining

After gel electrophoresis the DNA fragments were visualized by staining the gel with an aqueous ethidium bromide solution, 1% (10 mg / ml, Carl Roth) for 30 min. The fluorescent dye ethidium bromide pitched itself as an intercalating substance in the double strands of DNA. The excess dye in the gel was removed by swirling with distilled water (10 min at room temperature). Subsequently, the DNA bands were under UV-illumination visualized and photographically documented using a gel-video documentation system (Image Master VDS, Pharmacia Biotech, Freiburg, Germany). The DNA fragments were found to be bright bands against a dark background. Finally, the DNA bands were quantified with ImageJ 1.42q software.

4.11. Oxidative stress determination

HPCT-1E3 cells were seeded at (1×10^6 cells) on 35 mm Petri dishes and pre-incubated overnight. Then, the cells were pre-treated with and without silibinin

at 260 µM 2 h prior to the administration of 50 µM OTA, ActD/TNF-α 200/20 ng/ml and 10 mM H_2O_2 for 6 h. The cells were harvested after 6 h without trypsinisation by a cell scraper from the incubation medium and centrifuged at 3000 rpm for 3 min at 25ºC. The final pellet was used for measuring MDA and ROS according to the company´s instructions.

4.11.1. Lipid Peroxidation (MDA) assay

In order to quantify the MDA as natural end product of lipid peroxidation, the collected pellets from treated samples were homogenized in 300 µl of MDA lysis buffer containing 3 µl BHT 100x, then centrifuged at 11000 rpm for 10 min, 200 µl of the supernatant from each homogenized sample were placed into microcentrifuge tubes. The colour in samples were developed by adding 600 µl of TBA solution, incubated at 95ºC for 60 min then cooled to room temperature in an ice bath for 10 min. Subsequently, 200 µl from each reaction mixture were transferred into a 96-well microplate for analysis. Finally, the MDA-TBA adduct was quantified fluorometrically at (Ex/Em=532/553).

4.11.2. Reactive oxygen species assay

Reactive oxygen species was quantified by applying the competitive enzyme immunoassay technique utilizing a monoclonal anti-ROS antibody and an ROS-HRP conjugate. Briefly, the cells in collected pellets were re-suspended in 300 µl PBS then disrupted physically by repeated cycles of freezing and thawing

through ice crystal formation, then centrifuged at 3000 rpm for 15 min at 4ºC. 100 µl from each sample were transferred in the antibody pre-coated microtiter plate. Subsequently, 10 µl of balance solution and 50 µl of conjugate were added to each well, mixed well, then incubated for 1 h at 37ºC. The incubation mixture was aspirated and the microtiter plates washed, 50 µl of substrate A and B were added to each well, incubated in dark for 15 min at 23ºC. The reaction was stopped by adding 50 µl of stop solution. Finally, the resulted yellow colour was colorimetrically measured by ELISA reader at 450 nm.

4.12. Statistical analysis

Rat primary hepatocytes were obtained from 3 to 5 different animals as indicated by ″n″. Experiments were performed with each cell preparation three times in case of caspase assay, eight times in case of MTT test and once in case of DNA laddering. HepG2 and HPCT-1E3 cells were used from three independent passages of the cell line. The results were expressed as mean ± SD. Statistical analysis was done using D'Agostino-Pearson omnibus test, then one-way of variance (ANOVA) followed by Tukey's multiple comparison test with Graphpad Prism 5.0 software (San Diego, CA, USA).

V. Results

The following results were obtained with hepatocytes after preparation by the EDTA-perfusion method. Hepatocytes prepared by the classical enzymatic digestion method by collagenase perfusion yielded unfavourable results regarding apoptosis experiments. Such cells exhibited apoptotic events already observable shortly after cell preparation and in early 6 to 24 h cultures even in the absence of ochratoxin A **(Fig.9a)**. In contrast, hepatocytes prepared by EDTA-perfusion exhibited non-degraded DNA as long as 96 h in culture **(Fig.9b)**.

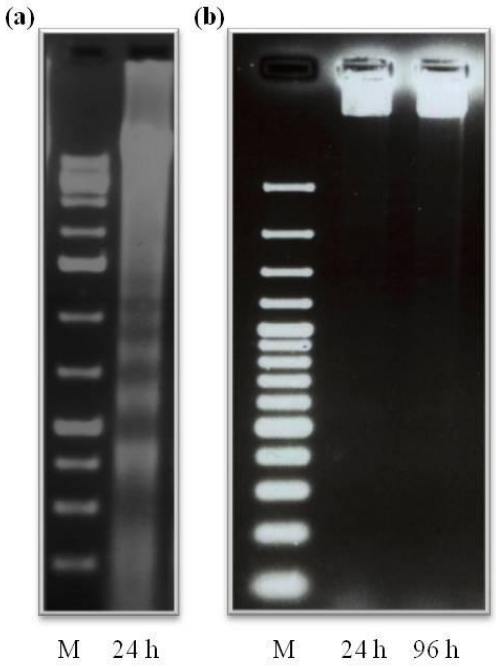

Fig. 9: DNA-stability in hepatocytes prepared by two different isolation methods

Agarose gel loaded with DNA obtained at 24 or 96 h from cultured primary rat hepatocytes. (a) The fragmented DNA bands after electrophoresis of DNA obtained from 24 h cultured primary rat hepatocytes, which were prepared by the collagenase perfusion method. (b) The non-fragmented DNA remained on the top of the gel, which were prepared by the EDTA-perfusion method. All experiments were repeated with five different cell preparations.

5.1. Cytotoxicity and viability

The cytotoxicity assay (MTT-test) and viability assay (Live/Dead kit) were performed to investigate the effects of the hepatotoxins OTA, LPS, ActD/TNF-α, H_2O_2, and UVC radiation on the vitality of cultured primary rat hepatocytes, HepG2, and HPCT-1E3. In addition, to which extent, silibinin exerts its preventive effect.

5.1.1. Effects of OTA, LPS, ActD/TNF-α, H_2O_2, and UVC on cytotoxicity in primary rat hepatocytes

The cells were treated with different concentrations of OTA 0, 0.5, 2.5, and 12.5 µM for 24 and 36 h to give a concentration–time-response curve for its cytotoxicity in cultured primary hepatocytes.

The OTA showed statistically significant a dose-dependent cytotoxic effect compared to the DMSO control after 36 h **(Fig. 10)**. At 2.5 and 12.5 µM (1 µg/ml and 5µg/ml), it decreased cell viability after 36 h incubation to 65% and 55% respectively. Since a concentration of 12.5 µM exhibited detectable cytotoxicity, I've chosen this concentration for further experiments.

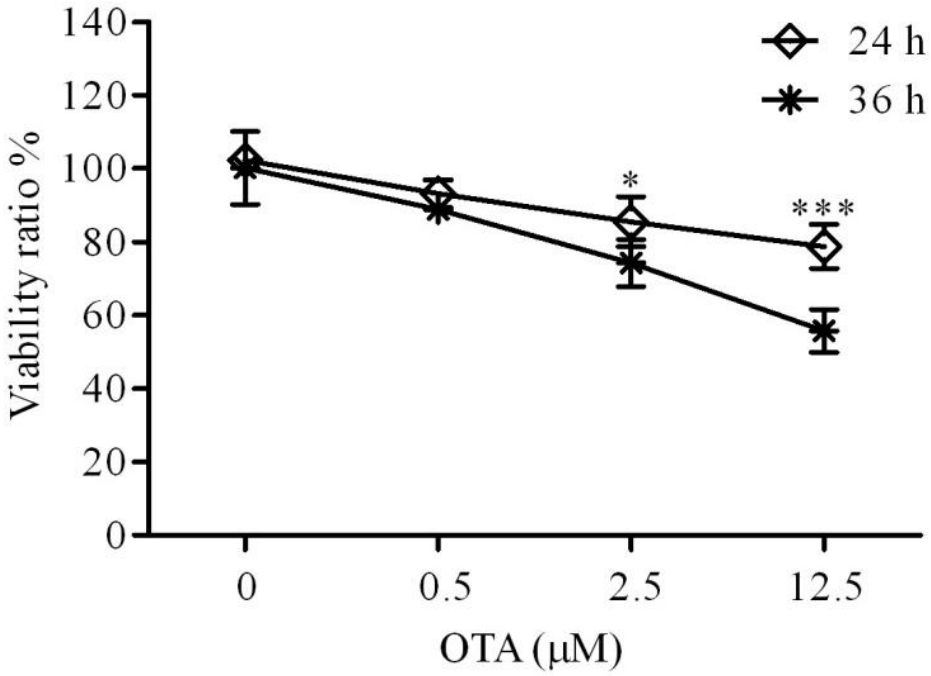

Fig. 10: OTA effect on cytotoxicity in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were treated with various concentrations of Ochratoxin A (OTA) for 24 or 36 h. Cell viabilities were measured by MTT test. Decreased cell viability under OTA was observed on 36 h cultures. All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group). *P* values *≤ 0.05, **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

LPS, up to 12.5 μg/ml did not alter the viability of cultured hepatocytes, in comparison with untreated cells **(Fig. 11)**. In cultured primary rat hepatocytes, cytotoxic effect was not evident under incubation with 0.1 to 12.5 μg/ml LPS during 24 and 36 h.

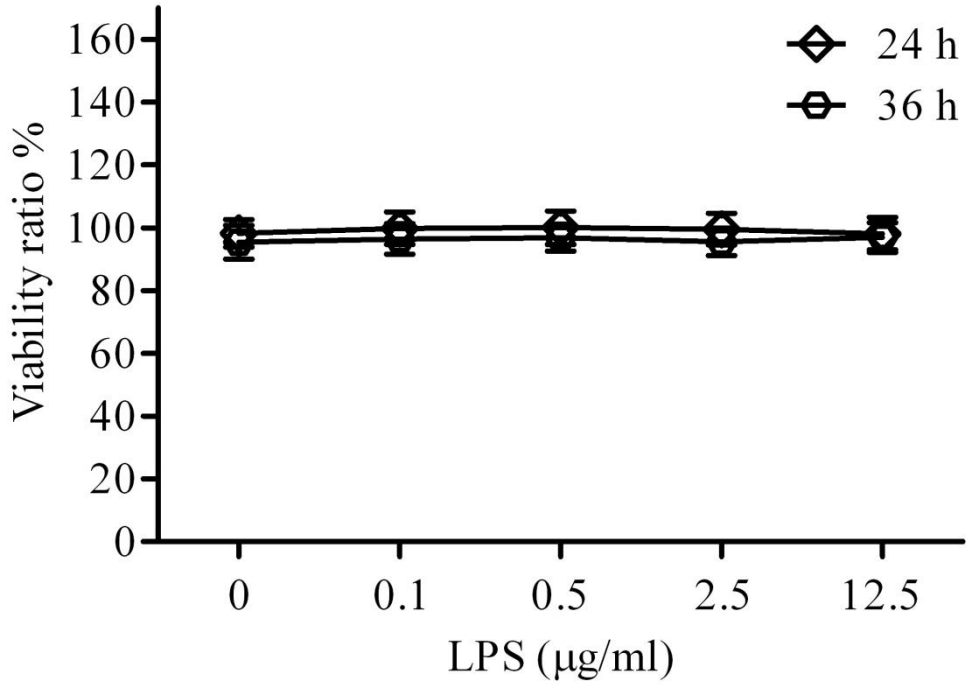

Fig. 11: Failure of toxic effects on cell viability by LPS in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were treated with various concentrations of lipopolysaccharide (LPS) for 24 or 36 h. Then, cell viability was measured by MTT assay. Viabilities were not significantly different from controls (0). All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group).

Cell death by TNF-α in cultured hepatocytes becomes manifested only under the metabolic condition of the transcriptional arrest. Therefore, we added ActD separately and in combination with TNF-α to cultured hepatocytes and measured cell toxicity by MTT-test. First, a dose-finding study with ActD in primary rat hepatocyte cultures was performed. Cultures of hepatocytes tolerated 50 to 333 ng/ml of ActD for 24 h without any change in cell viability **(Fig. 12)**.

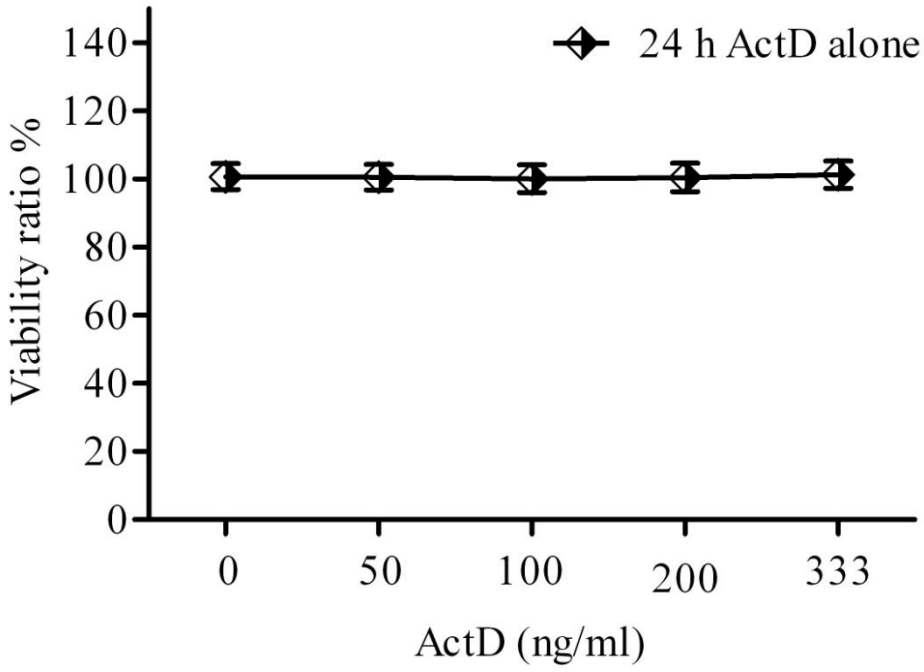

Fig. 12: ActD effect on cytotoxicity in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were treated with various concentrations of actinomycin D (ActD) for 24 h. Then, cell viability was measured by MTT assay. Viabilities were not significantly different from controls (0). All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group).

Next, a high and tolerated concentration of ActD (200 ng/ml) was applied for 30 min followed by the addition of increasing concentrations of TNF-α (10–30 ng/ml). The used TNF-α concentrations were tested separately, and found to be non-toxic **(Fig. 13)**.

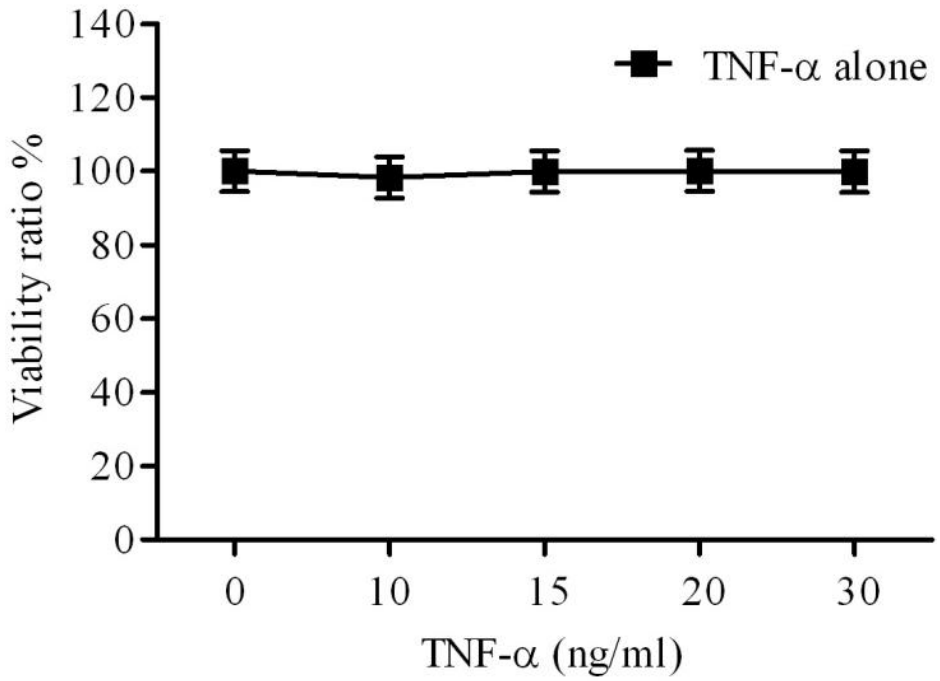

Fig. 13: TNF-α effect on cytotoxicity in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were treated with various concentrations of tumour necrosis factor alpha (TNF-α) for 24 h. Then, cell viability was measured by MTT assay. Viabilities were not significantly different from controls (0). All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group).

However, sensitization by ActD caused a vast reduction in cell viability by TNF-α in a dose-dependent manner leaving 36 % of viability at the highest applied TNF-α concentration at 24 h **(Fig. 14)**.

