HEPATOTOXICITY OF THE PHYTOMEDICINES KAVA KAVA AND CIMICIFUGA RACEMOSA

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"Nearly all men die of their medicines, not of their diseases."

Molière (1622-1672)

Kava Kava (*Piper methysticum* Forst.)

Cimicifuga racemosa

TABLE OF CONTENTS

1 SUMMARY

In this thesis the hepatotoxic properties of the two phytomedicines kava kava and cimicifuga racemosa have been investigated. This topic is a prevailing problem as herbal medicines are quite popular today and not much is known about their toxicological profile. Especially hepatotoxicity is a wide-spread problem of medicines in general as most of them are metabolized in the liver and the liver therefore represents a near target. Ingestion of kava has been associated with liver damage and therefore Kava has been withdrawn from the market in different countries. For cimicifuga so far not much evidence for liver toxicity in humans exists. However, lately it was found that rats fed with high doses of cimicifuga developed microvesicular liver steatosis.

In order to examine the *in vitro* toxicity of the two herbs the hepatocarcinoma cell line HepG2, the hepatoblastoma cell line HUH6, and isolated rat liver mitochondria have been used. Three kava extracts (a methanolic and an acetonic root extract; a methanolic leaf extract) and an ethanolic cimicifuga extract have been investigated.

In the first project (chapter 6) toxicity of kava kava was investigated. It was found that all three kava extracts showed concentration-dependent toxicity in the general cytotoxicity tests. Experiments in mitochondria revealed that the kava extracts inhibited and uncoupled (root extracts) or only uncoupled (leaf extract) the respiratory chain and decreased the mitochondrial membrane potential; in addition, beta-oxidation was inhibited. Furthermore, oxidized glutathione was slightly increased and ATP content of the cells was maintained. Apoptosis was found in HepG2 cells for all three kava extracts. These findings of mitochondria-related toxicity (affected respiratory chain, decreased mitochondrial membrane potential and increased reactive oxygen species (ROS) production) might result in the opening of the permeability transition pore and consequently in the rupture of the outer mitochondrial membrane, thus possibly leading to the release of cytochrome c and subsequently to apoptosis. These events might contribute to kava associated hepatotoxicity, especially in predisposed patients having mitochondrial damages.

In the second project (chapter 7) two methods for cell death determination were applied and compared in the hepatoma cell lines HepG2 and HUH6 utilizing kava as death-inducing agent: the annexin V / propidium iodide (PI) stain and the green fluorescent protein (GFP)-method. The annexin V / PI stain has already been established as a method to detect apoptosis and necrosis elicited by kava kava in the study mentioned above. The GFP-method had been described in different cell lines by other groups and is based upon the phenomenon that GFP decreases its fluorescence when apoptosis and/or necrosis occur. We found that the annexin V / PI stain and the GFP-method provided similar results. These results on the one

hand confirm that kava indeed induces cell death; on the other hand they show that the GFP-method can also be employed in liver cell lines. Therefore, the GFP-method is suited as an easy, reliable, cost-effective method to screen substances for their hepatotoxic potential.

In a further study (chapter 8) the toxicity profile of the phytomedicine cimicifuga was assessed. High doses of cimicifuga extract caused microvesicular liver steatosis in rats. Based on these findings *in vitro* experiments were performed to further investigate the hepatotoxic potential of this plant. General cytotoxicity tests revealed concentration-dependent toxicity. In mitochondria, cimicifuga extract was able to inhibit β-oxidation, to uncouple the respiratory chain and to reduce the mitochondrial membrane potential. In addition, HepG2 cells underwent apoptosis when incubated with cimicifuga. It might therefore be assumed that the impairment of the respiratory chain causes a decrease of the mitochondrial membrane potential, which in turn could lead to the opening of the permeability transition pore and to apoptosis. Whereas the inhibition of the β-oxidation may lead to accumulation of fatty acids and subsequently to liver steatosis. Toxicity was discovered at concentrations higher than the ones expected in humans. It is therefore conjectured, that hepatotoxicity in humans only occurs under certain conditions.

Finally, it can be concluded that although kava kava and cimicifuga racemosa displayed clear toxicity in the *in vitro* situation it has to be further investigated whether these toxicities can be extrapolated to humans. Factors which have to be taken into account when estimating the toxic potential for humans include the concentrations used in the *in vitro* tests in comparison to portal venous concentrations in humans after intake of the extracts, the metabolism in the body, predisposing factors for liver toxicity like age, gender and pre-existing liver diseases, and poly-medication. Nevertheless, these cytotoxicity tests represent a valuable tool for evaluating the toxic potential of herbal products before they reach the market. Of course, other experiments (animal experiments, for example) would have to be added, if an assessment corresponding to the rules of the regulatory offices is sought. These in-depth investigations are needed, if botanicals are to persist on the market in future.

2 ZUSAMMENFASSUNG

In dieser Arbeit wurden die hepatotoxischen Eigenschaften der beiden Phytopharmaka Kava Kava und Cimicifuga racemosa untersucht. Dieses Thema ist ein aktuelles Problem, da pflanzliche Medikamente heutzutage sehr beliebt sind und nicht viel über ihr toxikologisches Profil bekannt ist. Vor allem Hepatotoxizität ist ein weit verbreitetes Problem bei Medikamenten im Allgemeinen, da die meisten über die Leber metabolisiert werden und die Leber deshalb einen nahe liegenden Angriffsort darstellt. Die Einnahme von Kava Kava wurde mit Leberschäden in Zusammenhang gebracht und deshalb wurde es in verschiedenen Ländern vom Markt genommen. Für Cimicifuga existieren bis jetzt kaum Hinweise auf Lebertoxizität beim Menschen. Es wurde jedoch kürzlich beobachtet, dass Ratten, die mit hohen Dosen Cimicifuga-Extrakt gefüttert wurden, eine mikrovesikuläre Lebersteatose entwickelten.

Um die *in vitro*-Toxizität der zwei Pflanzen zu untersuchen, wurden die Hepatocarcinoma-Zelllinie HepG2, die Hepatoblastoma-Zelllinie HUH6 und isolierte Rattenleber-Mitochondrien verwendet. Es wurden drei Kava-Extrakte – ein methanolischer und ein acetonischer Wurzelextrakt sowie ein methanolischer Blattextrakt – und ein ethanolischer Cimicifuga-Extrakt untersucht.

Im ersten Projekt (Kapitel 6) wurde die Toxizität von Kava Kava untersucht. Dabei wurde gefunden, dass alle drei Kava-Extrakte konzentrationsabhängige Toxizität in den allgemeinen Toxizitätstests zeigten. Experimente in Mitochondrien enthüllten, dass die Kava-Extrakte die Atmungskette inhibieren und entkoppeln (Wurzelextrakte) oder nur entkoppeln (Blattextrakt); ausserdem war die β-Oxidation gehemmt. Des Weiteren war das oxidierte Glutathion leicht erhöht und der ATP-Gehalt der Zellen blieb aufrechterhalten. In HepG2 wurde für alle drei Kava-Extrakte Apoptose gefunden. Diese Ergebnisse mitochondrieller Toxizität (beeinträchtigte Atmungskette, erniedrigtes Mitochondrien-Membranpotential und erhöhte ROS-Produktion) könnten zur Öffnung der Permeabilitäts-Transitions-Pore und infolgedessen zum Aufreissen der äusseren Mitochondrienmembran führen, was möglicherweise die Freisetzung von Cytochrom c und daraus folgend Apoptose verursacht. Diese Ereignisse könnten zur Kava-assoziierten Hepatotoxizität beitragen, vor allem bei prädisponierten Patienten mit mitochondriellen Schäden.

Im zweiten Projekt (Kapitel 7) wurden zwei Methoden zur Zelltod-Bestimmung in den Hepatoma-Zelllinien HepG2 und HUH6 mit Kava-Extrakt als Zelltod-auslösendem Agens angewendet und verglichen: die Annexin V / Propidiumiodid (PI)-Färbung und die GFP-Methode. Die Annexin V / PI-Färbung wurde schon in der oben erwähnten Arbeit zur Detektion von Kava-verursachter Apoptose und Nekrose etabliert. Die GFP-Methode wurde durch andere

Gruppen in verschiedenen Zelllinien beschrieben und basiert auf dem Phänomen, dass sich die GFP-Fluoreszenz reduziert, wenn Apoptose und/oder Nekrose auftreten. Wir haben gefunden, dass die Annexin V / PI-Färbung und die GFP-Methode ähnliche Resultate lieferten. Diese Resultate bestätigten auf der einen Seite, dass Kava tatsächlich Zelltod induziert. Andererseits zeigten sie, dass die GFP-Methode auch in Leber-Zelllinien angewendet werden kann. Aus diesem Grund kann die GFP-Methode als eine einfache, Kosten sparende, verlässliche Methode für das Screenen von Substanzen bezüglich ihres hepatotoxischen Potentials verwendet werden.

In einer weiteren Studie (Kapitel 8) wurde das Toxizitätsprofil des Phytopharmakons Cimicifuga racemosa abgeschätzt. Hohe Dosen von Cimicifuga verursachten mikrovesikuläre Steatose in Ratten. Basierend auf diesen Ergebnissen wurden *in vitro*-Experimente durchgeführt um das hepatotoxische Potential dieser Pflanze weiter zu untersuchen. Allgemeine Zytotoxizitätstests zeigten konzentrations-abhängige Toxizität. In Mitochondrien vermochte der Cimicifuga-Extrakt die β-Oxidation zu hemmen, die Atmungskette zu entkoppeln und das mitochondrielle Membranpotential zu reduzieren. Ausserdem wurde in HepG2-Zellen Apoptose induziert. Es könnte deshalb angenommen werden, dass die Beeinträchtigung der Atmungskette eine Erniedrigung des mitochondriellen Membranpotentials verursacht, was zur Öffnung der Permeabilitäts-Transitions-Pore und daraus folgend zu Apoptose führen könnte. Dahingegen könnte die Hemmung der β-Oxidation zur Akkumulation von Fettsäuren und anschliessend zu Lebersteatose führen. Die gefundene Toxizität trat bei höheren Konzentrationen auf als bei denen, die im Menschen erwartet würden. Es wird deshalb spekuliert, dass nur unter bestimmten Voraussetzungen Hepatotoxizität im Menschen auftritt.

Obwohl Kava Kava und Cimicifuga racemosa eine deutliche Toxizität in der *in vitro*-Situation zeigten, kann abschliessend gefolgert werden, dass weiter untersucht werden muss, ob diese Toxizitäten auf den Menschen übertragen werden können. Faktoren, die beachtet werden müssen, wenn das toxische Potential für den Menschen abgeschätzt werden soll, sind die in den *in vitro*-Tests verwendeten Konzentrationen im Vergleich zu Portalvenenkonzentrationen im Menschen nach Einnahme der Extrakte, der Metabolismus im Körper, prädisponierende Faktoren für Lebertoxizität wie Alter, Geschlecht und vorbestehende Lebererkrankungen und Polymedikation. Dennoch stellen diese Zelltoxizitätstests ein wertvolles Hilfsmittel für die Evaluation des Toxizitätspotentials pflanzlicher Produkte dar, bevor sie auf den Markt kommen. Natürlich müssten noch weitere Experimente, wie z. B. Tierexperimente, beigefügt werden, wenn eine Beurteilung gemäss den Regeln der Zulassungsbehörden angestrebt wird. Diese vertieften Untersuchungen sind nötig, wenn Phytopharmaka auch in Zukunft auf dem Markt bestehen sollen.

3 ABBREVIATIONS

293T	human embryonal kidney cell line
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
Apaf-1	apoptotic protease activating factor 1
AR	acetonic root extract
ATP	adenosine triphosphate
BPDS	bathophenanthrolinedisulfonic acid
BSA	bovine serum albumin
Cy3	a fluorescent cyanine dye
CYP	cytochrome P450 enzymes
DHK	dihydrokavain
DHM	dihydromethysticin
Diablo	= Smac
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DMY	demethoxyyangonin
DNP	2',4'-dinitrophenol
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FACS	fluorescence activated cell scanning
FADD	Fas associated death domain
FADH	flavin adenine dinucleotide
Fas	Fas-receptor (= CD95)
FasL	soluble Fas ligand cellular supernatant
FSC	forward scatter
GFP	green fluorescent protein
GSH	reduced glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HepG2	human hepatocarcarcinoma cell line
HRT	hormone replacement therapy

HtrA2	high temperature requirement protein A2 = OMI
HUH6	human hepatoblastoma cell line
IAP	inhibitor of apoptosis proteins
IgG	immunglobuline G
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide
K	kavain
LDH	lactate dehydrogenase
M	methysticin
ML	methanolic leaf extract
MPT	mitochondrial permeability transition
MR	methanolic root extract
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OMI	= HtrA2
PBS	phosphate buffered saline pH 7.4
PCA	perchloric acid
Pefa	Pefablock
PI	propidium iodide
RCR	respiratory control ratio
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 medium
SE	standard error of the mean
Smac	second mitochondria-derived activator of caspase = Diablo
SRB	sulforhodamine B
SSC	side scatter
TNB	5-thio-2-nitrobenzoic acid
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor (TNF)-related apoptosis-inducing ligand
Y	yangonin
zFA-fmk	Z-Phe-Ala-fluormethylketone
zVAD-fmk	Z-Val-Ala-Asp-fluoromethylketone

4 INTRODUCTION

The use of plants as medicines has a very long tradition. Already the earliest humans availed themselves of herbs for the treatment of manifold ailments and diseases. On the other hand, people were also aware of their negative sides and used them as poisons. Over the course of the centuries new substances in the field of chemistry and pharmacology were discovered and people enforced the advancement of these findings. Especially in the 20th century a great leap in the discovery of chemical agents was made and the herbal knowledge and medicines fell into oblivion, particularly in the Western countries. Only in the 80ties and 90ties of the 20th century, as people were increasingly tired of the chemical drugs with their side effects etc., more and more people remembered the old herbal remedies and fell back on these traditional medicines. Phytopharmaceuticals became popular all around the world. In the course of this development studies examining the efficacy of these herbal medicines were undertaken – but the safety aspect has been neglected to a great deal. Only as cases of toxicity emerged in man attention was paid to this aspect.

The aim of this work was to shed light on the toxicity potential and mechanisms of kava kava and cimicifuga racemosa extracts, representing two examples of herbal remedies where toxicity has not been elucidated yet.

The following chapters are meant to give a short introduction into the field of toxicology, with emphasis to toxicity mechanisms, liver toxicity and toxicity of phytomedicines.

4.1 Toxicology

Toxicology is a young science that developed only during the last 40 years as concern for consumer and worker health and for the environment increased. Currently, toxicology encompasses mainly activities to determine the potential for adverse effects from chemicals (both natural and synthetic), with the objective of assessing hazard and risk to humans and animals. Based on this appropriate precautionary, protective, restrictive and therapeutic measures can be defined [1].

In toxicology the emphasis has changed from acute, particularly human, toxicology to long-term and non-target species toxicology. The following two key issues are embedded in the term toxicology: First, a close structural or functional contact of chemicals, or their conversion products, to tissues or organs and second a quantitative relationship between the triggering agent and the effect. Such a dose-response relationship is important for a causality assessment and to predict the dimension of risk and hazard.

The term "toxicity" is used to describe the nature of adverse effects produced and the conditions required for their induction; i.e. toxicity is the potential for a material to cause harm in biological systems [1]. For pharmacologically active and therapeutic agents undesired effects are described by different terms: side-effects, overdosage, underdosage, loss of effect, intolerance, idiosyncrasy, secondary effects, and adverse drug interactions.

The nature and magnitude of a toxic effect of a compound depend on several factors like its physicochemical properties, its pharmacokinetic behavior, the conditions of exposure and the presence of bioprotective mechanisms. Examples for the expression of a toxic response are inflammation, necrosis, apoptosis, enzyme inhibition, biochemical uncoupling, lethal synthesis, lipid peroxidation, covalent binding, receptor interaction, immunosuppression or neoplasmia [1].

4.2 Mechanisms of Toxicity

4.2.1 Apoptosis and Necrosis

Cell proliferation and cell demise are processes which are tightly regulated in normal functioning cells and tissues. The balance between dying and growing is sustained by various mechanisms. A disequilibrium of this system would lead to different detrimental outcomes. On the one hand, when cell death is preponderant, cell loss is high, for example in conditions like reperfusion injury, heart failure, AIDS, neurodegeneration, ostheoarthritis. On the other hand, when cell death is hindered, i.e. cells can proliferate uncontrolled, dangerous conditions like cancer, viral infections, restenosis, autoimmunity occur [2, 3]. From this follows that cell death and its regulation has an important role in all creatures.

In physiological conditions, cell death is mediated by a process called apoptosis and is a strictly regulated course of events removing superfluous, aged, or damaged cells. In this context, mitosis can be regarded as the opposite process in the regulation of the cell population. Apoptosis is derived from the ancient Greek and means "the falling of petals from a flower" or "of leaves from a tree in the autumn" [4]. The term apoptosis was first used by John Kerr in 1972 [5], although this morphological phenomenon has been described already more than hundred years earlier by Carl Vogt in 1842 [4]. However, cells can not only die from apoptosis, but also from another kind of cell death termed necrosis. Necrosis is always induced after exposure to (high doses of) external factors or drastic changes in the physiological environment, such as heat, anoxia, loss or increase of ions, ethanol, toxic substances and usually is a pathogenic process [6]. Also apoptosis can be triggered not only by growth regulation of tissues but also by external factors, which can be the same as the ones causing necrosis. In general, the trigger needs to be more massive to provoke necrosis instead of apoptosis. Apoptosis and

necrosis not only differ in the triggers and the course of events, but also in the picture they present during this process.

The image of apoptosis is characterized by the shrinkage of the cell and the cell nucleus, condensation of chromatin, DNA fragmentation, and blebbing of the cell membrane with the constriction of apoptotic bodies without loosing the structural integrity and most of the plasma membrane function [6, 7]. Whereas necrosis is represented by swelling of the cell and the cytoplasmatic organelles and disrupture of the cell membrane leading to inflammation and thus causing further tissue damage (Figure 1) [6]. In apoptosis, inflammation is usually prevented by the engulfment and lysis of apoptotic bodies by phagocytes, which in addition results in the release of anti-inflammatory cytokines and immune tolerance [8]. Also scar formation usually does not take place. However, if apoptosis is massive or phagocytic cells are lacking, apoptosis can eventually turn into necrosis [9].

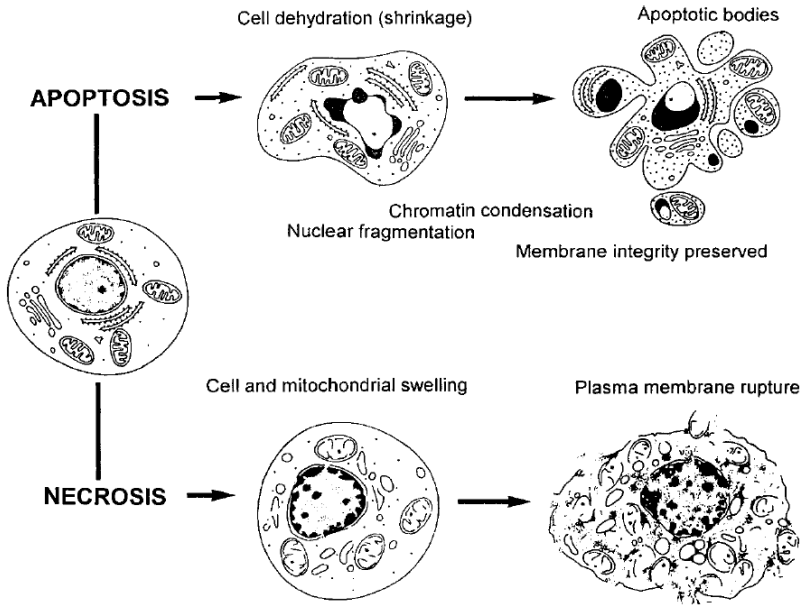

Figure 1: Scheme illustrating morphological and biochemical changes during apoptosis and necrosis [7].

Apoptosis can be triggered mainly by two pathways [4, 10, 11]: the intrinsic and the extrinsic pathway. The intrinsic pathway is starting from the mitochondria as consequence of extra- or intracellular stress, the other is triggered by an extracellular ligand activating a death receptor in the cell membrane (Figure 2). In both pathways so-called caspases are usually involved. Caspases are cysteine containing aspartic acid-specific proteases and can be divided into initiator caspases (caspases 8, 9 and 10) and downstream or executioner caspases (caspases 3, 6, 7,) [4, 12]. Upon activation of a death receptor (Fas-, TRAIL- or TNF-receptor) recruiting of adaptor molecules (such as FADD: Fas associated death domain proteins) and procaspases occurs, forming an "apoptosome", a so-called DISC (death-inducing signaling complex).

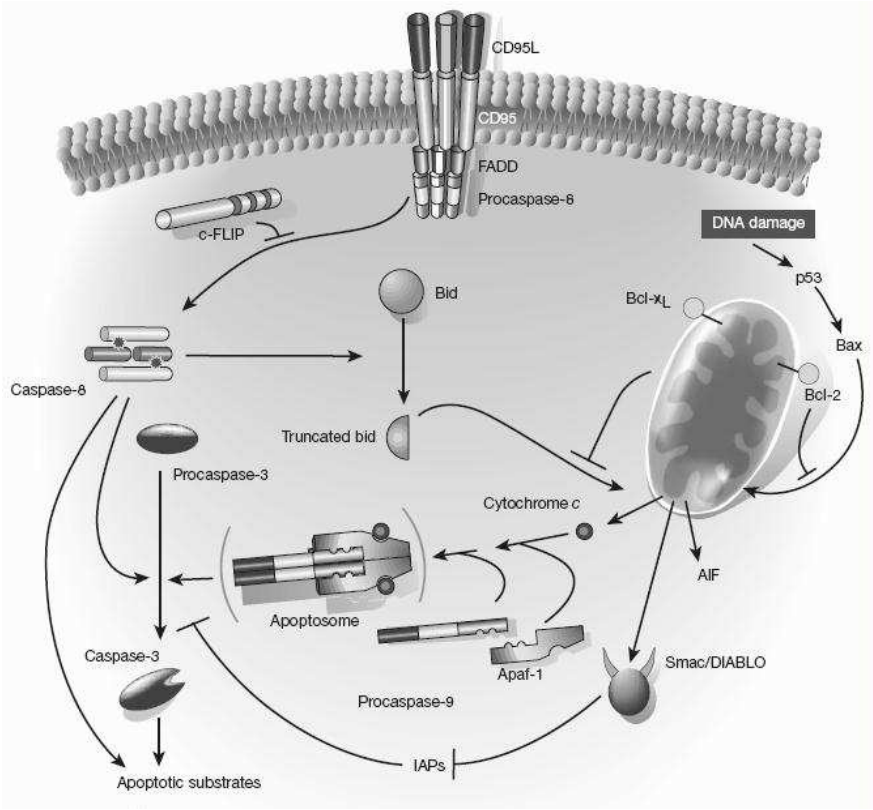

Figure 2: The death-receptor pathway (left pathway) is triggered by members of the death-receptor superfamily (such as CD95 (= Fas) and tumour necrosis factor receptor I). The mitochondrial pathway (right) is used extensively in response to extracellular cues and internal insults such as DNA damage. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family. In addition, pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome *c* exit. If the pro-apoptotic camp wins, an array of molecules is released from the mitochondrial compartment. The death-receptor and mitochondrial pathways also converge at the level of caspase-3 activation. Caspase-3 activation and activity is antagonized by the IAP proteins, which themselves are antagonized by the Smac/DIABLO protein released from mitochondria [13].

In this complex procaspases are transactivated and subsequently cleaved, resulting in active caspases, which activate downstream caspases [10]. These effector caspases then cleave cellular substrates leading to the phenomena of the apoptotic picture. The intrinsic pathway is initiated by some kind of stress, like oxidative stress or treatment with cytotoxic drugs, and starts with the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. Cytochrome c aggregates with apoptotic protease activating factor 1 (Apaf-1), procaspase-9 and ATP to form an apoptosome [14]. Upon procaspase-9 activation an effector caspase, e.g. caspase-3, is activated. The latter can trigger the evolvement of the apoptotic morphology, like in the extrinsic pathway. Apoptosis, and especially caspase activation, is an energy-requiring process. In case ATP is decreased apoptosis is blocked and turns into necrosis [15, 16].

In reality, the whole situation is much more complex. The two pathways are not only connected by the convergence at the level of the executioner caspases but can also be at an earlier stage. The activated initiator caspase-8 not only cleaves and activates caspase-3 but also Bid, a proapoptotic member of the Bcl-2 family. Bid acts on mitochondria to trigger the release of cytochrome c with the above described consequences. Under most conditions, this cross-talk is minimal, and the two pathways operate largely independently of each other. During the whole process of apoptosis also further pro- and anti-apoptotic molecules are involved which regulate the death process at several places (see Figure 2) [10, 11, 13].

The whole course of events can be expressed as a three-step model of apoptosis: a pre-mitochondrial phase during which signal transduction cascades and damage pathways are activated; a mitochondrial phase during which mitochondrial membrane function is lost; and a post-mitochondrial phase during which proteins released from mitochondria elicit the activation of catabolic proteases and nucleases [17]. However, also caspase-independent death processes exist, in which no caspases but other proteases, including cathepsins, calpains and endonucleases, are activated [18].

4.2.2 Mitochondria

Mitochondria are key players in the functioning of a cell as their primary role is providing cells with energy in form of ATP, which is produced by the coupling of the ATP synthase with the electron transport chain. The respiratory chain is fed with reductive equivalents (NADH and $FADH_2$) from the citric acid cycle, β-oxidation, and glycolysis. Mitochondria encompass the important metabolic pathways of tricarboxylic acid cycle and fatty acid β-oxidation. In addition, they have a central role in ion homeostasis and redox regulation [19].

It is assumed that mitochondria originally evolved from aerobic prokaryotic bacteria who underwent symbiosis with anaerobic host cells. This co-living represented a benefit for both parties as their survival was increased due to the better exploitation of energy in foodstuff [20]. Mitochondria consist of two compartments, the matrix, circumvented by the inner membrane, and the intermembrane space, which is delineated by the outer membrane. The inner membrane is folded forming so-called cristae, which clearly enlarge the surface, and is hardly permeable under physiological conditions. This tightness ensures the maintenance of the electrochemical gradient, comprising a membrane potential (negative inside) and a pH gradient (basic inside), which is the basis of the coupling of the respiratory chain to oxidative phosphorylation. The protein complexes of the respiratory chain are embedded in the inner membrane. The outer membrane is much more permeable for low-molecular-weight solutes. Mitochondria consume more than 90 % of the cells oxygen and supply most of our ATP [21, 22]. Most cells

contain hundreds of mitochondria [23]. Organs with high energetic requirements contain more mitochondria to cope with this situation, e.g. heart, muscle.

A drawback of the production of ATP is the generation of reactive oxygen species (ROS) by the respiratory chain, which is especially pronounced at complexes I and III [24]. Mitochondria are the major source of ROS and as mentioned above play also a pivotal role in apoptosis. They are the central element in the intrinsic apoptosis pathway. Upon the impact of a trigger they release several molecules from the intermembrane space, which initiate the apoptotic answer including Apaf-1, cytochrome c, apoptosis inducing factor (AIF) (see chapter 4.2.1). Concomitantly, an opening of the mitochondrial permeability transition (MPT) pore and a dissipation of the mitochondrial membrane potential occurs, both of them early signs of cell death. Mitochondrial apoptogenetic factors can be divided into two groups: the caspase-dependent substances and the caspase-independent factors (e.g. AIF, high temperature requirement protein A2 (HtrA2/OMI) and endonuclease G) [25]. Summarized it can be stated that mitochondria are able to trigger both caspase-dependent and caspase-independent apoptotic cell death.

Induction of the MPT is sufficient to trigger apoptosis or necrosis and the pharmacological inhibition of MPT prevents cell death [26]. As a consequence of MPT pore opening, solutes < 1500 Da diffuse across the inner mitochondrial membrane, causing mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude swelling, which in turn can lead to depletion of ATP and cell death [16]. MPT can be favored by high calcium concentration in the mitochondrial matrix, NAD(P)H oxidation and mitochondrial generation of ROS, whereas magnesium, low pH and cyclosporine A inhibit MPT (Figure 3) [15].

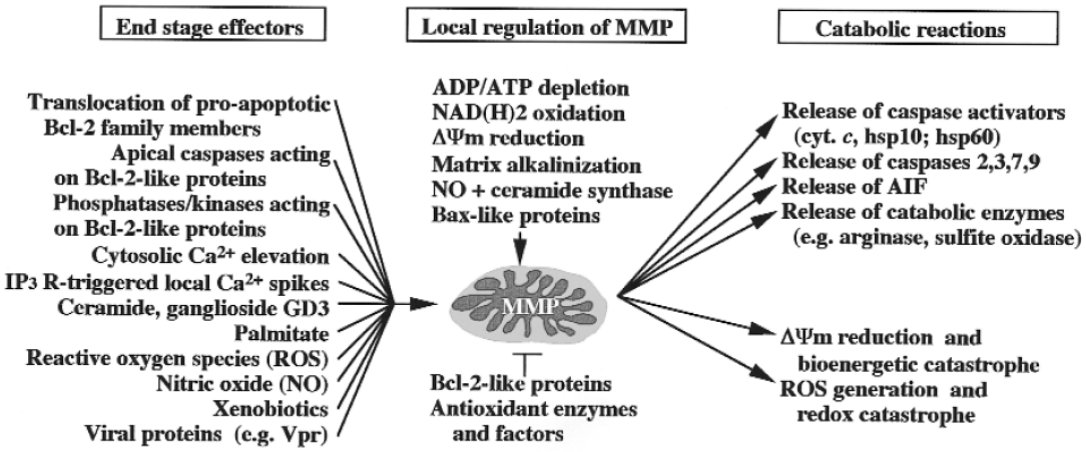

Figure 3: Mitochondrial involvement in cell death, with focus on events proximal to the regulation of mitochondrial membrane permeabilization (MMP = MPT). Mitochondrial membrane effectors may cause MMP, depending on local regulators which sensitize mitochondria to MMP or inhibit MMP. Permeabilization of the outer and/or inner mitochondrial membranes then triggers a series of catabolic reactions that entail cell death, either by apoptosis or by necrosis [27].

Massive induction of MPT leads, via depletion of ATP, to necrosis, whereas a more subtle, regulated induction of MPT gives time for the activation and action of proteases ending in apoptosis [6]. As possibility, one could imagine that the respective outcome of the bioenergetic and redox "catastrophe" on one side and the activation of catabolic enzymes (caspases and nucleases) on the other side might compete in some sort of race. This could explain, why some substances induce apoptosis as well as necrosis, depending on the applied concentration. In this context, a new term can be introduced: necrapoptosis. Necrapoptosis describes death processes which begin with a common stress or death stimulus, share the same pathways, but finally lead to either necrosis or apoptosis, depending on modifying factors like ATP (Figure 4) [15, 16]. Formigli described the same phenomenon as aponecrosis [28].

Once cytochrome c is released and mitochondrial membrane potential is disrupted, the cell is committed to die either by apoptosis – through Apaf-1 mediated activation of caspases – or by necrosis – as a result of the collapse of the respiratory function due to increased ROS and insufficient supply of ATP [20, 29-31].

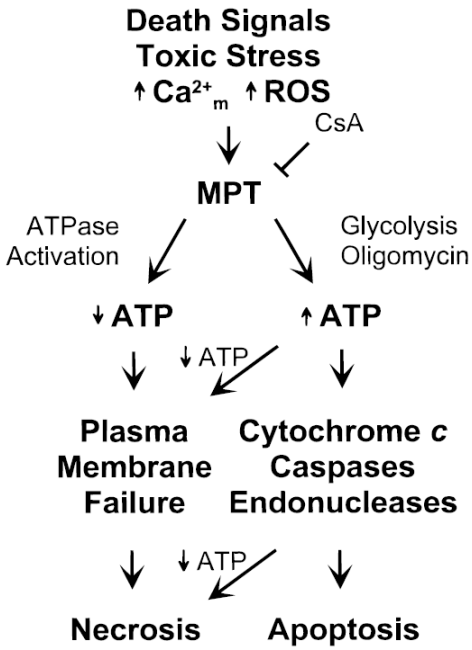

Figure 4: Scheme showing the role of ATP in necrapoptosis mediated by the mitochondrial permeability transition. When the MPT occurs abruptly, activation of mitochondrial ATPases causes ATP depletion, which leads to plasma membrane rupture and necrotic cell death. In case MPT progresses relatively slowly ATP levels remain relatively preserved even after onset of the MPT. Under such conditions, cytochrome *c* release activates a cascade of caspases, endonucleases, and other degradative enzymes, causing apoptotic rather than necrotic cell death. At any time, ATP depletion can supervene to cause secondary necrosis [15].

When cells are exposed to environmental stress cell cycle is arrested at G_0 or G_1 phase and mitochondria are motivated to produce more ATP in order to provide more energy for repairing

the damage sustained. Signals, like H_2O_2, are sent to the nucleus to initiate proliferation of mitochondria and concomitantly mitochondrial DNA (mtDNA) amplification in order to allocate more mitochondria to fulfill this task [20].

The mtDNA is more susceptible to mutations than nuclear DNA, e.g. caused by ROS, as it is a naked compact DNA molecule without protective histones, is rapidly and frequently replicated without proofreading or efficient DNA repair systems and is located near the major ROS source [19, 20, 32]. This leads to an increased percentage of damaged mtDNA (somatic mutations) with increasing age, possibly associated with decreased mitochondrial function and enhanced ROS production (Figure 5) [19]. The resultant age-related decline in oxidative phosphorylation would lead to a reduction of bioenergetic capacity until a certain threshold is undershot and symptoms or senescence occur [23].

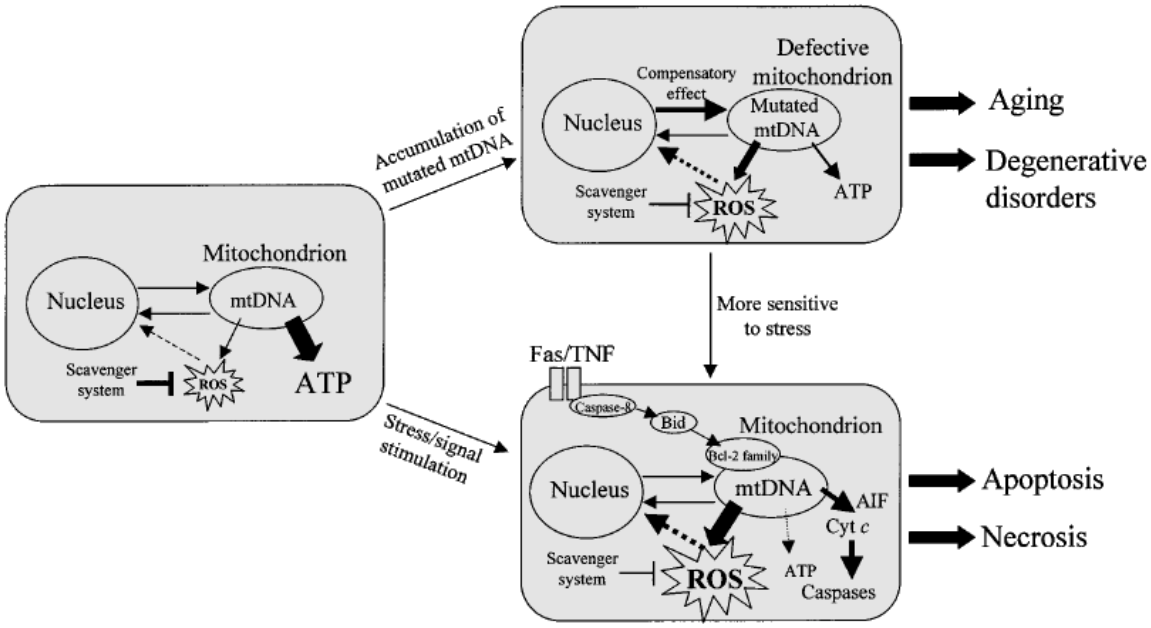

Figure 5: Mitochondrial role in the determination of life and death of the cell. Under normal physiological conditions, mitochondria are the major source of bioenergy and ROS. To cope with the ROS, human cells have developed an efficient scavenger system, which includes antioxidant enzymes and small molecular-weight antioxidants (left panel). Although this system can dispose of ROS and free radicals, a small proportion of them may escape these defense mechanisms and cause damage to cellular constituents including DNA, RNA, proteins, and lipids. The ratio of damaged or mutated mtDNA to wild-type mtDNA can result in bioenergetic and/or redox alteration of tissue cells. Therefore, in a synergistic manner, all mutations and oxidative damage to mtDNA cause a deleterious effect on the respiratory function of mitochondria and lead the affected individual to aging and degenerative disorders (right upper panel). On the other hand, mitochondria can sense and respond to extracellular and intracellular signals and stresses. Once beyond a threshold of stress or challenge (e.g., ROS-elicited oxidative stress), mitochondria may drive the cell into an irreversible cell death process (right lower panel) [20].

4.2.3 Reactive oxygen species

Another important factor influencing cell functioning is the occurrence of reactive oxygen species. Cells are constantly generating ROS during aerobic metabolism. ROS is an umbrella term for several reactive molecules containing oxygen, including $O_2^{\bullet -}$ (superoxide anion), $^{\bullet}OH$ (hydroxyl radical), and H_2O_2 (hydrogen peroxide). They origin mainly from the mitochondrial respiratory chain, where about 1 – 5 % of the electrons lose their way and directly interact with oxygen to form $O_2^{\bullet -}$ [31]. Other sources are the microsomal electron transport chain or oxidative enzyme systems like xanthine oxidase, cyclooxygenase, lipoxygenase, cytochrome P450, and NADPH oxidase [33].

Most of the produced ROS are caught by the endogenous antioxidant system. This system consists of enzymes like catalase, superoxide dismutase, glutathione peroxidase, and the molecule glutathione. Glutathione is a tripeptide (γ-Glu-Cys-Gly) occurring in two forms in the cell: reduced (GSH) and oxidized (GSSG) glutathione, with GSH being the predominant form. Depletion of GSH is sufficient to trigger apoptosis in some cell types and is able to make cells more susceptible to other apoptosis inducing stimuli [34, 35].

Oxidative stress is induced if the natural antioxidant defense cannot cope anymore with the incidental ROS. The effects that ROS may have on the cell are manifold. First of all, ROS are able to oxidize DNA with subsequent wrong or hampered synthesis of proteins leading to malfunctioning and structural changes of these proteins (see chapter 4.2.2). Moreover, they may also peroxidize membrane lipids causing dysfunction of the cell membrane. By modifying the DNA bases they are able to impair the DNA repairing mechanisms and through hydrolysis they lead to malondialdehyde production from deoxyribose [33].

Oxidative stress is involved in the pathogenesis of several diseases including AIDS, Huntington's, Parkinson's disease, Alzheimer's disease, sepsis, artherosclerosis, and retinal degenerative disorders [36].

4.3 Drug-induced liver toxicity

Liver toxicity is a very common adverse event of a drug. More than 1100 drugs world-wide are thought to be potentially hepatotoxic [37]. Drug-induced liver injury is the most frequent cause for the after-marketing withdrawal of a drug, despite rigorous preclinical and clinical testing [38]. There are three reasons for this high frequency: better diagnostic possibilities, a growing number of potentially hepatotoxic medicaments coming to the market, and the liver's central role in the metabolism and disposition of drugs [39].

The clinical picture of liver toxicity corresponds to the common liver diseases and can vary from reversible, mild liver enzyme elevation to fatal liver failure, including necrosis, hepatitis,

cholestasis, steatosis, veno-occlusive disease, cholangitis, cirrhosis, hepatocellular carcinoma, with acute hepatitis being the most frequent presentation (90 % of cases). Several risk factors are known for the development of hepatotoxicity of drugs, like gender, age, lack of food, adipositas, diabetes mellitus, liver diseases, renal diseases, heart diseases, hyperthyreosis, psoriasis, rheumatic diseases, AIDS, pregnancy, long-term therapies, dosage, polymedication, alcohol abuse, genetic factors, and others. The underlying causes can be of allergic-immunologic or metabolic-toxic nature, but the hepatotoxicity mechanisms of most drugs remain unknown [40, 41]. Formation of reactive metabolites is relatively frequent. Despite several protective mechanisms, this may lead to the covalent binding of electrophilic metabolites to proteins, the mutation of lipid peroxidation by free radicals, and the depletion and/or oxidation of glutathione. A number of structural and functional lesions, including a sustained release in cytosolic calcium, eventually leading to cell death, follow the before mentioned events [41].

Immunologic responses are rare (often less than 1 case per 1000 treated patients), unexpected, delayed (from 2 weeks to several month), host-dependent and not dose-related reactions, which can be persistent; autoantibodies or antibodies against modified proteins in the blood of patients frequently occur. As mechanism for this immunoallergic hepatitis has been postulated that the respective drug is enzymatically converted to a reactive metabolite, which binds to the enzyme that produced it. This complex is called neoantigen, which once presented to the immune system, causes an immune response by the production of antibodies against both the natural protein and/or the modified protein. Examples of drugs causing an immunoreaction in the liver are halothane, tienilic acid, anticonvulsants [38, 39, 42, 43].

Causes for the development of hepatotoxicity of metabolic-toxic origin consist of the following mechanisms: metabolite-mediated toxicity and endothelial lesions, inhibition of biliary secretion, decreased secretion of lipoproteins, inhibition of fatty acid mitochondrial β-oxidation, or activation of Ito cells [41]. Activation of Kupffer cells leads to the release of cytotoxic mediators, such as reactive oxygen species, and proinflammatory mediators, such as cytokines and chemokines [44].

Directing damaged cells to apoptosis, rather than necrosis, might be very beneficial as by this way consequential reactions like inflammation can be prevented, provided cell death is not massive. On the other hand interfering with the apoptosis program risks to direct cells to necrosis with secondary consequences which can further aggravate the injury [45].