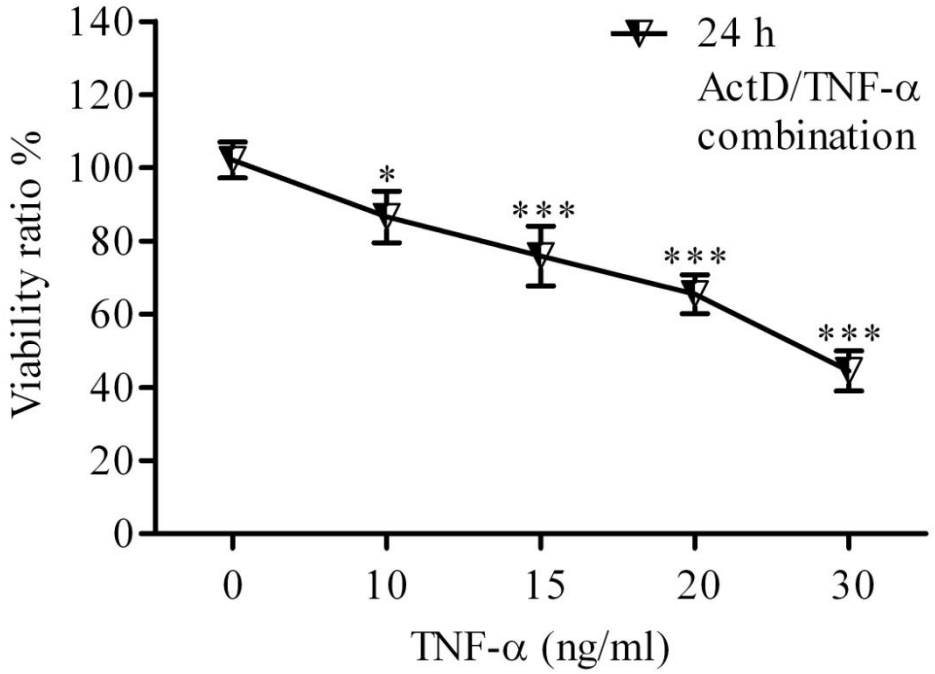

Fig. 14: ActD/TNF-α effect on cytotoxicity in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were pre-treated with 200 ng/ml actinomycin D (ActD) 30 min prior to tumour necrosis factor alpha (TNF-α) up to 30 ng/ml for 24 h. Cell viabilities were measured by MTT test. Decreased cell viability under OTA was observed on 36 h cultures. All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group). *P* values *≤ 0.05, **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

Besides, exposure of primary rat hepatocytes to H_2O_2 and UVC resulted in a dose-and time-dependent cytotoxicity **(Fig. 15a and b)**.

The cell damage caused decrease of cell viability that was strongest at 15 mM H_2O_2 or 50 mJ/cm^2 UVC at 36 h.

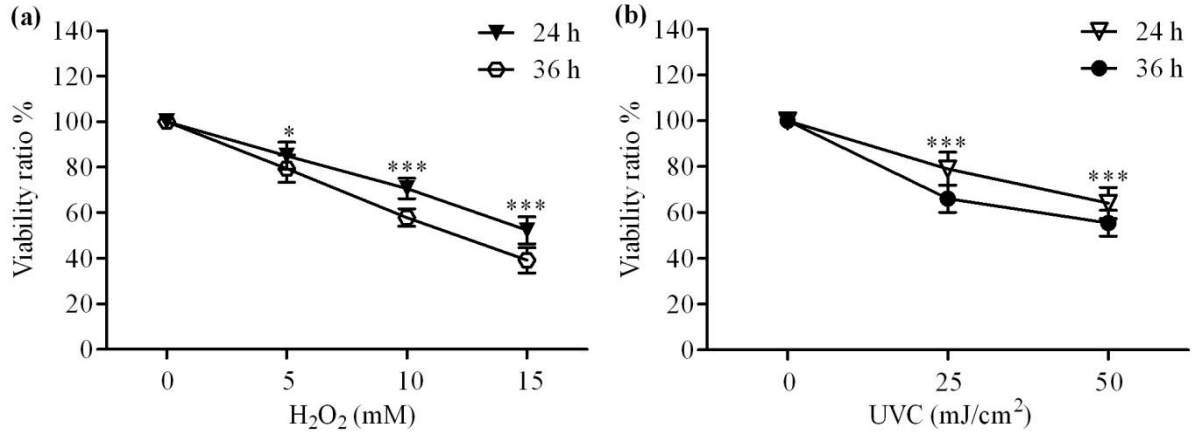

Fig. 15: H_2O_2 and UVC effect on cytotoxicity in cultured primary rat hepatocytes

Primary rat hepatocytes in cell culture were treated with various concentrations of hydrogen peroxide (H_2O_2) for 24 h (a) and irradiated with ultraviolet (UVC) at different doses for 24 h. Cell viabilities were measured by MTT test. All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group). *P* values *≤ 0.05, **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

5.1.2. Effect of silibinin on OTA, ActD/TNF-α, H_2O_2, and UVC cytotoxicity

Silibinin acts on liver cell membranes to prevent the entry of toxic substances and on the nucleus to accelerate cell regeneration by stimulating protein synthesis (Luper, 1998). In this study, we tested the protective effects of silibinin versus the late-term OTA cytotoxicity in hepatocyte cultures after 24 and 36 h incubation with 12.5 µM OTA. Silibinin pre-treatment at 130 µM 2 h prior to OTA exposure protected against OTA-mediated hepatotoxicity as revealed by MTT-test **(Fig. 16)**.

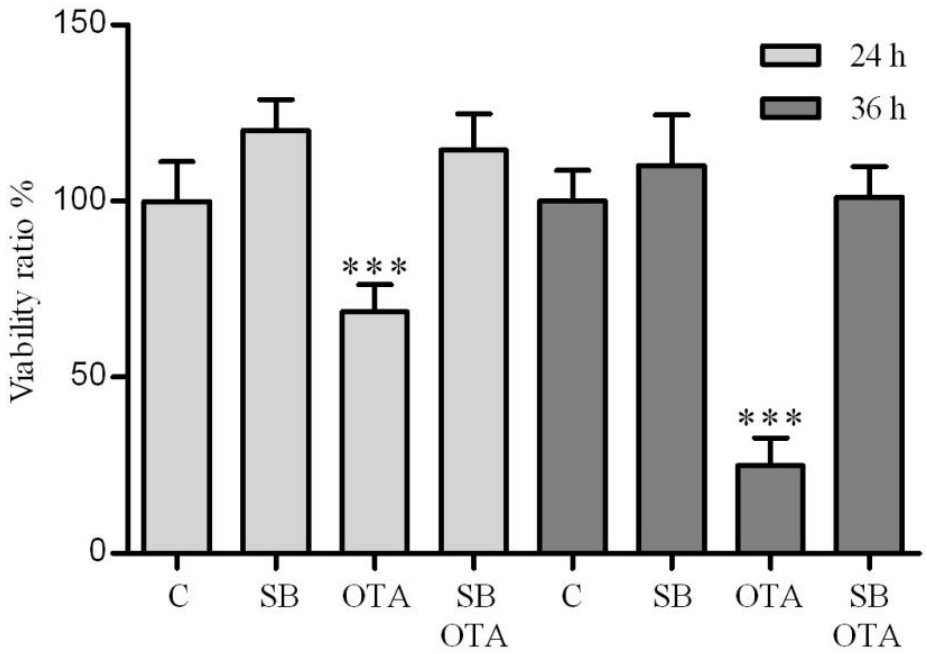

Fig. 16: Protective effect of silibinin on OTA-mediated cytotoxicity in cultured primary rat hepatocytes

Cultured primary rat hepatocytes were pre-treated with silibinin (SB) at 130 µM 2 h prior to ochratoxin A (OTA) at 12.5 µM for 24 and 36 h. Then, the decrease in cell viability was measured by MTT assay. All experiments were repeated with five different cell preparations. Data presents the value of the mean ± SD (n=5 / group). *** is significantly different from control and from SB+OTA values at $P \leq 0.001$.

In addition, we determined the protective effects of silibinin against cytotoxicity in hepatocyte cultures after 24 h incubation with 200/20 ng/ ml ActD/TNF-α, with 10 mM H_2O_2 or radiated with 50 mJ/cm^2 UVC. Under all these conditions pre-treatment for 2 h with silibinin at 130 µM protected the cells as confirmed by MTT-test. Under the same experimental conditions, even the ActD/TNF-α-mediated hepatotoxicity was blocked by silibinin albeit at a higher concentration of 260 µM **(Fig.17)**.

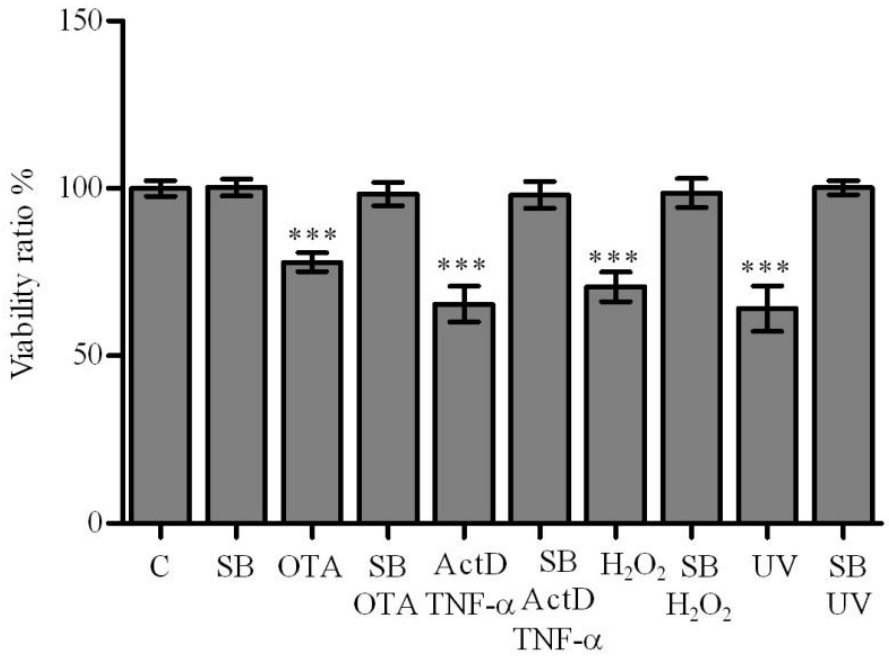

Fig. 17: Protective effect of silibinin on cytotoxicity mediated by OTA, ActD/TNF-α, H_2O_2, and UVC in cultured primary rat hepatocytes

Silibinin (SB) protection was measured by MTT assay after pre-treatment of cultured primary rat hepatocytes with it at 260 µM 2 h prior to 200/20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α) and 130 µM 2 h prior to 12.5 µM ochratoxin A (OTA), 10 mM hydrogen peroxide (H_2O_2), and 50 mJ/cm^2 ultraviolet (UVC) for 24 h. All experiments were repeated with five different cell preparations. Data presents the value of the mean ±SD (n =5/group). *** is significantly different from control at $P \leq 0.001$.

5.1.3. Viability decrease by OTA and prevention by silibinin

Furthermore, under same conditions, the staining of cultured primary rat hepatocytes with LIVE/DEAD kit confirmed the MTT-test results. In **figure 18**, such cells also detached under OTA treatment from the Petri dish bottom and many of the remaining cells stained red. Red cells were, however, almost completely absent in controls and in silibinin-treated hepatocyte cultures.

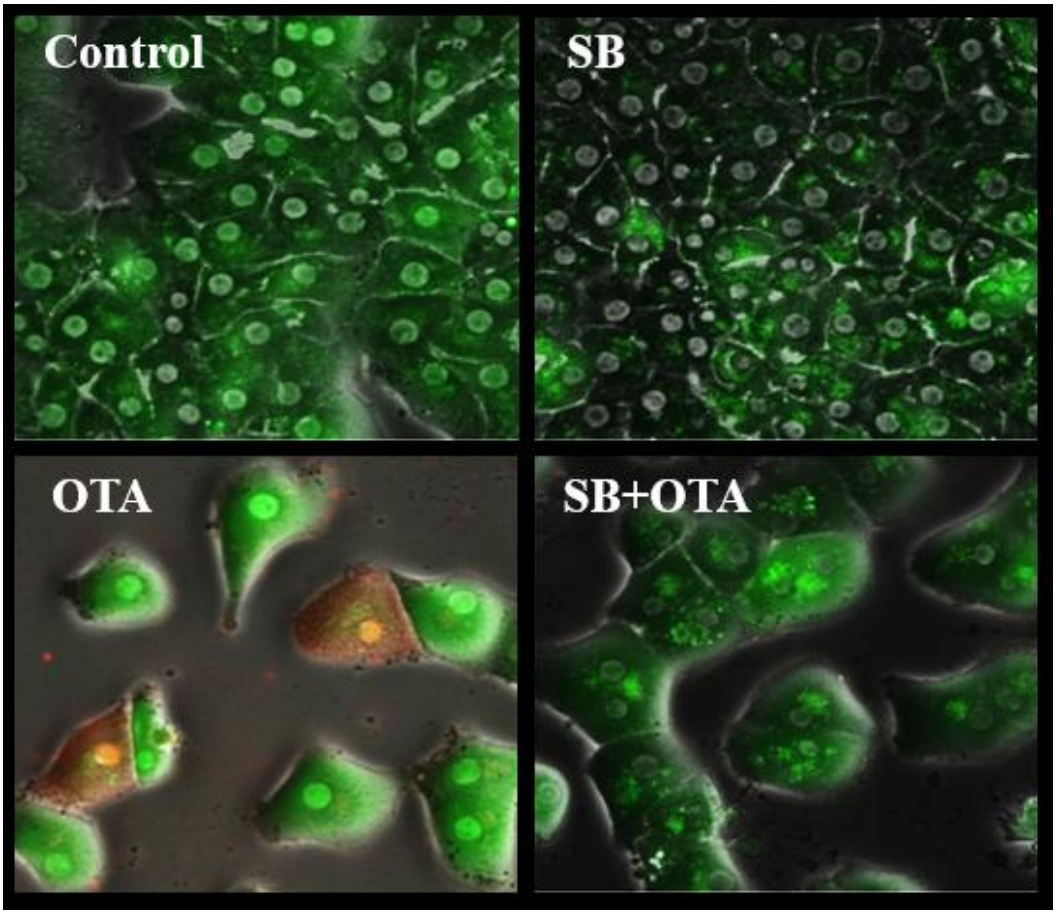

Fig. 18: Staining by LIVE/DEAD kit dyes of cultured primary rat hepatocytes

Protective effect of silibinin on OTA-mediated cytotoxicity in primary rat hepatocytes cell cultures at 24 h was observed after pre-treatment with 26 µM silibinin (SB) given 2 h prior to in 12.5 µM ochratoxin A (OTA) for 24 h. The pictures are representing at least five fields from a cover slip; each experiment was performed at least 3 times. Live cells fluorescent green and dead cells red. Dead cells treated with OTA also detached from the bottom.

5.1.4. Effects of OTA, LPS, CPT and H_2O_2 on cytotoxicity in HepG2 cells

One of the most meaningful experiments of this study is investigating the effects of OTA, LPS, CPT and H_2O_2 on cytotoxicity in HepG2 cells. Therefore, MTT assay was performed to examine whether the OTA and LPS treatment had cytotoxic effect on HepG2 cells. As shown in **figure 19**,

decreased cell viability under OTA was observed in dose-time dependent manner, reached 49.4% at highest concentration after 36 h exposure.

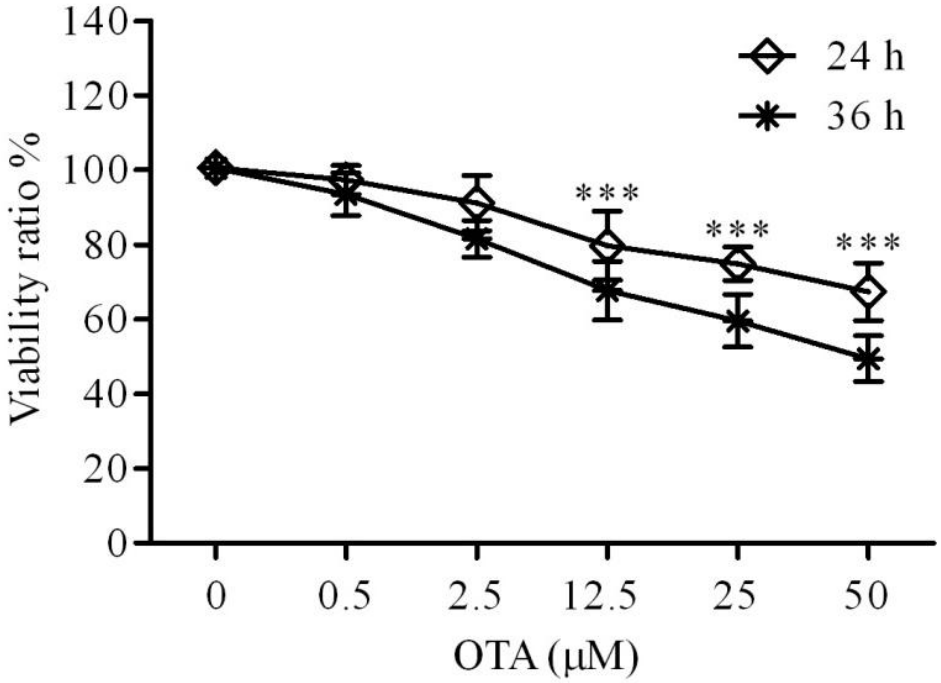

Fig. 19: OTA effect on cytotoxicity in cultured HepG2 cells

HepG2 in cell culture were treated with various concentrations of ochratoxin A (OTA) for 24 or 36 h. Cell viabilities were measured by MTT test. All experiments were repeated with five different cell passages. Data presents the value of the mean ± SD (n=5 / group). *** is significantly different from control at $P \leq 0.001$.

Besides, exposure of cultured HepG2 cells to various concentrations of LPS for 36 h showed no alteration in cell viability. On the other hand, incubating of HepG2 cells with 10 mmol H_2O_2 and 2 µg/ml CPT showed statistically significant decrease in cell viability in time-dependent manner **(Fig. 20)**.