4.4 Toxicity of Phytomedicines

At all times, people tried to treat and cure themselves with some kind of medicine. Originally, treatment occurred with natural substances, lately moving to industrially synthesized drugs. Nowadays, phytomedicines enjoy great popularity again. The reasons for this attractiveness

are the perceiving that natural medicines are safe, gentle and cost-effective, the easy accessibility, and the desire of self-medication [46]. Out of this, people often use herbal drugs as self-medication, i.e. without the doctor's knowing. The problem is that people take them not only as exclusive medication but also in combination with other – practitioner-prescribed – drugs. Especially in the USA, where herbal medicines are categorized as dietary supplements and therefore are available in the supermarket, the dispensing and sales and distribution is inadequately controlled and their regulatory requirements do not correspond to the standards of conventional drugs, i.e. the efficacy, quality control and safety of a product [47]. In other countries like Germany a series of herb recommendations, the Commission E Monographs, had been implemented, detailing dosages and indications of herbs whose efficacy is supported by the literature.

There are several typical problems in connection with botanicals: contamination (e.g. heavy metals), adulteration, interaction with other drugs (e.g. Hypericum perforatum), and toxicity itself (e.g. kava kava) [48]. Contaminations can occur unintentionally as well as deliberately, whereas adulterations are committed fraudulently. Clinical relevant adulterants and contaminants consist of toxic botanicals and botanical substances, pathogenic microorganisms and microbial toxins, pesticides and fumigation agents, toxic metals, and pharmaceuticals [49]. Examples for adulterants found in traditional Chinese medicines are acetaminophen, corticosteroids, caffeine, diazepam, diclofenac, hydrochlorothiazide, fluocinoline, glibenclamide, mefenamic acid, and phenytoin [50].

Recently, efforts have been undertaken to study the efficacy and mechanism of action of botanicals, but the safety aspect has been neglected to a high degree. Especially, about the way by which toxicity is elicited only few is known and investigated so far. Often, cases of toxicity caused by a botanical are becoming known through single case reports and not based on clinical trials. Therefore, adverse reactions to herbals are probably underrecognized and underreported [51]. Herbal preparations contain quite an amount of pharmaceutically active ingredients, which potentially could all produce negative effects and make it not easy to denominate the actually side effect-causing constituent. Increasingly, it seems that the health hazards outnumber the beneficial effects of herbal medicinals [52]. Most herbs share the pharmacological mechanisms of action with already existing prescription or over-the-counter drugs [47], potentially leading to some kind of interaction.

Phytomedicinals can evoke a big variety of physical detrimental effects. An overview of these effects is given in Table 1, which is adopted from Winslow and Kroll, and a summary of adverse events of the 20 best-selling herbs in the USA is provided by De Smet [49]. Herbs can effect quasi every organ or function in the body. In the following, emphasis is placed on liver toxicity of herbal medicinals. For establishing an accurate diagnosis of hepatotoxicity, several aspects should be considered including thorough anamnesis, pattern recognition, awareness of

the spectrum of herbal liver injury and botanic identification [53]. Before a liver disease can be associated with a phytomedicine, other causes, like hepatitis viruses and autoimmune disorders, should be excluded. In cases of botanical-induced liver toxicity, the clinical picture is usually not different from other forms of liver diseases, thus encompassing a wide spectrum of liver pathology including elevated liver enzymes, hepatitis, fibrosis, cirrhosis, and acute liver failure [53] (see also chapter 4.3). Examples of herbal medicines suspected to cause liver injury are chaparral (Larrea tridentata), Dai-saiko-to (Sho-saiko-to, TJ-9), greater celandine, Ma-huang (from Ephedra species), pennyroyal oil, saw palmetto (Serenoa repens), germander (Teucrium chamaedris), valerian, mistletoe [52-54]. Pyrrolizidine alkaloids, contained in plants like Heliotropium, Senecio, Symphytum (Compositae), are able to induce veno-occlusive disease.

In some cases, (hepato)toxic effects do not originate from sole intake of botanicals but from the combination with other drugs. These interactions can occur via the induction of CYPs (like CYP3A4 or CYP2E1) by other drugs or alcohol, which may enhance the production of toxic herbal metabolites [54]. On the other hand, herbal components may act on CYPs by inhibiting these, thus potentially leading to accumulation of concomitantly taken drugs. In an *in vitro* study, inhibition of CYP 2C9, 2C19 and 3A4 by kava kava, Ginkgo biloba, garlic, and St. John's wort was found [55]. But interactions may not only occur via CYPs, but also by other pathways, including the immune system (Echinacea – corticosteroids, cyclosporine), the clotting system (garlic, ginger, ginkgo – warfarin), the cardiac system (herbs containing cardiac glycosides – digoxin), and the renal system (licorice – spironolactone) [56].

In this work, two herbs will be investigated regarding their hepatotoxic potential: kava kava and cimicifuga racemosa. Details concerning these plants will be discussed in chapter 6 and chapter 8 later in this thesis, however a short introduction will be given at this place.

Kava kava is a shrub originating from Oceania. It became popular some years ago as herbal alternative to the classically used benzodiazepines as anxiolytic and mild sedative. A few years ago, kava has been withdrawn form the market in many countries because of its association with liver toxicity. First, only acetonic extracts have been taken from the market, but later all products have been banned.

Cimicifuga racemosa is a herb native to the Eastern United States and Canada. It is now widely used as herbal alternative to the standard hormone replacement therapy (HRT) in the treatment of menopausal complaints. As HRT was linked to breast cancer as well as cardiovascular disease and many women asked for more natural medication cimicifuga gained great popularity in the last years. However, so far not much is known about the hepatotoxic potential of cimicifuga and as it is a widely used phytomedicine it would be interesting to learn more about its toxicological profile.

Table 1: Reported adverse effects of some common herbs [47]

Organ System	Toxic Effects	Herb	Comments
Gastrointestinal	Hepatotoxic (from asymptomatic enzyme elevation to fulminant necrosis)	Chinese herbal teas, mistletoe, germander, chaparral, or comfrey	First reported case of hepatic veno-occlusive disease was caused by comfrey
	Nausea/vomiting	Dandelion, garlic, ginseng, or chaparral	
	Diarrhea	Herbal teas, aloe, ligustrum, dandelion, prunella, garlic, or ginseng	
Hematologic	Anticoagulant/antiplatelet	Yarrrow, red clover, tang-kuei, pau d'arco, or salvia	
Central nervous system	Nervousness, agitation, insomnia, mood changes, depression, confusion, or hallucinations	Ginseng	With long-term use and higher doses
	Cholinergic toxicity	Jimson weed	Contains atropine, scopolamine, hyoscyamine
	Hallucinogenic	Catnip, hops, kava kava, khat, lobelia, mandrake, nutmeg, jimson weed, valerian, or yohimbe	
	Sedation	Peony, salvia, or tang-kuei	
	Seizures, psychosis, or coma	Ephedra	
Pulmonary	Pulmonary hypertension	Chinese herbal teas	
Allergic/Immunologic	Contact dermatitis	Propolis, garlic, echinacea, or melaleuca oil	
	Systemic lupus erythematosis	Alfalfa	
Endocrinologic	Gynecomastia, vaginal bleeding	Ginseng	Contains estrogen
	Goiters, hyperthyroidism and hypothyroidism	Kelp	Contains iodine
	Inhibition of iodine uptake	Garlic	
	Hypoglycemia	Atractylodes, scrofularia, lycium, or burdock	
Renal	Diuresis	Burdock, astragalus, peony, or dandelion	
	Hypertension, sodium and water retention, or hypokalemia	Licorice	*Glycyrrhiza glabra*, *Glycyrrhiza radix* can have same effects and is found in 74 % of Chinese herbal teas
Cardiovascular	Hypotension	Astragalus, codonopsis, prunella, scrofularia, or salvia	
	Hypertension	Ginseng	
	Hypertension, coronary spasm, palpitations, or tachycardia	Ephedra	

5 AIMS OF THE THESIS

The goal of this thesis was to shed light on the mechanisms of hepatotoxicity of the phytomedicines kava kava and cimicifuga racemosa.

Herbal medicines have been increasingly consumed over the last two decades. People using them were appealed by the idea that they have self-control over their medication and by the thought of taking safe medicines. A lot of herbal products have been used for all sorts of ailments. But over the years growing evidence for adverse effects outcropped and cases of toxicity were reported. This was also the case for the Oceanian plant kava kava, which was used for its anxiolytic effect. Kava was suspected to be related to liver toxicity with different pictures of liver damage.

For this reason, in a first project (chapter 6), we aimed to elucidate whether kava is really hepatotoxic and if so, by which mechanism. So far, no detailed investigations of the mechanisms possibly leading to hepatotoxicity on cell organelle level have been conducted. For this purpose, the hepatocarcinoma cell line HepG2 was used as an *in vitro* model for studying hepatotoxicity. During this work, the focus was put on the determination of apoptosis and necrosis and on the function of mitochondria.

In a further project (chapter 7), two methods assessing cell death (i.e. apoptosis and necrosis) were compared: the annexin V / propidium iodide stain and the GFP-method. The GFP-method was recently described by Strebel et al. [57] and Steff et al. [58] and is based on the phenomenon that intracellular GFP decreases its fluorescence when apoptosis and/or necrosis occur. The aims were not only the comparison of the two methods but also the characterization and establishment of the GFP-method in liver cell lines. For this purpose HepG2 cells and the hepatoblastoma cell line HUH6 were used as model for hepatic cells and kava extracts as test compounds which can trigger a toxic response.

The third project (chapter 8) focused again on the hepatotoxicity of a medicinal herb, namely on cimicifuga racemosa. Cimicifuga was so far not related to any relevant, and in the narrower sense hepato-, toxicity. However, this herb is often used by women for menopausal complaints and a detailed assessment of adverse events has not been performed until now. Recently, Prof. Udo Spornitz from the Anatomic Institute of the University of Basel found liver steatosis in rats after feeding of high doses of cimicifuga extract. This interesting result led us to further evaluate the hepatotoxicity of this herb on cellular level.

6 HEPATIC TOXICITY OF KAVA LEAF AND ROOT EXTRACTS

Short title: Hepatotoxicity of Kava Kava

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6.1 Abstract

Kava is used since ages in Polynesian ceremonials, social occasions or as a medicine. Some years ago, kava became popular also in Western countries. It was thought to be a safe drug, but lately, several cases of liver toxicity have been published and kava was subsequently banned from the market in many countries. Since not much is known about the mechanisms of hepatotoxicity of kava, we compared three different kava extracts (a methanolic and an acetonic root and a methanolic leaf extract) with respect to their toxic potential on HepG2 cells. It was found that all three extracts showed cytotoxicity starting from about 75 µg/mL on. The mitochondrial membrane potential was decreased and the respiratory chain inhibited and uncoupled (root extracts) or only uncoupled (leaf extract) at 150 µg/mL, and β-oxidation was inhibited by all extracts. Oxidized glutathione (GSSG) was increased, whereas the cellular ATP content was maintained, and apoptosis was found at concentrations of 150 µg/mL. These results indicate that the kava extracts are toxic to mitochondria, leading to inhibition of the respiratory chain, increased ROS production and to a decrease in the mitochondrial membrane potential. This in turn could result in the opening of the permeability transition pore and consequently to the rupture of the outer mitochondrial membrane with the release of cytochrome c and apoptosis. In predisposed patients, mitochondrial toxicity of kava extract may explain hepatic adverse reactions of this drug.

Keywords: kava, piper methysticum, hepatotoxicity, apoptosis, HepG2

6.2 Introduction

Kava kava (*Piper methysticum* Forster) is a plant originating from Oceania (Polynesia, Melanesia and Micronesia). In these regions, kava beverages are consumed for ages at social or ceremonial occasions and also as medicines. Medicinal indications are induction of relaxation and sleep, but also counteraction of fatigue, congestion in the urinary tract, chronic cystitis, asthma, rheumatism, weight reduction, headache, cold, fever, syphilis, gonorrhea and others [59]. Traditionally, kava extracts are prepared from kava roots macerated with water or coconut milk [60, 61].

Some years ago, the medicinal plant kava became popular also in Western countries as a herbal alternative to commonly used drugs such as benzodiazepines. Depending on the country, kava was used also for urinary tract infection, as a general tonic, but also against stress, anxiety and nervous disorders [62]. Until recently, apart from a so called kava dermatopathy, kava was reputed not to cause severe adverse reactions [63]. Occasionally, yellowing of the skin, red eyes, and episodes of allergic reactions were noticed, which were all spontaneously reversible on stopping of the medication [59].

Lately, however, 82 cases of liver toxicity apparently associated with the use of kava dating from 1990 – 2002 were reported from several countries [64]. For twenty of these cases, there was obviously no connection to kava intake. In 21 reports, patients were treated concomitantly with potentially hepatotoxic drugs. In seven cases, the causality of kava could be doubted considerably, whereas in 31 other cases, the available data were too fragmentary for an assessment. This left three cases, in whom the hepatotoxic effects associated with kava could be established as probable [64]. In only one of these cases, kava was ingested according the dosage recommendations of the German commission E monograph [64]. Nevertheless, based on these findings, kava was banned from the market in the European countries, Canada and Australia. It was hypothesized that these cases of liver failure might have an immunological origin [64-66].

To further evaluate the reasons for the toxicity associated with kava, possible mechanism(s) have been investigated by various authors. Several mechanisms and/or risk factors for the toxicity of kavalactones were proposed, including glutathione depletion [67], *in vitro* formation of electrophilic metabolites such as quinones [68] and genetic polymorphisms for CYP2D6 [65, 69]. While kavalactones are the pharmacological principle of kava extracts, it remains unclear whether they are also responsible for their toxicity.

Kavalactones, also called kavapyrones, are the major lipophilic compounds in the kava root and have been found to be responsible for the anxiolytic effect of kava [69]. Accordingly, kava containing a high amount of kavalactones is generally considered to be of high quality [70]. The amount of kavalactones varies with the different parts, the age and the cultivar of the

plant [71, 72]. Duve et al. [73] found that the total kavalactone content is typically highest in the lateral roots and decreases continuously towards the aerial parts.

It was also speculated whether other components of kava extracts and/or the extraction method could be the cause for kava toxicity. Nerurkar et al. [74] and Dragull et al. [70] have investigated the alkaloid pipermethystine and found a stronger toxicity in HepG2 cells than for kavalactones, which were not toxic at the same concentrations. Coté et al [75] compared the effect of an acetonic, an ethanolic and methanolic root extract with a (traditional) aqueous extract on cytochrome P450 enzymes (CYP). The aqueous extract was the least potent inhibitor for the examined CYPs. It can also be imagined that kava toxicity originates from interactions with CYPs, because kavalactones and also kava extracts have been shown to interact with and to inhibit several CYPs [76].

The aim of this study was to gain further insight into the cellular mechanisms of toxicity of kava, as so far no extensive investigations of the mechanism of toxicity have been performed. In addition, we wanted to find out whether extracts from different parts of the plant and extracted with different solvents display a different toxicity. These investigations were also carried out with the idea that in the future modified extracts – prepared with different solvents and/or extracts from specific parts of the plant - could be utilized in the treatment of humans.

6.3 Materials and methods

6.3.1 Chemicals

JC-1 and propidium iodide were from Molecular Probes (Eugene, OR, USA); Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Z-Phe-Ala-fluormethylketone (zFA-fmk) were from Enzyme Systems Products (Livermore, CA, USA); Alexa Fluor 633 labeled annexin V was a kind gift of Dr. Felix Bachmann, Aponetics Ltd. (Witterswil, Switzerland). [1-^{14}C]palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). The scintillation cocktail was from Perkin Elmer (Boston, MA, USA). All other chemicals were from Sigma (Buchs, Switzerland) and of highest quality available when not otherwise stated.

6.3.2 Preparation and analysis of the three kava extracts

A methanolic and acetonic root, and a methanolic leaf extract deriving from the same cultivar, harvested from 3 years old greenhouse kava plants, were produced. Dried leaves and lateral roots were pulverized in a laboratory mill and extracted twice, either with methanol or acetone, in an ultrasonic bath for 15 minutes. The solvents were evaporated to dryness and the residue was diluted in methanol and filtered (regenerated cellulose, 0.45 µm) for HPLC analysis.

A reversed phase HPLC analysis was carried out on a Spherisorb-5 ODS column (5 µm, 250 x 4.6 mm; Waters, Milford, MA, USA) using a Jasco HPLC system (Easton, MD, USA) equipped with an auto sampler and a diode array detector. The samples were chromatographed with 22 % acetonitril, 18 % methanol and 60 % H_3PO_4 (85 %) as solvent, at a flow rate of 0.8 ml/minute and at 60 °C. The identification a nd quantification of the six kavalactones was based on comparing the retention times, UV spectra and peak areas with external standards (PhytoLab, Hamburg, Germany). Methysticin, dihydromethysticin, kavain and dihydrokavain were detected at 240 nm, demethoxyyangonin and yangonin at 360 nm. Solutions of the dried extracts and of kavain were prepared in DMSO.

6.3.3 Cell culture

The human hepatocarcinoma cell line HepG2 was kindly provided by Dr. Dietrich von Schweinitz (Department of Pediatric Surgery, Childrens Hospital, University of Basel). The cell line was grown in RPMI 1640 medium (supplemented with GlutaMAX^{TM}-I, 25 mM HEPES, 10 % (v/v) heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin; all from Gibco, Paisley, UK). Culture conditions were 5 % CO_2 and 95 % air atmosphere at 37 °C. Experiments were performed when the cells had reached a confluence of about 80 %.

6.3.4 Cytotoxicity tests

The sulforhodamine B (SRB) test was performed according to the protocol of Skehan [77]. For the lactate dehydrogenase (LDH) assay, cells were incubated with the extracts in a 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) for the indicated time period. 200 µL of the supernatant were used for the detection of the LDH activity according to the method of Vassault [78].

To examine cell viability and activity of the mitochondrial electron transport chain, the dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used [79]. This assay is based on the ability of living cells to (reductively) convert the dissolved MTT (yellow) into the insoluble formazan (blue). The latter can be measured colorimetrically and is proportional to the amount of living cells. For this assay, 50'000 cells/well were incubated in a 96-well plate in the presence of kava extracts for 24 h. After washing, 0.5 % MTT was added for 4 h. The reaction was stopped with 100 µL sodium dodecyl sulfate 20% and absorption was measured at 550 nm (Spektra Max 250, Molecular Devices, Sunnyvale, CA, USA).

6.3.5 Mitochondrial function

To assay the mitochondrial membrane potential, the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethyl-benzimidazolylcarbocyanine iodide) assay was performed according to the protocol of Molecular Probes. After detachment of the cells with 10 mM EDTA, they were filtered through a 40 µm mesh. 100'000 cells were incubated in the presence of the kava extracts and JC-1 (7.5 µM). Subsequently, cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

6.3.6 Animals

Male Sprague Dawley rats (Charles River, Les Onins, France) were used for all experiments. They were fed ad libitum and hold on a 12-hour dark and light cycle. The study protocol had been accepted by the Animal Ethics Committee of the Canton of Basel.

6.3.7 Isolation of rat liver mitochondria

Rats were anesthetized with carbon dioxide and killed by decapitation. The liver was extirpated, rinsed, minced and washed with ice-cold MSM buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L 3-[N-Morpholino]propanesulfonic acid (MOPS), pH 7.4). Mitochondria were isolated by differential centrifugation according to the method of Hoppel et al [80]. The mito-

chondrial protein content was determined using the Biuret method with bovine serum albumin as standard [81].

6.3.8 Oxygen consumption

Polarographic monitoring of oxygen consumption was carried out in a 1 mL chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30 °C as described previously [82]. The oxygen content of respiration buffer equilibrated with air was assumed to be 223 nmol O_2/mL at 30 °C [83]. The final concentration of the substrate L-glutamate was 20 mmol/L. The respiratory control ratio (RCR) was determined as a marker of the functional integrity, i.e. the coupling of oxidative phosphorylation of the mitochondria. The RCR was calculated according to Estabrook [84] as the ratio between the rate of oxygen consumption in the presence of a substrate and added ADP (state 3) and the rate obtained after complete conversion of ADP to ATP (state 4).

After depletion of endogenous substrates by the addition of ADP, the substrate to be tested was added to the incubation. Subsequently, state 3 respiration was initiated by adding ADP (final concentration 100 µM). The test compounds were added to the mitochondrial incubations before the addition of the substrate L-glutamate. Subsequent experiments with the F_1F_0-ATPase inhibitor oligomycin (5 µg/mL) were performed to check whether a possible drop in the RCR derives from uncoupling or increased intramitochondrial metabolism of ATP. An uncoupler is able to dissipate the proton gradient over the inner mitochondrial membrane and thereby to reinitiate the electron and oxygen flow even when the mitochondrial ATPase is inhibited.

6.3.9 *In vitro* mitochondrial β-oxidation

Beta-oxidation with freshly isolated liver mitochondria was assessed as the formation of ^{14}C-acid-soluble β-oxidation products from [1-^{14}C]palmitic acid in the presence of the kava extracts. Experiments were performed as described previously [85] with the modifications described by Spaniol et al [86]. After 5 minutes of preincubation with the kava extracts, the reaction was started by adding 100 µL 400 µM [1-^{14}C]palmitic acid (0.925 Bq/100 µL) and the incubations were carried out at 30 °C for 15 min with slow shaking. We have shown previously that the reaction is in the linear phase at this time point [86]. The reaction was stopped with 200 µL 20 % perchloric acid and by placing the tubes on ice. After centrifugation for 5 minutes at 12000x g, the ^{14}C-acid-soluble products in the supernatant were counted. It has been verified under the conditions used that the formation of ketone bodies accounts for at least 80 % of the [1-^{14}C]-palmitate oxidized [87]. The formation of [^{14}C]CO_2 by isolated liver mitochondria was considered as negligible.