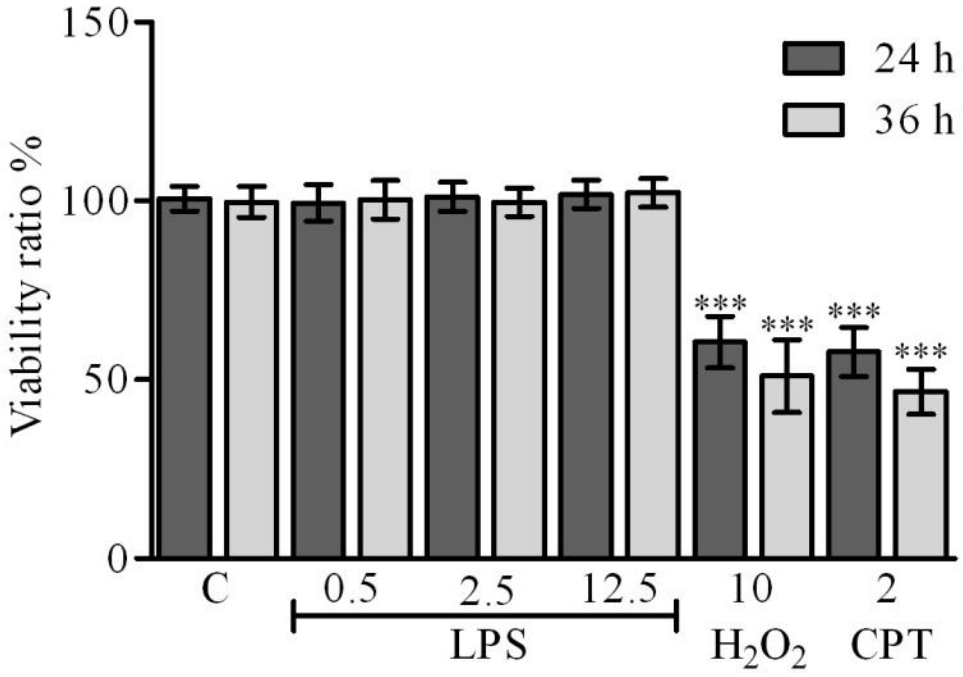

Fig. 20: Effects of LPS, CPT and H_2O_2 on cytotoxicity in cultured HepG2 cells

Cultured HepG2 cells were treated with lipopolysaccharide (LPS) at 0.5, 2.5, and 12.5 µg/ml, hydrogen peroxide (H_2O_2) at 10 mM, and camptothecin (CPT) at 2 µg/ml for 24 and 36 h. Then, the decrease in cell viability was measured by MTT assay. All experiments were repeated with five different passages. Data presents the value of the mean ± SD (n=5 / group). *** is significantly different from control values at $P \leq 0.001$.

In addition, under same conditions, the staining of cultured HepG2 cell with LIVE/DEAD kit confirmed the MTT-test results. In **figure 21** showed a dose-dependent toxic effect on HepG2 cells, in presence of OTA for 24 h, increased numbers of dead cell (red cell) can be observed at 12.5 µM. On the other hand, LPS at 12.5 µg/ml showed no cytotoxic effect on HepG2 cell. Red cells were, however, almost completely absent in controls and LPS treated cultures.

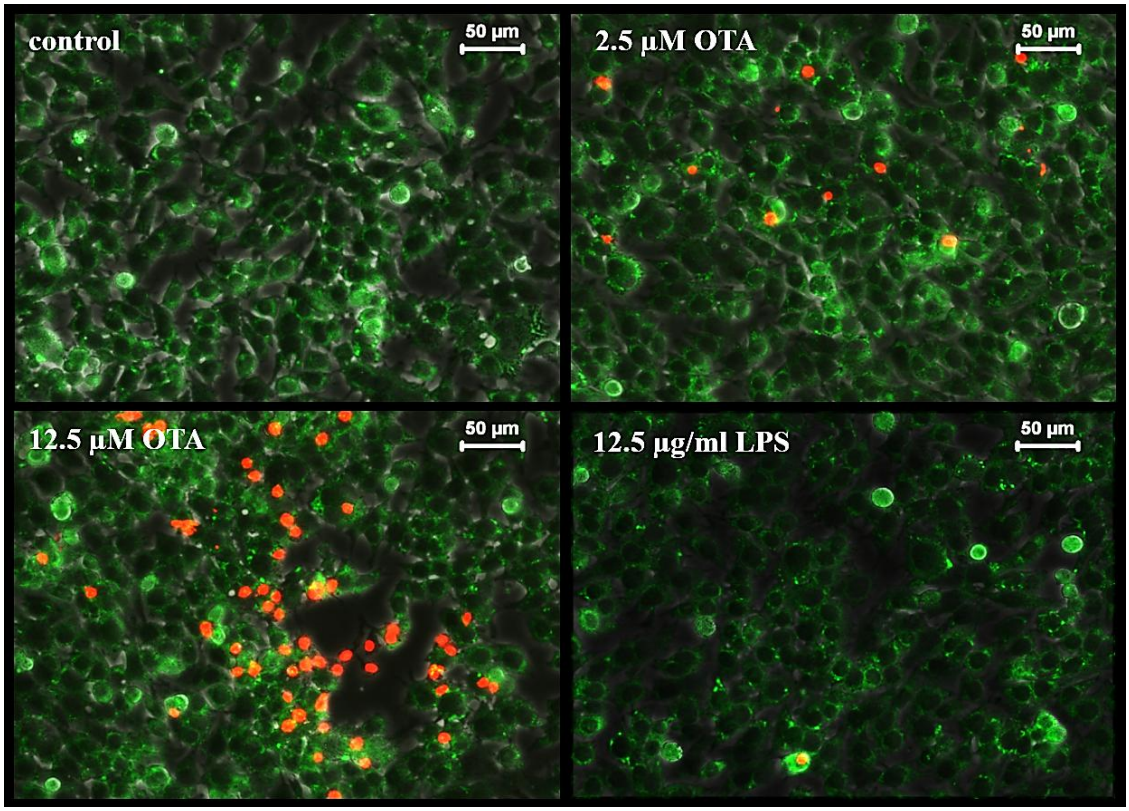

Fig. 21: Dose depended cytotoxicity mediated by OTA in HepG2 cell cultures

HepG2 cells cultures were stained by Live/Dead kit after treatment with 2.5 and 12.5 µM ochratoxin A (OTA) and 12.5 µg/ml lipopolysaccharide (LPS) for 24 h. The pictures are representing at least five fields from a cover slip; each experiment was performed at least 3 times. Live cells fluorescent green and dead cells red.

5.1.5. Effects of OTA, LPS, ActD/TNF-α, and H_2O_2 on cytotoxicity in HPCT-1E3 cells

Also the cytotoxic effect of OTA, LPS, ActD/TNF-α, and H_2O_2 was investigated in HPCT-1E3 cells as a model for rat hepatocytes. After treatment with various concentration of OTA for 24 hours, the MTT assay showed a concentration-dependent decrease in viability of the cultured HPCT-1E3 cells **(Fig. 22)**.

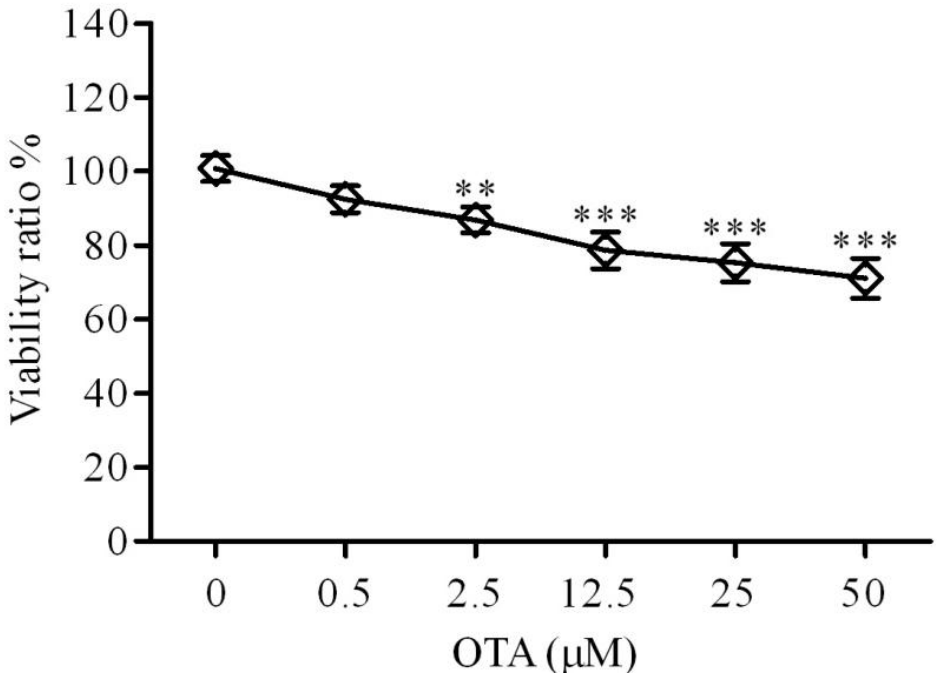

Fig. 22: Cytotoxic effect of OTA on cultured HPCT-1E3 cells

HPCT-1E3 cells in cell culture were treated with various concentrations of ochratoxin A (OTA) for 24 h. Cell viabilities were measured by MTT test. All experiments were repeated with five different cell passages. Data presents the value of the mean ± SD (n=5 / group). *P* values *≤ 0.05, **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

In contrast, HPCT-1E3 cultures were incubated with LPS up to 12.5 µg/ml for 24 h showed no alteration in cell viability. Whereas, another HPCT-1E3 cultures were subjected to H_2O_2 at 10 mM and ActD/TNF-α at 200/20 ng/ml for 24 h, showed statistically significant decrease in cell viability **(Fig. 23)**.

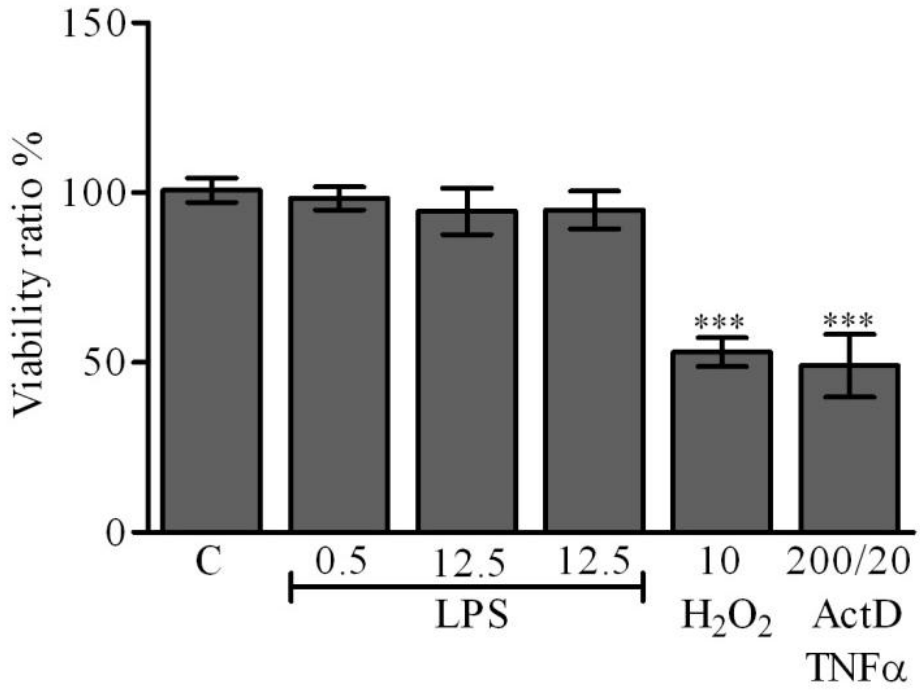

Fig. 23: Effects of LPS, H_2O_2, and ActD/TNF-α on cytotoxicity in cultured HPCT-1E3 cells

Cultured HPCT-1E3 cells were treated with lipopolysaccharide (LPS) at 0.5, 2.5, and 12.5 µg/ml, hydrogen peroxide (H_2O_2) at 10 mM, and actinomycin D/tumour necrosis factor alpha (ActD/TNF-α) at 200/20 ng/ml for 24 h. Then, the decrease in cell viability was measured by MTT assay. All experiments were repeated with five different cell passages. Data presents the value of the mean ± SD (n=5 / group). *** is significantly different from control values at $P \leq 0.001$.

5.2. Apoptosis markers

5.2.1. Development of DNA ladders by OTA but not by LPS in primary rat hepatocytes

The cytotoxic effect of hepatotoxins on the primary hepatocytes and HepG2 cells was further characterized to determine whether the observed reduction in viability was of necrotic or apoptotic origin. Therefore, internucleosomal DNA fragmentation, representing one of the hallmarks of apoptosis was measured by separation of fragmented DNA on an agarose gel, creating a characteristic

banding pattern, a so-called DNA ladder with bands at a distance of approximately 200 bps, the intensity of DNA fragmentation reflects the degree of apoptosis. Treatment of cultured primary rat hepatocytes with OTA of 2.5 up to 12.5 µM caused the development of DNA ladders after 24 h and more pronounced after 36 h **(Fig. 24a and b)** confirming results from Chopra et al. 2010.

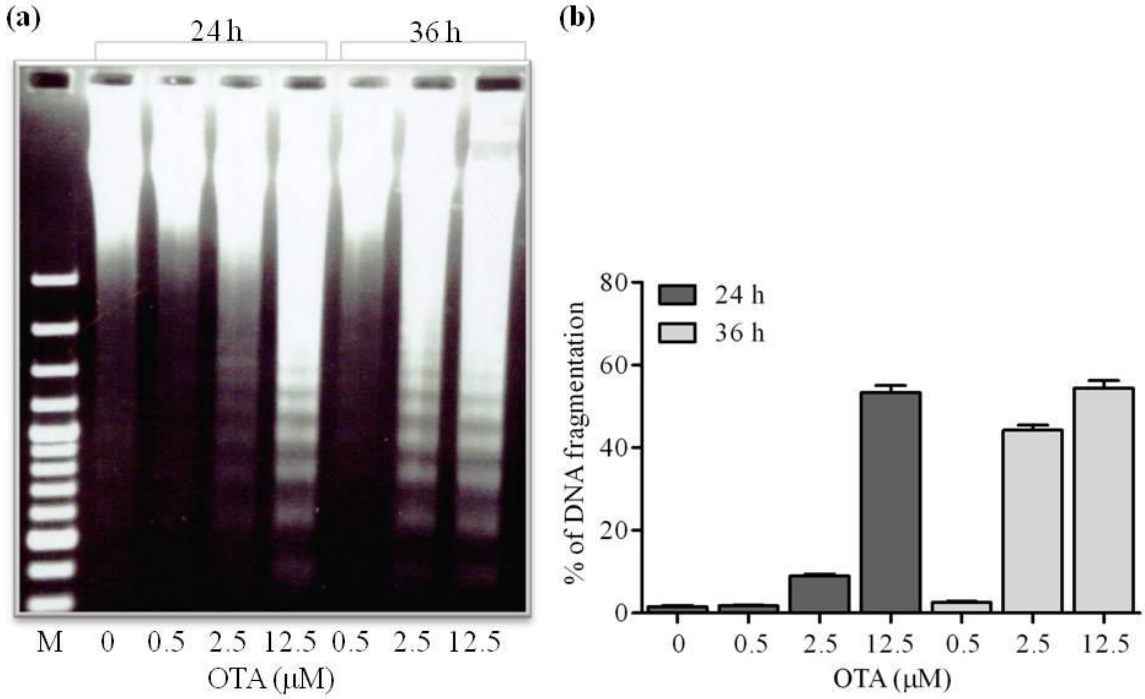

Fig. 24: Dose- and time-dependent DNA fragmentation by OTA in a representative experiment

Cultures of primary rat hepatocytes were treated with various concentrations of ochratoxin A (OTA) for 24 and 36 h. Then, the DNA was isolated and visualized by gel electrophoresis (a) and quantified by ImageJ 1.42q software (b). All experiments were repeated with five different cell preparations.

On the other hand, treatment of primary hepatocytes with LPS up to 12.5 µg/ml for 24 and 36 h did not cause DNA ladders **(Fig. 25a and b)**.

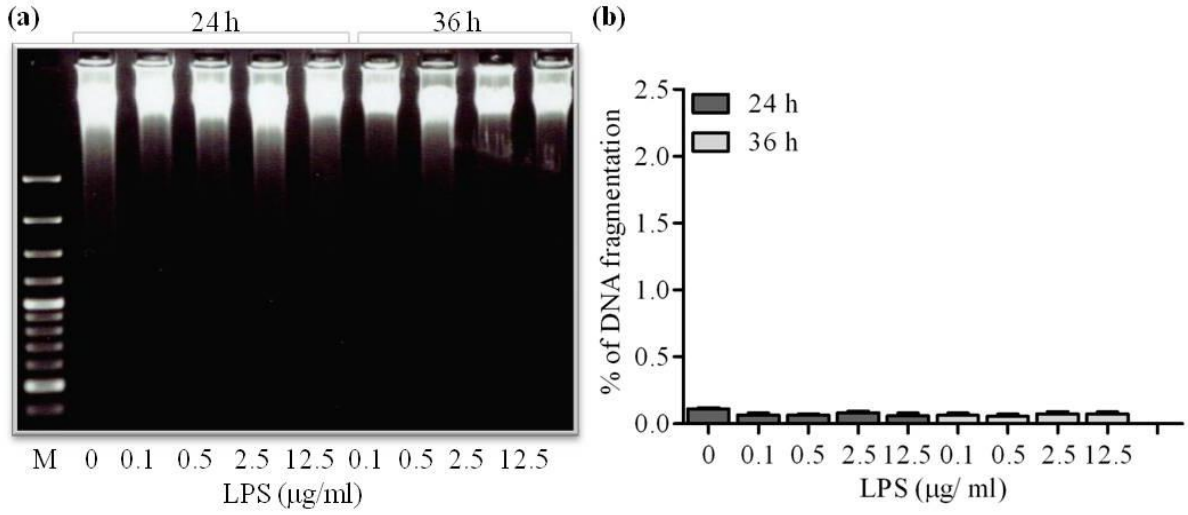

Fig. 25: Lack of DNA fragmentation by LPS in cultured primary rat hepatocytes in a dose- and time-dependent experiment

Cultured primary rat hepatocytes were treated with various concentrations of lipopolysaccharide (LPS) for 24 and 36 h. Then, the DNA was isolated and visualized by gel electrophoresis (a) and quantified by ImageJ 1.42q software (b). All experiments were repeated with five different cell preparations.

5.2.2. Development of DNA ladders by OTA and CPT but not by LPS in HepG2 cells

The cytotoxicity results guided me to investigate the apoptosis also in cultured HepG2 cells. Further experiments were done to see whether these results are consistence with DNA ladder results or not.

Cultured HepG2 cells were incubated with 12.5 µM OTA generated DNA fragmentation after 36 h. By contrast, incubation of HepG2 cells with LPS at 12.5 µg/ml for 36 h showed no DNA ladder, this confirmed the DNA ladder results of primary rat hepatocytes.

Under same conditions, CPT HepG2 cultures showed typical DNA ladder, and thus, was used as control positive for apoptosis **(Fig. 26)**.

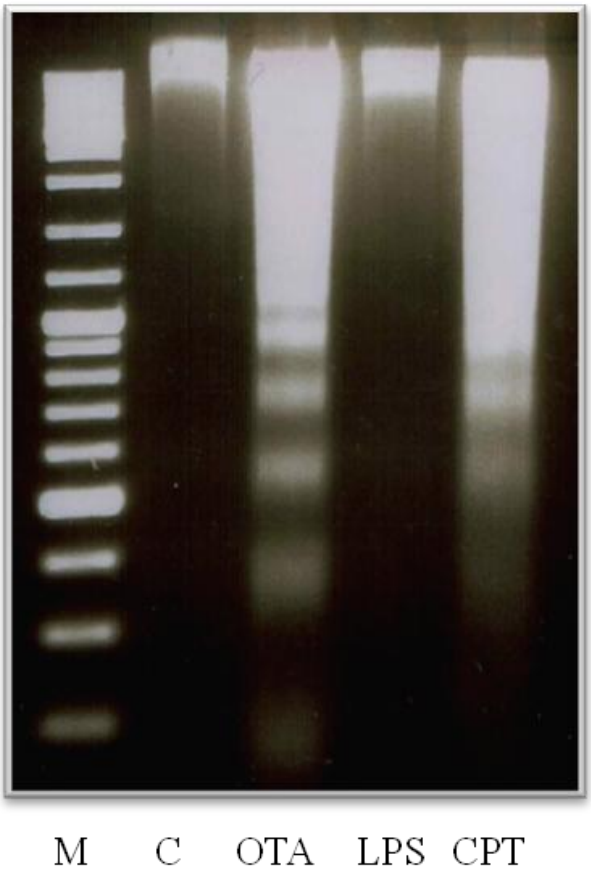

Fig. 26: DNA ladder mediated by OTA and CPT but not LPS in HepG2 cells

Cultured HepG2 cells were incubated with ochratoxin A (OTA) at 12.5 µM, lipopolysaccharide (LPS) at 12.5 µg/ml, and camptothecin (CPT) at 2 µg/ml for 36 h. Then, the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell passages.

5.2.3. Induction of DNA fragmentation by TNF-α in presence of a transcriptional inhibitor ActD or CPT

In fact, the cytotoxic effect of TNF-α in cultured hepatocytes becomes manifested only under the metabolic condition of transcriptional arrest (Leist et al., 1994).

Therefore, in this study, the apoptotic origin of cytotoxicity was further investigated. Firstly, primary rat hepatocytes were incubated with two transcriptional inhibitors separately at various concentrations to be sure that ActD or CPT are able to sensitize cultured hepatocytes to TNF-α mediated DNA ladder. **Figure 27 a** and **b** showed that CPT up to 100 µM and ActD up to 333 ng/ml did not induce DNA fragmentation of cultured hepatocytes at 24 h.

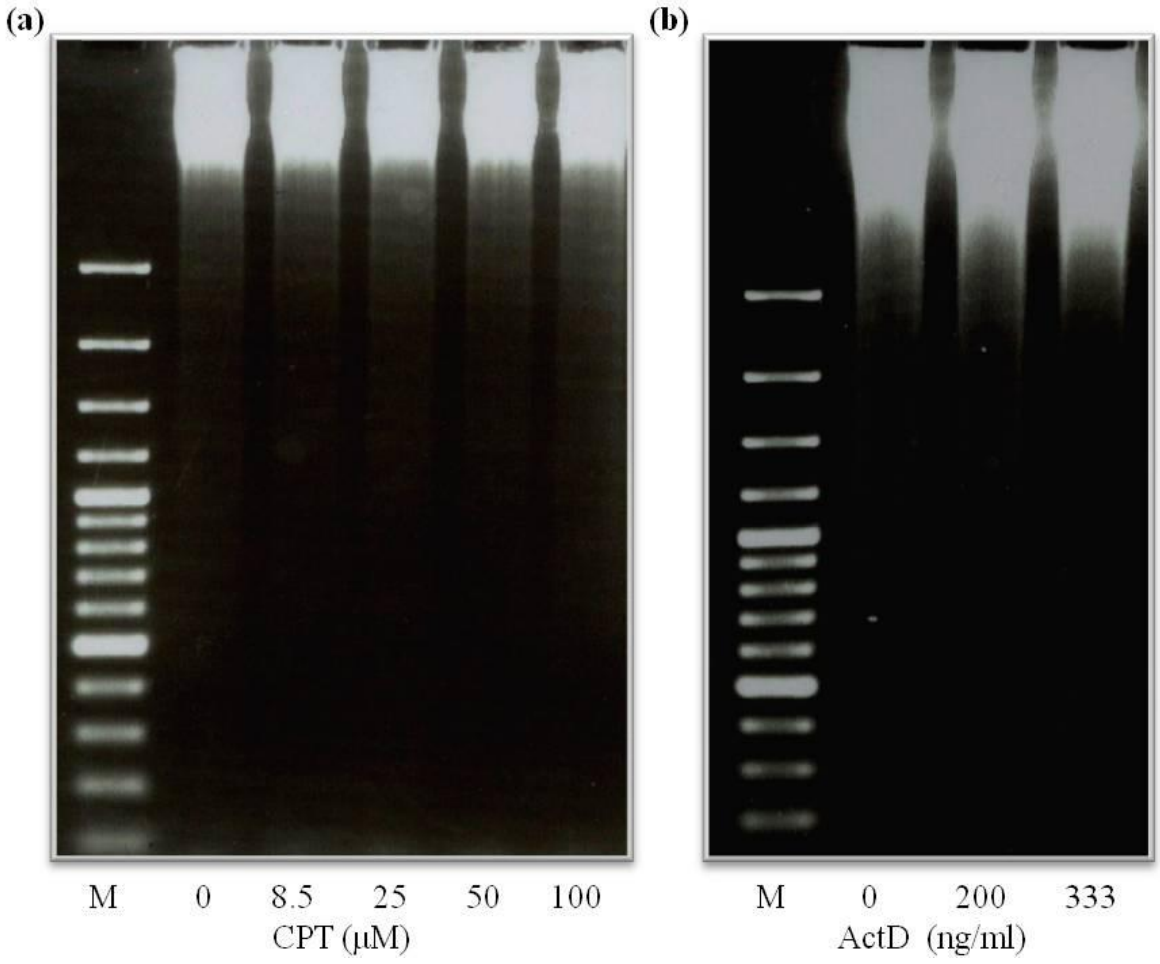

Fig. 27: Lack of DNA ladder by transcriptional inhibitors in primary rat hepatocytes
Cultures of primary rat hepatocytes were treated with (a) various concentrations of camptothecin (CPT) 0, 85, 25, 50, and 100 µM, (b) three concentrations of actinomycin D (ActD) 0, 200, and 333 ng/ml, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

Whereas, the pre-treatment of cultured hepatocytes with 100 μM CPT or 200 ng/ml ActD for 30 min before the addition of TNF-α at 20 ng/ml developed pronounced DNA Ladder **(Fig. 28)**, indicating that TNF-α-mediated apoptosis in cultured primary rat hepatocytes also needs to transcriptional arrest.

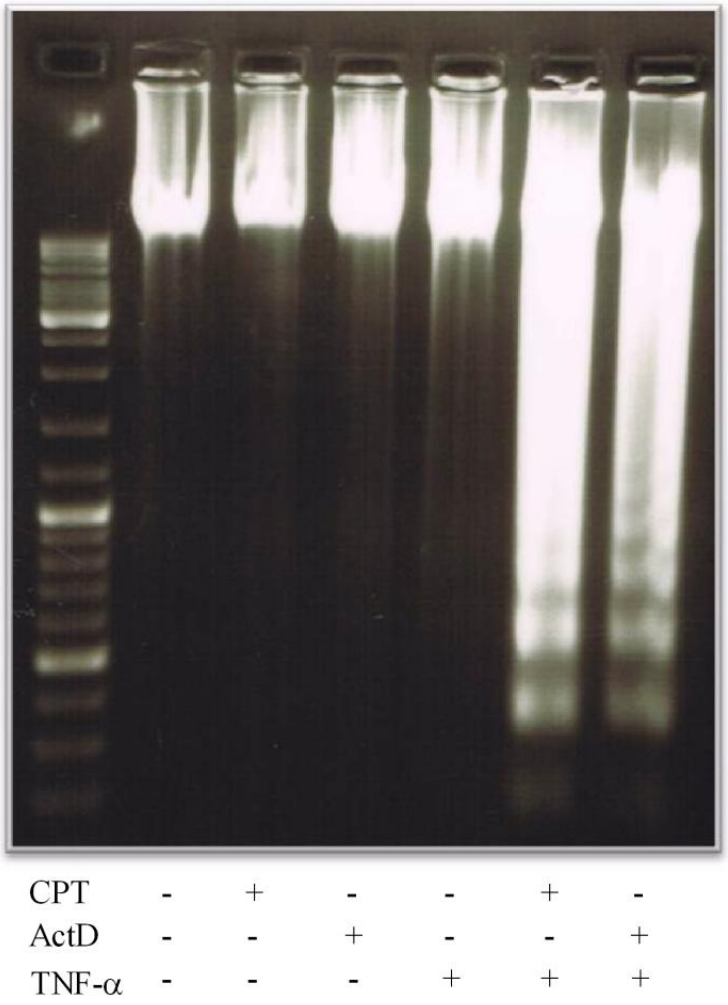

Fig. 28: Development of DNA ladder by TNF-α only in presence of transcriptional inhibitors

Cultures of primary rat hepatocytes were treated with camptothecin (CPT) 100 μM, actinomycin D (ActD) 200 ng/ml, tumour necrosis factor alpha (TNF-α) 20 ng/ml, CPT 100 μM 30 min prior to 20 ng/ml, and ActD 200 ng/ml 30 min prior to 20 ng/ml TNF-α; the first four lanes show high-molecular, non-fragmented DNA, the last two lanes show DNA ladder developed by TNF-α in presence of transcriptional inhibitors, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

5.2.4. Prevention of DNA fragmentation by silibinin

Silibinin has been used over ages due to its non-toxic and mechanism-based strong preventive/therapeutic efficacy. So, I tested in this study the anti-apoptotic property of silibinin on hepatotoxins-mediated apoptosis in cultured primary rat hepatocytes and HepG2 cells. The applied concentrations (26 and 130 µM) of silibinin did not prevent OTA-mediated DNA-laddering if applied 30 min prior to OTA in cultured primary rat hepatocytes after 24 h **(Fig. 29a)**. Moreover, silibinin at 26 µM did not prevent OTA-CPT-mediated DNA-fragmentation if applied 30 min prior to OTA in cultured HepG2 cells after 36 h **(Fig. 29b)**.

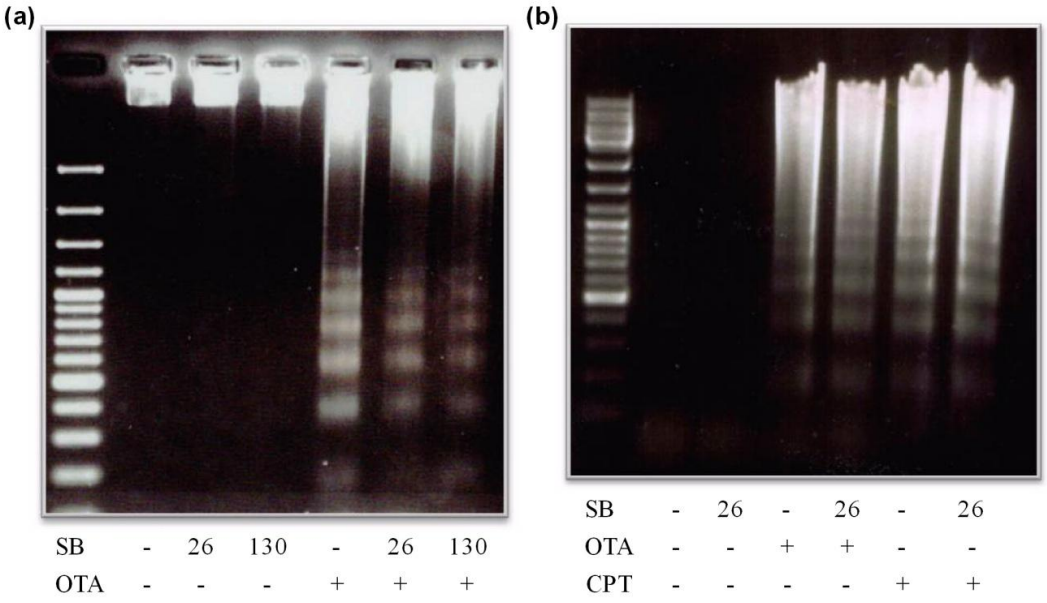

Fig. 29: Silibinin short pre-incubation did not prevent OTA-mediated DNA fragmentation

Primary rat hepatocytes were pre-treated with silibinin (SB) at 26 and 130 µM 30 prior to 12.5 µM ochratoxin A (OTA) for 24 h (a), HepG2 cells were pre-treated with silibinin at 26 µM 30 prior to 12.5 µM OTA and 2 µg/ml camptothecin (CPT) for 36 h (b). Then, the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations or passages.

After that, silibinin longer pre-incubation experiments were done with primary rat hepatocytes. **Figure 30a** and **b** showed that longer pre-incubation of silibinin concentrations 26 and 52 µM did not prevent OTA-mediated DNA-laddering at 24 and 36 h. By contrast, longer pre-incubation of silibinin concentration (≙ 130 µM) showed complete inhibition of OTA-mediated apoptotic DNA laddering at 24 and 36 h.

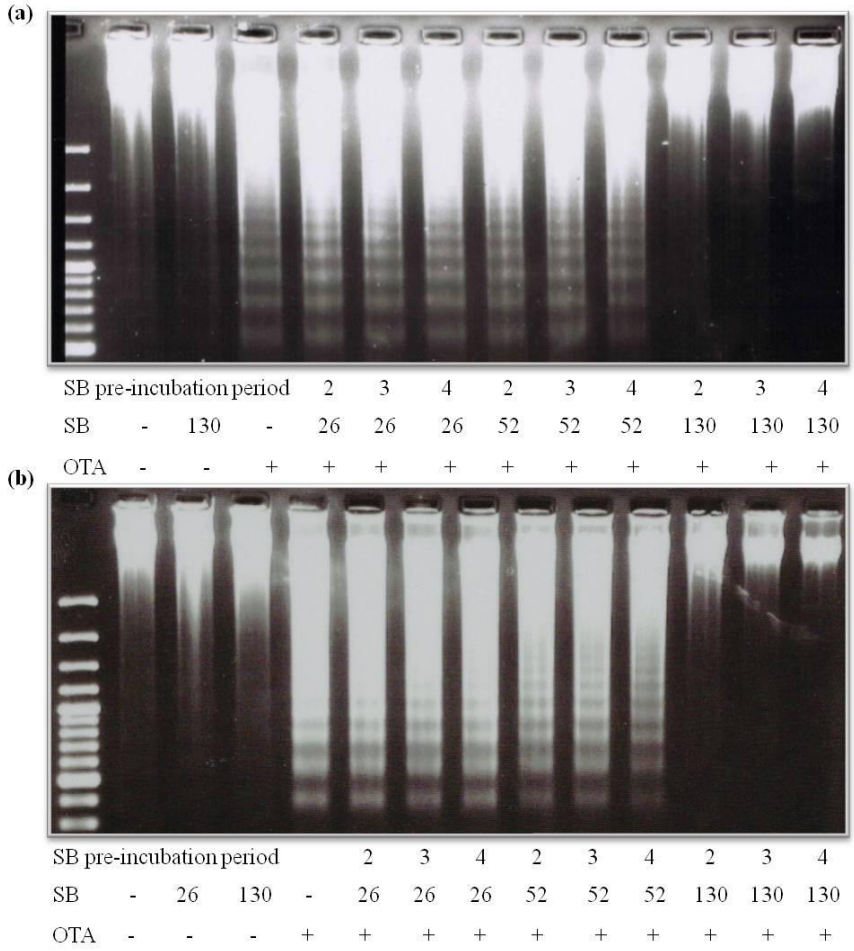

Fig. 30: Longer pre-incubation of silibinin prevented OTA-mediated DNA fragmentation

Primary rat hepatocytes were pre-treated with silibinin (SB) at 26, 52, and 130 µM 2, 3, and 4 h prior to 12.5 µM ochratoxin A (OTA) for 24 h (a) or 36 h (b). Then, the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

Silibinin does not show preventive effect against OTA-mediated apoptotic DNA-laddering unless pre-incubated in a minimum period of 2 h and at concentration not less than 130 µM (**Fig. 31a** & **b)**.