6.3.10 Determination of intracellular GSH and GSSG content

In order to assess the redox status of the treated cells and possible formation of reactive metabolites, determination of GSG (glutathione) and GSSG (oxidized glutathione) was done as follows. HepG2 cells were co-incubated with kava for 2 hours, scrapped from the dish, suspended in 1 mL PCA/BPDS (1 mM bathophenanthrolinedisulfonic acid in 10 % perchloric acid) and sonicated for 30 seconds (sonicator from Heat Systems Ultrasonics Inc., Farmingdale, NY, USA, setting 4.5) in order to lyse them. After centrifugation, the pellet was used for protein determination (BCA protein assay kit Pierce, Rockford, IL, USA) and the supernatant for the determination of glutathione using the enzymatic recycling assay of Tietze [88], with the modifications of Griffith [89]. For the determination of total glutathione, the pH of the supernatant was adjusted to pH 6 to 7 by the addition of triethanolamine. For GSSG determination, a derivatization with 2-vinylpyridine was performed prior to the pH adjustment. For the enzymatic reaction, NADPH (0.21 mM), DTNB (5,5'-dithiobis(2-nitrobenzoic acid), 0.6 mM), glutathione reductase (1 U/mL) and sample or standard, respectively, were mixed. The formation of TNB (5-thio-2-nitrobenzoic acid) was monitored at 30 ℃ using a plate reader at 412 nm during 2 min. Glutathione was quantified by comparing the slope of the samples with the corresponding standard curve.

6.3.11 Apoptosis / Necrosis

Discrimination between apoptosis and necrosis was done with an annexin V / propidium iodide stain and analysis by flow cytometry (FACSCalibur, Becton Dickinson) as described previously. HepG2 cells were incubated for 24 hours with the different extracts. After trypsinization and centrifugation, cells were resuspended in RPMI 1640 medium (adjusted to 2.5 mM calcium), stained with Alexa Fluor 633 labeled annexin V and propidium iodide (final concentration 1 µg/mL) and analyzed by FACS. Annexin V-positive cells are in early apoptosis, whereas annexin V and propidium iodide double positive cells can be in both, late apoptosis or necrosis. For the analysis, cells in three regions were added up and the percentage of cells in each region was calculated. The three regions were defined as "living cells" (annexin V- and propidium iodide-double negative), "early apoptotic" (annexin V-positive) and "late apoptotic / necrotic" (annexin V- and propidium iodide-double positive).

6.3.12 ATP determination

The ATP content of HepG2 cells treated with kava extract was determined with the luciferin/luciferase method using an ATP bioluminescence assay kit (Sigma, Buchs, Switzerland) as described previously [90]. After treating the cells with the three kava extracts (each 10

or 150 µg/mL) for 24 hours, the cells were trypsinized, resuspended in 600 µL water and snap-frozen in liquid nitrogen. To extract the ATP from the cells, the samples were incubated in boiling water for 10 minutes and centrifuged (20'000x g, 5 min, 4 ℃) [91]. 100 µL of the supernatant were used for the determination of ATP according to the user manual of Sigma. The ATP content was calculated by comparison to a standard curve.

6.3.13 Statistical methods

Data represent mean ± standard error of the mean (SE) of at least n = 3 replicates. Statistical analysis of differences between control incubations and incubations with kava extract was performed using analysis of variance (ANOVA) and Dunnett's multiple comparison test as posthoc test to localize differences obtained by ANOVA. A p-value < 0.05 was considered to be statistically significant.

6.4 Results

6.4.1 HPLC analysis of the three kava extracts

The chromatograms of the extracts showed several peaks which could be identified as kavalactones. No clear difference in the total amount of kavalactones was observed in the methanolic and the acetonic root extract, respectively. Each of them contained about 80 % (w : w) kavalactones (Table 2). However, the leaf extract was clearly distinguishable from the root extracts, containing only 24 % kavalactones (Table 2). In the root extracts, the amounts of the single kavalactones were distributed more or less equally, meaning that the quantity of each kavalactone ranged between 10 – 17 %, with methysticin and kavain being the most prevalent. In contrast, the leaf extract contained only about 0.5 – 1 % of each kavalactone, apart from dihydromethysticin and dihydrokavain, which were present at about 10 %. Furthermore the leaf extract showed an additional peak which could not be identified and was not detectable in the root extract.

Table 2: Kavalactone content in % (w : w) of the three kava extracts, analyzed with reversed phase HPLC

Extract	M	DHM	K	DHK	Y	DMY	Total content
Methanolic root	16.6	12.0	16.2	13.4	13.6	9.8	**81.6**
Acetonic root	17.3	12.4	14.4	13.7	13.1	10.6	**81.4**
Methanolic leaf	0.6	11.3	0.4	10.2	1.1	0.5	**24.1**

Abbreviations: M = methysticin, DHM = dihydromethysticin, K = kavain, DHK = dihydrokavain, Y = yangonin, DMY = demethoxyyangonin

6.4.2 Cytotoxicity

The cytotoxicity of the three kava extracts was investigated using the hepatocarcinoma cell line HepG2. The sulforhodamine B assay (SRB) showed a concentration-dependent toxicity for all three kava extracts from a concentration of 50 µg/mL on, when incubated for 24 hours (Figure 6). These results were verified with the lactate dehydrogenase (LDH) test, which revealed toxicity already after incubation for 1, 3.5 and 5.5 hours at similar concentrations (results not shown).

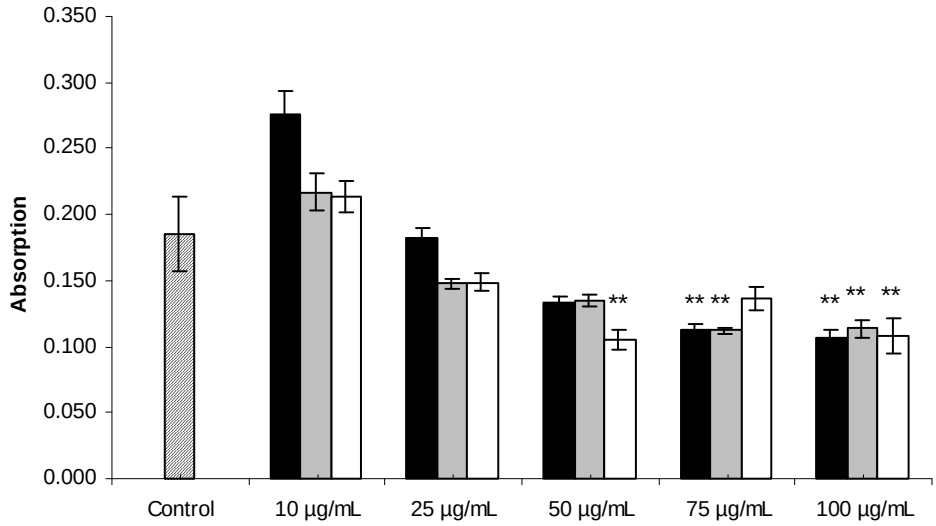

Figure 6: Absorption of sulforhodamine B, a marker for cytotoxicity. The lower the absorption, the higher the toxicity of the compound. 10'000 cells were incubated for 24 hours with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. Proteins of the living cells were stained with sulforhodamine B (0.4 %) and the absorption was measured at 540 nm. Results are expressed as mean ± SE of 5 determinations. **p < 0.01 vs. control.

After having observed a general toxic effect of the three kava extracts using the SRB and the LDH test, further steps were undertaken to clarify the mode of action. As mitochondria are often a target of toxicity, the MTT test was performed. This test reflects mainly the mitochondrial reductive activity [79]. As shown in Figure 7, reductive activity of HepG2 cells was clearly decreased by the two root extracts in a concentration-dependent fashion from 1 µg/mL (acetonic root extract) or 25 µg/mL (methanolic root extract). In comparison, the leaf extract had only a moderate effect on reductive capacity of HepG2 cells, but showed also a significant toxicity from 10 µg/mL on.

Kavain, a kavalactone and thus a main component of kava, was tested as well at concentrations according to its content in the kava plant and to its content in the kava extracts. At concentrations between 0.77 - 7.7 µg/mL, kavain did not show any toxicity on HepG2 cells, neither in the LDH, nor in the MTT test (data not shown).

6.4.3 Mitochondrial toxicity of kava extracts

Since the MTT test suggested a toxic effect of kava extracts on mitochondria, the mitochondrial membrane potential was determined in HepG2 cells. Using JC-1 as a marker [92], both root extracts dissipated the mitochondrial membrane potential (the methanolic root extract at 100 µg/mL and the acetonic root extract starting from 50 µg/mL, see Figure 8). In contrast, the leaf extract did not affect the mitochondrial membrane potential up to 100 µg/mL.

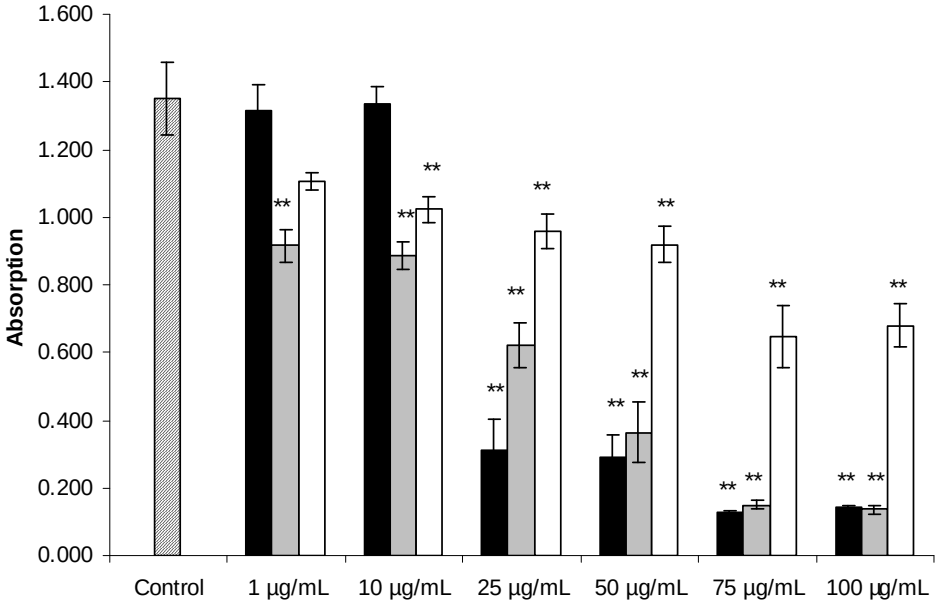

Figure 7: Absorption of MTT, a marker for viability and reductive activity of the cells. The lower the absorption, the lower the reductive capacity of the cells. Cells were incubated for 24 hours with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract, MTT (0.5 %) was added and color intensity was measured at 550 nm. Results are expressed as mean ± SE of 10 determinations. **p < 0.01 vs. control.

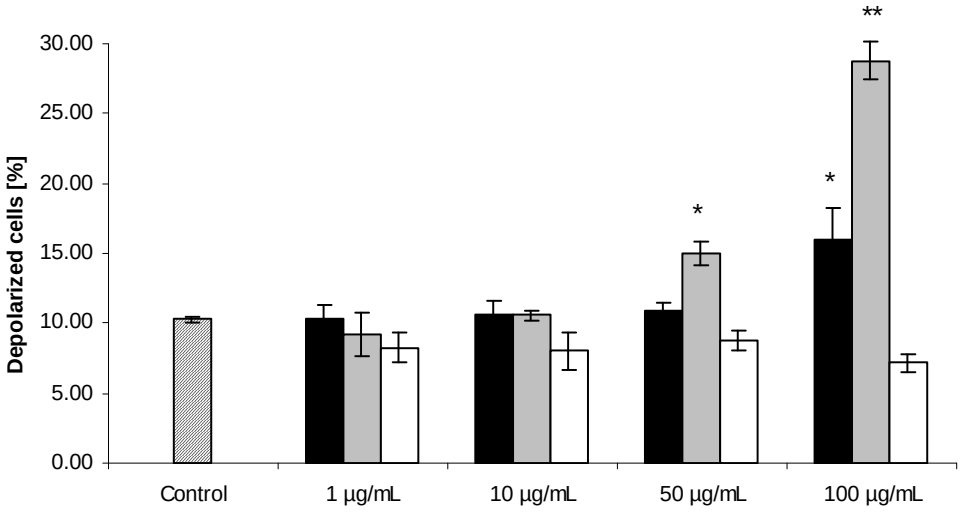

Figure 8: Detection of the mitochondrial membrane potential by the dye JC-1. Mapped is the percentage of depolarized cells. Cells were incubated for 10 minutes with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. Results are expressed as mean ± SE of 3 determinations. *p < 0.05 vs. control, **p < 0.01 vs. control.

Since the function of the respiratory chain is important for the maintenance of the mitochondrial membrane potential, oxidative metabolism was tested in the presence of kava extracts using isolated rat liver mitochondria. In the presence of 150 µg/mL root extract, state 3 oxidation rates were significantly inhibited, but not in the presence of the leaf extract at the same concentration or in the presence of 7.7 µg/mL kavain. In contrast, neither of the root extracts affected state 4 oxidation rates, whereas state 4 showed a significant increase in the presence of 150 µg/mL leaf extract (Figure 9).

In order to investigate uncoupling of oxidative phosphorylation the F_1F_0-ATPase inhibitor oligomycin was added. The latter is able to induce an artificial state 4 also called state 4u. Under such conditions, uncouplers are able to increase oxygen consumption, reflected in an increase of state 4u. As shown in Figure 9C, both root extracts and also the leaf extract led to a significant increase in state 4u oxygen consumption at 150 µg/mL. In contrast, such an increase could not be shown in the presence of 7.7 µg/mL kavain.

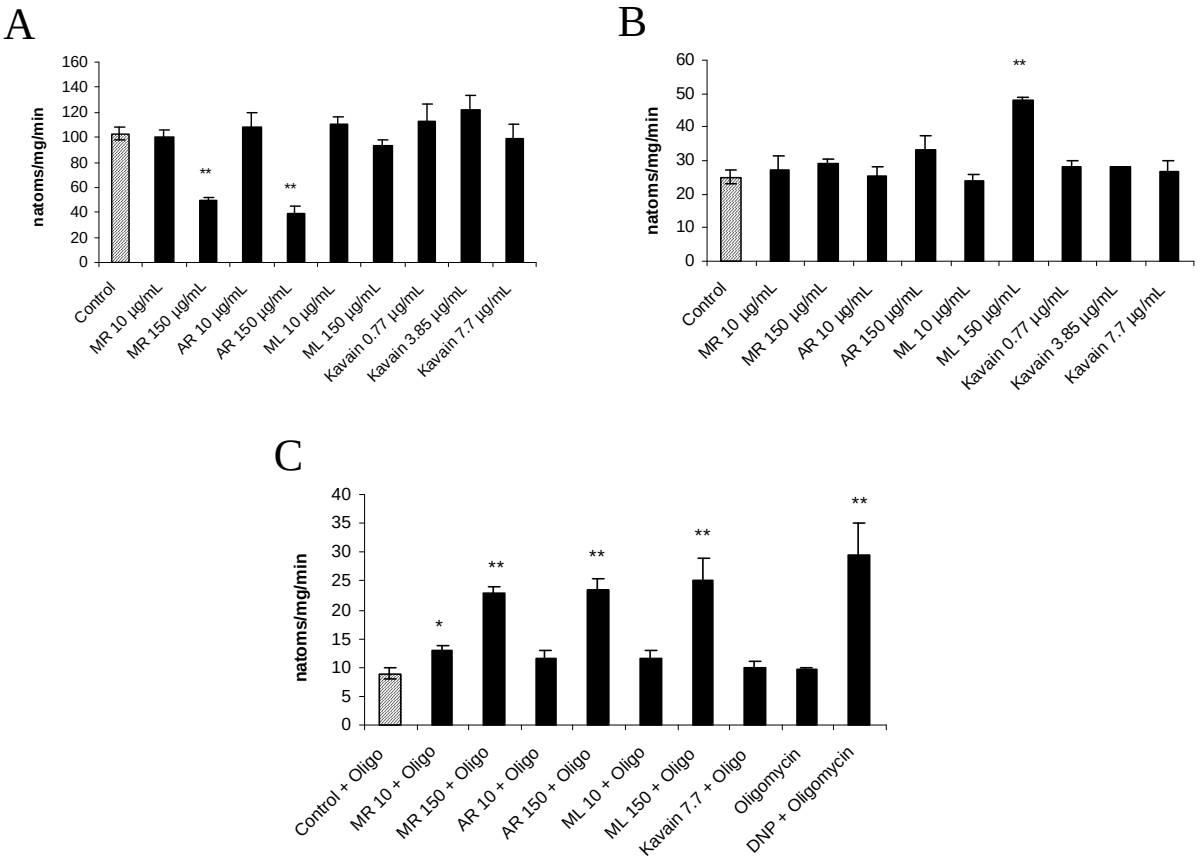

Figure 9: Oxygen consumption (natoms O/mg protein/min) was measured using isolated rat liver mitochondria and glutamate as a substrate. The rate of oxygen consumption in the presence of added ADP (state 3, Figure 9A) and the rate obtained after complete conversion of ADP to ATP (state 4, Figure 9B) was determined for the three kava extracts and kavain. In a second experiment the mitochondria were preincubated with the F_1F_0-ATPase inhibitor oligomycin (5 µg/mL) in order to investigate uncoupling of oxidative phosphorylation (Figure 9C). MR = methanolic root, AR = acetonic root, ML = methanolic leaf extract. Results are expressed as mean ± SE of 3 determinations. *p < 0.05 vs. control, **p < 0.01 vs. control.

Besides the respiratory chain, β-oxidation of fatty acids represents another target of mitochondrial toxicity. Using freshly isolated rat liver mitochondria, all three extracts showed a concentration-dependent inhibition of the activity of mitochondrial β-oxidation as compared to the control (0.61366 nmoles/min/mg = 100 %) (Figure 10). For the two root extracts, inhibition could be demonstrated starting from a concentration of 50 µg/mL, whereas the leaf extract showed toxicity starting at 200 µg/mL. At 200 µg/mL, the extent of inhibition was similar for all extracts, with the residual activity ranging from 14.7 % to 35.9 % of the activity of the control value.

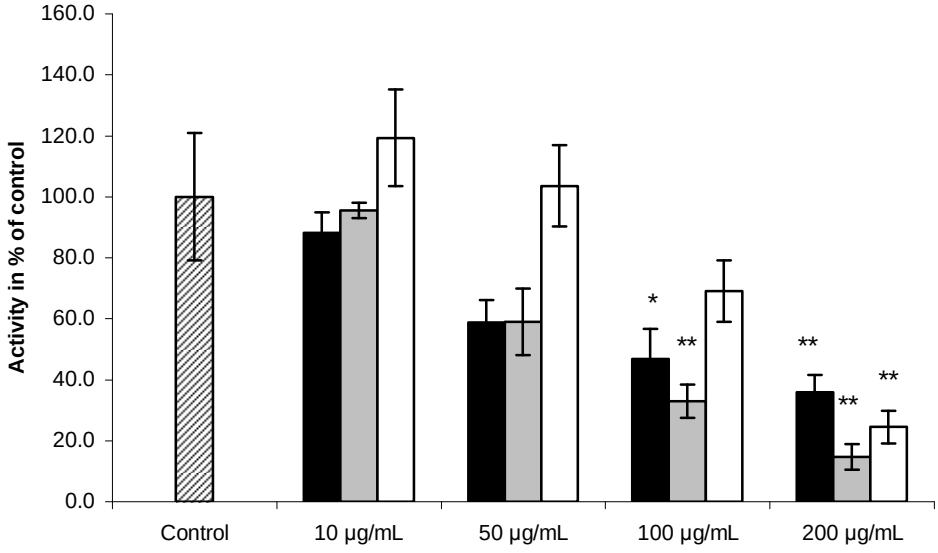

Figure 10: Activity of β-oxidation of palmitic acid after incubation with a methanolic root (solid bars), an acetonic root (grey bars) or a methanolic leaf (open bars) extract. Results are expressed as mean ± SE of 3 determinations. *p < 0.05 vs. control, **p < 0.01 vs. control.

6.4.4 Glutathione determination

The experiments described so far have shown that kava extracts can inhibit and uncouple the respiratory chain. Inhibition of the respiratory chain often leads to the generation of ROS [90]. To assess whether ROS production takes place, the redox status was determined by measuring the glutathione content and ratio (GSH/GSSG) of the treated cells. The measured glutathione ratio of the cells treated with kava were in the range of 1.5, whereas the ratio of the control was 2.90 (Table 3). Therefore, all kava incubations showed a tendency for lowering the GSH/GSSG ratio as compared to the control, suggesting production of reactive metabolites such as ROS. However, these differences did not reach statistical significance, with the exception for the leaf extract ($p < 0.05$).

Table 3: Glutathione pool of HepG2 cells treated with kava extracts

	GSH	GSSG	GSH/GSSG
	µg GSH/mg protein		
Control	12.6 ± 0.70	4.48 ± 0.42	2.90 ± 0.41
Antimycin A 10 µg/mL	8.88 ± 1.69	7.62 ± 2.24	1.23 ± 0.14 *
MR 10 µg/mL	9.82 ± 1.24	5.82 ± 0.70	1.80 ± 0.45
MR 150 µg/mL	7.98 ± 1.29	5.30 ± 1.00	1.73 ± 0.63
AR 10 µg/mL	10.2 ± 1.2	7.49 ± 0.97	1.45 ± 0.32
AR 150 µg/mL	10.6 ± 1.6	6.89 ± 1.17	1.75 ± 0.63
ML 10 µg/mL	11.5 ± 0.8	8.47 ± 1.10 *	1.43 ± 0.29 *
ML 150 µg/mL	8.65 ± 2.42	9.47 ± 0.93 *	0.99 ± 0.39 *

Antimycin A was used as positive control. GSH = reduced glutathione, GSSG = oxidized glutathione; MR = methanolic root, AR = acetonic root, ML = methanolic leaf extract; *$p < 0.05$ vs. control

6.4.5 Discrimination between apoptosis and necrosis by annexin V and propidium iodide staining

Mitochondria play a key role in the mechanisms of apoptotic but also necrotic cell death [6, 15, 16, 31]. In the view of the toxicity of the kava extracts on this organelle, the kind of cell death, namely apoptosis or necrosis, was assessed. Therefore, staining with fluorescence labeled annexin V in combination with propidium iodide was performed on HepG2 cells exposed to the kava extracts. As shown in Figure 11, both root extracts (Figure 11A and B) and the leaf extract (Figure 11C) induced a concentration-dependent increase in the apoptotic and/or necrotic/late apoptotic cell population at 150 µg/mL. The specifity of this mechanism was shown by adding the pancaspase-inhibitor zVAD-fmk to the incubations, which was able to reduce early and/or late apoptosis; this was not the case with the negative control zFA-fmk. The serine protease inhibitor Pefablock did not seem to have this effect, since it reduced toxicity only in combination with zVAD-fmk.

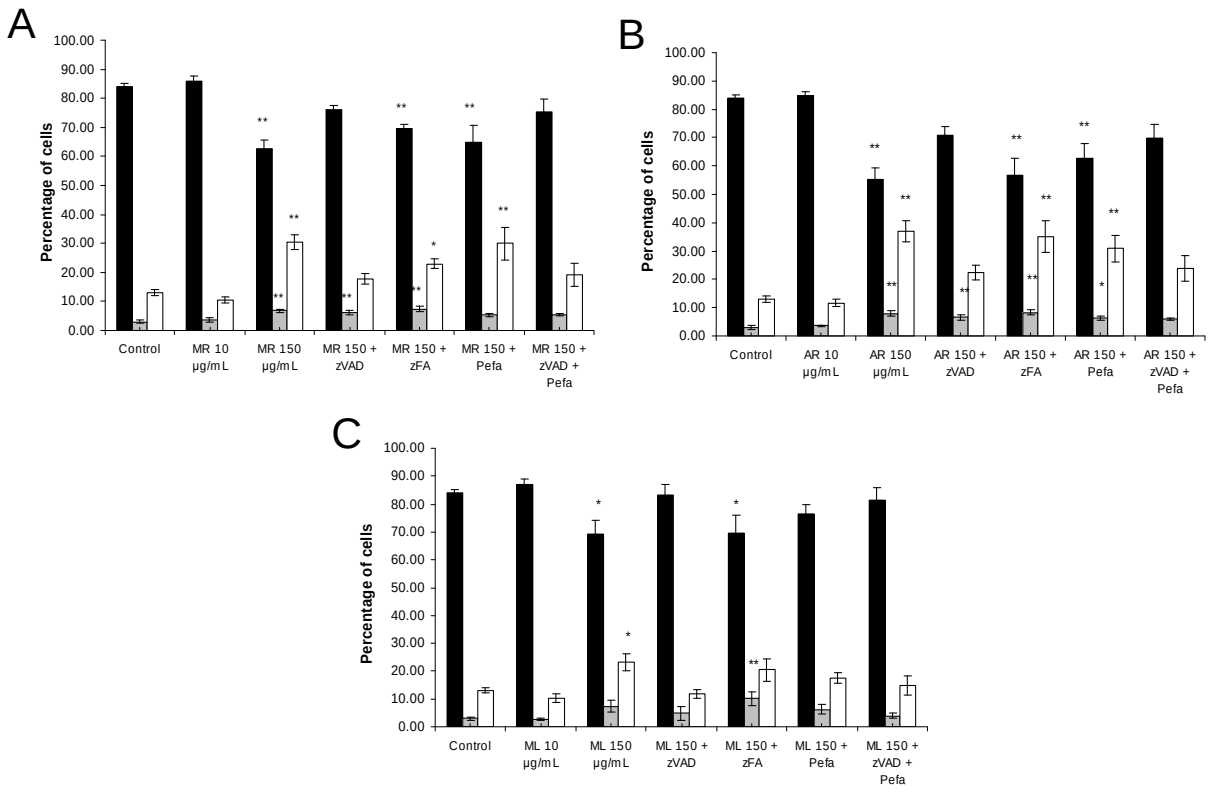

Figure 11: HepG2 cells were separated into the three categories living cells (black bars), early apoptotic cells (grey bars) and late apoptotic/necrotic cells (open bars). Distinction between these three groups has been done by a combined annexin V / PI stain, analyzed with FACS as described in Methods. Cells have been incubated for 24 hours with a methanolic root (Figure 11A), an acetonic root (Figure 11B) or a methanolic leaf (Figure 11C) extract. MR = methanolic root, AR = acetonic root, ML = methanolic leaf extract. Additionally, 3 hours-preincubations with the pancaspase-inhibitor zVAD, the corresponding negative control zFA, and the serine protease inhibitor Pefablock have been performed. Results are expressed as mean ± SE of at least 3 determinations. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control.

6.4.6 Cellular ATP content

When stained with annexin V / propidium iodide necrosis or (late) apoptosis could be detected. To make a further discrimination between these two modes of cells death, the ATP content of treated cells was determined. For the occurrence of apoptosis, normal levels of ATP are necessary, whereas low level ATP status is indicative for necrosis. In our experiments, ATP levels of the cells treated with the three kava extracts were not decreased compared to the control (data not shown). This suggests that cell death in the presence of the kava extracts is due to apoptosis and not necrosis.

6.5 Discussion

In the last few years, cases of hepatotoxicity associated with the ingestion of kava kava have been published in several countries, including Switzerland, Germany and Canada. In most cases, the trigger of the liver toxicity was not clearly identifiable as other medications were taken concomitantly or the given data were too fragmentary for an unambiguous assessment. Nevertheless, in 3 of the 82 cases, the relationship between intake of kava and hepatotoxicity was declared as probable [64] and it could not be ruled out that kava may have led to liver damage. For this reason, we examined the toxicity of total kava extracts, prepared from different parts of the plant and extracted with different solvents.

After having demonstrated toxic effects resulting in cell death using HepG2 cells, additional tests were performed with the aim to find out the mechanisms of toxicity. One of these assays was the MTT test, a colorimetric method used to assess cytotoxicity and therefore viability of cells. Since reduction of MTT is dependent on the activity of the mitochondrial electron transport chain, MTT absorbance is usually interpreted as indicative for mitochondrial damage. To determine whether the reduced production of formazan was indeed due to the impairment of mitochondrial function by the kava extracts, the mitochondrial membrane potential was measured using the fluorescent dye JC-1. Depolarization of mitochondria could be demonstrated for both root extracts, but not for the leaf extract, a result agreeing well with the MTT test, which showed also only a marginal toxicity for the leaf extract.

The experiments with isolated liver mitochondria definitively demonstrated that kava extracts are toxic for mitochondria. The root extracts were shown to act as inhibitors and also uncouplers of the respiratory chain and of β-oxidation and the leaf extract as an inhibitor of β-oxidation and uncoupler of oxidative phosphorylation. In the view that mitochondria are important players in cell death induction, apoptosis and necrosis were examined directly. Annexin V FACS stains revealed that all kava extracts induced early apoptosis and also late apoptotic/necrotic cell death. Cell death could be reduced by the pancaspase inhibitor zVAD-fmk, therefore clearly demonstrating the involvement of caspases, which are key players of apoptosis. It can therefore be speculated that the interaction of the kava extracts with the electron transport chain and β-oxidation and uncoupling of oxidative phosphorylation result in a decrease of the mitochondrial membrane potential, possibly also via opening of the permeability transition pore [15, 16]. This may lead to the rupture of the outer mitochondrial membrane with the release of cytochrome c and other factors and thus, depending on the energetic status of the cell, to apoptosis or necrosis [15, 16].

In order to find out whether ROS and/or other active metabolites are essential in cell death associated with kava extracts, we determined the glutathione pool of the cells. An increased GSSG and/or a decreased GSH/GSSG ratio would be compatible with reactive metabolites. At

least a tendency pointing in this direction could be observed for all three kava extracts, most probably indicating increased production of ROS. After an initial insult, the energetic status of the cell decides, whether the cell is going into the direction of apoptosis or necrosis. Procaspase 9 is activated in an ATP-dependent way to caspase 9, which then activates caspase 3 to initiate the final executioner phase of apoptosis [16]. When ATP is depleted, apoptosis is blocked, since procaspase 9 cannot be activated. Instead, necrosis is induced by the same upstream proapoptotic signals. Our results, showing no reduction of the ATP level, are therefore compatible with apoptosis as the principle death mechanism rather than necrosis.

Our findings suggest that the leaf extract exerts a lower toxicity in some tests as compared to the root extracts. This rises the question of whether extracts from other parts of the kava plant could be used instead of the root. Public reports indicate that also so-called peelings, scrapings, and shavings from the base of the plant are being used for kava trade due to ample supply, low cost, and increased demand on the pharmaceutical and herbal supplement markets. The pharmacologically active kavalactones are present in the underground parts of the plants in high quantities, while alkaloids, like pipermethystine, are present in these parts only in trace amounts [70]. Instead, alkaloids appear to be concentrated in stem peelings and leaves [70, 72, 93]. In traditional kava use, these latter parts of plants are obviously rather avoided for preparing kava drinks. Alkaloids are in general considered to be toxic components of plants, possibly associated with animal and human hepatotoxicity [94]. Taking into account that aboveground plant parts tend to contain piperidine alkaloids, Dragull et al advise for caution in using these parts for human consumption [70]. This advice is supported by the observation that in HepG2 cells, pipermethystine exerted more toxicity as compared to kavalactones [74]. Considering the results of our studies, however, showing a higher toxicity of root as compared to leaf extracts, mitochondrial toxicity of kava kava appears to be associated with the kavalactone content of the plant parts investigated.

Finally, it can be estimated whether the used *in vitro* concentrations of the kava extracts correspond to relevant tissue levels in humans. According to the recommendation of the German Commission E, the daily consumption of kava should correspond to 60 – 120 mg kavalactones. Based on the kavalactone content of our root extracts (i.e. ca. 80 %) and leaf extract (i.e. 24 %), 120 mg kavalactones correspond to 150 mg kava root and 500 mg leaf extract, respectively. Since kinetic data of kava extracts in humans have not been published, our assumptions are based on kinetic data of kavain and its first metabolite OH-kavain [95]. After oral ingestion of 3.2 g kavain, maximal plasma concentrations of kavain and OH-kavain were in the range of 10 – 40 ng/mL. Assuming a good bioavailability, this corresponded to a volume of distribution in the range of 10^4 L, suggesting an almost exclusive accumulation in tissues [95]. After ingestion of 120 mg of kavalactones, tissue concentrations in the range of 1 - 2 µg/g (corresponding to 2 - 10 µg extract per g of tissue) can be expected, which is in the

lower range of where we started to observe mitochondrial toxicity. Since only a minority of persons developed hepatotoxicity after ingestion of kava extracts, an individual predisposition appears also to be important, as described for other mitochondrial toxins such as valproate [96].

The toxicity of kava should not be regarded isolated, however. In comparison with other substances used for the same indication, i.e. benzodiazepines and neuroleptic agents, kava seems to exert not more adverse effects, even when the worst case scenario regarding hepatotoxicity is assumed [97].

In conclusion, our data show that kava extracts exert some toxic effects in a model for liver toxicity, i.e. HepG2 cells. Disturbance of the mitochondrial metabolism, possibly leading to apoptosis, could be seen. Whether these findings can be directly transferred to the human conditions cannot be answered with certainty, as the pharmacokinetic properties of the kava extracts are not known exactly. Taking into account the *in vitro* effects on mitochondria and HepG2 cells, it can be speculated that under certain conditions, e.g. mitochondrial diseases, individual patients may develop hepatotoxicity due to ingestion of kava extracts.

6.6 Acknowledgement

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7 A GFP-METHOD AS AN ALTERNATIVE TO ANNEXIN V / PROPIDIUM IODIDE STAIN IN THE DETECTION OF HEPATIC TOXICITY OF KAVA KAVA

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7.1 Abstract

Background

Apoptosis and necrosis are important processes in the physiology and pathology of cells. Several methods have been used so far to detect and discriminate them, including annexin V / propidium iodide (PI) stain. As these methods are quite time and material consuming, other investigators have described a method that discriminates the two forms of cell death by measuring the decrease of enhanced green fluorescent protein (EGFP) as a marker. So far this method was not established on cells of hepatic provenience, therefore the aim of this work was to compare the GFP-method to annexin V / PI stain on above mentioned cells.

Methods

HepG2 and HUH6 cells have been transduced with a retroviral vector containing EGFP to obtain GFP-expressing cell lines. These cells have been incubated with three different kava extracts as apoptosis inducing agents. Apoptosis and necrosis have then been detected by using annexin V / PI stain and the GFP-method.

Results

It was found that also liver cell lines could be used to apply the GFP-method in the detection of cell death, as the results from the annexin V / PI stain and the GFP-method corresponded to each other.

Conclusions

In this report we show the first time that the GFP-method can also be used with cells of hepatic provenience to screen for hepatotoxicity.

Key words: apoptosis, GFP, annexin V, propidium iodide, kava

7.2 Introduction

Apoptosis and necrosis are important events in the live cycle of a cell. Apoptosis is a tightly regulated process, also called programmed cell death, which is important for the regulation of the cell homeostasis in the body. Old or damaged cells are discarded by this course of events without eliciting an inflammatory response. In contrast to this, necrosis is always a pathological event which is associated with inflammation. Necrosis is usually caused by external agents (toxins), physical damage or drastic changes of the physiological condition. Apoptosis may also be provoked by external substances as well. Apoptosis can represent a strategy against cancer cells, sometimes, however, it is an adverse reaction of a drug. Apoptosis is characterized by typical morphological changes like chromatin condensation, membrane blebbing, and DNA fragmentation, whereas in necrosis, cell swelling develops, finally ending in cell rupture [6].

Consequentially, it is also interesting for scientists to study these incidents. Several methods have been established and used which detect the characteristic features described above, including terminal deoxynucleotidyl transferase (TdT)-mediated end-labeling of DNA strand breaks (TUNEL) [98], annexin V / propidium iodide stain [99], and DNA fragmentation laddering on agarose gels. All these methods are associated with several handling and washing steps, leading to time and material-intensive procedures.

For this reason, Strebel et al. described a novel tool to measure apoptosis and necrosis [57]. This method is based on the property of green fluorescent protein (GFP)-containing cells to lower or loose their green fluorescence in case of apoptosis or necrosis and will be called GFP-method herein. Several human and mouse immune cell lines were found in whom the method could be applied, including Jurkat, A20.2J, and PB3c cells. Another research group found at the same time similar results using different cell lines [58]. However, the method has so far not been applied to liver cell lines.

GFP is a protein originally stemming from the bioluminescent jellyfish Aequoria victoria. To improve its fluorescence many strategies have been followed, including modulation by human codon optimization and amino acid mutations [100-102]. In this study we used the enhanced GFP (EGFP) which sometimes is also depicted as GFPmut1. EGFP carries two mutations (L64F and T65S) which shifts its excitation peak to 488 nm and enhances its fluorescence [102, 103]. EGFP exhibits a spontaneous, stable fluorescence, which is independent of cofactors or substrates and is easy to monitor and quantify. These properties make it an ideal marker for several applications [104-107].

Lately we investigated the potential toxicity of kava extracts. We have shown that kava extracts are toxic on the hepatocarcinoma cell line HepG2 and induce also apoptosis (Lüde et al. 2005, unpublished results). Kava kava is a drug, which was widely used in the Western countries as an alternative to benzodiazepines for the treatment of anxiety or as mild sedative.

But lately, it has been withdrawn from the market as several cases of liver toxicity have been reported [64].

The aim of this investigation was to compare the GFP-method with the annexin V / PI assay and to establish the GFP-method as a procedure to detect apoptosis and necrosis in liver cell lines.

7.3 Materials and methods

7.3.1 Materials

Soluble Fas ligand cellular supernatant (FasL), Alexa Fluor 633 labeled annexin V and Pefa-block (Pefa), were kind gifts of Dr. Alessandro Strebel, Aponetics Ltd. (Witterswil, Switzerland). Z-Val-Ala-Asp-fluoromethylketone (zVAD) and Z-Phe-Ala-fluormethyl-ketone (zFA) were from Enzyme Systems Products (Livermore, CA, USA). Propidium iodide (PI) was from Molecular Probes (Eugene, OR, USA). The three kava extracts (a methanolic (MR) and an acetonic (AR) root , and a methanolic leaf (ML) extract) were kindly provided by Dr. Karin Berger Büter from the Institute for Pharmaceutical Biology of the University of Basel. All other chemicals were from Sigma (Buchs, Switzerland) and of highest quality available when not otherwise stated.

7.3.2 EGFP-expressing cell lines

HepG2 and HUH6 cells were retrovirally transfected with an EGFP-expressing vector. The vector was constructed based on the vector HLox-CMV-EGFP-IRES-TK [108], which was a kind gift of Dr. Didier Trono (Swiss Institute of Technology, Lausanne, Switzerland). The thymidine kinase (TK) gene was replaced by a second EGFP sequence. Retrovirus containing the newly constructed vector was produced using the cell line 293T (kindly provided by Dr. Christian Kalberer, Experimental Hematology, University Hospital Basel) according to the method of Salmon et al [108]. Subsequently, the human hepatocarcinoma cell line HepG2 and the hepatoblastoma cell line HUH6 (both cell lines kindly provided by Dr. Dietrich von Schweinitz, Department of Pediatric Surgery, Children Hospital, University of Basel) were stably transduced with virus containing the bicistronic pLOX-EGFP-IRES-EGFP. The resulting HepG2-GFP and HUH6-GFP cells were three times batch-sorted on a FACS Vantage SE cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to obtain cells with a high EGFP signal. All cells were grown in RPMI 1640 medium (supplemented with GlutaMAX^{TM}-I, 25 mM HEPES, 10 % (v/v) heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin; all from Gibco, Paisley, UK). Culture conditions were 5 % CO_2 and 95 % air atmosphere at 37 °C. Experiments were performed with cells which were 80 % confluent.

7.3.3 Detection of apoptotic and necrotic cells

HepG2-GFP and HUH6-GFP cells were incubated in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) for 24 hours with kava extracts. As positive control of necrosis the detergent NP40 was used and as positive control for induction of apoptosis, soluble FasL cell supernatant

[57]. As a negative control for apoptosis the supernatant of cells transfected with the empty vector (Sup) was employed. In order to verify apoptotic cell death 3 hour-pre-incubations with the pancaspase inhibitor zVAD (final concentration 40 µM) were performed; zFA (final concentration 40 µM) was the according negative control. Additional experiments in HepG2-GFP cells with the serine protease inhibitor Pefablock (final concentration 50 µM) were performed to assess caspase independent apoptosis. After trypsinization and centrifugation, cells (floating and detached cells combined) were resuspended in RPMI 1640 medium (adjusted to 2.5 mM calcium) and stained with Alexa Fluor 633 labeled annexin V and propidium iodide (final concentration 1 µg/mL). GFP, annexin V, and propidium iodide were detected simultaneously by flow cytometry (FACSCalibur, Becton Dickinson) and the results were analyzed using the CELLQuest software (Becton Dickinson). Annexin V was acquired on the FL-4, propidium iodide on the FL-2 and GFP on FL-1 channel, respectively. Annexin V-positive cells are in early apoptosis, whereas annexin V and propidium iodide double positive cells can be in both, late apoptosis or necrosis. For the analysis, cells in three regions (R1 – R3) were added up and set to 100 %. The percentage of cells in each region was calculated. The three regions were defined as "living cells" (annexin V- and propidium iodide-double negative, R1), "early apoptotic" (annexin V-positive, R2) and "late apoptotic / necrotic" (annexin V- and propidium iodide-double positive, R3). For the analysis of the GFP-method, cells displayed in the histogram were assigned to three regions: High GFP (living cells, R4), intermediate GFP (apoptotic cells, R5) and low GFP (necrotic cells, R6). However, during the performance of the experiments it was found that the GFP-method could not clearly distinguish between apoptotic and necrotic cell death in the used cell lines. For this reason, the two regions R5 and R6 were combined to create a new region called "dying cells" R8. Cells in this new region now corresponded to the sum of R2 and R3 (= R9) in the annexin V / PI stain. From now on, results were therefore analyzed comparing these two broader regions.