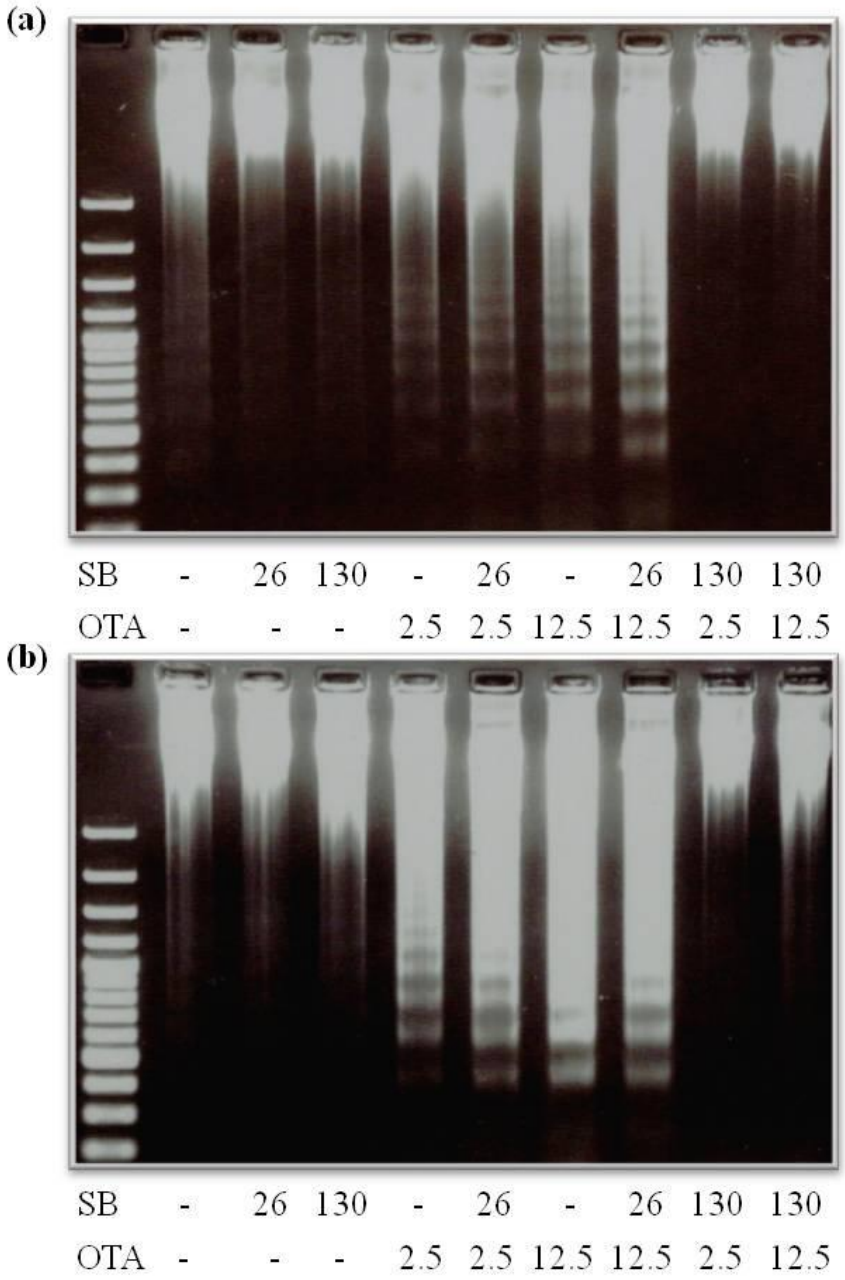

Fig. 31: Silibinin preventive effect conditions against OTA-mediated DNA laddering
Primary rat hepatocytes were treated with silibinin (SB) at 26 and 130 µM alone and 2 h prior to 2.5 or 12.5 µM ochratoxin A (OTA) for 24 h (a) or 36 h (b). Then, the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

Furthermore, Silibinin dose-dependently decreased OTA-mediated DNA fragmentation and even completely abolished fragments at 130 µM at 24 h **(Fig. 32a and b)**.

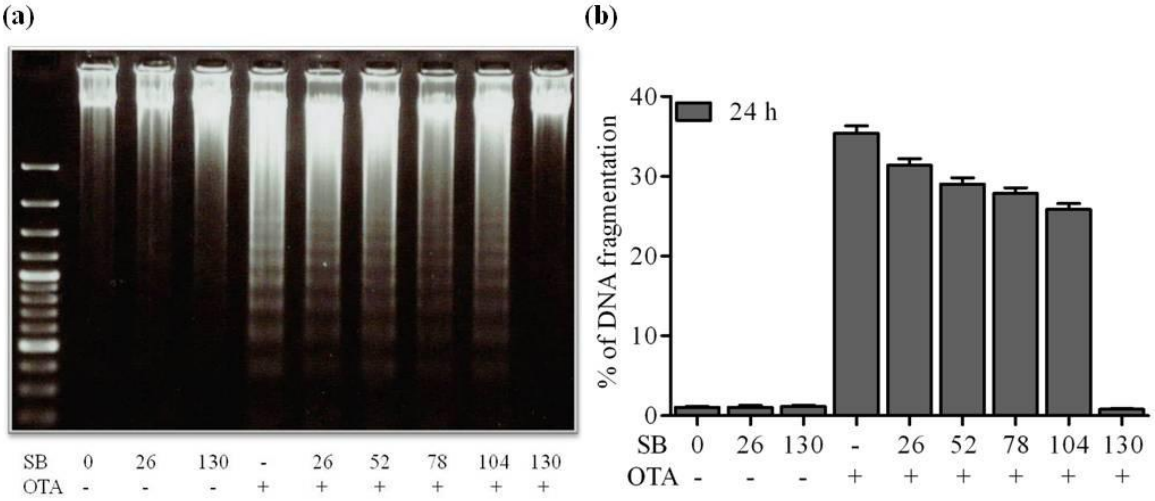

Fig. 32: Preventive effect by silibinin on OTA-mediated DNA fragmentation related to silibinin concentrations

Primary rat hepatocytes were pre-treated with silibinin (SB) at various concentrations up to 130 µM 2 h prior to 12.5 µM ochratoxin A (OTA). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis (a) and quantified by ImageJ 1.42q software (b). All experiments were repeated with five different cell preparations.

Interestingly, under same conditions, silibinin exerted complete hepatoprotection effect against H_2O_2- and UVC-mediated apoptotic fragmentation at 130 µM. These finding confirmed that silibinin is strong antioxidant where H_2O_2 and UVC were used in this study as control positive **(Fig. 33a and b)**.

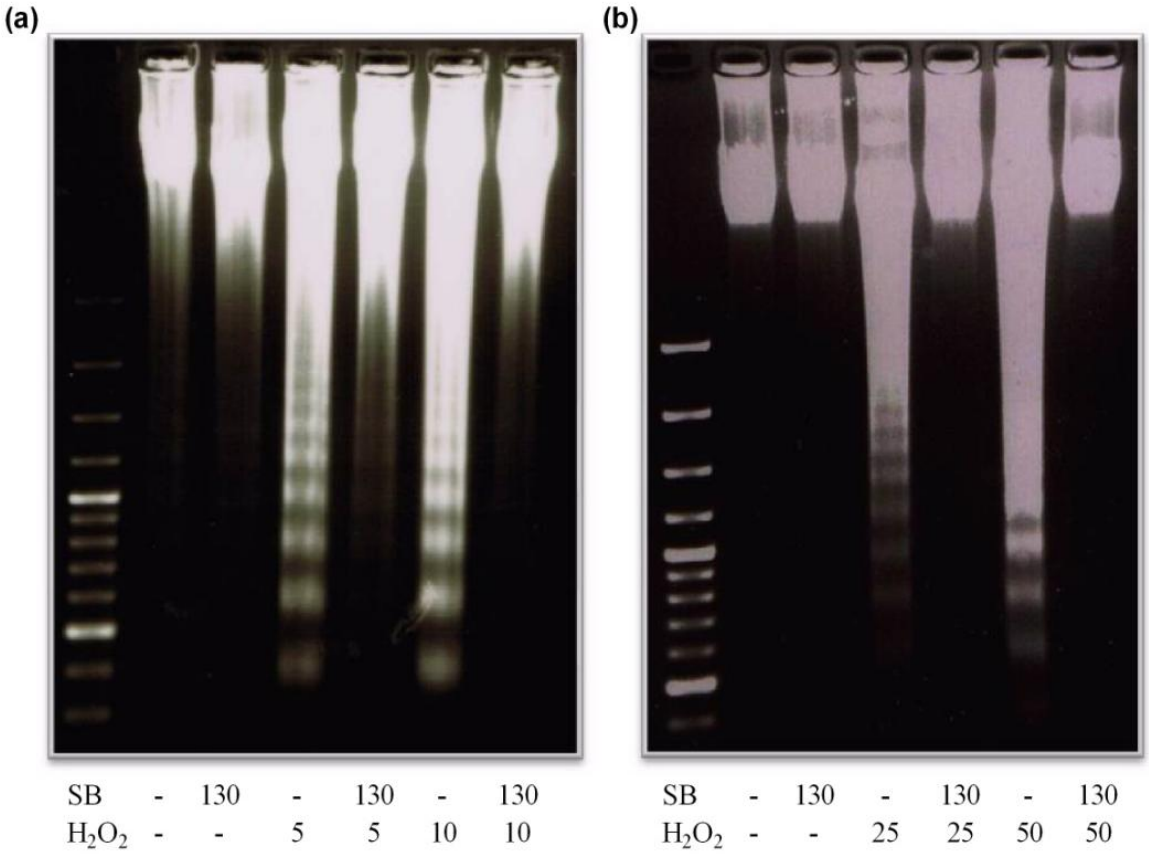

Fig. 33: Development of DNA ladder by H_2O_2 and UVC and prevention by silibinin

Primary rat hepatocytes were pre-treated with silibinin (SB) at 130 µM 2 h prior to hydrogen peroxide (H_2O_2) at 5 and 10 mM (a) and ultraviolet (UVC) at 25 and 50 mJ/cm^2 (b). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

Additionally, silibinin dose-dependently decreased ActD/TNF-α-mediated DNA apoptotic DNA ladder and even completely abolished fragments at 260 µM concentration at 24 h **(Fig. 34)**.

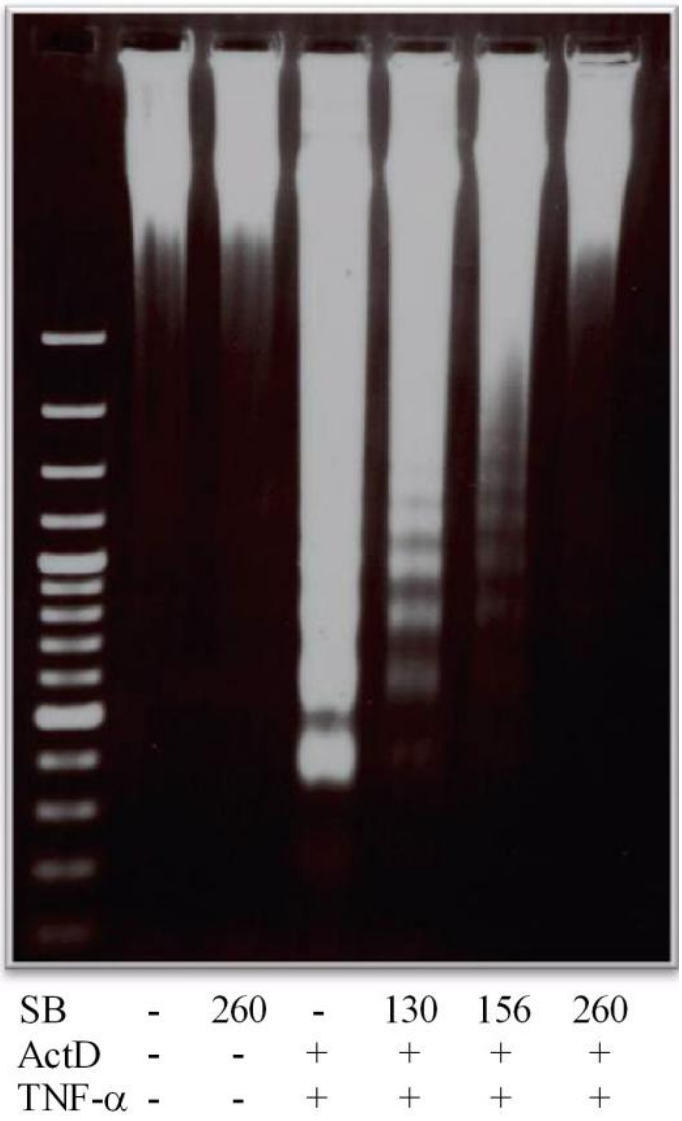

Fig. 34: Development of DNA ladder by ActD/TNF-α and prevention by silibinin
Cultures of primary rat hepatocytes were pre-treated with silibinin (SB) at increasing concentrations 2 h prior to 200/20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

Finally, **figure 35** showed silibinin as a quite strong anti-apoptotic compound that exerts protection against OTA, ActD/TNF-α, H_2O_2 and UVC mediated apoptosis most likely by its antioxidant and membrane stabilizing effect on primary rat hepatocytes.

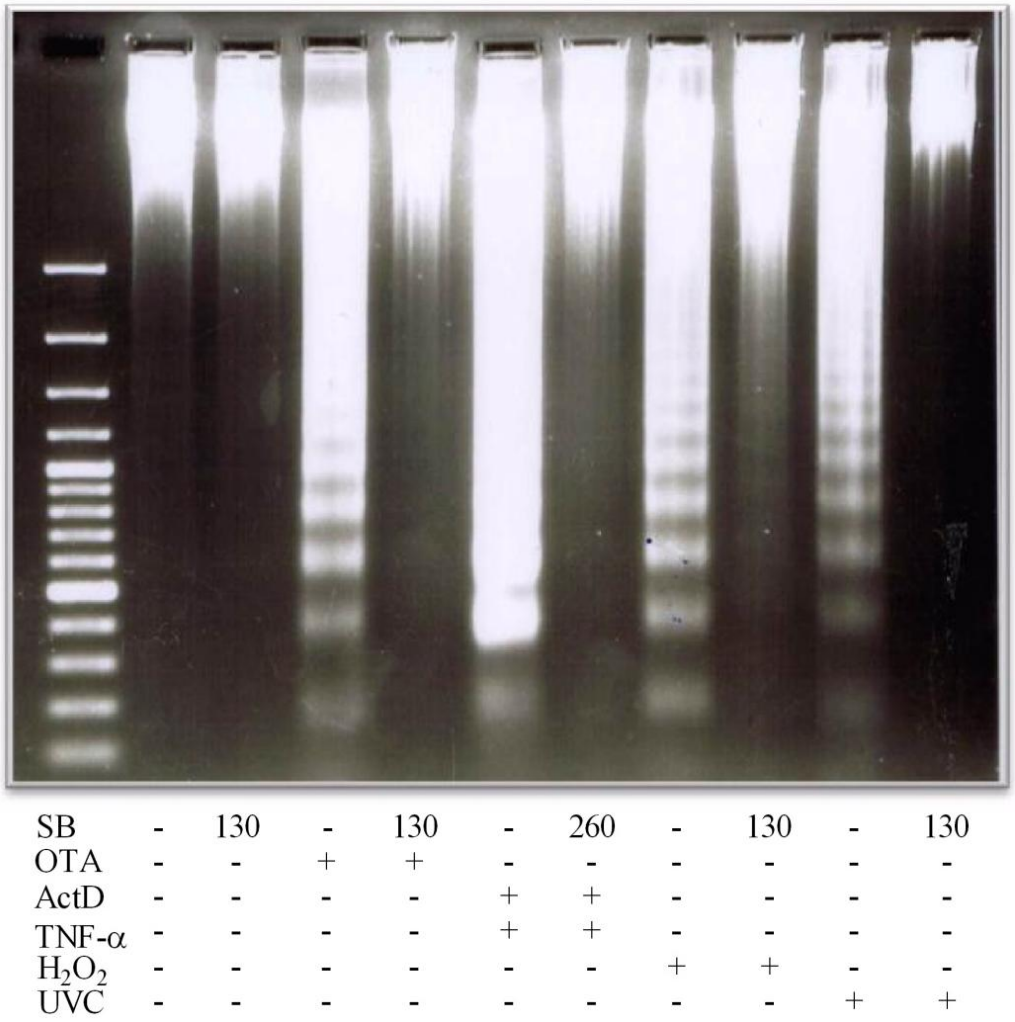

Fig. 35: Development of DNA ladder by OTA, ActD/TNF-α, H_2O_2, and UVC and prevention by silibinin

Primary rat hepatocytes were pre-treated with silibinin (SB) at 130 µM 2 h prior to 12.5 µM ochratoxin A (OTA), 10 mM hydrogen peroxide (H_2O_2), and 50 mJ/cm^2 ultraviolet (UVC) and at 260 µM 2 h prior to 200/20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with five different cell preparations.

5.2.5. Effects of TNF-α antagonists, sTNFR1, and sTNFR2 on OTA- and ActD/TNF-α mediated DNA fragmentation

Tumour necrosis factor alpha exerts its effects through two distinct receptors, TNFR1 and TNFR2. But one prominent path toward apoptosis is the ligand-

induced association of TNF receptor 1 with death domain adaptor proteins (Beutler and van Huffel, 1994).

In this study, in order to made progress in understanding the molecular mechanisms that mediate TNF-α induced apoptosis in liver. Primary rat hepatocytes were pre-incubated with 25 µg/ml of sTNFR1 or sTNFR2 2 h prior to 200/20 ng/ml ActD/TNF-α. The results indicated that TNF-α uses mainly TNFR1 to induce apoptosis in cultured primary rat hepatocytes **(Fig. 36)**.

Besides, cultured primary rat hepatocytes were pre-incubated with two different TNF-α antagonists at 25 µg/ml 2 h prior to 200/20 ng/ml ActD/TNF-α.

These antagonists used to mimic the most critical tumour necrosis factor alpha recognition loop on TNF receptor 1 and prevent interactions of TNF-α with its receptor. The antagonist I is a synthetic TNF receptor fragment 55kD peptide and suppose to inhibit TNF-α- cytotoxicity *in vitro*, the antagonist II designed to inhibit TNF-α mediated apoptosis. **Figure 36** lanes 5 and 6 showed no prevention of TNF-α-mediated DNA ladder by these antagonists.