7.4 Results

The hepatoma cell lines HepG2 and HUH6 were transduced with a retroviral vector in order to express EGFP. Subsequently, experiments were carried out in which annexin V / PI staining was compared to the GFP-method by the use of flow cytometry. The GFP-method bases on the phenomenon that living cells exert a higher GFP signal than apoptotic/necrotic cells [57, 58].

After exposure of the GFP-expressing cells to the test substances, annexin V / PI stain was performed and subsequently all three fluorescences were analyzed on a FACS. Figure 12 represents exemplary data of this analysis and demonstrates that a discrimination between living and dying cells can be undertaken, whereas necrotic and apoptotic cells can only be clearly discriminated with annexin V / PI but not with the GFP-method.

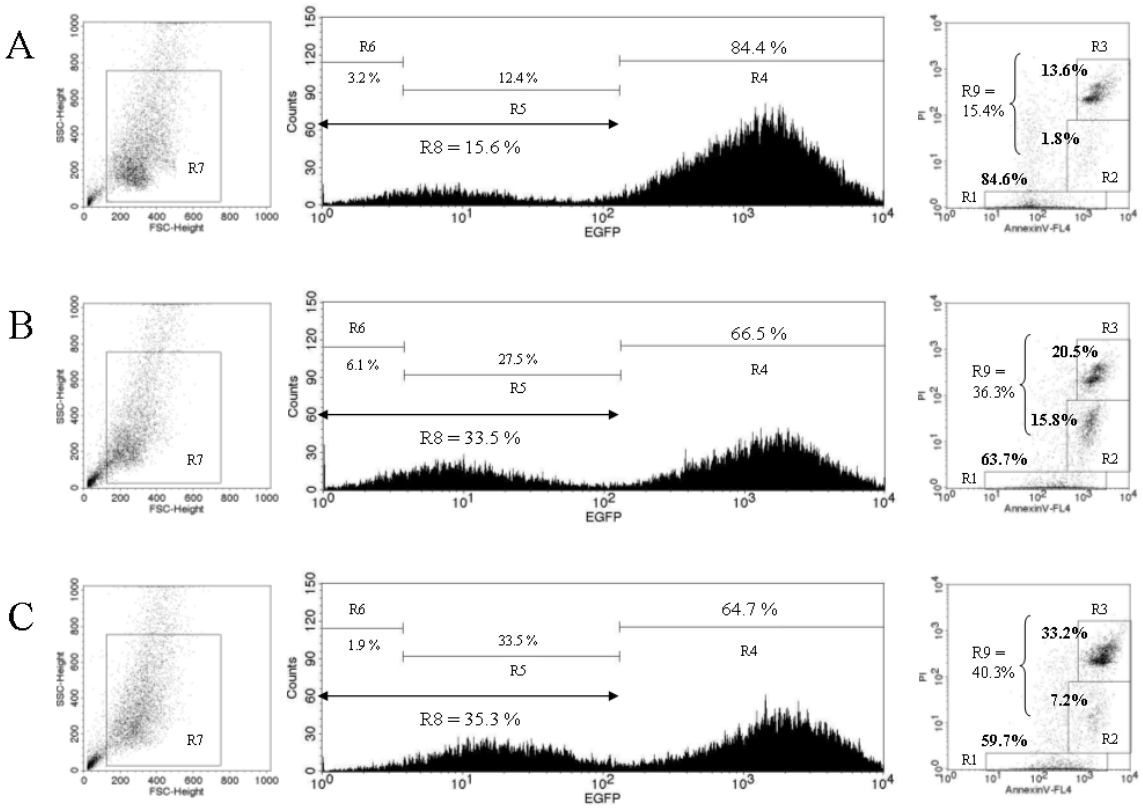

Figure 12: Comparison of GFP and annexin V / PI signal in HepG2-GFP cells. (A) corresponds to the control, (B) to FasL and (C) to MR 150 µg/mL. A cell pre-selection was done by only further analyzing the cell population in R7 (see left dotplot) in order to exclude cell debris and cell aggregates. In the middle histogram, EGFP distribution is mapped, R4 being high GFP or living cells, R8 being dying cells (consisting of R5 = intermediate GFP or apoptotic cells and R6 = low GFP or necrotic cells). In the right dotplot annexin V / PI stain is represented, where R1 corresponds to the living, R2 to the early apoptotic and R3 to late apoptotic/necrotic cells. R9 represents dying cells (= R2 + R3), corresponding to R8 in the EGFP-histogram.

From the total cell population (depicted in the SSC-FSC-Dotplot) only selected cells were further analyzed (R7) to exclude cell debris and cell clusters. These selected cells were mapped in the EGFP histogram for analysis of the EGFP fluorescence and in the dotplot for annexin V / PI examination. It was found with the GFP-method that FasL as well as methanolic root extract elicited cell death with a proportion of about 35 % dying cells (R8); with the annexin V / PI stain a similar amount was seen (35 – 40 %, R2 + R3 = R9). In the latter it could also be discriminated between early apoptotic (R2) and late apoptotic/necrotic cell death (R3). Whereas in the GFP-method this was not possible, as necrotic and apoptotic cell population merged (see R5 and R6).

Experiments with different incubation conditions (e.g. different kava extracts, FasL) were performed with HepG2-GFP and HUH6-GFP cells and analyzed as described above. The results are summarized in Figures 13 and 14. In Figure 13 the results of the experiments with HepG2-GFP cells are depicted. For reasons of clearness, neither data of the living population are shown, nor the results of the methanolic leaf extract, which were similar to those of the root extracts. Figure 13A represents the amount of dying cells detected with annexin V / PI stain and the GFP-method, respectively. It could be shown that the two methods provided almost the same results. For all three kava extracts, the methanolic root, the acetonic root and the methanolic leaf extract, the same pattern of changes in living and dying cells was found. At a concentration of 10 µg/mL, kava extracts were not toxic, whereas 150 µg/mL were associated with toxicity, which could be inhibited by the pancaspase inhibitor zVAD. The control zFA was not able to reduce the toxicity of the extracts, demonstrating the specificity of our findings. Kava 150 µg/mL + Pefablock had less effect than kava 150 + zVAD, whereas kava 150 µg/mL + zVAD + Pefablock corresponded to the incubation kava 150 µg/mL + zVAD. The decrease of dying cells in the incubation kava 150 µg/mL + zVAD was not only observed with annexin V / PI but also with the GFP-method, suggesting the presence of caspase-dependent apoptosis and not only necrosis.

With annexin V / PI (Figure 13B), a maximum of only about 10 % early apoptotic cells was detected, even in the presence of FasL. Nevertheless, incubations with the higher kava concentration showed significant increases in early apoptotic cells compared to the control. A clear decrease in the population of the living cells (R1) was seen with the different incubation conditions (data not shown), which was reflected in the increasing proportion of late apoptotic/necrotic cells (R3). With the GFP-method, the dying cell population (R8) revealed clear changes with the different incubation conditions, which were also reflected in a corresponding decrease of the living cells (R4, data not shown). These changes were almost identical to the ones seen with the annexin V / PI stain in the apoptotic/necrotic cells (R9).

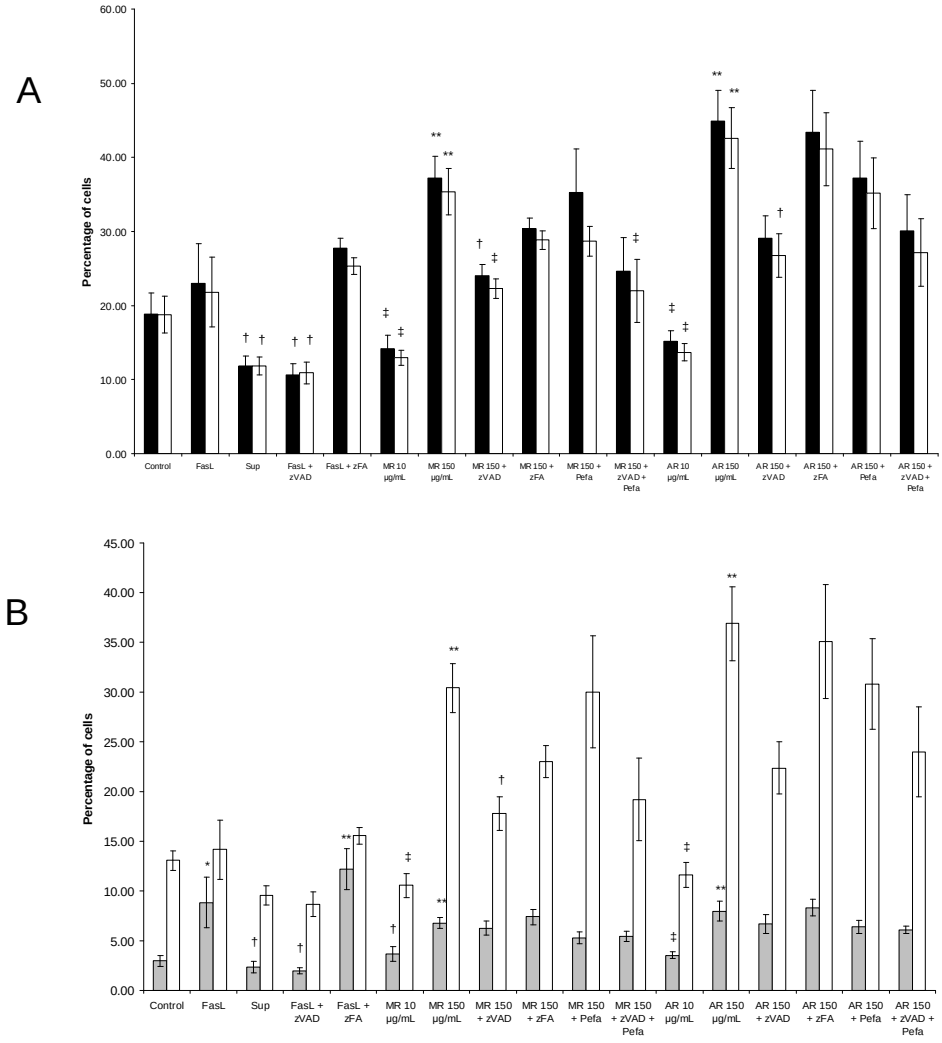

Figure 13: Comparison of the annexin V / PI- with the GFP-method in HepG2-GFP cells after incubation with different substances (A). Black bars represent dying cells of the annexin V / PI stain (R9), open bars dying cells of the GFP-method (R8). Discrimination between apoptotic and necrotic cell death was performed with annexin V / PI stain (B). Grey bars represent early apoptotic (R2) and open bars late apoptotic/necrotic cells (R3). Results are expressed as mean ± SE of at least 3 individual determinations. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control; † $p < 0.05$ vs. FasL, MR 150 µg/mL, AR 150 µg/mL, respectively; ‡ $p < 0.01$ vs. FasL, MR 150 µg/mL, AR 150 µg/mL, respectively.

The comparison of the two methods was repeated with the second hepatic cell line HUH6-GFP (Figure 14). These cells showed similar results as obtained with the HepG2-GFP cells and both methods revealed comparable percentages of dying cells (Figure 14A). However, when compared to HepG2-GFP cells, the amount of dying cells was much higher, especially for the acetonic root and the methanolic leaf extract, which showed a dying population of about 80 – 100 % with the annexin V / PI stain. Using the GFP-method this amount turned out to be less, i.e. about 70 %. This high percentage may be explained by the increased base line level of dead cells in HUH6-GFP cell cultures, which could be seen in all populations studied.

With the annexin V/PI stain (Figure 14B) even the early apoptotic cell population (R2) reached relatively high amounts when incubated with the high kava concentration, i.e. up to 42 % for the methanolic leaf extract. This is much higher than with the HepG2-GFP cells.

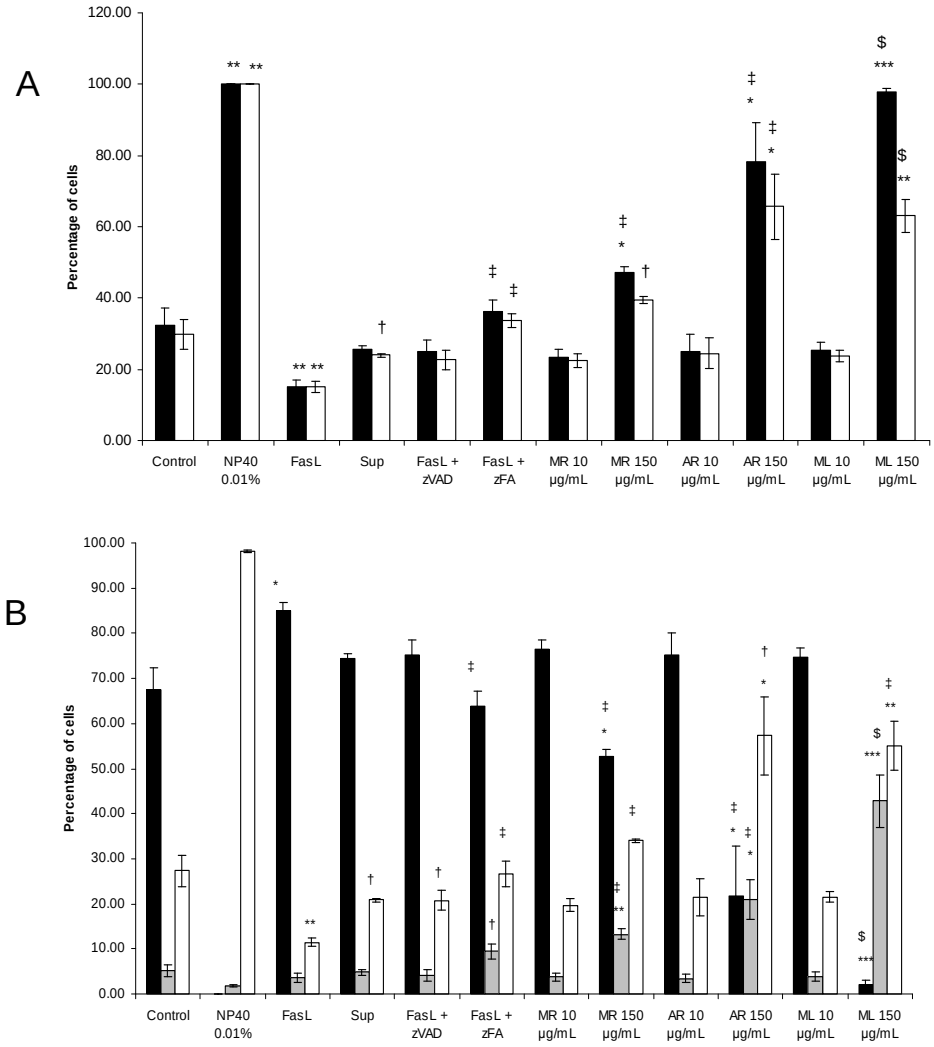

Figure 14: Comparison of the annexin V / PI- with the GFP-method in HUH6-GFP cells after incubation with different substances (A). Black bars represent dying cells of the annexin V / PI stain (R9), open bars dying cells of the GFP-method (R8). Discrimination between apoptotic and necrotic cell death was performed with annexin V / PI stain (B). Solid bars represent living (R1), grey bars early apoptotic (R2), and open bars late apoptotic/necrotic cells (R3). Results are expressed as mean ± SE of 3 individual determinations. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control; † $p < 0.05$ vs. FasL, MR 10 µg/mL, AR 10 µg/mL, ML 10 µg/mL, respectively; ‡ $p < 0.01$ vs. FasL, MR 10 µg/mL, AR 10 µg/mL, ML 10 µg/mL, respectively; $ $p < 0.001$ vs. MR 10 µg/mL, AR 10 µg/mL, ML 10 µg/mL, respectively.

Experiments with freshly isolated hepatocytes from GFP-transgenic mice (C57BL/6-TgN (ACTbEGFP)1Osb, obtained from the Jackson Laboratory Bar Harbor, Maine, USA) have also been performed. But the results were hardly interpretable, as the GFP expression of the hepatocyte preparations were scattered over a very broad range and therefore the shift in the fluorescence pattern could not be reliably quantified (data not shown).

7.5 Discussion

Cell death investigations have been conducted using several time and material consuming methods, including annexin V / PI stain and DNA fragmentation pattern analysis. In order to ease these procedures, Strebel et al [57] and Steff et al [58] described a method where several handling steps could be omitted and which was easy to interpret. This method is based on the property of GFP to decrease in fluorescence intensity when death-evoking agents act on a cell. Such cells were previously prepared by transfection for stable expression of GFP.

The mechanism by which the GFP fluorescence decreases is still not known. The speculation that GFP would leak out of the cell or be degraded by proteases such as caspases upon activation of cell death was ruled out. The GFP-method even worked when cell death was mediated by a caspase-independent pathway and the GFP amount in the cell was similar in living and dying cells as could be shown by Western blot experiments [58]. Another explanation for the decrease of GFP could be the influence of the intracellular pH. It was found that most GFP mutants rapidly respond to pH changes in the way that cytosolic acidification leads to reduced GFP fluorescence [109]. This makes them an ideal tool to monitor intracellular pH [110, 111]. A decrease in pH (around 0.4 U) was indeed observed in cells undergoing the apoptotic process [112-114]. In addition, it could be evidenced that redox changes influence the fluorescence of GFP [115, 116]. Therefore, redox events occurring during apoptosis may also contribute to the loss of GFP fluorescence.

Our experiments were performed with different kava extracts, substances found to be cytotoxic in an earlier study (Lüde et al., unpublished results). Their ability to trigger cell death was investigated in this study by comparing and using the GFP and the annexin V / PI method. It was found that these two methods provided similar results, when comparing the percentage of dying cells. With the GFP-method, a clear discrimination between apoptotic and necrotic cells, like with the annexin V / PI stain, could not be obtained, however. Using the GFP-method the apoptotic and necrotic cell populations merged and the region setting was therefore not feasible. A distinction between apoptotic and necrotic cell death, like it could be shown by Strebel et al. [57], might be achievable by using other liver cell lines. Therefore, also a HUH6-GFP cell line was investigated and showed a better discrimination between necrosis and apoptosis, which could not be quantified separately, however.

When comparing toxicity of the kava extracts on HepG2 and HUH6 cells, respectively, it could be seen that the toxic effect on HUH6 cells was higher than on HepG2 cells. Obviously, HUH6 cells seem to be more susceptible to damage by kava extracts. This might be due to the different origin of these cell lines, HepG2 being a hepatocarcinoma and HUH6 a hepatoblastoma cell line. The exact reason for this difference could only be studied if the mechanisms of hepatotoxicity of kava kava were clear, what is not the case so far, however.

It was found in HUH6 cells that apoptosis was not provokable by FasL. This may be explained by the fact that HUH6 cells have a low expression of Fas receptor [117].

As was shown by [57] and [58], the GFP-method could also be used for plate reader application. This is advantageous when screening high numbers of substances. Consequently, the plate reader method could represent an alternative to the enzyme (lactate dehydrogenase test) or staining tests (sulforhodamine B) used for screening of cell viability. However, the GFP method itself will not allow to discriminate between apoptotic and necrotic cell populations in the plate reader assay, since the mean of the fluorescence of all cells will be measured. However, co-incubations of cell death inducing agents with caspase inhibitors, like zVAD, could help to specify the mode of cell death in the same incubation. Nevertheless, other apoptosis-detecting assays should be used in addition, including annexin V / PI stain, when apoptosis is desired to be confirmed. Even so, the GFP-method can be used as an easy, reliable method to detect cell death, also in liver cell lines.

With the annexin V / PI method apoptosis was observed (increase of the early apoptotic population), but only to a relatively low – although significant – extent. Instead, the late apoptotic / necrotic population displayed a greater accumulation of cells. Out of this it was speculated that the early apoptotic pool is rather a transient stage where apoptotic cells pass through on the way to the late apoptotic / necrotic stage. In case no significant increase in the early apoptotic population occurs a rise of the late apoptotic / necrotic cell pool is interpreted to be caused by necrotic cells.

It can be concluded that the GFP-method is an easy, reliable and stable method which is ideal to detect cell death, i.e. apoptosis and necrosis. It was shown in this study that this method also works with hepatoma cell lines, but without being able to differentiate between apoptosis and necrosis. Nevertheless, in combination with caspase inhibitors and/or other assays distinguishing between apoptosis and necrosis, the method is usable to screen for hepatotoxicity.

8 HEPATOTOXICITY OF CIMICIFUGA RACEMOSA

Short title: Hepatotoxicity of Cimicifuga Racemosa

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8.1 Abstract

Background/Aims:

Extracts of cimicifuga racemosa became popular in the last years as natural medicines for the treatment of menopausal complaints since hormone replacement therapy was found to be associated with breast cancer. Several trials studying the efficacy of cimicifuga have been performed, but side effects were not investigated profoundly or considered to be mild. This study was designed to assess the potential hepatotoxicity of cimicifuga racemosa.