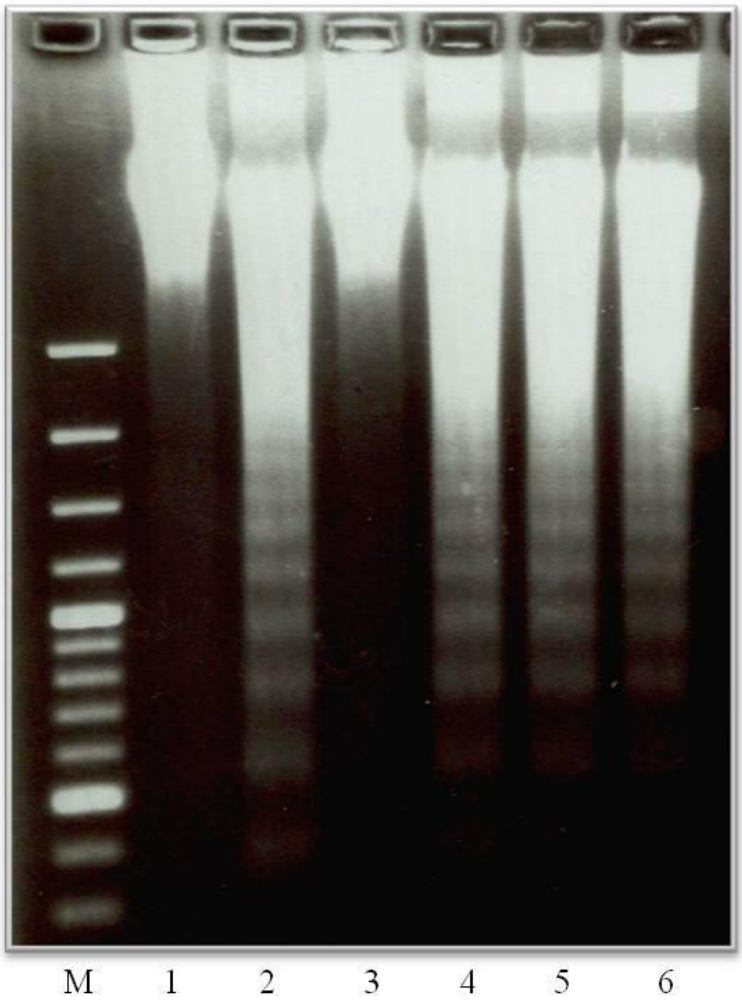

Fig. 36: Effect of TNF-α antagonists, sTNFR1, sTNFR2 on ActD/TNF-α-mediated DNA fragmentation

Primary rat hepatocytes were negative control for experiment (1), cells treated with 200/20 ng/ml actinomycin D/ tumour necrosis factor alpha (ActD/TNF-α) (2), cells pre-treated with 25 µg/ml sTNFR1 2 h prior to 200/20 ng/ml ActD/TNF-α (3), cells pre-treated with 25 µg/ml sTNFR2 2 h prior to 200/20 ng/ml ActD/TNF-α (4), cells pre-treated with 25 µg/ml TNF-α antagonist I 2 h prior to 200/20 ng/ml ActD/TNF-α (5), cells pre-treated with 25 µg/ml TNF-α antagonist II 2 h prior to 200/20 ng/ml ActD/TNF-α (6). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with three different cell preparations.

In order to gain insight into the mechanism of apoptosis by OTA, primary rat hepatocyte cultures were pre-incubated with soluble sTNFR1 as decoy strategy

to reduce the binding of OTA to the membrane bound receptor by competition and to decrease the potency for OTA-mediated apoptosis. **Figure 37** shows that addition of 500 µg/ml sTNFR1 2 h prior to 12.5 µM OTA had no effect on OTA-mediated DNA laddering. However, only 25 µg/ml sTNFR1 2 h prior to 200/20 ng/ml ActD/TNF-α completely prevented TNF-α-mediated DNA fragmentation **(Fig. 36)**.

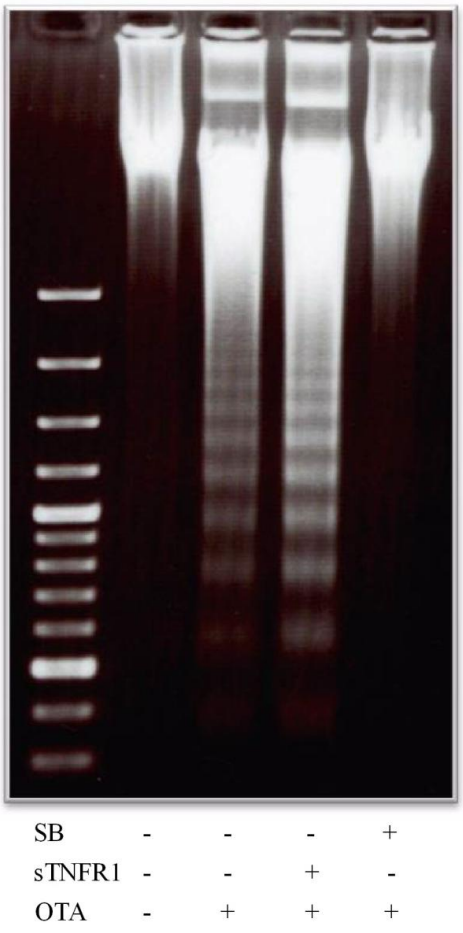

Fig. 37: Effect of sTNFR1 and silibinin on OTA-mediated DNA fragmentation

Primary rat hepatocytes were pre-treated with silibinin (SB) at 130 µM or sTNFR1 at 500 µg/ml 2 h prior to 12.5 µM ochratoxin A (OTA). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with three different cell preparations.

In addition, primary rat hepatocytes were treated with the TNF-α antagonists I and II, soluble sTNFR1 and 2 alone and 2 h prior the addition of 12.5 µM OTA. Neither TNF-antagonists I and II nor soluble sTNFR1 and 2 showed no preventive effect on OTA-mediated DNA ladder **(Fig. 38)**.

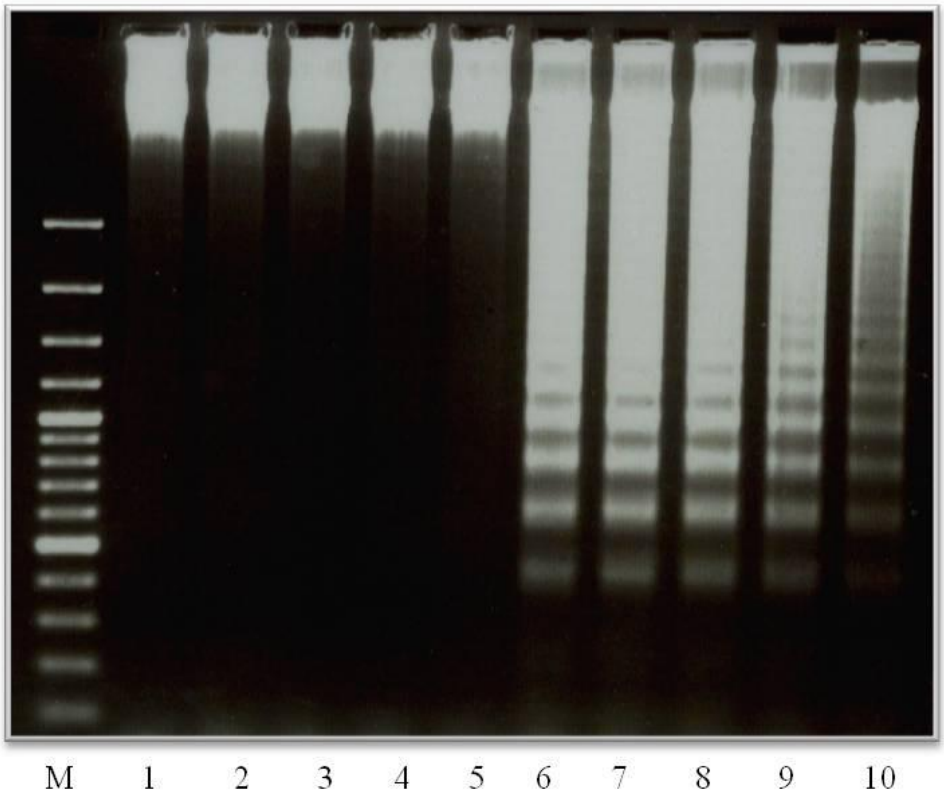

Fig. 38: Effect of TNF-α antagonists, sTNFR1, sTNFR2, on OTA-mediated DNA fragmentation

Primary rat hepatocytes were negative control for experiment (1), cells were treated with 25 µg/ml soluble tumour necrosis factor 1 (sTNR1) (2), cells were treated with 25 µg/ml TNF-α antagonist I (3), cells were treated with 25 µg/ml TNF-α antagonist II (4), cells were treated with 25 µg/ml soluble tumour necrosis factor 2 (sTNR2) (5), cells were treated with 12.5 µM OTA (6), cells pre-treated with 25 µg/ml sTNFR1 2 h prior to 12.5 µM OTA (7), cells pre-treated with 25 µg/ml TNF-α antagonist I 2 h prior to 12.5 µM OTA (8), cells pre-treated with 25 µg/ml TNF-α antagonist II 2 h prior to 12.5 µM OTA (9), cells pre-treated with 25 µg/ml sTNFR2 2 h prior to 12.5 µM OTA (10). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with three different cell preparations.

The pre-incubation of sTNFR2 at its highest applied concentration (375 µg/ml) neither prevented TNF-α-mediated DNA fragmentation nor OTA-induced DNA development **(Fig. 39)**.

Indicating that TNFR1 is generally associated with TNF-α-induced cell death and TNFR2 most likely with cell proliferation. In addition, both heterogenic TNF receptors 1 and 2 are not implicated in OTA-mediated apoptosis.

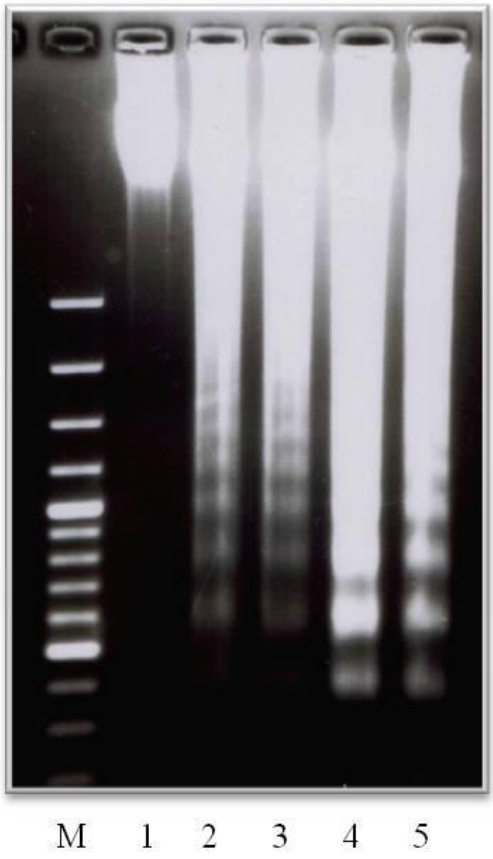

Fig. 39: Effect of sTNFR2 on DNA fragmentation induced by OTA and TNF-α

Primary rat hepatocytes were negative control for experiment (1), cells treated with 12.5 µM OTA (2), cells pre-treated with 375 µg/ml sTNFR2 2 h prior to 12.5 µM OTA (3), cells treated with 200/20 ng/ml ActD/TNF-α (4), cells pre-treated with 375 µg/ml sTNFR2 2 h prior to 200/20 ng/ml ActD/TNF-α (5). Then, at 24 h the DNA was isolated and visualized by gel electrophoresis. All experiments were repeated with three different cell preparations.

5.2.6. The Nuclear Damage caused by OTA, ActD/TNF-α, H_2O_2 and UVC and prevention by silibinin

In order to investigate the extent of nuclear apoptosis, experiments were carried out: for a Hoechst stain, experiment was undertaken to determine the percentage of apoptotic nuclei. Nuclei with condensed chromatin and fragmented were counted under a fluorescence microscope to determine the percentage of apoptotic nuclei. Apoptotic nuclei were determined by counting cells with: 1) highly condensed, 2) crescent-shaped or 3) fragmented nuclei. **Figure 40a** shows micrographs of apoptotic nuclei (white arrows) in cultured primary rat hepatocytes exposed to OTA, ActD/TNF-α, H_2O_2, and UVC. Treatment of primary rat hepatocytes by OTA, ActD/TNF-α, H_2O_2, and UVC led to a statistically significant induction rate of nuclear apoptotic events on approximately 25, 62, 55, and 30% cells, respectively **(Fig. 40b)**. These correlate with cytotoxicity as examined with MTT-test.

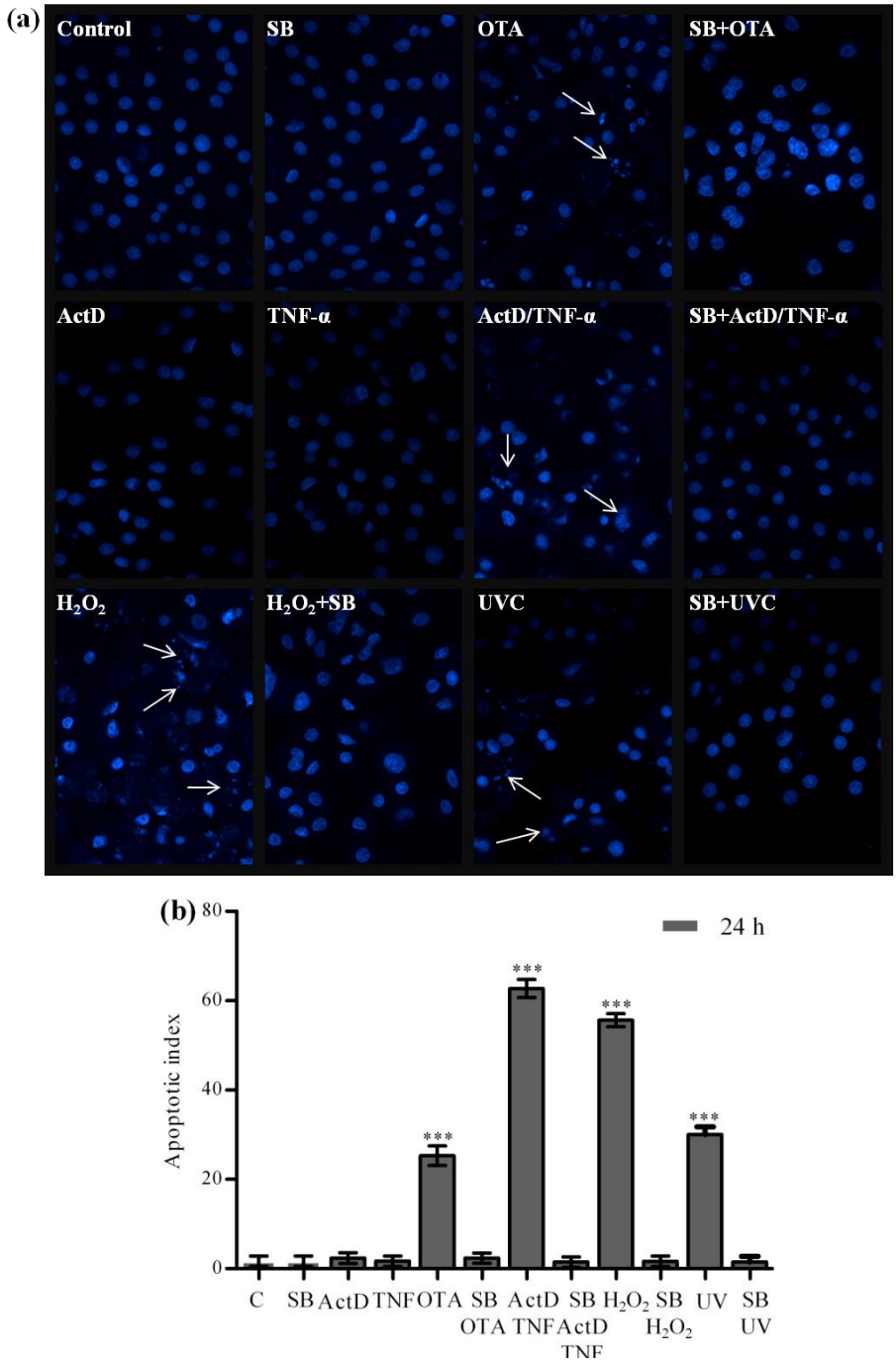

Fig. 40: Protective effect of silibinin on nuclear damage caused by OTA, ActD/TNF-α, H_2O_2, and UVC

Cultures of primary rat hepatocytes were pre-treated with 260 µM silibinin (SB) given 2 h prior to 12.5 µM ochratoxin A (OTA), 200/20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α), 10 µM hydrogen peroxide (H_2O_2), and 50 mJ/cm^2 ultraviolet (UVC) for 24 h. Cultured cells were fixed, chromatin was stained with Hoechst, and apoptotic nuclei were counted. (a) Fluorescence micrographs of Hoechst stained nuclei of primary rat hepatocytes (×400). The white arrows indicate apoptotic nuclei exhibiting fragmented chromatin whereas other nuclei are intact. The pictures are representing at least five fields from a slide; each experiment was performed at least 3 times. (b) The percentage of apoptotic nuclei is given as apoptotic index. N=3, treatment groups were compared to control using one-way ANOVA. *** is significantly different from control (0) at $P \leq 0.001$.

5.2.7. Effect of OTA, LPS, ActD/TNF-α, H_2O_2, and UVC on Caspase 3 activity

Next, we performed caspase activity assay in order to examine to what extent caspase activation is involved in apoptosis in primary rat hepatocytes by hepatotoxins.

The activation of caspase 3 represents an early hallmark during apoptosis. In this study, OTA treatment led to an up to 2.85-fold increase of executioner caspase 3 activity in a dose- and time-dependent manner in cultured primary rat hepatocytes **(Fig. 41)**.

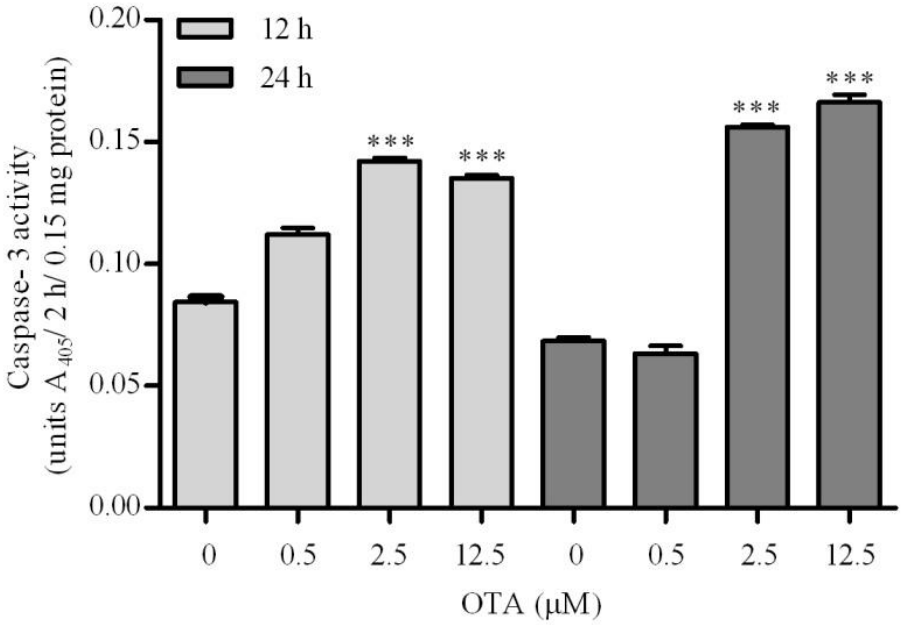

Fig. 41: Activation of caspase 3 by OTA in cultured primary rat hepatocytes

Cultured primary rat hepatocytes were treated with various concentrations of ochratoxin A (OTA) for 12 and 24 h. Then, the caspase 3 activity was measured by Caspase-3/CPP32 Colorimetric Assay Kit. All experiments were repeated with three different cell preparations. Data presents the value of the mean ± SD (n=3 / group). *** is significantly different from control (0) at $P \leq 0.001$.

As well, the exposure of hepatocyte cultures to ActD/TNF-α, H_2O_2, and UVC, caused activation of caspase 3. The most prominent activation was observed after treatment of primary rat hepatocytes by ActD/TNF-α **(Fig. 42)**.

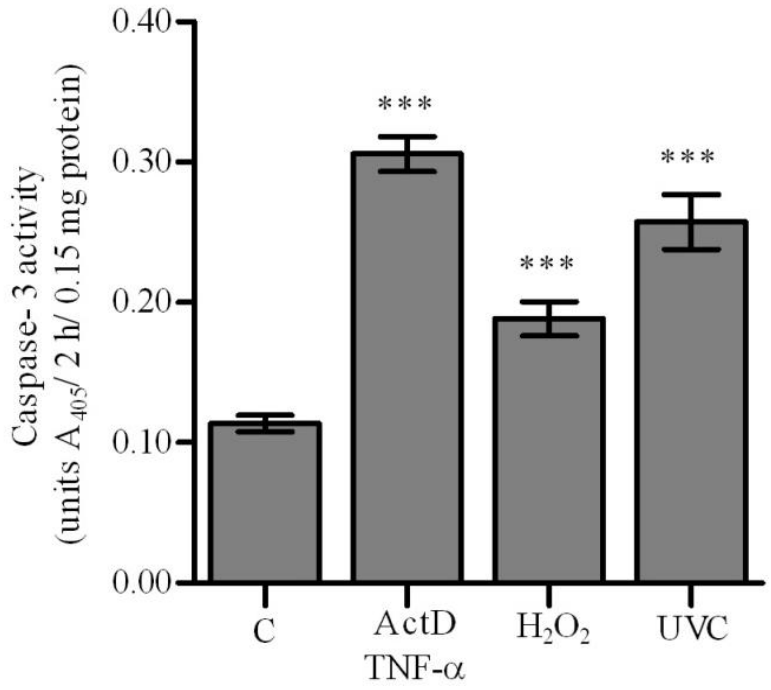

Fig. 42: Activation of caspase 3 by ActD/TNF-α, H_2O_2, and UVC in cultured primary rat hepatocytes

Cultured primary rat hepatocytes were treated with 200/ 20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α), 10 mM hydrogen peroxide (H_2O_2), and 50 mJ/cm^2 ultraviolet (UVC) for 12 h. Then, the caspase 3 activity was measured by Caspase-3/CPP32 Colorimetric Assay Kit. All experiments were repeated with three different cell preparations. Data presents the value of the mean ± SD (n=3 / group). *** is significantly different from control (0) at $P \leq 0.001$.

In contrast, in cultured rat hepatocytes, caspase 3 activity was not altered under incubation with 0.1–12.5 μg/ml LPS during 12 and 24 h **(Fig. 43)**.

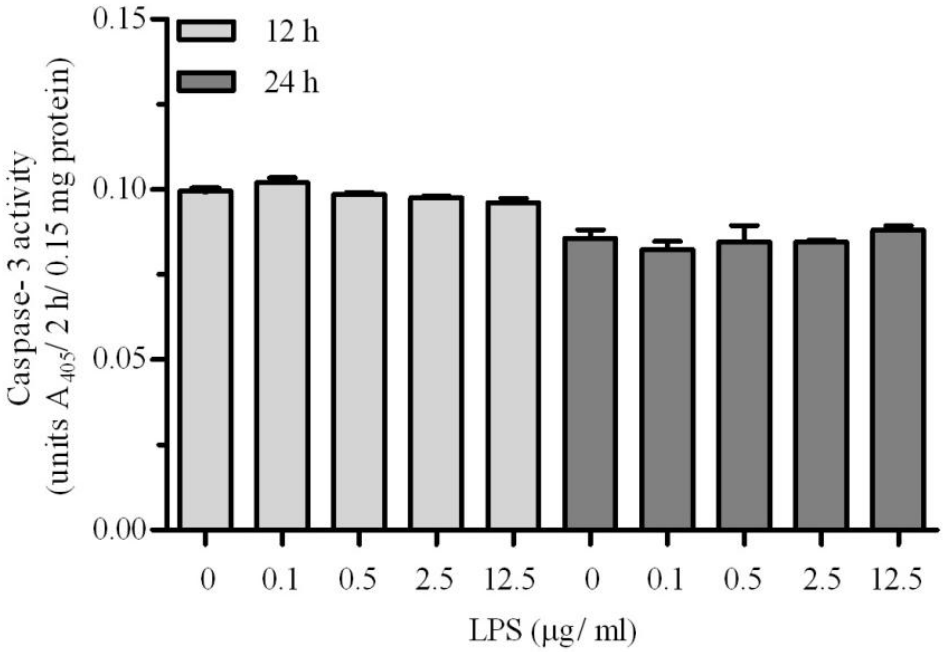

Fig. 43: Lack of effect on caspase 3 activity by LPS in cultured primary rat hepatocytes
Cultured primary rat hepatocytes were treated with various concentrations of lipopolysaccharide (LPS) for 12 and 24 h. Then, the caspase 3 activity was measured by PromoKine Caspase-3/CPP32 Colorimetric Assay Kit. Caspase 3 activity was not significantly different from control (0). All experiments were repeated with three different cell preparations. Data presents the value of the mean ± SD (n=3 / group).

5.2.8. Effect of silibinin on Caspase 3 activation

Silibinin shows various effects on initiators caspases (caspase 8 and 9) and on executioner caspases (e.g caspase 3). In this study, I tested the preventive effect of silibinin on caspase 3 activation induced by hepatotoxins treatment in cultured primary rat hepatocytes. Rat primary hepatocytes were pre-treated with and without 130 µM silibinin 2 h prior of 12.5 µM OTA and were cultured for 12 and 24 h. Silibinin completely abrogated the OTA-mediated caspase 3 activation **(Fig. 44)**.

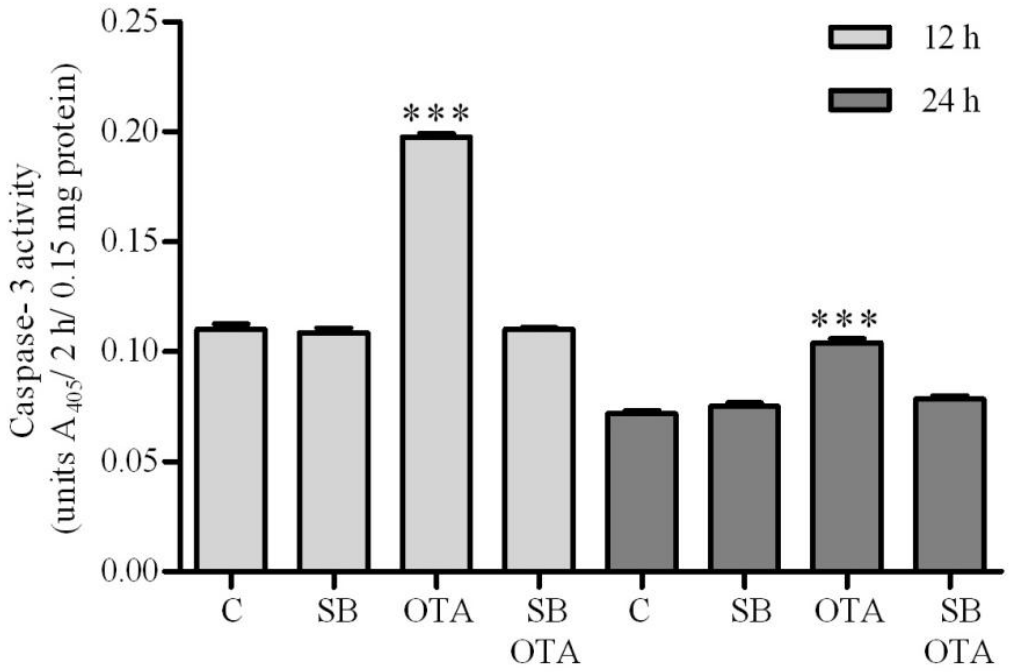

Fig. 44: Activation of caspase 3 by 12.5 µM OTA and prevention by silibinin

Cultures of primary rat hepatocytes were pre-treated with 130 µM silibinin (SB) for 2 h prior to ochratoxin A (OTA) at 12.5 µM. Caspase 3 activity was measured on 12 and 24 h cultures by Caspase-3/CPP32 Colorimetric Assay Kit. All experiments were repeated with three different cell preparations. Data presents the value of the mean ± SD (n=3 / group). *** is significantly different from control (0) and from SB+OTA at $P \leq 0.001$.

Under same conditions, silibinin exerted complete hepatoprotection against H_2O_2- and UVC-mediated caspase 3 activation at 130 µM whereas twice the concentration (260 µM) prevented caspase 3 activation by ActD/TNF-α at 24 h **(Fig. 45)**.

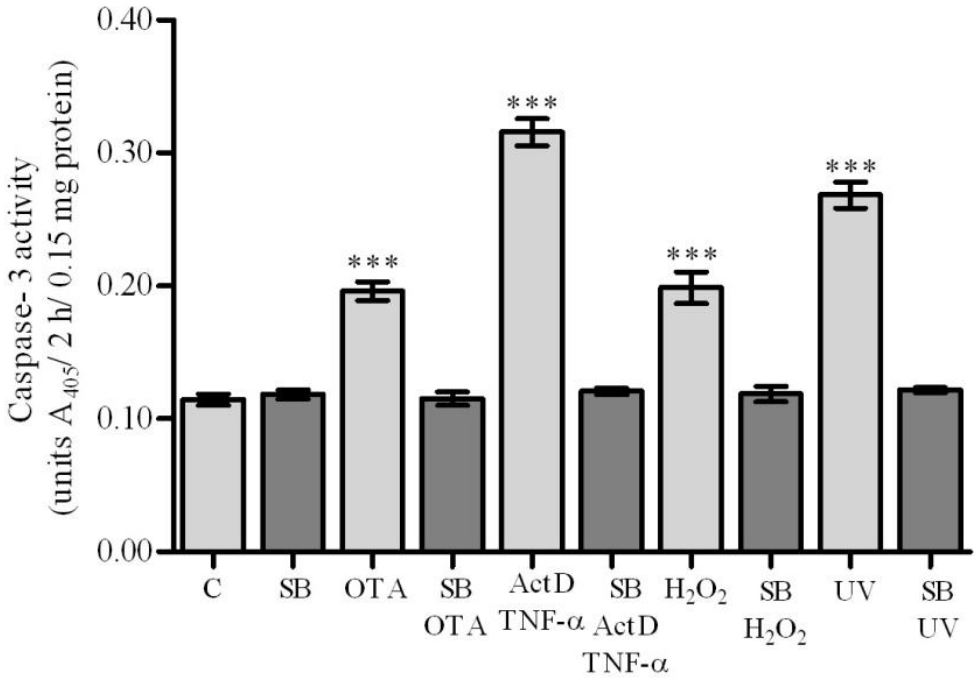

Fig. 45: Caspase 3 activation by OTA, ActD/TNF-α, H_2O_2, and UVC and prevention by silibinin in cultured primary rat hepatocytes

Caspase 3 activity was measured by a Caspase-3/CPP32 Colorimetric Assay Kit after pre-treatment of cultured primary rat hepatocytes with silibinin (SB) at 130 µM 2 h prior to12.5 µM ochratoxin A (OTA), 10 mM hydrogen peroxide (H_2O_2), and 50 mJ/cm^2 ultraviolet (UVC), and 260 µM 2 h prior to 200/20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α) for 12 h. All experiments were repeated with three different cell preparations. Data presents the value of the mean ± SD (n=3 / group). *** is significantly different from control (0) at $P \leq 0.001$.

5.3. Oxidative stress markers

Besides, apoptosis induction, oxidative stress is defined as structural and/or functional injury produced in tissues by the uncontrolled formation of pro-oxidant free radicals. Oxidative stress usually develops when the pro-oxidant action of an inducer exceeds the anti-oxidant capacity of the cell defence system, altering its homeostatic capacity.

5.3.1. Lipid peroxidation induction by OTA, ActD/TNF-α, and H_2O_2 in HPCT-1E3 cells

The findings so far, point out that alternatively to cytokine mediated apoptosis and cytotoxicity, oxidative stress reactions may have caused cell damage and that silibinin has prevented them as an antioxidant. A test was performed to detect malondialdehyde as a natural end product of membrane lipid peroxidation and also reactive oxygen species generation. Cell cultures of immortalized rat hepatocytoma cells (HPCT-1E3) were exposed to OTA and ActD/TNF-α. For control, samples were also analysed after incubation with H_2O_2. The results indicated that OTA generates, as with the other hepatotoxins, oxygen radicals in a dose depended manner in cell cultures after 6 h **(Fig. 46)**.

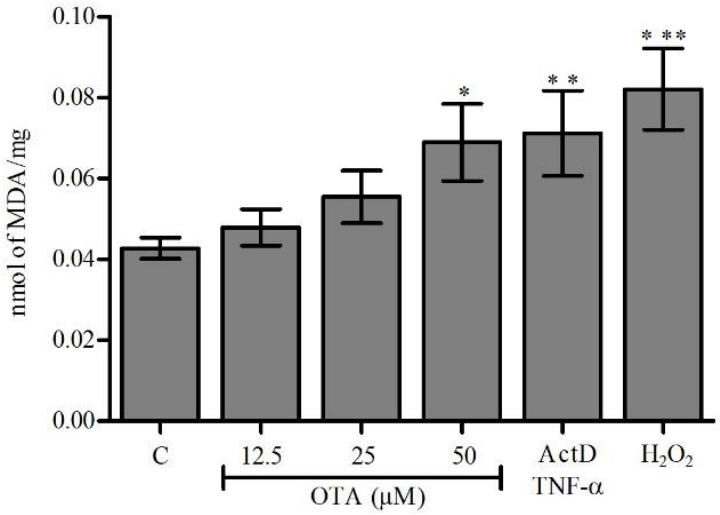

Fig. 46: Lipid peroxidation induced by OTA

HPCT-1E3 cells were treated with various concentrations of ochratoxin A (OTA), 200/20 ng actinomycin D/tumour necrosis factor alpha (ActD/TNF-α), and 10 mM hydrogen peroxide (H_2O_2). Then, at 6 h the lipid peroxidation was determined by MDA Fluorometric Assay Kit. All experiments were repeated with 3 different cell cultures. *P* values *≤ 0.05, **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

5.3.2. Oxidative stress induction by OTA, ActD/TNF-α, and H_2O_2 in HPCT-1E3 cells and prevention by silibinin

Moreover, the silibinin pre-treatment at 260 µM 2 h prior to hepatotoxins restored significantly the lipid peroxidation and ROS induction by 50 µM OTA, 200/20 ng/ml ActD/TNF-α and 10 mM H_2O_2 **(Fig. 47a and b)**.

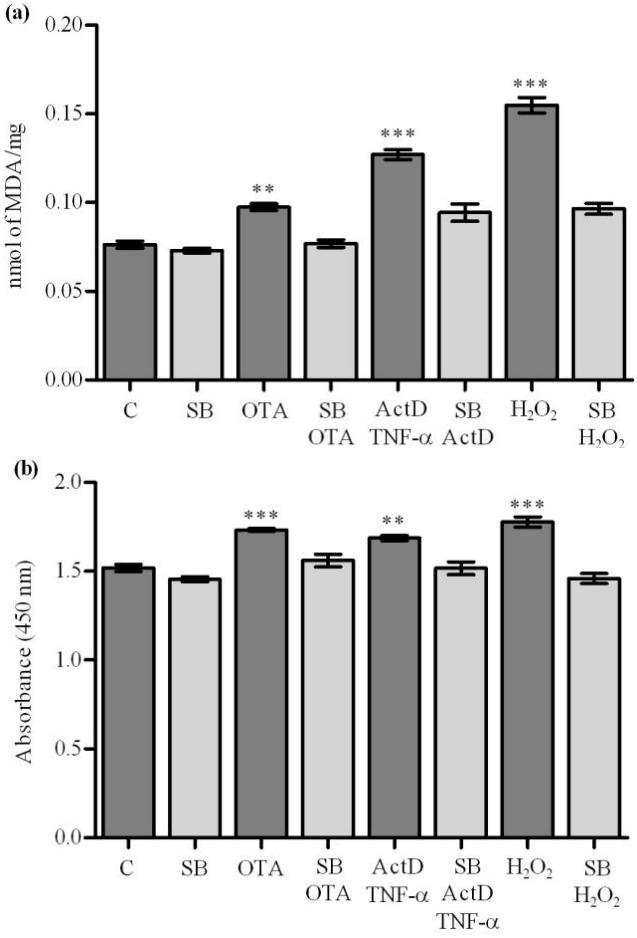

Fig. 47: Silibinin effect on oxidative stress induced by OTA, ActD/TNF-α, and H_2O_2

HPCT-1E3 cells were pre-treated with silibinin (SB) at 260 µM 2 h prior to 50 µM ochratoxin A (OTA), 200/ 20 ng/ml actinomycin D/tumour necrosis factor alpha (ActD/TNF-α), and 10 mM hydrogen peroxide (H_2O_2). Then, at 6 h the lipid peroxidation was determined fluorometrically (a) and the ROS generation was measured colorimetrically (b). All experiments were repeated with 3 different cell cultures. *P* values **≤ 0.01, ***≤ 0.001 compared with control values were considered statistically significant.