Methods:

Cimicifuga racemosa was fed to rats and liver slices were analyzed by electron microscopy. Cytotoxicity tests, apoptosis/necrosis investigations, redox status assessment, and mitochondrial membrane potential determination were conducted in the hepatocarcinoma cell line HepG2 after incubation with cimicifuga extract. Oxygen consumption and β-oxidation were assessed using isolated rat liver mitochondria.

Results:

Microvesicular steatosis was found in rats fed with cimicifuga extract (1000 mg/kg). Clear cytotoxic effects were detected *in vitro* from 300 µg/mL on. At this concentration, the mitochondrial membrane potential was decreased and apoptosis was induced. Further, oxidative phosphorylation was uncoupled and β-oxidation was inhibited.

Conclusions:

Cimicifuga racemosa exerts toxicity *in vivo* and *in vitro*. Although the concentrations associated with toxicity were higher than the expected plasma levels in humans, it can not be excluded that cimicifuga extracts are hepatotoxic also in humans.

Key words: Cimicifuga racemosa, Actaea racemosa, black cohosh, hepatotoxicity, apoptosis, HepG2

8.2 Introduction

Hormone replacement therapy (HRT) is currently the standard treatment of menopausal ailment. Association of HRT with breast cancer and the request of many women for natural treatment were the reasons why alternative therapies became popular. Especially extracts of the plant cimicifuga racemosa are now widely used for this indication. Cimicifuga racemosa, also called Actaea racemosa or black cohosh, is a member of the buttercup family (ranunculaceae) and ethanolic or isopropanolic extracts of it are being used. Cimicifuga racemosa is a herb native to the Eastern United States and Canada. Traditionally, the rhizome was used by North American Indians for joint aches, myalgias, and neuralgias, as well as menopausal complaints, pain during labour, and also rheumatism. Nowadays, it is most commonly used for menopausal symptoms and the premenstrual syndrome, in particular dysmenorrhea [118].

Data from clinical studies and from spontaneous reporting programs suggest that adverse events with cimicifuga racemosa are rare, mild, and reversible. Gastrointestinal upsets and rashes are the most common adverse events reported [119]. These findings were confirmed by another review of safety of cimicifuga racemosa: Uncontrolled reports, postmarketing surveillance, and human clinical trials of more than 2,800 patients demonstrated a low incidence of adverse events (5.4%). Of the reported adverse events, 97% were minor and did not result in discontinuation of therapy, and the only severe events were not attributed to cimicifuga treatment [120]. In larger doses, cimicifuga racemosa can cause dizziness, headaches, giddiness, nausea, and vomiting [121]. One case report describes a woman who developed a seizure after taking a combination of black cohosh, chasteberry, and evening primrose oil, but no clear cause-and-effect relationship was documented [122]. Huntley et al [119] found the following cases of more severe adverse events, when taking all the surveillance programs into consideration. There was one case of hepatic failure, two or three cases of hepatitis (one case may be a duplicate in two databases), and three cases with increased activity of hepatic enzymes (one of them is identical with one of the hepatitis cases mentioned above). In addition, also cardiovascular events were reported, including two cases of thrombosis, three cases of hypertension, one with circulatory failure, one with bradycardia, and one with palpitations, ventricular and supraventricular extrasystoles. Since these programs based on spontaneous reporting, the true adverse rate is most probably higher.

Since the association of hepatotoxicity with cimicifuga appears to be likely, we tried to reproduce such findings in an animal model. Indeed, rats treated with 1000 mg/kg cimicifuga extract developed microvesicular steatosis, which may be a mechanism of hepatic toxicity also in humans. Using HepG2 cells and isolated mitochondria, we subsequently clarified the mechanism of this adverse drug reaction.

8.3 Material and methods

8.3.1 Chemicals

The dry ethanolic cimicifuga extract was obtained from Zeller AG (Romanshorn, Switzerland, batch number V2009). Solutions of the extract were made by dissolving the extract in DMSO. Caffeic acid and ferulic acid were purchased from Fluka (Buchs, Switzerland) and cimiracemoside A was from ChromaDex (Santa Ana, CA, USA). JC-1 and propidium iodide were from Molecular Probes (Eugene, OR, USA); Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Z-Phe-Ala-fluormethylketone (zFA-fmk) were from Enzyme Systems Products (Livermore, CA, USA); Alexa Fluor 633 labeled annexin V was a generous gift of Dr. Felix Bachmann, Aponetics Ltd. (Witterswil, Switzerland). [1-^{14}C]palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). The scintillation cocktail was from Perkin Elmer (Boston, MA, USA). The Cy3^{TM} conjugated anti-sheep IgG was purchased from Jackson Laboratories (West Grove, PA, USA). All other chemicals were from Sigma (Buchs, Switzerland) and of highest quality available when not otherwise stated.

8.3.2 Liver sections of rats treated with cimicifuga

Groups of five female Wistar rats were fed each with daily doses of 1, 10, 100, 300 or 1000 mg per kg body weight of cimicifuga racemosa extract. The extract was given suspended in 1 mL of an aqueous solution of gum arabic by means of esophageal tube (gavage) over a period of 21 days. After anaesthesia and decapitation animals were perfused for fixation, tissue blocks were excised and prepared for microscopy [123].

8.3.3 Cell culture

The human hepatocarcinoma cell line HepG2 was kindly provided by Dr. Dietrich von Schweinitz (Department of Pediatric Surgery, Children's Hospital, University of Basel). The cell line was grown in RPMI 1640 medium (supplemented with GlutaMAX^{TM}-I, 25 mM HEPES, 10 % (v/v) heat-inactivated fetal bovine serum and 100 U/mL penicillin/streptomycin; all from Gibco, Paisley, UK). Culture conditions were 5% CO_2 and 95 % air atmosphere at 37 ℃. Experiments were performed when the cells had reached a confluence of about 80 %.

8.3.4 Cytotoxicity tests

To examine cell viability and reductive activity, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed in HepG2 as described previously [124], with a washing step included [79].

The sulforhodamine B (SRB) test was performed according to the protocol of Skehan [77] and the lactate dehydrogenase (LDH) assay was carried out according to the method of Vassault [78].

8.3.5 Isolation of rat liver mitochondria

Male Sprague Dawley Rats (Charles River, Les Onins, France) were anesthetized with carbon dioxide and killed by decapitation. Liver mitochondria were isolated by differential centrifugation according to the method of Hoppel et al [80]. The mitochondrial protein content was determined using the biuret method with bovine serum albumin as standard [81]. The study protocol had been accepted by the Animal Ethics Committee of the Canton of Basel.

8.3.6 *In vitro* mitochondrial β-oxidation

Beta-oxidation with freshly isolated liver mitochondria was assessed as the formation of ^{14}C-acid-soluble β-oxidation products from [1-^{14}C]palmitic acid in the presence of the cimicifuga extracts. Experiments were performed as described previously [85] with the modifications described by Spaniol et al [86].

8.3.7 Oxygen consumption

Polarographic monitoring of oxygen consumption by rat liver mitochondria was carried out in a 1 mL chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30 ℃ as described prev iously [82]. Subsequent experiments with the F_1F_0-ATPase inhibitor oligomycin (5 µg/mL) were performed to check for uncoupling of oxidative phosphorylation [90].

8.3.8 Mitochondrial function

To check the mitochondrial membrane potential, the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) was used according to the protocol of Molecular Probes. After incubation with JC-1 cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

8.3.9 Determination of intracellular GSH and GSSG content

In order to assess the redox status of the treated cells and to check for possible formation of reactive metabolites, determination of GSH (glutathione) and GSSG (oxidized glutathione) was performed using the enzymatic recycling assay of Tietze [88], with the modifications of Griffith [89].

8.3.10 Apoptosis / Necrosis

Discrimination between apoptosis and necrosis was done with the annexin V / propidium iodide stain. HepG2 cells were incubated for 24 hours with the extract as described in the results section. After trypsinization and centrifugation, cells were resuspended in RPMI medium (adjusted to 2.5 mM calcium), stained with Alexa Fluor 633 labeled annexin V and propidium iodide (final concentration 1 µg/mL) and analyzed by FACS (FACSCalibur, Becton Dickinson).

8.3.11 ATP determination

The ATP content of HepG2 cells treated with cimicifuga extract was determined with the luciferin/luciferase method using the ATP bioluminescence assay kit from Sigma. After treating the cells with the cimicifuga extract (each 10 or 500 µg/mL) for 24 hours, the cells were trypsinized, resuspended in 600 µL water and snap-frozen in liquid nitrogen. To extract the ATP from the cells, the samples were incubated in boiling water for 10 minutes and centrifuged (20'000x g, 5 min, 4 ℃) [91]. 100 µL of the supernatant were used for the determination of ATP according to the user manual of Sigma. The ATP content was calculated by comparison to a standard curve.

8.3.12 Hoechst staining

To confirm apoptosis HepG2 cells were incubated with cimicifuga extract for 24 hours, stained with 0.1 mM Hoechst 33342 dye and visualized with an inverted fluorescent microscope (Olympus IX50, Hamburg, Germany).

8.3.13 Cytochrome c staining

100'000 HepG2 cells were seeded into a 8-well chamber slide (Nunc Labtek, Naperville, IL, USA) and cultured for two days. Subsequently, cells were incubated for 24 hours with cimicifuga extracts as described in the result section. The cells were fixed with 4 % paraformaldehyde and rinsed three times with blocking buffer (0.05 % saponin and 3 % BSA in PBS). 75 µL

of diluted anti-cytochrome c antibody (1 : 300) were added and incubated for 1 hour at 37 ℃. After rinsing with blocking buffer, 75 µL diluted Cy3 conjugated anti-sheep IgG (1 : 100) were added and incubated for another hour at 37 ℃. Cell s were washed once with blocking buffer and twice with PBS and then analyzed in the inverted fluorescent microscope with the AnalySIS^D software (Olympus).

8.3.14 Statistical methods

Data represent mean ± standard error of the mean (SE) of at least 3 replicates. Statistical analysis of differences between control incubations and incubations with cimicifuga extract was performed using analysis of variance (ANOVA) and Dunnett's multiple comparison test to localize differences obtained by ANOVA. A p-value < 0.05 was considered to be statistically significant.

8.4 Results

Animals fed with lower doses of cimicifuga racemosa extract (1 or 10 mg/kg body weight) showed slight alterations of their hepatic morphology, like a moderate increase in mitochondrial volume and a slight enlargement of the bile canaliculi (data not shown). The animals fed with 100 or 300 mg cimicifuga extract/kg body weight showed more dramatic changes like mitochondrial vacuoles (data not shown) and, finally, the animals fed with 1000 mg/kg body weight developed either a general or a rather circumscribed steatosis. Most obvious signs were an accumulation of lipid droplets in their hepatocytes (microvesicular steatosis), glycogen depletion and fragmentation of the rough endoplasmic reticulum to a predominantly vesicular type (see Figure 15).

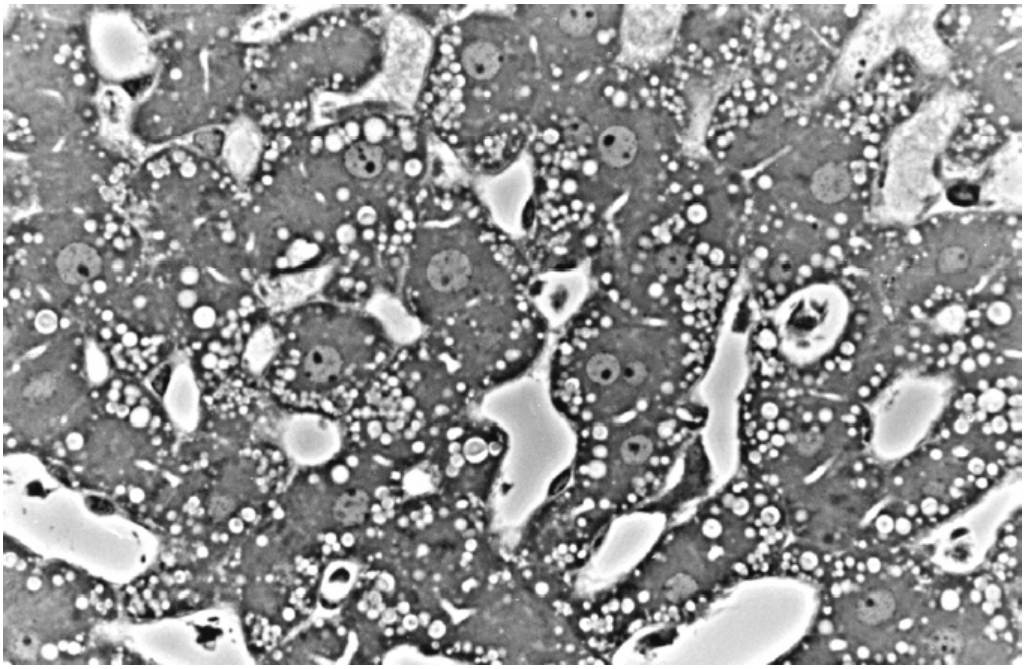

Figure 15: Semi-thin section of a liver from a rat treated with 1000 mg/kg body weight cimicifuga extract for 21 days. The majority of the intracellular vesicles are lipid droplets, they are part of a typical steatotic morphology. The sections have been stained with paraphenylene diamine (PPD).

In view of this findings *in vitro* tests were performed to investigate the mechanism of cimicifuga triggered liver steatosis. Since microvesicular steatosis usually reflects mitochondrial damage [125], the MTT test was carried out on HepG2 cells to find out whether the cimicifuga extract affects reductive capacity of the cells and exerts cytotoxicity. It could be shown that the cimicifuga extract displayed a concentration-dependent toxicity, starting from 75 µg/mL (Figure 16).

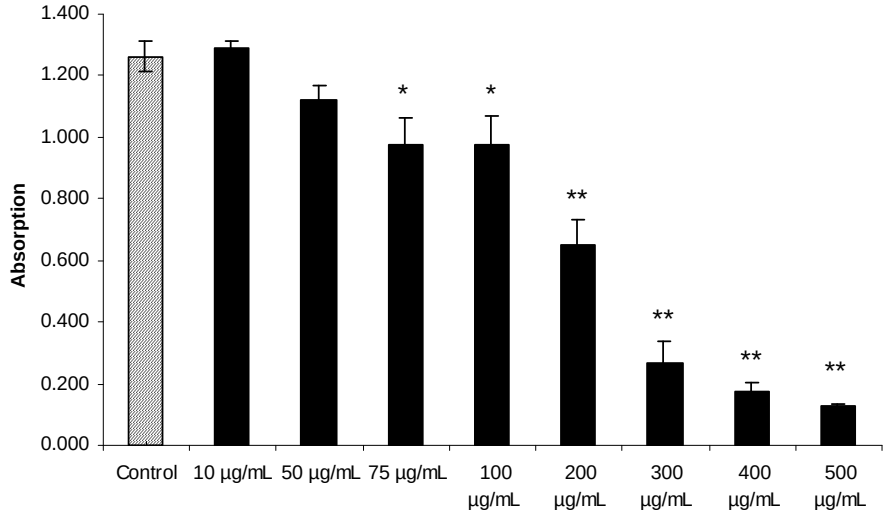

Figure 16: Cytotoxicity and reductive capability of HepG2 cells after incubation with cimicifuga extracts for 24 hours. In intact cells MTT is metabolically converted to its blue formazan, whose absorption can be measured at 550 nm and is represented here. Results are expressed as mean ± SE of 10 determinations. *p < 0.05, **p < 0.01 vs. control.

Cytotoxicity of cimicifuga racemosa could be confirmed with the LDH and the sulforhodamine B test. Both showed also a concentration-dependent cytotoxicity, similar to the MTT test (data not shown). Specific components of the cimicifuga extract, namely caffeic acid, ferulic acid and cimiracemoside A, showed neither in the MTT test nor in the LDH test any toxicity.

The mitochondrial membrane potential is influenced by many mitochondrial functions, and can therefore be used as a test for screening for mitochondrial toxicity. The mitochondrial membrane potential was determined in HepG2 cells in the presence of cimicifuga extract using JC-1 as a marker. These experiments revealed a decrease in the membrane potential starting from 300 µg/mL cimicifuga extract (Figure 17).

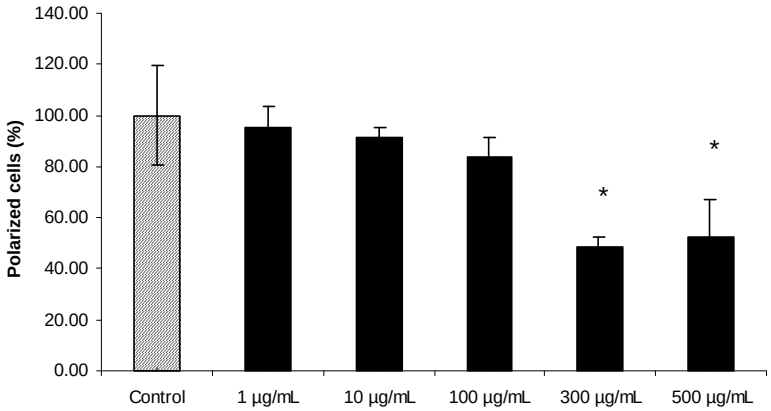

Figure 17: Percentage of polarized HepG2 cells after 10 min incubation with cimicifuga and the dye JC-1 (final concentration 7.5 µM). Results are expressed as mean ± SE of 3 determinations. *p < 0.05 vs. control.

In order to find out the reason for the observed decrease in the mitochondrial membrane potential, the function of isolated rat liver mitochondria was studied more closely in the presence of cimicifuga. Determination of β-oxidation revealed that cimicifuga racemosa inhibited β-oxidation starting from 10 µg/mL on (Figure 18). The residual activity at a concentration of 500 µg/mL was only 8.5 %.

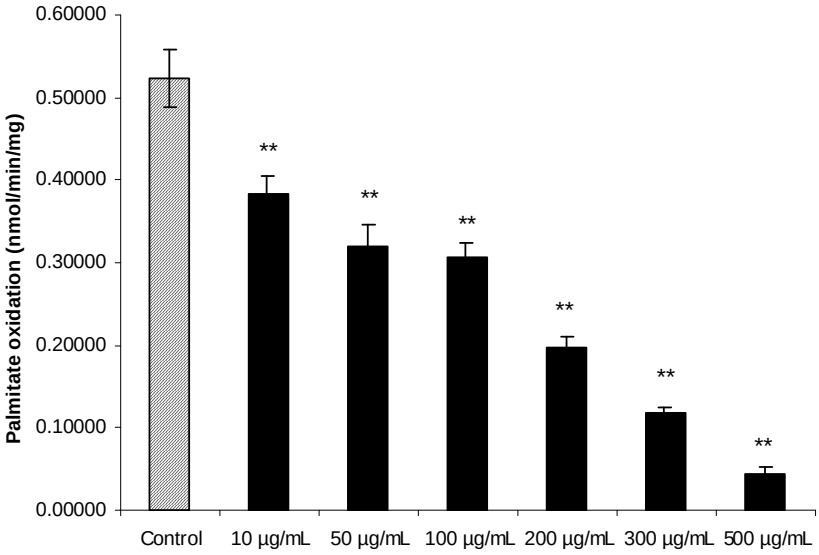

Figure 18: Activity of the β-oxidation of palmitic acid by isolated rat liver mitochondria after incubation with cimicifuga. Results are expressed as mean ± SE of 3 determinations. **p < 0.01 vs. control.

Oxidative phosphorylation is another important metabolic process in mitochondria. To test its functioning, oxygen consumption by isolated mitochondria was assessed after incubation with cimicifuga extracts. State 3 oxidation rates were not decreased by any concentration of the extract (Figure 19A), whereas state 4 oxidation rates were increased with increasing concentration (Figure 19B), suggesting uncoupling of oxidative phosphorylation. The component cimiracemoside did not affect state 3 or state 4 oxidation rates (Figure 19A+B). In order to investigate uncoupling of oxidative phosphorylation, state 4u was induced by the addition of oligomycin, an inhibitor of F_1F_0-ATPase. Under such conditions, uncouplers are able to increase oxygen consumption. As shown in Figure 19C, cimicifuga extract led to a significant increase in state 4u oxygen consumption at 500 µg/mL. Such an increase could not be shown in the presence of up to 5 µg/mL cimiracemoside.