VI. Discussion

In this study, the mechanism of apoptosis induction by OTA was investigated in liver cell systems of rats and humans. It addresses the questions; whether the apoptotic effect of OTA is mediated by the TNF alpha receptors and whether silibinin can prevent it and by which mechanisms.

6.1. Cytotoxicity

Although the liver is not the main target organ for OTA toxicity, hepatocytes are exposed to OTA since it has to pass the liver after intestinal absorption. Despite that liver toxicity is one of the consequences of OTA, the mechanisms by which OTA exerts its hepatotoxicity are still scantly understood. One possible detrimental cause could be liver cell damage exerted by the release of TNF-α, since OTA provokes TNF-α release in the intact rat liver model from Kupffer cells (Al-Anati et al., 2009). Therefore, I investigated cytotoxic effects by OTA, LPS, and TNF-α when applied separately on pure cultured liver cells. OTA appeared cytotoxic in two used test systems (MTT-test and Live/Dead kit assay) in a dose- and time-dependent manner to the liver cells. But TNF-α was toxic only after induction of a transcriptional arrest performed by actinomycin D or camptothecin. A successive combined administration of ActD or CPT with TNF-α mediated a statistically significant decrease in the viability of cultured liver cells as confirmed by MTT-test.

Neither TNF-α nor transcriptional inhibitors alone had a toxic effect on all used liver cells (primary rat hepatocytes and HPCT-1E3 cells) in comparison with untreated cells. Hence, the rat hepatocytes are extremely resistant to direct toxic effects of TNF-α unless pre-sensitized by a transcriptional inhibitor e.g. ActD or CPT confirming a previous finding (Leist et al., 1994). Despite LPS-induced leakage of cytotoxic markers GLDH and LDH from isolated blood-free perfused rat liver (Al-Anati et al., 2009), surprisingly, the long incubation of cell cultures with LPS alone at high concentration has no toxic effect on all used liver cells. Cytotoxicity of LPS is mainly due to binding of the endotoxin to Toll-like receptor 4. Since in liver cells this receptor is only marginally expressed (Matsumura et al., 2003) no or only weak cytotoxicity of LPS to liver is expected.

Silibinin pre-treatment in the MTT and Live/Dead test systems restored significantly the decrease in cell viability caused by OTA as compared to untreated control. Additionally, silibinin completely prevented the toxic effects caused by ActD/TNF-α combination in cultured primary rat hepatocytes. Silibinin preventive effects refers to the ability of silibinin to prevent the absorption of toxins into the hepatocytes by occupying the binding sites, as well as, inhibiting also transport proteins at the membrane (Faulstich et al., 1980). In addition, silibinin exerts a regulatory action on cellular and mitochondrial membrane permeability due to with an increase in membrane stability against oxidative toxin injury. In this study silibinin was used as an antioxidant that

completely prevented the H_2O_2 and UVC-mediated cytotoxicity in liver cells. Silibinin acted as a potent antioxidant that neutralized harmful free radicals. The mentioned actions along with the anti-oxidative property, makes silibinin a promising remedy for the prevention and treatment of toxic liver diseases.

6.2. Apoptosis markers and prevention by silibinin

The other event which was investigated apart from cytotoxicity was apoptosis. The present study confirmed previous results (Chopra et al., 2010b) that OTA induced apoptosis in primary rat hepatocytes and in human hepatoma cells. Both hepatotoxins, OTA and TNF-α resulted in concentration-dependent apoptosis of characteristic effects: chromatin condensation and fragmentation, and internucleosomal DNA fragmentation. The internucleosomal DNA fragmentation caused by TNF-α, however, could be seen only in the presence of the transcriptional inhibitors ActD or CPT. The third toxic compound, LPS induced no apoptosis in rat and human hepatocytes. This is in contrast to pro-apoptotic effects on hepatocytes in the intact rat liver *in vivo* in the presence of LPS (Hamada et al., 1999). Finally, incubation of liver cells with H_2O_2 or UVC irradiation caused pronounced apoptosis.

The present study identified the flavonolignan silibinin as an anti-apoptotic agent counteracting the induction of programmed cell death by the used hepatotoxins on primary rat hepatocytes cultures. This effect required pre-incubation of primary rat hepatocytes with silibinin in order to prevent the

nuclear and internucleosomal DNA fragmentation. The pronounced DNA ladder was completely reduced by the pre-treatment with silibinin. This anti-apoptotic effect was time and dose dependently. In primary rat hepatocytes, it made a difference whether the cells had been pre-incubated with silibinin for less than 2 hours or more than 2 h prior to hepatotoxins treatment. The influence of silibinin on preventing pronounced DNA cleavage was observed only after long pre-incubation greater than or equal to 2 h. The pro-apoptotic effects of silibinin were achieved at concentrations greater than or equal to 130 μM. Only the TNF-α induced apoptosis required a double concentration of silibinin of 260 μM for prevention at the same pre-incubation period of 2 h. The results indicated that silibinin prevented (i) extrinsic apoptosis pathway by inactivation the cell surface death receptors belonging to TNF superfamily and (ii) intrinsic apoptosis pathway by preventing the permeabilization of the mitochondrial outer membrane.

Furthermore, in this study caspase 3 activity was examined as apoptosis biomarker. OTA treatment resulted in 2.85-fold increase of executioner caspase 3 in primary rat hepatocytes. This result confirmed earlier studies, which found this mycotoxin to enhance caspase activities in monkeys (Scibelli et al., 2003), and rats (Sauvant et al., 2005), as well as, in cultured primary rat hepatocytes (Chopra et al., 2010a, Essid and Petzinger, 2011). Moreover, OTA potentiates the effect of TNF-α on the caspase 3 activity in MDCK-C7 cells (Gekle et al., 2000). In contrary, LPS had no effect on caspase 3 activity under same

conditions. This is explained (i) by the absence of TNF-α because of the absence of Kupffer cells in cultured primary rat hepatocytes and (ii) by almost complete lack of Toll-like receptors 4 (TLR4) that also could mediate LPS induced apoptosis. The other pro-apoptotic stimuli TNF-α, H_2O_2, and UVC however, increased significantly the caspase 3 activity in primary rat hepatocytes. This activation of caspase 3 was completely inhibited by silibinin pre-incubation. Silibinin has shown inhibitory effect on caspase 9 and 3-mediated intrinsic apoptosis pathway (Jiang et al., 2009) and caspase 8 and 3-mediated extrinsic apoptosis pathway (Wang et al., 2005, Li et al., 2006). In other words, these data are also in accordance with other studies concerning silibinin, showing protection of cells from apoptosis signal pathways.

6.3. Effect of TNF-α antagonists and soluble TNFR1 and 2 on nuclear apoptosis

The generally accepted picture of receptor-mediated apoptosis by TNF-α claims that, the cell membrane receptor TNF-α receptor 1 (TNFR1) triggers apoptosis by binding TNF-α with its extracellular domain and by activation of its intracellular domain, named adaptor protein TNFR1 associated death domain (TRADD). Following activation of TRADD, the recruitment and activation of the initiator aspartate specific cysteine protease caspase 8 occurs, that in turn precipitates the activation of downstream effector caspases 3 and 7 which triggers further events of receptor induced apoptosis.

TNF-α originates in the intact liver model only from Kupffer cells (Al-Anati et al., 2005), and accordingly, OTA does not cause TNF-α release in cultured hepatocytes (Al-Anati et al., 2010). Since Kupffer cells were absent in primary hepatocyte cultures, we can thus conclude that the apoptosis pathway triggered by OTA *in vitro* in hepatocytes follows a TNF-α-independent pathway and that silibinin did not act via the suppression of cytokine interactions. TNF-α dependent apoptosis, however, occurs in the intact liver by LPS (Hamada et al., 1999), since hepatocytes express type I TNF-α receptors (TNFR1) (Libert et al., 1991). In the primary rat hepatocytes culture model, and in the absence of TNF-α, cell apoptosis was not observed under LPS. Thus, the trigger for apoptosis on primary cultured hepatocytes by OTA and LPS from apoptosis in intact liver was different. The pre-incubation of primary rat hepatocytes with sTNFR1 completely inhibits TNF-α mediated apoptosis, whereas, pre-incubation with sTNFR2 did not prevent TNF-α-mediated apoptosis. Since TNF-α uses its TNFR1 to induce apoptosis in cultured primary rat hepatocytes, therefore, and in order to confirm the previous result, rat primary hepatocytes were pre-incubated with two different TNF-α antagonists: (I) a peptide contains the specific TNF-α binding site of the human 55 kD TNF receptor, which binds TNF and blocks its actions *in vitro* and *in vivo* and (II) a cyclic peptide called WP9QY that is designed to prevent interaction of TNF with its receptor. Surprisingly, these TNF-α antagonists did not prevent TNF-α induced apoptosis despite their applications were at the same concentration of sTNFR1.

This contradicts that these peptides have bound to all functional sites of TNFR in rat hepatocytes. Taken together, TNF-α mediates apoptosis *in vitro* in pure hepatocyte cultures via complete functional TNFR1. So, I supposed that OTA uses TNF-α receptors to induce apoptosis in cultured primary rat hepatocytes. But actually, the observations indicated that neither pre-incubation of cultured hepatocytes with sTNFR1 and 2 nor TNF-α antagonists prevent OTA-mediated apoptosis at all. As also results of Chopra and his co-workers indicated that caspase 8 activity under OTA was not statistically significant (Chopra et al., 2010b), the extrinsic apoptotic pathway is less likely to be involved in OTA-mediated apoptosis in primary rat hepatocytes.

6.4. Oxidative stress and prevention by silibinin

OTA circulating in liver activates Kupffer cells and hepatocytes to produce free radicals. These free radicals increase lipid peroxidation which can lead to liver toxicity and subsequently liver dysfunction. Therefore, the most likely possibility is oxidative stress of liver cells induced by OTA via lipid peroxidation, since the release of ROS under OTA was reported from rat liver (Meki and Hussein, 2001), and hepatotoxicity in rats is probably as a consequence of oxidative stress (Gagliano et al., 2006). This leads to apoptosis-inducing factor (AIF) release from mitochondria as result from mitochondrial depolarization (Chopra et al., 2010b). In this study, OTA treatment caused lipid peroxidation and ROS generation in a dose-dependent manner and was

comparable to H_2O_2 that was completely prevented by silibinin. These results concluded that OTA induced apoptosis most likely by intrinsic signalling pathway. Interestingly, TNF-α caused also lipid peroxidation and ROS generation, thus these findings indicate that both the extrinsic and the intrinsic pathway are involved in TNF-α-induced apoptosis. Moreover, silibinin is generally capable of inhibiting apoptosis and oxidative damage produced by H_2O_2. This candidates silibinin as a potent anti-oxidant additive, and its anti-apoptotic effects may attribute to its antioxidative effect.

6.5. Conclusion

In this thesis, I showed that liver cell apoptosis by the mycotoxin ochratoxin A *in vitro* is not mediated by cytokine TNF-alpha nor by direct binding to the receptor TNFR1, but by oxygen radical formation that causes lipid peroxidation and was, thus, different from *in vivo* conditions in the intact rat liver. TNF-α produces apoptosis in liver cells via the extrinsic apoptosis pathway by using TNFR1 and causes oxidative damage. However, OTA seems to cause apoptosis not by activating the extrinsic pathway, but, rather the intrinsic pathway. The herbal flavanolignan silibinin counteracted the cytotoxicity and induction of apoptosis by OTA, TNF-α, H_2O_2, and UVC on primary rat hepatocytes cultures and thus was a potent cytoprotective and anti-apoptotic agent. This distinguishes silibinin as a prophylactic hepatoprotective remedy.

VII. Zusammenfassung

Primäre Hepatozyten aus Ratten wurden nach zwei verschiedenen Methoden isoliert: der klassischen enzymatischen Methode nach künstlicher Verdauung durch Kollagenase-Perfusion und einer neuen EDTA-Perfusionsmethode. Die EDTA-Perfusionsmethode ergab Hepatozyten, die in Zellkulturen bis zu 96 h stabil ohne DNA-Fragmentierung kultiviert wurden, während die Kollagenase präparierten Hepatozyten Apoptoseereignisse bereits von Beginn ihres Kultivierung auch in Abwesenheit von OTA zeigten. Experimentell wurde Apoptose durch 20 ng/ml Tumor-Nekrose-Faktor-alpha (TNF-α) nur in Gegenwart von 200 ng/ml des transkriptionellen Inhibitors Actinomycin D (ActD) ausgelöst. Diese Apoptosewirkung wurde vollständig in Gegenwart von 25 µg/ml löslichem TNF-α-Rezeptor 1 (sTNFR1) verhindert. Der transkriptionelle Inhibitor Actinomycin D (ActD) verursachte alleine keine Apoptose. Die durch ActD/TNF-α ausgelöste Apoptose wurde dagegen nicht in Gegenwart von 25-375 µg/ml löslichem TNF-α Rezeptor 2 (sTNFR2) im Überstand von Zellkulturen verhindert. Dies zeigt, dass die TNF-α-erzeugte Apoptose in kultivierten Hepatocyten nur durch TNFR1 vermittelt wird. Apoptose trat auch nach Anwendung von 12,5 µM Ochratoxin A (OTA) sowhol in kultivierten Hepatozyten als auch in HepG2-Zellen auf. Allerdings wurde diese weder von sTNFR1 bis 500 µg/ml noch von sTNFR2 bis zu 375 µg/ml verhindert. Dies zeigt, dass TNFR1 und 2 an der OTA vermittelten

Apoptose in kultivierten Hepatozyten nicht beteiligt sind. Eine Inkubation von kultivierten Hepatozyten und HepG2 Zellen mit dem klassischen TNF-α Induktor Lipopolysaccharid (LPS) (von 0,1 bis 12,5 µg/ml) hatte keine cytotoxische oder apoptotische Wirkung unter diesen Bedingungen.

Das antioxidative Flavanolignan Silibinin in Dosen von 130 bis 260 µM verhinderte an den Zellkulturen eine Chromatin-Kondensation, Caspase-3-Aktivierung und apoptotische DNA-Fragmentierung, die durch OTA sowie durch 10 mM Wasserstoffperoxid (H_2O_2), durch ultraviolettes (UVC) Licht (50 mJ/cm2) und durch ActD/TNF-α induziert wurden. Um eine Schutzwirkung durch Silibinin zu erreichen, wurde das Medikament zu den Hepatozyten für 2 h im voraus gegeben. Als Verursacher der OTA stimulierten Apoptose wird keine TNF-α vermittelte Apoptose, sondern eine durch Lipidperoxidation ausgelöste Apoptose angenommen. Unter OTA trat an den kultivierten menschlichen Lebertumorzellen HepG2 sowie an immortalisierten Rattenleber HPCT-Tumorzellen eine Sauerstoffradikal- und Malondialdehyd (MDA)-Bildung auf. Dieser Indikator für die Lipidperoxidation trat auch durch H_2O_2 und ActD/TNF-α Inkubation auf. Sämtliche oxidativen Reaktionen, nämlich ROS (reactive oxygen species) und MDA wurden durch Silibinin Vorbehandlung unterdrückt. Wir folgern, dass die anti-apoptotische Aktivität von Silibinin gegen OTA, H_2O_2 und ActD/TNF-α durch die antioxidativen Wirkungen des Flavanolignans verursacht wird.

Neben der Apoptose-Schutzwirkung vermittelte Silibinin auch einen Schutz vor Zytotoxizität. Dieser wurde gegenüber allen pro-apoptotischen Stoffen durch den MTT-Test-und Live/Dead Kit offenbart. Wenn einzeln angewandt, zeigte ActD und TNF-α keine zytotoxische Wirkung nach 24 h, die aber in Kombination auftrat. Die verwendeten Konzentrationen von OTA, H_2O_2 und die Dosis von UVC verursachte einen deutlichen Rückgang der Zellvitalität innerhalb von 36 h, der von Silibinin verhindert wurde. Zusammengenommen läßt sich feststellen, dass die durch OTA vermittelte Apoptose in kultivierten primären Rattenhepatozyten entgegen früherer Annahmen nicht durch TNF-α/ TNFR1, sondern durch Sauerstoffradikale und Lipidperoxidation verursacht wird. Silibinin ist eine sehr wirksame anti-apoptotisch wirkende Verbindung, die auch vor einer Zytotoxizität durch OTA und die anderen untersuchten Stoffe schützt.

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Ebtisam Essid received her bachelor degree in veterinary medicine in 1999 from faculty of veterinary medicine in Tripoli, Libya. After graduating she was appointed as a demonstrator to the faculty of veterinary sciences. In 2006, she was granted a scholarship by the university to pursue a PhD degree in Veterinary sciences. In 2007, she started her doctoral thesis at the Institute of Pharmacology and Toxicology, faculty of veterinary medicine, Justus-Liebig-University Giessen, Germany. The objective of her thesis is shedding the light on the hepatotoxicity of OTA in vitro, viz apoptotic pathway by comparing it with well known hepatotoxin apoptosis inducers and ist protection by drug Silibinin on hepatocytes.

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VVB LAUFERSWEILER VERLAG

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STAUFENBERGRING 15
D-35396 GIESSEN

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