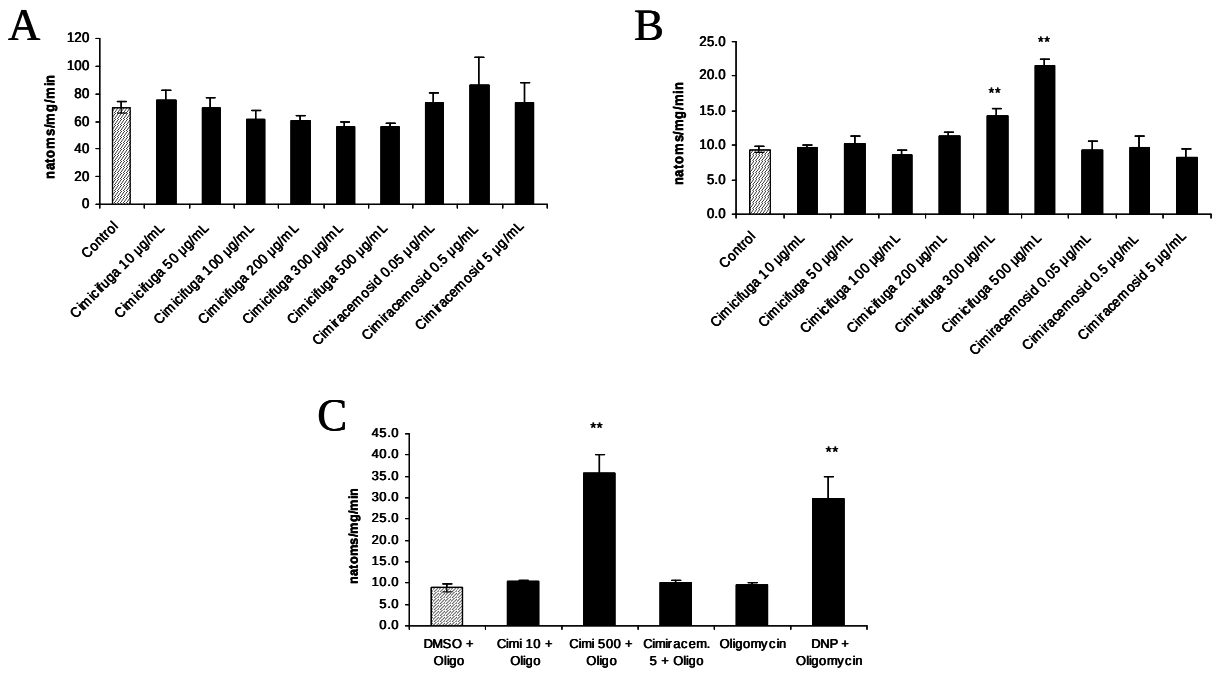

Figure 19: Oxygen consumption by isolated rat liver mitochondria in the presence of L-glutamate (20 mmol/L) as substrate and added ADP (state 3; Figure 19A) and after complete conversion of ADP to ATP (state 4; Figure 19B). The test compounds were added to the mitochondrial incubations before the addition of the substrate L-glutamate. In an additional experiment, oxygen consumption after the addition of the F_1F_0-ATPase inhibitor oligomycin and, subsequently, cimicifuga was determined to study uncoupling of oxidative phosphorylation (state 4u; Figure 19C). Results are expressed as mean ± SE of at least 3 determinations. **p < 0.01 vs. control. Oligo = oligomycin 5 µg/ml; Cimi = cimicifuga; DNP = 2',4'-dinitrophenol (an uncoupler).

Since an inhibited or uncoupled respiratory chain often goes along with an increased ROS production, the redox status of HepG2 cells was assessed by determining their glutathione content. However, a raised GSSG level or an increased GSSG/GSH ratio were not found in HepG2 cells, suggesting that ROS do not play a significant role in cimicifuga hepatotoxicity (data not shown).

Mitochondrial damage can be associated with both apoptosis and necrosis [90]. Hence, we assessed these possibilities with annexin V and propidium iodide staining of HepG2 cells. As shown in Figure 20, the cimicifuga extract induced a concentration-dependent increase in apoptotic and/or late apoptotic/necrotic cells, starting from 300 µg/mL. The specificity of this mechanism was shown by adding the pancaspase-inhibitor zVAD-fmk to the incubations, which was able to reduce early apoptosis significantly.

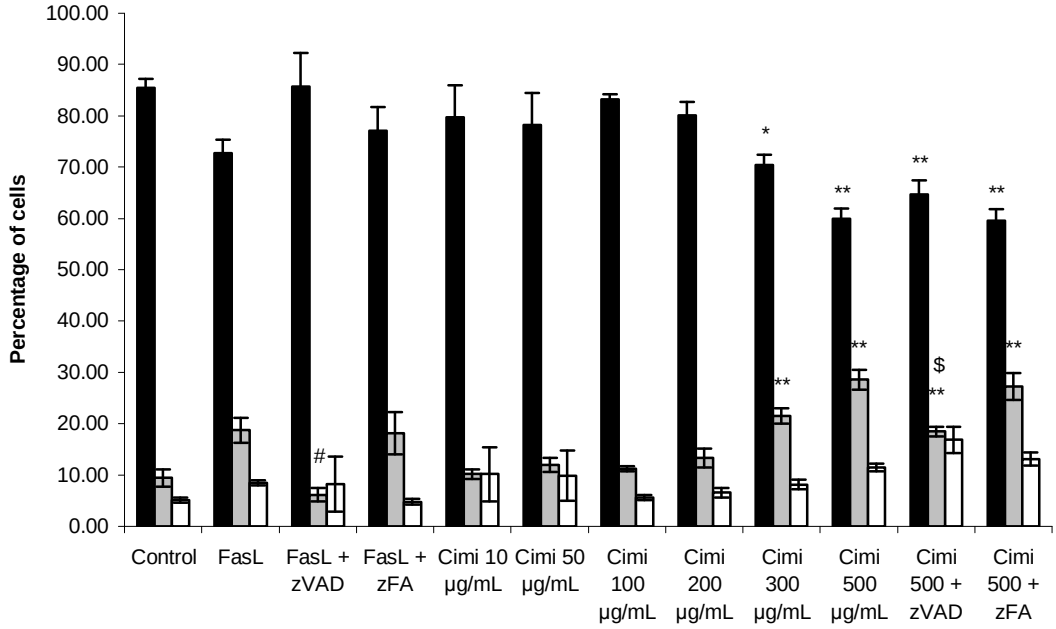

Figure 20: Amount of living (black bars), early apoptotic (grey bars), and late apoptotic/necrotic (open bars) cells after 24 hour incubation with the test compounds. The total number of cells analyzed (set as 100 %) have been proportionally allotted to the three categories "living cells" (annexin V / propidium iodide-double negative), "early apoptotic" (annexin V-positive) and "late apoptotic/necrotic" cells (annexin V / propidium iodide-double positive). Results are expressed as mean ± SE of 3 determinations. *$p < 0.05$, **$p < 0.01$ vs. control; #$p < 0.05$ vs. FasL; \$$p < 0.05$ vs. Cimi 500 µg/mL.

To further discriminate the late apoptotic/necrotic cell population, the cellular ATP content was determined. For the occurrence of apoptosis, normal levels of ATP are necessary, whereas a low level ATP status is indicative for necrosis. In our experiments, the ATP levels of HepG2 cells treated with the cimicifuga extract were not different from control levels (data not shown), suggesting that apoptosis is likely to occur.

From the results of the annexin V / propidium iodide stain and the ATP measurements, it was assumed that apoptosis occurs during incubation with cimicifuga extract. To further confirm these results, Hoechst 33342 stains were performed. These stains revealed that, at a concentration of 500 µg/mL, cimicifuga caused apoptosis as judged from the higher occurrence of condensed nuclei (Figure 21).

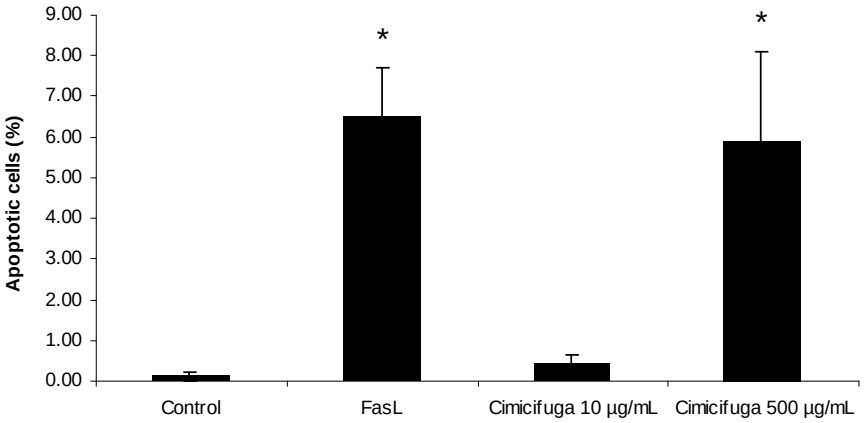

Figure 21: Percentage of apoptotic cells after incubation with cimicifuga and soluble FasL (positive control) for 24 hours and detection with Hoechst 33342 dye. 5 pictures of different areas of each incubation were taken, and the total amount of cells as well as the apoptotic cells were counted. Results are expressed as mean ± SE of 3 independent determinations. *$p < 0.05$ vs. control.

Cytochrome c leakage is another marker of apoptosis triggered by mitochondrial damage [90]. However, we found in our experiments that cimicifuga 500 µg/mL provoked no leakage of cytochrome c (data not shown).

8.5 Discussion

Although several studies concerning the efficacy of cimicifuga were carried out not much is known about the safety of the extracts used. Either it was not looked at the adverse events or there was nothing found/reported or only minor complaints were registered in the studies conducted. So, reviews regarding the safety of cimicifuga racemosa did come to the conclusion that cimicifuga is a safe herbal medicine when used for short time [118-120, 126]. These conclusions were based on the clinical findings in case series and randomized or non-randomized studies about the efficacy of cimicifuga. *In vitro* tests looking at hepatotoxicity have not been published so far. Since hepatotoxicity has been reported in some patients, we performed *in vivo* and *in vitro* studies to reproduce such findings in animals and to find out possible mechanisms.

We found that cimicifuga racemosa, in the concentrations used by us, inhibits the mitochondrial β-oxidation to a large extent. A severe inhibition of hepatic β-oxidation *in vivo* leads to an accumulation of fatty acids in hepatocytes. Fatty acids can be converted to triglycerides, which will accumulate and form small vesicles, leading to the picture of microvesicular steatosis. This outcome was found in rats fed with high doses of cimicifuga. Microvesicular steatosis can be caused by drugs through different mechanisms, such as sequestration of CoA, inhibition or inactivation of enzymes involved in hepatic fatty acid metabolism, or modifications of mtDNA [127]. Indeed, cimicifuga extract inhibits β-oxidation in isolated mitochondria, offering an explanation for the *in vivo* findings.

A mode of cell death that is often found after exposure to different stimuli (chemicals, drugs, ionizing irradiation, viruses, depletion of growth factors, hormones and cytokines) is apoptosis [17]. Apoptosis can be regarded as a physiological process to control the proliferation of cells and tissues. On the other hand, apoptosis associated with drugs can lead to organ damage [90]. Considering cimicifuga extract, apoptosis was found in human breast carcinoma cell lines after incubation with isopropanolic or ethanolic extracts [128]. Our work demonstrates that cimicifuga extracts can trigger apoptosis also in hepatocytes, offering a possible explanation for hepatotoxicity of this drug. The concentrations causing 50 % growth inhibition of human breast carcinoma cell lines found by Hostanska et al [128] are lower then the ones we found for HepG2 cells, however. Our values, obtained using the SRB and MTT test, were in a range of 200 µg/mL, theirs between 30 and 80 µg/mL. A reason for this difference may be the different cell lines used.

To assess whether the concentrations used are in the range of the blood concentrations reached after ingestion of cimicifuga tablets, a rough estimation is made. Assuming that a tablet containing 6.5 mg dried ethanolic extract (contained in Climavita®, Permamed, Switzerland) is ingested, the whole extract is absorbed and distributed to body water, a plasma concentration in the range of 0.2 µg/mL could be expected. This value is much below the cytotoxic concen-

trations found in our experiments, but hepatic accumulation of toxic substances during long-term ingestion of cimicifuga cannot be excluded.

In comparison to humans, higher doses of cimicifuga were used in the rat experiments. For a rat weighing 300 g, daily doses of 0.3 – 300 mg were administered, resulting in plasma concentrations in the range of 1.5 – 1500 µg/mL (assuming distribution in the body water). In rats *in vivo*, mitochondrial toxicity started to manifest itself at 100 mg/kg (resulting in plasma concentrations of about 150 µg/mL), which corresponds quite well with our *in vitro* findings. The dosages used in humans appear to be too low, however, to be associated with hepatic mitochondrial toxicity.

Cimicifuga racemosa is - as all other plants - a multicomponent drug. The main constituents of cimicifuga racemosa are triterpene glycosides (actein, 27-deoxyactein, cimifugoside, cimiracemoside), phenolic acids (isoferulic acid, fukinolic acid), flavonoids, volatile oils, and tannins [129]. The components responsible for the pharmacological effect are not known so far. Hostanska et al [130] investigated the main classes of components for their effect on apoptosis. The two main classes tested were triterpene glycosides and, due to their similarity to synthetic estrogenic compounds, the cinnamic acid esters. Both classes tested were found to induce apoptosis in human breast cancer cells. It remains unclear, however, whether these substances are also the cause for the hepatotoxicity observed in our experiments.

The formation of quinoid metabolites has also been considered to be a cause of toxicity of cimicifuga [131]. Although reactive quinoid metabolites are formed by phase I reactions, *in vivo* studies did not confirm the formation of these products.

In vivo, cimicifuga toxicity could occur when cimicifuga components are metabolized through cytochrome P450 or other enzymes, producing reactive metabolites. But so far, only very few data have been published concerning the metabolism of cimicifuga ingredients. In one investigation, cimicifuga extract was subjected to microsomal metabolism before cytotoxicity tests were performed. However, cytotoxicity was not different to incubations without previous treatment with microsomes, strongly suggesting that formation of metabolites is not essential for cimicifuga toxicity [130]. These findings are in accordance with our studies, showing a good agreement between *in vivo* and *in vitro* toxicity and no influence on the glutathione pool in HepG2 cells.

In conclusion, cimicifuga extract is hepatotoxic in rats starting at 100 mg/kg body weight. *In vitro* studies show that this toxicity is due to inhibition of mitochondrial β-oxidation and uncoupling of oxidative phosphorylation, which is associated with hepatocellular apoptosis. In humans, the dosages used are too low to explain hepatotoxicity entirely by this mechanism, suggesting the presence of additional risk factors.

8.6 Acknowledgement

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9 CONCLUSIONS AND OUTLOOK

Today, phytomedicinals are very popular and are therefore widely used. From the consumers point of view these medicines are a safe, good working and easily accessible alternative to the conventional pharmaceuticals with their side effects and other suspicious properties. From the view of a scientist, these herbal products would require a second look. The aspect of efficacy has after all been investigated to some extent, but partly there is only the traditional knowledge from ancestors or from some tribes. As opposed to this, the issue of safety and quality control has been neglected to a great deal. Recently, more and more reports of herb-connected adverse reactions became public and the concepts of regulatory authorities point into the direction of gaining more information about phytomedicines as well. For this reason, efforts are undertaken to elicit the mechanisms of action of wanted as well as unwanted effects. This is surely highly needed if companies want to further market their phytopharmaceuticals in a legally regulated environment. This thesis comprises two projects which support these efforts. One work comes actually to late, from the manufacturer's point of view, as kava has already been withdrawn from the market in many countries. Despite of this, the results could give hints about the precise location where the plant or parts of it act on liver cells in a negative fashion. These negative effects might be avoided in future developments when taking the above mentioned results into account and when advancing the research based upon the here obtained findings. This might include testing of different extracts (i.e. using other solvents or extraction procedures), single constituents and/or different parts of the plant. It is important to consider that the plants used have the same characteristics and that the raw material is of constant quality.

In the case of cimicifuga or also other phytomedicines the same fate as for kava could maybe be anticipated by testing the extracts in an early stage. This means, that the herein used methods could represent a basis for testing herbal products regarding their hepatotoxic potential before they reach market. Of course, other methods should also be added, like animal experiments, to evaluate concerns about hepatotoxicity. In addition, similar tests should be performed to assess the toxicity for other organs, e.g. brain and kidney. Also metabolic investigations like attributes concerning CYPs are of major interest, as the greatest part of xenobiotics are metabolized by these enzymes. Interactions leading to potentially toxic levels of any of the involved substances could thus be predicted and hence be avoided to some part. These assessments have been performed for kava extracts and kavalactones, which is important. For cimicifuga racemosa this has not been done so far. Anyway, not much is known about the toxicity of cimicifuga. However, in contrast to kava only very few cases of liver toxicity have been reported until now. Nevertheless, clarifications regarding cimicifuga's (liver) toxicity are of great

interest, as it is a widely used medicine against menopausal ailments. For this reason, assessment of *in vitro* hepatotoxicity of cimicifuga was done in this work and toxicity was found indeed. What this means, when extrapolated to humans is ambiguous, as only few cases of toxicity of cimicifuga were assumed in humans, i.e. one case of autoimmune hepatitis, one case of acute liver failure, two cases of "hepatitis", and two cases of mild elevation in liver function enzymes [132]. As no further cases have been published, despite the widespread use of cimicifuga products, it is assumed that the herein toxic concentrations are higher than the concentrations obtained *in vivo* and that other factors might contribute to this liver toxicity. These factors may also play a role in liver toxicity cases of other plants and consist of genetic predisposition, pre-existing liver or mitochondrial diseases, gender, age, induced or inhibited CYP function, and alcohol (see also chapter 4.3).

That mitochondria may play an important role in the development of toxicity could be deduced from the results of the investigations regarding kava kava and cimicifuga racemosa. Mitochondria are central elements in the energy metabolism as well as in live and death of cells. Interference with these functions may lead to detrimental outcomes like necrosis or apoptosis. However, since lately, not only mitochondria are thought to be involved in the development of apoptosis, but also the endoplasmic reticulum (ER). The ER is the place where protein synthesis, protein folding and trafficking, cellular response to stress and intracellular calcium levels are regulated. Alterations in calcium homeostasis and accumulation of misfolded proteins in the ER cause stress, finally leading to apoptosis [133]. Calcium seems to be involved in apoptosis in a manner, that release of calcium from the ER and subsequent mitochondrial calcium uptake play an important role in the regulation of the release of cytochrome c from the mitochondria [134]. These apoptosis-associated calcium fluxes are under the control of the Bcl-2 family of proteins, which also regulate cellular pH and ER resident proteins [134, 135]. So, further examinations of toxicity mechanisms should also focus on this organelle and on calcium levels in the different compartments of cells (i.e. ER, mitochondria, cytosol). Maybe some herbal extracts could also be used for the treatment of diseases with diminished apoptosis rate like cancer or autoimmunity to induce apoptosis and thus leading to the demise of redundant and altered cells. For cimicifuga this purpose was already proposed in the context of breast cancer [128].

In this thesis, hepatoma cell lines (HepG2 and HUH6) have been used to assess the hepatotoxic potential of the two plant extracts. Cell lines are an easy to handle model for the investigation of toxicological questions. Despite, attention has to be paid when interpreting data stemming from experiments with these cell lines, as carcinoma cells have altered characteristics and metabolism compared to normal liver cells. Therefore, it would be more adequate to use freshly isolated liver cells. The best model would be human cells, but this is of course not feasible for routine assays. Freshly isolated rat or mouse liver cells could be gained more

easily, but then the field of human tissue would have been abandoned. Thus, compromises will have to be done when performing (routine) toxicity assays.

Based on the herein gained results one could ask oneself provocatively whether it is not too dangerous to apply phytomedicines at all. Either toxicity has been found *in vitro* or in clinical use or herbal products have not been investigated thoroughly enough. This does not lay a very trustful basis for the use of botanicals. Despite, one can not lump together all the phytomedicines and ban all the herbs that have been used for ages without clear toxicity. What probably needs to be done is to set rules how herbal remedies should be handled in the future. A start would be to first clarify the entitlement of the botanical in question to be further used as evidence-based medicine, i.e. with controlled studies. When the further marketing is justified from the efficacy point of view then measures to evaluate and report the safety of a phytomedicine should be taken. These measures could first consist of a general obligation to perform (*in vitro*) tests to estimate the toxicity of a herbal product. In addition, instruments to monitor the occurrence of negative effects should be established and the attention of medical doctors and consumers should be drawn to the fact that also phytomedicines are able to elicit side effects in a direct or indirect way and that surveillance of liver enzymes could be appropriate. Additionally, products should be standardized to some main component in order to offer a basis for dosage recommendations, methods to test for contamination and adulteration but also for confirmation of the presence of the herb in question should be defined, and finally compliance with the new regulations has to be monitored.

In summary, it can be concluded that there needs a lot to be done in the field of phytomedicines including enhancement of the awareness of potential toxicity and of the research concerning herbal drugs. To finally make a balanced assessment of value of any herbal extract, evidence of efficacy has to be weighed against information on safety [48]. In the case of kava kava, more investigations should be done when a reintroduction into the pharmaceutical market is sought. These investigations would have to consist of additional toxicological experiments including different extracts and single compounds to denominate the toxic part of kava. Based on these findings new products might be developed to put on the market. Concerning cimicifuga racemosa, *in vitro* investigations suggest some toxicity. However, in reality quasi no cases of liver toxicity have been reported. For this reason, further experiments will have to be performed in order to scrutinize the (hepato)toxic potential and to define situations in which cimicifuga might lead to detrimental outcomes.

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Publications

Papers

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Lüde, S., Török, M., Dieterle, S., Jäggi, R., Berger Büter, K., and Krähenbühl, S. Hepatic toxicity of kava leaf and root extracts. Submission in 2005.

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Lüde, S., Bachmannn, F., Krähenbühl, S., and Török, M. A GFP-method as an alternative to annexin V / propidium iodide stain in the detection of hepatic toxicity of kava kava. Submission in 2005.

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Abstracts

Kaufmann, P., Török, M., Lüde, S., and Krähenbühl, S. Statin induced mitochondrial toxicity. Kardiovaskuläre Medizin 2004;7:35.

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Congresses and Posters

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